

Advances in Food Diagnostics

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*This book is dedicated to my mother,
Francina Vilardell, who passed
away during the final preparation of this
book. She was very enthusiastic and great
supporter of my research activity.
I will always remember her.*

Fidel Toldrà

Contents

List of Contributors *xvii*

Preface *xxiii*

1	Assuring Safety and Quality along the Food Chain	1
	<i>Gerhard Schiefer</i>	
1.1	Quality and safety: issues	1
1.2	Tracking and tracing through chains and networks	2
1.3	Food safety – the baseline	3
1.4	Food quality – delivery concepts	4
1.5	Quality programs – steps towards sector quality agreements	5
1.5.1	Overview	5
1.5.2	A closed system concept – the case of IKB	6
1.5.3	An open sector system concept – the case of Q&S	6
1.5.4	Trade initiatives	7
1.6	The information challenge	7
1.6.1	Information clusters	7
1.6.2	Organisational alternatives	9
1.6.3	Data ownership and data markets	10
1.6.4	Added value of emerging information infrastructures	10
1.7	Conclusion	10
	References	11
2	Methodologies for Improved Quality Control Assessment of Food Products	13
	<i>Manuel A. Coimbra, Sílvia M. Rocha, Cátia Martins and António S. Barros</i>	
2.1	Introduction	13
2.2	Use of FT-IR spectroscopy as a tool for the analysis of polysaccharide food additives	14
2.2.1	Identification of polysaccharide food additives by FT-IR spectroscopy	16
2.2.2	Influence of hydration on FT-IR spectra of food additive polysaccharides	20
2.3	Use of outer product (OP) and orthogonal signal correction (OSC) PLS1 regressions in FT-IR spectroscopy for quantification purposes of complex food sample matrices	23

2.3.1	Outer product (OP)-PLS1 regression applied to the prediction of the degree of methylesterification of pectic polysaccharides in extracts of olive and pear pulps	23
2.3.2	Orthogonal signal correction (OSC)-PLS1 regression applied to white and red wine polymeric material extracts	29
2.4	Screening and distinction of coffee brews based on headspace – solid phase microextraction combined with gas chromatography in tandem with principal component analysis (HS-SPME/GC-PCA)	33
2.5	Comprehensive two-dimensional gas chromatography (GC × GC) combined with time-of-flight mass spectrometry (ToFMS) as a powerful tool for food products analysis	38
2.5.1	GC × GC-ToFMS principles and advantages	38
2.5.2	Beer volatile profiling by HS-SPME/GC × GC-ToFMS	41
2.6	Study of cork (from <i>Quercus suber</i> L.) – wine model interactions based on voltammetric multivariate analysis	44
2.6.1	Evaluation of the voltammetric analysis in what concerns the cyclic and square wave technique	46
2.6.2	Cyclic voltammetric analysis for cork classification	49
2.7	Concluding remarks	52
	References	52
3	Developments in Electronic Noses for Quality and Safety Control	63
	<i>John Bosco Balaguru Rayappan, Arockia Jayalatha Kulandaisamy, Madeshwari Ezhilan, Parthasarathy Srinivasan and Ganesh Kumar Mani</i>	
3.1	Introduction	63
3.2	Overview of classical techniques for food quality testing	65
3.2.1	Chromatographic techniques	70
3.2.2	Spectroscopic techniques	70
3.2.3	Imaging techniques	74
3.2.4	Biological techniques	75
3.3	Electronic Nose	75
3.3.1	Various definitions of eNose reported in literature	75
3.3.2	Aroma as biomarker	76
3.4	Instrumentation of eNose (Loutfi <i>et al.</i> , 2015)	77
3.4.1	Sampling system	77
3.4.1.1	Analytical distillation methods	77
3.4.1.2	Headspace analysis methods (HS)	78
3.4.1.3	Direct extraction methods	78
3.4.2	Detection system (Loutfi <i>et al.</i> , 2015)	78
3.4.2.1	Types of chemical sensors for gaseous environment	78
3.4.3	Data processing system	79
3.5	Recent developments in electronic nose applications for food quality	79
3.5.1	Meat	79
3.5.2	Milk	80
3.5.3	Fish and seafood	81
3.5.4	Fruits and vegetables	82
3.5.5	Adulterants	83

3.5.6	Beverages	84
3.5.6.1	Non-alcoholic beverages	84
3.5.6.2	Alcoholic beverages	84
3.6	Conclusion	85
	References	85
4	Proteomics and Peptidomics as Tools for Detection of Food Contamination by Bacteria	97
	<i>Dina Rešetar, Tamara Martinović, Sandra Kraljević Pavelić, Uroš Andjelković and Djuro Josić</i>	
4.1	Introduction	97
4.2	Bacteria as food-borne pathogens	98
4.3	Gram-positive bacteria	101
4.4	Gram-negative bacteria	106
4.5	Bacterial toxins	110
4.5.1	Endotoxins	110
4.5.2	Exotoxins	111
4.6	Detection of bacterial contamination in food	114
4.6.1	Omics methods for detection of bacteria	116
4.6.1.1	Proteomic and peptidomic methods	116
4.6.1.2	Affinity-based methods	117
4.6.1.3	Mass spectrometry-based methods	120
4.7	Analysis of bacterial toxins	121
4.8	Conclusions	126
4.9	Acknowledgements	127
	References	127
5	Metabolomics in Assessment of Nutritional Status	139
	<i>Kati Hanhineva</i>	
5.1	Introduction	139
5.2	Usability of metabolomics in nutrition sciences	139
5.3	The metabolite complement in human studies	140
5.4	Metabolomics within the analysis of relationship between diet and health	141
5.5	Individual differences in metabolic and nutritional phenotype	142
5.6	Assessment of nutritional status, example studies	143
5.6.1	Malnutrition	143
5.6.2	Deficiencies in particular nutrients	145
	References	148
6	Rapid Microbiological Methods in Food Diagnostics	153
	<i>Catherine M. Logue and Chantal W. Nde</i>	
6.1	Introduction	153
6.1.1	Why the need for rapid methods – their benefits and potential limitations	153
6.2	Quantitative vs qualitative	154
6.3	Culture dependent vs independent	154

6.4	Automation and multi-pathogen detection	155
6.5	Separation and concentration	156
6.5.1	Filtration	156
6.5.2	Stomacher	156
6.5.3	Pulsifier	157
6.6	Rapid methods that are currently in the market	157
6.6.1	Microscopic-based	157
6.6.1.1	DEFT – direct epifluorescent filter technique	157
6.6.1.2	FISH – fluorescent <i>in situ</i> hybridisation	158
6.6.1.3	Live dead assay	158
6.6.1.4	Enzyme-linked immunosorbent assay (ELISA)	159
6.6.1.5	MALDI-TOF MS	159
6.6.1.6	Flow cytometry	160
6.6.1.7	Solid phase cytometry	161
6.6.2	Metabolism-based detection	161
6.6.2.1	Head space analysis	162
6.6.3	Luminescence-based	162
6.6.3.1	Bioluminescence/ATP detection	162
6.6.4	Immunological/ serological based	163
6.6.4.1	Antibody-based latex agglutination assay	163
6.6.4.2	Immunoprecipitation	163
6.6.4.3	Immunomagnetic separation (IMS)	163
6.6.5	Nucleic acid-based (molecular)	164
6.6.5.1	DNA microarrays	164
6.6.5.2	DNA colony hybridisation	165
6.6.5.3	Polymerase chain reaction (PCR)	166
6.6.5.4	Nested PCR	167
6.6.5.5	Loop-mediated isothermal amplification (LAMP)	167
6.6.5.6	Real-time PCR	167
6.6.5.7	Quantitative PCR (qPCR)	168
6.6.5.8	Digital PCR	168
6.6.5.9	Droplet digital PCR	169
6.6.5.10	16S Riboprinting	169
6.6.6	Next-generation technologies	169
6.6.7	Immunosensors or biosensors	170
6.6.7.1	Electronic nose sensors	170
6.6.7.2	Mass-sensitive biosensors	170
6.6.7.3	Surface plasmon resonance (SPR)	171
6.6.7.4	Raman and Fourier transform spectroscopy	171
6.6.7.5	Fourier transform infrared spectroscopy (FTIR)	171
6.6.7.6	Fibre optic biosensor	172
6.6.7.7	Aptamer-based biosensors	172
6.6.7.8	Nanotechnology for pathogen detection	172
6.7	Conclusion	173
	References	173

7	Molecular Technologies for the Detection and Characterisation of Food-Borne Pathogens	187
	<i>Geraldine Duffy</i>	
7.1	Introduction	187
7.2	Hybridisation-based methods	188
7.2.1	DNA hybridisation methods	188
7.2.2	RNA hybridisation methods	189
7.2.2.1	Fluorescent <i>in situ</i> hybridisation (FISH)	189
7.2.3	DNA microarrays	189
7.3	Nucleic acid amplification methods	190
7.3.1	Polymerase chain reaction	190
7.3.1.1	Real-time PCR	190
7.3.1.2	Quantitative PCR	192
7.3.1.2.1	Digital PCR	192
7.3.1.3	Multiplex PCR	192
7.3.2	RNA-based amplification assays	193
7.3.2.1	Reverse transcriptase polymerase chain reaction	193
7.3.2.2	Viability dyes in RT-PCR	193
7.3.3	Isothermal amplification	194
7.3.3.1	Loop-mediated isothermal amplification (LAMP)	194
7.3.3.2	Nucleic acid sequence-based amplification (NASBA)	194
7.4	Molecular characterisation methods	195
7.4.1	Pulse field gel electrophoresis (PFGE)	195
7.4.2	Amplified fragment length polymorphism (AFLP)	195
7.4.3	Restriction fragment length polymorphism (RFLP)	196
7.4.4	Multi-locus variable-number tandem repeat analysis (MLVA)	196
7.4.5	Multi-locus sequence typing (MLST)	196
7.4.6	Whole genome sequencing (WGS)	197
7.5	Conclusion	198
	References	198
8	DNA-based Detection of GM Ingredients	205
	<i>Patrick Guertler, Alexandra Hahn, Ulrich Busch and Karl-Heinz Engel</i>	
8.1	Introduction	205
8.2	Analysis of GMO	205
8.2.1	Sampling and DNA extraction	206
8.2.2	Choice of target sequences	207
8.2.3	Conventional end-point PCR	208
8.2.4	Real-time PCR	208
8.2.5	Digital PCR	210
8.2.6	Multiplex approaches	212
8.3	Quantification of GMOs	215
8.4	Validation	217
8.5	Challenges in GMO detection	218
8.5.1	Influences of food composition and processing	218

- 8.5.2 Copy numbers 219
- 8.5.3 Certified reference material 219
- 8.5.4 Sequence information 220
- 8.5.5 Stacked events 220
- 8.5.6 GM animals 221
- 8.6 Outlook 221
- References 222

9 Enzyme-based Sensors 231

Anastasios Economou, Stephanos K. Karapetis, Georgia-Paraskevi Nikoleli, Dimitrios P. Nikolelis, Spyridoula Bratakou and Theodoros H. Varzakas

- 9.1 Introduction to enzymatic biosensors 231
- 9.2 Types of transducers 235
- 9.3 Enzymatic biosensors and the food industry 238
- 9.4 Biosensors for the analysis of main food components 239
 - 9.4.1 Sugars 239
 - 9.4.2 Acids 241
 - 9.4.3 Amino acids 242
 - 9.4.4 Alcohols 243
- 9.5 Biosensors for contaminants 244
 - 9.5.1 Pesticides 244
 - 9.5.2 Heavy metals 245
- 9.6 Food freshness indicators, antinutrients and additives 246
- 9.7 Future perspectives 247
- References 248

10 Immunology-based Biosensors 251

Theodoros H. Varzakas, Georgia-Paraskevi Nikoleli and Dimitrios P. Nikolelis

- 10.1 Introduction 251
- 10.2 Antibodies and biosensors 251
 - 10.2.1 Immunochemiluminescence biosensors 252
 - 10.2.2 Site-directed antibody immobilisation techniques for immunosensors 253
 - 10.2.3 Label-free arrayed imaging reflectometry (AIR) detection platform 255
- 10.3 Immunoassays for detection of microorganisms 255
- 10.4 Immunosensors and cancer biomarkers-immunoarrays 259
 - 10.4.1 Microfluidic paper-based analytical devices (mPADs) 260
- References 261

11 Graphene and Carbon Nanotube-Based Biosensors for Food Analysis 269

Stephanos K. Karapetis, Spyridoula M. Bratakou, Georgia-Paraskevi Nikoleli, Christina G. Siontorou, Dimitrios P. Nikolelis and Nikolaos Tzamtzis

- 11.1 Introduction 269
- 11.2 Biosensing devices based on graphene and CNTs and their applications in food analysis 270
- 11.3 Future trends and prospects 274
- References 275

12	Nanoparticles-Based Sensors	279
	<i>Luís G. Dias, António M. Peres and Alfredo Teixeira</i>	
12.1	Introduction	279
12.2	Nanoparticles for sensor technology	280
12.2.1	Electrochemical techniques	281
12.2.2	Spectroscopic techniques	283
12.2.3	Nanoparticles characterisation	285
12.3	Nanoparticles-based sensors: applications	286
12.3.1	Nanoparticles based-sensors for pesticides detection in foods	287
12.3.2	Nanoparticles-based sensors for antibiotics, growth enhancers and other veterinary drugs detection in foods	287
12.3.3	Nanoparticles based-sensors for mycotoxins detection in foods	289
12.3.4	Nanoparticles based-sensors for microorganisms' detection in foods	290
12.3.5	Nanoparticles-based sensors for detecting food valuable constituents	291
12.3.6	Nanoparticles based-sensors for detecting food contaminants and adulterations	292
12.3.7	Nanoparticles-based sensors for detecting food dyes/additives	294
12.3.8	Nanoparticles based-sensors for detecting metal ions in foods	297
12.4	Conclusions and future trends	298
	References	299
13	New Technologies for Nanoparticles Detection in Foods	305
	<i>G. Castillo, Z. Garaiova and T. Hianik</i>	
13.1	Introduction	305
13.2	Nanoparticle properties and applications in food industry	306
13.2.1	Preparation of nanoparticles	306
13.2.1.1	Top-down strategy	306
13.2.1.2	Bottom-up strategy	307
13.2.2	Properties of nanoparticles	308
13.2.2.1	Organic nanoparticles	309
13.2.2.2	Inorganic nanoparticles	310
13.2.2.3	Combined nanoparticles	312
13.2.3	Applications of nanoparticles in food industry	312
13.2.3.1	Food functionalisation	313
13.2.3.2	Food packaging and quality preservation	315
13.3	Toxicity of food-related nanoparticles	317
13.3.1	Biological fate of ingested nanoparticles	317
13.3.2	Toxicity studies of engineered nanoparticles	318
13.4	Methods of nanoparticle detection in food	321
13.4.1	Direct visualisations of nanomaterials	322
13.4.2	Measurement of nanoparticles by light-scattering methods	323
13.4.3	Electrochemical methods in nanoparticle analysis	327
13.4.4	Food monitoring and safety controls	329
13.5	Conclusion	330
13.6	Acknowledgments	330
	References	330

14 Rapid Liquid Chromatographic Techniques for Detection of Key (Bio)chemical Markers 343

M-Concepción Aristoy, Milagro Reig and Fidel Toldrá

- 14.1 Introduction 343
- 14.2 The fundamentals of liquid chromatography 344
 - 14.2.1 Adsorption HPLC 345
 - 14.2.2 Ion exchange HPLC 345
 - 14.2.3 Size exclusion HPLC 345
 - 14.2.4 Partition HPLC 345
- 14.3 Advances in modern HPLC 346
- 14.4 Analysis of biochemical markers: applications for nutritional quality 347
 - 14.4.1 Amino acids 347
 - 14.4.2 Carbohydrate and carboxylic acids 348
 - 14.4.3 Vitamins 350
 - 14.4.4 Minerals and trace elements 352
 - 14.4.5 Antioxidants 352
- 14.5 Analysis of biochemical markers: applications for food quality 354
 - 14.5.1 Biochemical compounds 354
 - 14.5.1.1 Amino acids 354
 - 14.5.1.2 Nucleotides and nucleosides 354
 - 14.5.2 Additives 355
 - 14.5.3 Markers for process control 356
- 14.6 Analysis of biochemical markers: applications for the detection of food adulterations 356
- 14.7 Analysis of biochemical markers: applications for food safety 357
 - 14.7.1 Biochemical compounds 357
 - 14.7.2 Veterinary drug residues in foods of animal origin 359
 - 14.7.3 Antibiotic residues in foods of animal origin 360
 - 14.7.4 Other residues 361
- References 362

15 Olfactometry Detection of Aroma Compounds 379

Mónica Flores and Sara Corral

- 15.1 Introduction 379
- 15.2 Extraction of volatile compounds from foods for GC-olfactometry analysis (GC-O) 380
- 15.3 Olfactometry techniques 382
 - 15.3.1 Methodologies 382
 - 15.3.1.1 Dilution analysis method 383
 - 15.3.1.2 Detection frequency method 383
 - 15.3.1.3 Direct intensity method 388
 - 15.3.2 Use of GC-O methodologies 388
- 15.4 Applications of GC-O in food industry 389
 - 15.4.1 Identification of key aroma compounds in different foods 389
 - 15.4.2 Identification of off-flavours for quality control 392
 - 15.4.3 Application of GC-O to production processes 393
 - 15.4.4 Application of GC-O to reformulation of food aromas 395

15.5	Conclusions	395
15.6	Acknowledgements	396
	References	396
16	Data Handling	401
	<i>Riccardo Leardi</i>	
16.1	Introduction	401
16.2	Data collection	402
16.3	Data display	403
16.4	Process monitoring and quality control	417
16.5	Three-way PCA	417
16.6	Classification	420
16.7	Modelling	423
16.8	Calibration	424
16.9	Variable selection	426
16.10	Conclusion: future trends and the advantages and disadvantages of chemometrics	428
	References	429
	Suggested Books	430
17	Automated Sampling Procedures	431
	<i>Semih Otles and Canan Kartal</i>	
17.1	Introduction	431
17.2	Extraction techniques for sample preparation	432
17.2.1	Extraction from liquid samples	432
17.2.1.1	Liquid-liquid extraction	433
17.2.1.2	Solvent microextraction (SME)	433
17.2.1.2.1	Single-drop microextraction (SDME)	434
17.2.1.2.2	Dispersive liquid-liquid microextraction (DLLME)	438
17.2.1.2.3	Hollow-fibre microextraction (HF-LPME)	439
17.2.1.3	Solid-phase extraction (SPE)	440
17.2.1.3.1	Solid-phase microextraction (SPME)	442
17.2.1.3.2	In-tube solid-phase microextraction	443
17.2.1.3.3	Stir-bar-sorptive extraction	445
17.2.2	Extraction from solid samples	446
17.2.2.1	Matrix solid phase dispersion (MSPD)	446
17.2.2.2	Pressurised liquid extraction (PLE)	448
17.2.2.3	Super-heated water extraction (SHWE)	449
17.2.2.4	Supercritical fluid extraction (SFE)	449
17.2.2.5	Microwave- and ultrasound-assisted extraction	451
	References	453
18	The Market for Diagnostic Devices in the Food Industry	465
	<i>Mark Buecking, Hans Hoogland and Huub Lelieveld</i>	
18.1	Introduction	465
18.2	Food diagnostics	461
18.3	Product composition	466

18.3.1	Physical hazards	468
18.3.2	Biological hazards	468
18.3.3	Chemical hazards	468
18.3.3.1	Metals	468
18.3.3.2	Pesticides	469
18.3.3.3	Organic contaminants	469
18.3.3.4	Allergens	469
18.3.4	Metabolites	470
18.3.5	Desired product constituents	470
18.3.6	Source of constituents	470
18.4	Product structure	471
18.4.1	Viscosity	471
18.4.2	Air/gas	471
18.4.3	Crystal size	472
18.5	Influence of processing on product composition	472
18.5.1	Reactions between naturally present substances in food	472
18.5.2	Contamination with cleaning and disinfection agents	473
18.6	Processing parameters	473
18.6.1	General	474
18.6.2	Flow rate and velocity distribution/temperature and temperature distribution	474
18.6.3	Droplet, bubble, crystal size and distribution	475
18.6.4	Additional parameters for high-pressure processing	475
18.6.5	Pulsed electric field (PEF) processing	475
18.7	Packaging parameters	476
18.7.1	Sterility testing	477
18.8	Conclusion	477
	References	478
	Index	479

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Preface

The main goal of the book *Advances in Food Diagnostics* is to provide the reader with a comprehensive resource covering the field of diagnostics in the food industry. While it covers conventional (typically lab-based) methods of analysis, the book focuses on leading-edge technologies that are being (or are about to be) introduced in important areas like food quality assurance, nutritional value and food safety, and also on other relevant issues such as traceability and authenticity, which are strongly demanded by all sectors involved in 'farm to fork'. This means from the production of raw materials, through the processing food industry and distribution to markets, until reaching the consumer. Guaranteeing the health, well-being and safety of consumers is a must, and the response to any concern must be as immediate as possible, which is why on-line and at-line diagnostics applications or very rapid methodologies are so highly demanded. The field of diagnostics in the food industry is evolving very rapidly. A good example is the number of publications that is growing exponentially year by year. New diagnostics tools are being developed and finding new applications, while the existing ones are optimised, are often miniaturised and, increasingly, are becoming automated.

The first edition of this book dates from 2007, and contained topics spread through 16 chapters. This second edition brings 18 chapters, with new approaches in the dynamic field of food diagnostics. Thus, this second edition combines updated and revised versions of several old chapters, plus new chapters dealing with outstanding developments in recent years, on nanotechnology for sensor devices, or in the use of omics technologies like proteomics, metabolomics or genomics, and their applications in food quality, safety and nutrition.

The book looks at areas such as improved methodologies for safety and quality control; the use of nuclear magnetic resonance for quality control and traceability; the latest developments in 'electronic noses' for food safety and quality; proteomics applications in food safety; the use of metabolomics for nutritional assessment; newly developed molecular methods for microbiology monitoring and for detecting and characterising pathogens; DNA-based methods for the detection of GMO in composite and processed foods; the use of enzyme-based and immuno-based sensors for the detection of a variety of substances in foods; nanotechnology-developed sensors based on graphene, nanotubes and nanoparticles; tools for the effective detection of nanoparticles in foods; advances in increased-throughput high-performance liquid chromatography with less sample manipulation; the rapid techniques for olfactometry detection of aroma compounds; the latest developments in automation, especially on the efficient extraction of sample analytes; the fundamentals of chemometrics, especially the most

relevant techniques for data display, classification, modelling and calibration; and, lastly, a final discussion for the market of diagnostic devices in the food industry.

Once more, this second edition will find a large audience in the academia, administration and industry, and for all of those involved in food science and technology. We sincerely hope you will find this book of interest and that it provides you with a better understanding about new developed diagnostic tools, how they work and apply as well as their future trends.

The editors wish to thank all the contributors for their hard work and excellent results with the delivered chapters of this book, and also thank the production team at Wiley-Blackwell for their dedication and nice publication of this book.

Fidel Toldrá
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1

Assuring Safety and Quality along the Food Chain

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1.1 Quality and safety: issues

The term 'quality' has become a focus point in all discussions regarding the production and provision of food products to markets and consumers – quality in the broad sense of serving the consumers' needs (see also the early publication by Oakland, 1998) by providing them with the right product, at the right time, and with the right service. In today's competitive food markets, the quality approach is a precondition for sustainable market acceptance. It is a core pillar in the sustainability of enterprises and sectors, which builds on economic viability, quality orientation, ethical concerns, and an appropriate embedment in its environment.

In an enterprise, a *sustainable delivery of quality* is a result of a comprehensive effort. It involves the implementation of a quality approach at all levels of activities, ranging from enterprise management to process organisation, process management, and product control. Enterprise quality systems build on routine quality assurance and improvement activities that might encompass one or several of these levels. However, most food quality systems focus on system activities at several levels, involving process organisation, process management and product control.

Food safety is an inherent element of quality. It receives special attention not only by enterprises, but also by policy and legislation, because of its key importance for consumers' health, and the responsibility for food safety by enterprises and policy alike. Globalisation and industrialisation in the production and provision of food has increased the potential risk in food safety and has initiated increased efforts and controls in food safety assurance.

The efficient 'transportation' of quality from the farm, and any of the subsequent stages of processing and trade to the consumer as the final customer, requires efforts in cooperation along the chain. The dependency of food quality and safety from activities at all stages in the chain makes chain cooperation a prerequisite of any advanced quality assurance scheme, including food safety. Such cooperation might build on individual arrangements, sector agreements, or on any other way that avoids the loss and supports the gain of quality along the chain.

Chain cooperation has become a crucial element in quality assurance, and especially in food safety initiatives in the food sector. However, in the food sector, chains usually develop dynamically in a network of interconnected enterprises, with constantly changing lines of supplier-customer relationships. In this scenario, chain cooperation is based on network cooperation – or, in other words, on sector agreements.

The *quality guarantee* that one can derive from the implementation of a quality system depends on the evaluation of the system as a whole. Quality and food safety deficiencies at any stage might remain with the product throughout the remaining stages, until it reaches the consumer. The most crucial need for guarantees involves guarantees for food safety. These constitute the baseline guarantee level and the prerequisite for consumers' trust and market acceptance (Henson and Hooker, 2001; Verbeke, 2005).

The delivery of quality guarantees is based on controls, both, in the organisation of processes (process controls) and in process management (management controls). However, for the delivery of guarantees, these controls need to be integrated into a comprehensive scheme (quality program) that could serve as a cooperation platform for enterprises within supply chains and networks and provide a basis for communication with consumers.

Key issues involve agreements on *chain-encompassing quality assurance* schemes, and the ability to identify the product flow through the production chain clearly, by linking the different product entities that are being produced and traded at the different stages of the chain, from the farm to the consumer as the final customer, and their quality status (tracking and tracing capability).

The following sections cover the development path from tracking and tracing towards quality assurance in food chains, the organisational concepts and quality programs for implementation, and the role of information and communication systems for operational efficiency.

1.2 Tracking and tracing through chains and networks

The tracking and tracing of food products throughout the food chain has become a dominant issue in discussions on food quality and, especially, on the assurance of food safety (Lobb, 2005). They allow, for any product and from any stage within the chain, identification of the source (backward tracing) and its destination (forward tracing). This supports the (backward) identification of sources of product deficiencies, and the (forward) isolation of any other product that might have been affected by these sources. Tracking and tracing capabilities support consumer protection in case of food contamination. Furthermore, they support the communication of the quality status of products on their way through the food chain, and provide the basis for the delivery of quality guarantees at each stage of the chain and towards the consumers at the final stage.

However, it should be noted that, beyond this discussion line, the organisation of tracking and tracing schemes (TT schemes) has also a managerial dimension in supporting efficiency in the logistics chain (supply chain) from the source (farms) to the final destination (the consumer). In fact, the managerial dimension has been at the centre point of initial discussions on tracking and tracing schemes, not just in the food sector but in other sectors as well (Golan *et al.*, 2004).

This emphasises the global relevance of tracking and tracing schemes and their role as a baseline feature, not only for the delivery of guarantees for food safety and quality but also for logistics efficiency, which is at the core of enterprises' economic interests.

From a historical point of view, the TT schemes evolved from enterprise internal efforts and were subsequently extended to supply chains and networks. This historic development path also characterises a path of increasing complexity. The identification of product units and the monitoring of their movements inside an enterprise require less coordination efforts than is necessary in supply chains and, especially, in a sector as a whole, with its larger number of enterprises and different and ever-changing trade relationships.

The identification of product units and the monitoring of their movements is a problem that is easy to solve, if product modification during the various stages of a supply chain process do not affect the composition of the product. The most complex TT scenarios concern composite convenience products or commodity products, where an individual 'product unit' cannot be based on a physical product element (e.g. a piece of grain), but needs to be based on *logistics elements (batches)* that might involve production plots, transportation trucks, or storage units of any kind (Golan *et al.*, 2004; Schiefer, 2006; Fritz and Schiefer, 2009; Schiefer and Reiche, 2013). The linkage of these different batches in a batch sequence generates the production flow with its modifications, and provides the basis for tracking and tracing activities.

1.3 Food safety – the baseline

The general assurance of food safety is a prime concern and responsibility of society. Traditionally, food safety rests on the formulation and implementation of standards regarding the measurable quality of products – for example, the quantity of substances in the product with potentially negative effects on human health.

This approach is increasingly being supplemented (not replaced) by a proactive approach that intends to prevent food safety deficiencies from the beginning through regulations on the appropriate organisation and management of processes in production, trade and distribution.

For some time, policy discussions and legislative actions concerning pro-active food safety improvement initiatives have concentrated on:

- a) the assurance of tracking and tracing of products; and
- b) the implementation of the HACCP principles (USDA, 1997).

However, as both of these initiatives require enterprise activities for implementation, any regulations regarding their utilisation in the food sector require cooperation by enterprises. This is a crucial point in food safety assurance. Society (represented by policy) has responsibilities in the provision of food safety guarantees to its members, but has to rely on activities by enterprises to substantiate these guarantees (Figure 1.1).

In this scenario, the 'value' of society's guarantees depends on its ability to assure enterprises' cooperation (i.e. on the effectiveness of the sector control systems).

However, the enforcement of enterprises' cooperation through appropriate control systems has consequences for trade and constitutes, in principle, non-tariff trade

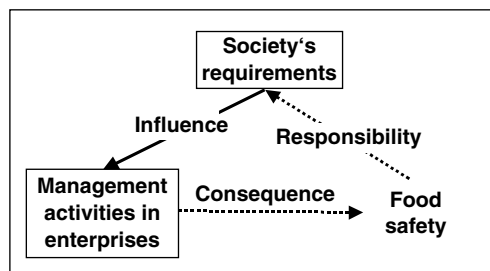


Figure 1.1 Chain of influence in food safety assurance.

barriers that have to adhere to European and international trade agreements. At the international level, the World Trade Organization (WTO) provides the umbrella for trade regulations, and allows introducing trade related regulations that avoid food safety hazards if backed by sufficient scientific evidence. An important reference in this context is the Codex Alimentarius Commission (FAO/WHO, 2003; Luning *et al.*, 2002), a joint initiative by FAO and WHO. In its Codes of Practice and guidelines, it addresses aspects of process management including, as its most prominent recommendation, the utilisation of the HACCP principles.

This is the background on which the European Community could introduce its food laws (van der Meulen, 2014), based on a White Paper on food safety (EU, 2000) and a baseline regulation (EU, 2002) which require enterprises all along the food chain to formally implement the HACCP principles in their food safety assurance activities. An exception is agriculture which is exempt from realising a formal HACCP concept, but which should, anyway, follow the principles of the HACCP concept in implementing appropriate food safety controls.

1.4 Food quality – delivery concepts

In enterprises and food chains, the delivery of quality and quality guarantees that reach beyond food safety traditionally builds on four principal areas of quality activities, integrated into a systematic process of continuous improvement. These include:

- a) the quality of enterprise management, as exemplified by the concepts of *total quality* or *total quality management* (TQM) (Oakland, 1998; Goetsch and Davis, 2012);
- b) the quality of process organisation, frequently captured in the phrase *Good Practice*;
- c) the quality of process management, usually phrased as *quality management*; and
- d) the quality of products that could be captured through sensor technology, etc.

Discussions on the assurance of food quality in the food sector concentrate primarily on the quality of *process organisation* and *process management*, and combine it with specific requirements on product quality characteristics. This integrated view is based on the understanding that not all food product characteristics with relevance for quality could be identified and competitively evaluated through inspection of the final product. It refocuses attention from traditional product inspection to the prevention of deficiencies in food quality.

However, it should be noted that successful quality initiatives of enterprises usually build on leadership initiatives related (even if phrased differently) to the TQM approach, and with a strong focus on continuous improvement activities. In this scenario, the quality-oriented process management is an integral part of the more comprehensive management approach, and not a 'stand-alone' solution for the elimination of quality problems.

A quality-oriented *process management* is characterised by management routines as, for example, audit activities that support the organisation and control of processes to assure desired process outputs, with little or no deviation from output specifications (*process quality*). The integration and specification of these routines constitutes a *management system* or, with a view on the quality-focused objectives, a *quality management system*. Well-known examples include the standard series ISO9000 (Hoyle, 2006) or the HACCP principles (USDA, 1997; Newslow, 2013).

The traditional view of quality assurance in supply chains of any kind builds on the isolated implementation of quality management systems in individual enterprises, and assumes a sufficient consideration of quality objectives through the chain of supplier-customer relationships, in which each supplier focuses on the best possible fulfilment of quality expectations of its immediate customers (Spiegel, 2004).

However, this traditional view does not match with the specifics of food production and the requirements on quality assurance in the food sector. These specifics suggest that substantial improvements can only be reached through increased cooperation between stages regarding the specification of quality levels, agreements on process controls, and the utilisation of quality management schemes. This requires agreements on information exchange and the establishment of appropriate communication schemes.

Initiatives towards integrated food supply chains were a focus of developments during the 1990s, especially in export-oriented countries such as the Netherlands and Denmark (Spiegel, 2004). These developments were primarily initiated for gaining competitive advantage in a quality-oriented competitive market environment while improvements in the sector's food quality situation were initially of secondary concern.

1.5 Quality programs – steps towards sector quality agreements

1.5.1 Overview

A variety of initiatives in different countries have focused on the formulation of comprehensive *quality programs*, which ask for the simultaneous implementation of a set of activities in process organisation and process management that assure a certain level of food quality and safety in enterprises and food chains. These programs, also referred to as *quality systems* or (if restricted to process management) *quality management systems*, are of a universal, regional or national scope.

Principal examples with focus on food chains include (Schiefer, 2003):

- a) initiatives on the basis of rather closed supply chains, such as the Dutch *IKB chains* (IKB for *Integrated Chain Management*) (Wierenga *et al.*, 1997); and
- b) sector-encompassing approaches that have little requirements on focused organisational linkages between enterprises, such as the German Q&S system (Nienhoff, 2003).

Specific alternatives are programs that evolved from retail trade. These do not involve the supply chain as a whole, but function as a quality filter for deliveries from supplier enterprises and the food chains to which these are connected.

1.5.2 A closed system concept – the case of IKB

The *IKB concept* is a chain management concept for food supply chains that was designed in the Netherlands in the 1980s for improvements in the efficiency and quality of food production. Its initial focus was on closed production chains, with a central coordinating body linked to processing industry (Wierenga *et al.*, 1997). Product deliveries into the IKB chains are restricted to enterprises that conform to certain quality requirements. A key example involves conformity to the Dutch standard series GMP (Luning *et al.*, 2002). Today's developments open the closed chain approach and move it closer towards a network system.

1.5.3 An open sector system concept – the case of Q&S

The system of Q&S addresses all stages of the vertical supply chain. However, it can be implemented by each individual enterprise on each stage, with the exception of agricultural enterprises that can only act as a group (Figure 1.2) and without any further coordination with the group's suppliers and/or customers.

The Q&S system is an open system, and its coordination is determined, in principle, by common agreements on the quality responsibility of the different stages. The approach tries to best adapt the food quality control activities to the actual market infrastructure that builds on open supply networks with continuously changing trade relationships. It places neither new organisational requirements on enterprise cooperation, nor restrictions on the development of individual market relationships within the supply chain.

The system preserves flexibility in market relationships between enterprises but, as an open flexible system, it does require substantial efforts to move the whole system to higher quality levels. Furthermore, the approach does not support the implementation of more advanced quality assurance systems of individual groups within the general

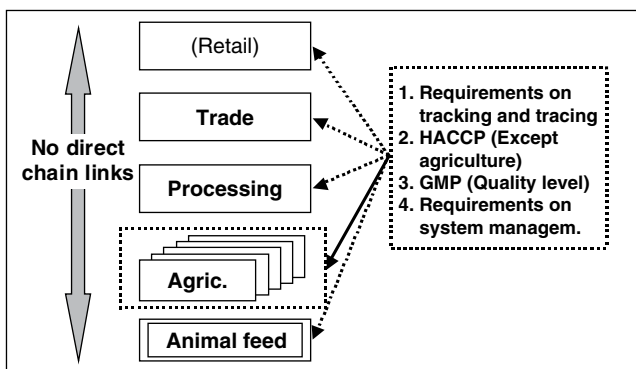


Figure 1.2 Q&S system organisation.

system environment. Such efforts would reduce the guarantee value of the general system for the remaining participants, and would contradict the interest of the system as a whole.

1.5.4 Trade initiatives

The retail sector has designed its own standards for requirements on quality activities in their supplier enterprises, including those from agriculture that deliver directly to the retail stage (for an overview see Hofwegen *et al.*, 2005; van der Meulen, 2011). Examples include: the international active standard, *GlobalG.A.P.*, which focuses on agricultural enterprises (GAP: Good Agricultural Practice; GAP, 2016; Newslow, 2013), initially in the production of fruits and vegetables, and today in most agricultural production lines, the *IFS* standard (the *International Featured Standard*; IFS, 2016; Newslow, 2013), with a stronghold in Germany and France; and the *BRC* standard (Kill, 2012), the standard of the British Retail Consortium, which has influenced many quality initiatives in food supply chains in the UK and elsewhere.

Furthermore, a global retail initiative, the *Global Food Safety Initiative* (GFSI; Newslow, 2013) has formulated requirements on food safety assurance activities for retailer-based standards which, if requirements are met, receive formal acceptance status by the GFSI (Figure 1.3).

1.6 The information challenge

1.6.1 Information clusters

Both tracking and tracing capabilities, as well as the fulfilment of quality expectations at the consumers' end, depend on activities in enterprises throughout the supply chain and, as a consequence, on the collection of information from chain participants and its communication throughout the chain, with the consumers as the final recipients. This requires the availability of a feasible sector-encompassing communication infrastructure.

Traditionally, the organisation of information in enterprises builds on a number of information layers that correspond with the different levels of business management and decision support. They reach from transaction information at the lowest level, to executive information at the highest level (Turban *et al.*, 1999). These layers are presently being complemented by two additional layers at the transaction level, that

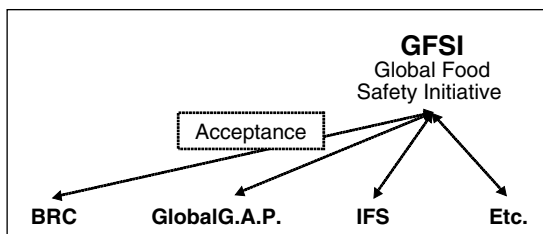


Figure 1.3 Relationships between retail quality initiatives.

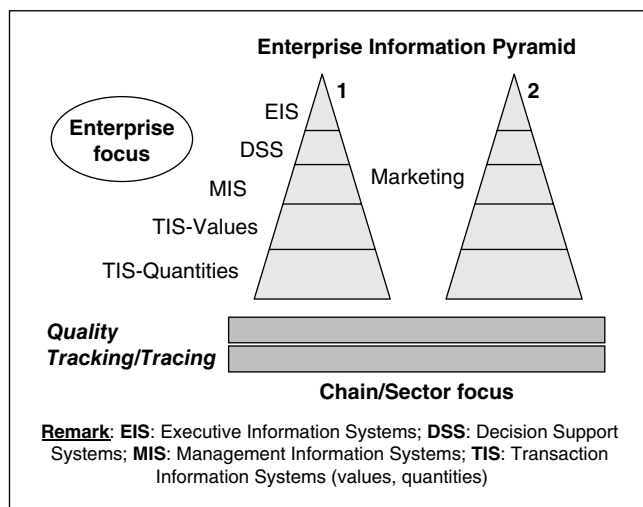


Figure 1.4 Information layers with enterprise (1, 2) and chain/sector focus.

incorporate information for tracking and tracing, as well as for quality assurance and improvement activities (Figure 1.4).

These new layers differ from traditional enterprise information layers due to their focus, which is not the individual enterprise but the vertical chain of production and trade. They are linked to the flow of goods and connect, in principle, the different stages of production and trade with each other and with the consumer. Their realisation depends on agreements between trading partners on responsibilities, content, organisation and technologies.

The layers were initiated by requirements for tracking and tracing capabilities from legislation (EU, 2002) and markets, and by increasing expectations of consumers regarding the quality of products and production processes. A number of European projects have dealt with tracking and tracing opportunities (e.g. project TRACE; www.tracefood.org), as well as with transparency requirements for meeting the emerging challenges towards sustainability, including food safety and quality (e.g. Project Transparent Food; www.transparentfood.eu; Schiefer and Reiche, 2013).

A sector encompassing general agreement is restricted to the lowest level of legal requirements. Any communication agreements beyond this level are subject to specific business interests, and might limit themselves to clusters of enterprises with common trading interests. In a network environment, individual enterprises might be members of different clusters, resulting in a future patchwork of interrelated and overlapping communication clusters (Figure 1.5).

The content of quality communication layers depends on the quality requirements of enterprises and consumers. However, the diversity of interests in a sector could generate an almost unlimited number of possible requirement sets – or, in other words, of needs for communication clusters. This is not a feasible approach.

In this situation, the quality requirements of quality programs could serve as a basic reference for the separation of communication clusters. First initiatives towards this end are under way. These developments will separate the sector's food production into

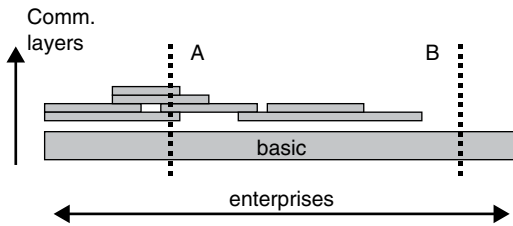


Figure 1.5 Agreed communication clusters with participation of enterprise A in five, and enterprise B in one of the clusters.

different segments with different quality guarantees. Examples are some of the retail-driven quality programs, such as the program ‘Proplanet,’ by a major retail group (Proplanet, 2016), which builds on the establishment of a clearly defined supplier chain reaching from agriculture to retail, and provides information from each stage of the chain on a number of selected sustainability characteristics.

1.6.2 Organisational alternatives

The principal alternatives for sector-wide information infrastructures focus on two different dimensions. The information may be communicated between enterprises directly, or it may be communicated between enterprises through a common data network that is linked with enterprises’ internal information systems. These approaches mirror classical network approaches, such as bus or ring network topologies (Turban *et al.*, 1999).

Apart from establishing data networks, there is an additional alternative form of communication that avoids the communication of data, but communicates assurances that certain information is true. If enterprises are assured that their suppliers fulfil the requirements of a certain quality system, information linked to the requirements do not have to be communicated, and the assurance (e.g. in terms of a certificate) is sufficient. As information infrastructures for quality assurance are not yet established sufficiently, this last approach is still attractive and utilised with a number of quality programs (Reardon *et al.*, 2001).

However, technological developments in internet technology, with its wireless networks and the *internet of things*, the establishment of *cloud services*, the ability to deal with *Big Data*, and the availability of advanced network devices such as sensors or intelligent smartphones with libraries of *Apps* for easy network access, are providing supporting means that will push the utilisation of information networks across the food sector. To this end, the European Commission has initiated the program *FI-PPP* (FI: Future Internet), which develops a European network and system development infrastructure (FIware; www.fiware.org), including stores of so-called *Generic Enablers* for supporting app development, and an experimental European-wide network for experimental use, as well as the simulation of scaling-up of applications. In addition, the program supports the development of more than 1000 apps that build on this technology, with more than 100 focusing on the food sector (see, for example, the accelerator projects FINISH – www.finish-project.eu; or SmartAgriFood – <http://smartagrifood.com>). It is expected that such initiatives will provide a major push towards the development of a transparent food sector.

1.6.3 Data ownership and data markets

With technology limitations becoming less a barrier, deficiencies in agreements on standardisation and content of data exchange receive increased attention. Dealing with data ownership and data utilisation has emerged as a major issue for clarification. At the moment, most data of interest are to be collected at early stages of the chain. These bear the costs of collection, while benefits of data utilisation are concentrated at later stages of the chain and, especially, at retail, with its link to consumers. A sustainable data exchange network needs to assure a balanced consideration of costs and benefits (Schiefer and Deiters, 2015).

One of the proposals discussed within the sector is to separate data from products, and to establish data markets separated from product markets. This may lead to products at retail with less or more information available, resulting in lower or higher market prices based on the argument that ‘information has its price’.

A specific model based on a separation of data from products is realised in the *book and claim approach* (Greepalm, 2016), which is suitable for quality issues linked to differences in production systems, not in quality issues linked to measurable food characteristics. In this model, quality certificates for products from highly valued production systems, such as systems with positive environmental effects, may be sold independently from the actual product. Later stages of the chain may purchase the certificates and link them with products from other sources, while the initial products are sold without any quality premium. In the end, the market may receive products that are sold as being from environmental production supported by the respective certificate, while they are not. However, as the initial products will be sold without any quality premium, the balance is unchanged. The quantity of products with certificate resembles exactly the quantity of products produced under the preferred condition.

1.6.4 Added value of emerging information infrastructures

The quality interest of customers and consumers, the chain efficiency aspect, and the legal requirements on the tracking and tracing capability of the food chain, together provide the argument for the establishment of a sector-wide information infrastructure. However, newly emerging aspects of quality communication schemes involve the potential for possible added values that these infrastructures could provide. As an example, chain-focused extension services might utilise information from various stages, to arrive at recommendations for improvements in chain quality performance or chain efficiency.

All these benefits combined are the long-term matching part for the costs of a sector-wide information infrastructure.

1.7 Conclusion

Initiatives to improve tracking and tracing capabilities, as well as the delivery of trustworthy and stable quality products, are the means to control risks and to assure and develop markets. From this point of view, they are prerequisites for a sustainable economic position of enterprises in the food market. Considerations of public health and legal requirements support the development, and are not contradictory.

Increased globalisation, industrialisation and sophistication of food production and trade increase the need for improved process control, process management and communication inside enterprises, but especially between enterprises along the vertical food production chain. This requires substantial investments in: the design of new quality assurance concepts; in cooperation agreements throughout the sector; in the identification of accepted quality levels; in the allocation of quality assurance responsibilities; in the design and implementation of communication systems; and in the distribution of investment and operations costs.

This makes the move from the traditional view on quality production to today's requirements difficult, and a challenge for the sector – but a challenge that needs to be met.

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2

Methodologies for Improved Quality Control Assessment of Food Products

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2.1 Introduction

The authentication of food is a major concern for the consumer and for the food industry at all levels of the food processing chain, from raw materials to final products. The search and development of fast and reliable methods is nowadays of upmost importance. Infrared spectroscopy, chromatographic techniques associated with solid phase microextraction and cyclic voltammetry in tandem with chemometrics are examples of methodologies that can be applied for the improvement of quality control assessment of food products.

Among the complex food constituents, the identification of the added polysaccharides could be a key factor if a rapid and reliable method is attainable. Classical chemical methods of polysaccharide determination are time-consuming and are not always straightforward for a widespread routine application in the food industry. In section 2.2 of the present chapter, it is shown how infrared spectroscopy, combined with principal component analysis (PCA), can be used as a rapid tool for the screening and analysis of polysaccharide food additives, and as probe for the detection of food adulterations. PCA and Partial Least Squares (PLS1) regression are well-consolidated chemometric methodologies that allow significant improvements in data analysis when compared with univariate analysis.

However, for the analysis of complex matrices, such as those of food products, other approaches are still required. In section 2.3, one example of the use of combined regions of the infrared spectra for quantification purposes is shown, by applying PLS1 regression to an outer product (OP) matrix, and an example of the application of orthogonal signal correction (OSC)-PLS1 regression to minimise the matrix effect in the spectra.

Gas chromatography is the main chromatographic tool used for food analysis. Due to the high complexity of food matrices, unidimensional gas chromatography equipment has been replaced by two-dimensional comprehensive gas chromatography (GC×GC-ToFMS). Screening and distinction of coffee brews can be done on the basis of combined headspace (HS) solid phase microextraction (SPME)-gas chromatography (GC)-PCA (HS-SPME-GC-PCA) methodology. Using this methodological approach,

presented in section 2.4, diagnostic global volatile profiles of coffees brews can be obtained, allowing their distinction precluding the use of mass spectrometry for the identification of the volatile compounds.

The advantage of the use of high throughput GC×GC-ToFMS is shown in section 2.5. Using beer as a case study, GC×GC-ToFMS revealed the complexity of the volatile profile of the matrix and the usefulness for screening of sensorially relevant compounds present in trace amounts.

Finally, section 2.6 of this chapter shows a methodological approach, based on cyclic voltammetry, for assessment of quality control for cork stoppers, proposed for the rapid screening of cork-wine model interactions in order to assess the potential contamination of wine by cork stoppers.

2.2 Use of FT-IR spectroscopy as a tool for the analysis of polysaccharide food additives

Polysaccharides and their derivatives are widely used in food processing technologies as gelling agents and thickeners. Starch, carrageenan and pectin are examples of the most widely used polysaccharides in food industry. Carbohydrates diversity has been systematised through the use of symbol notations. These have been proposed by the Consortium for Functional Glycomics (CFG) through the functional glycomics gateway (<http://www.functionalglycomics.org/static/consortium/Nomenclature.shtml>), and by the Glycopedia (<http://glycopedia.eu/e-chapters/the-symbolic-representation-of/article/at-the-instigation-of-glycobiology>). In this chapter, carbohydrates symbols notation, represented in figures, follows CFG nomenclature.

Starch, an important thickening and binding agent, is a mixture of two main glucan constituents, amylose, a linear polysaccharide composed of (1→4)- α -D-linked glucopyranose residues (Figure 2.1a) and amylopectin, a branched polysaccharide composed of (1→4)- and (1→4,6)- α -D-glucopyranose residues (Figure 2.1b).

Carrageenan utilisation in food processing is based on its ability to gel, to increase the solution viscosity and to stabilise emulsions and various dispersions. The carrageenans are characterised by an alternating repeating (1→4)-linked disaccharide structure, consisting of 3,6-anhydro- α -D-galactopyranosyl-(1→3)- β -D-galactopyranosyl residues. A sulphate group at positions C2, C4 or C6 can substitute each residue. The carrageenans, depending on the sulphate substitutions, can be defined as: kappa (κ), β -D-Galp-4-sulphate and 3,6-anhydro- α -D-Galp (Figure 2.2a), iota (ι), β -D-Galp-4-sulphate and 3,6-anhydro- α -D-Galp-2-sulphate (Figure 2.2b) and lambda (λ), non-gelling agent consisting of β -D-Galp-2-sulphate and α -D-Galp-2,6-disulphate (Figure 2.2c).

Pectins are polysaccharides composed of a linear backbone of (1→4)- α -D-GalpA interspersed by α -(1→2)-Rhap residues, with side chains consisting mainly of β -D-Galp and α -L-Araf residues (Figure 2.3a). Pectin with high ester content (Figure 2.3b) form gels in the presence of sucrose, as in marmalades, and low ester pectin can set into a gel in the presence of Ca^{2+} (Belitz *et al.*, 2009).

The authentication of food is a major concern for the consumers and for the food industry at all levels of the food processing chain, from raw materials to final products. Among the complex food constituents, the identification of the added polysaccharides could be a key factor if a rapid and reliable method is attainable.

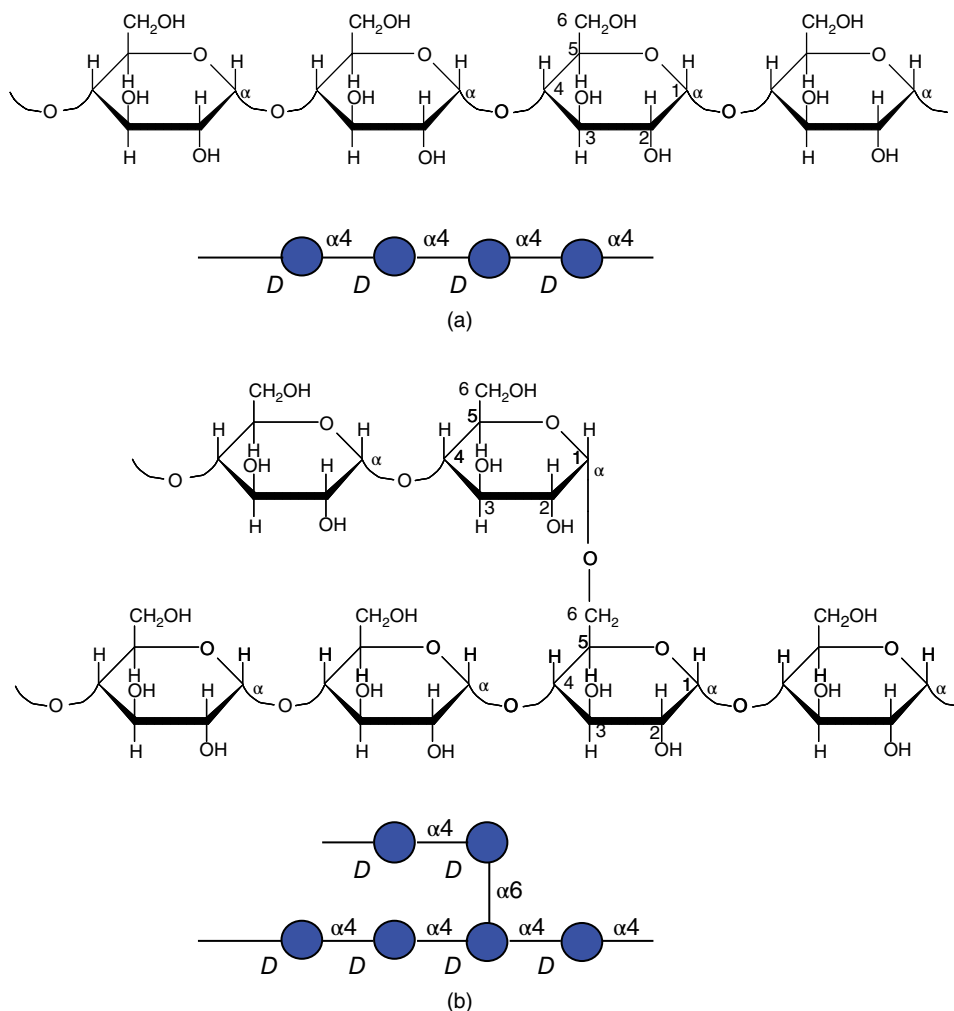


Figure 2.1 Schematic representation of the glycosidic structure of the polysaccharide constituents of starch: a) amylose; and b) amylopectin. (● Glc)

Vibrational spectroscopy has been found important applications in the analysis and identification of sugars in food industries (Mathlouthi and Koenig, 1986). Particularly, mid-infrared spectroscopy has been shown to be a rapid, versatile and sensitive tool for elucidating the structure, physical properties and interactions of carbohydrates (Kačuráková and Wilson 2001), to study pectic polysaccharides and hemicelluloses extracted from plants (Kačuráková *et al.*, 2000), and to detect structural and compositional changes occurring in the cell walls of grapes during processing (Femenia *et al.*, 1998). The carbohydrates show high absorbances in the 1200–950 cm^{-1} region, which is within the so-called ‘fingerprint’ region (Figure 2.4), where the position and intensity of the bands are specific for every polysaccharide (Filippov, 1992). Due to severe band overlapping in this region, it is very difficult to assign the absorbances at specific wave numbers to specific bonds or functional groups.

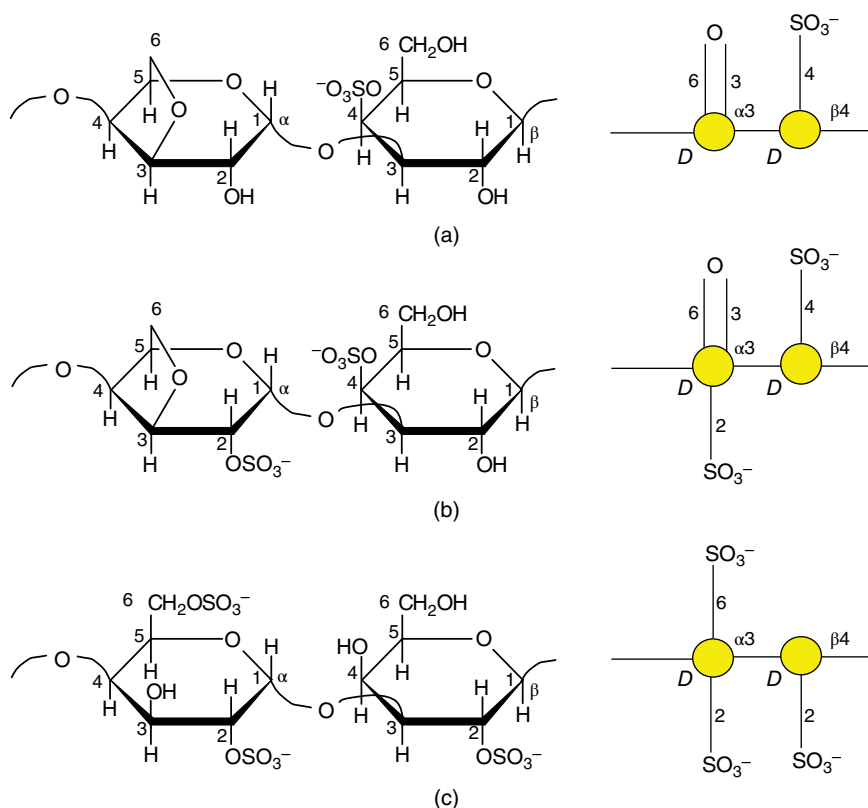


Figure 2.2 Schematic representation of the glycosidic structure of the disaccharide repetitive unit of carrageenans: a) kappa (κ); b) iota (ι); and c) lambda (λ). (● Gal)

The application of chemometrics to the FT-IR spectra have been shown to be a reliable and fast method for the characterisation of amidated pectins (Engelsen and Norgaard, 1996), and for classification of corn starches (Dupuy *et al.*, 1997) and commercial carrageenans (Jurasek and Phillips, 1998). Among many other applications, FT-IR and chemometrics have also been used for a quick evaluation of cell wall monosaccharide composition of polysaccharides of pectic (Coimbra *et al.*, 1998) and hemicellulosic origin (Coimbra *et al.*, 1999), for screening of *Arabidopsis* cell wall mutants (Chen *et al.*, 1998) and for evaluation of structural and compositional changes in the cell walls of pears with sun-dried processing (Ferreira *et al.*, 2001).

2.2.1 Identification of polysaccharide food additives by FT-IR spectroscopy

Figure 2.5a shows the PC1 vs. PC2 scores scatter plot of the FT-IR spectra in the 1200–800 cm^{-1} region (Figure 2.4b) of 27 carbohydrate standards: six monosaccharides (arabinose – Ara, fructose – Fru, galactose – Gal, galacturonic acid – GalA, glucose – Glc, and mannose – Man), three disaccharides (lactose, maltose, and sucrose), four glucans (amylose, amylopectin, barley β -glucan, and starch), five carrageenans (ι -, λ -, κ -carrageenan,

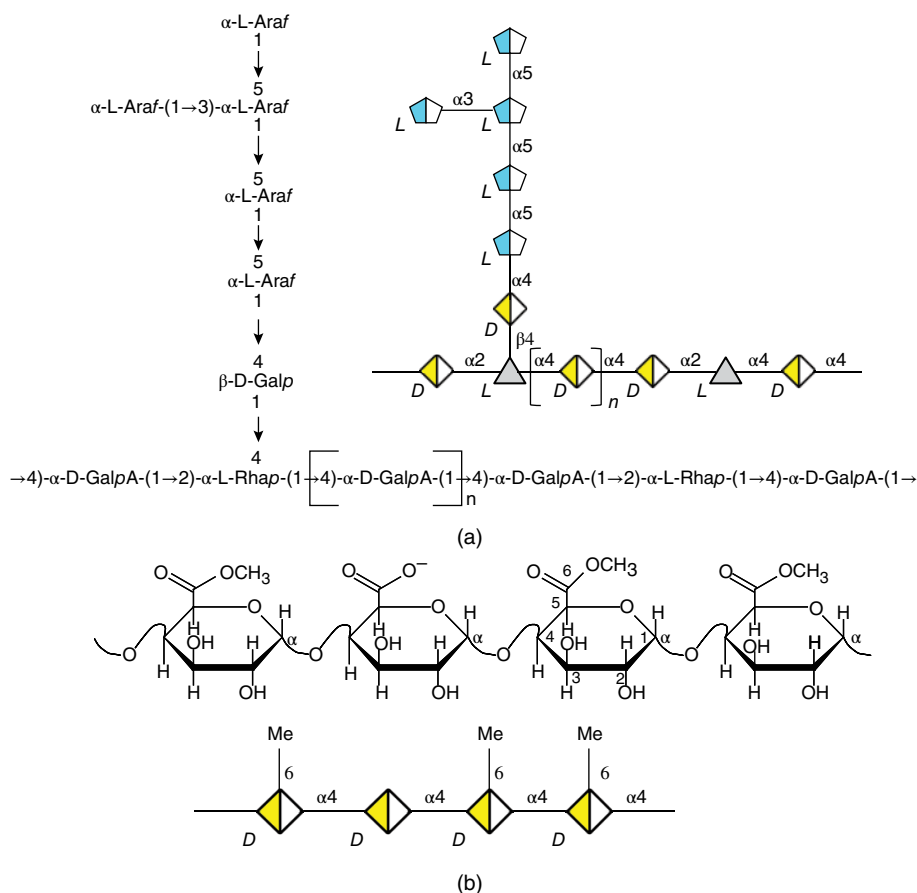


Figure 2.3 Schematic representation of the glycosidic structure of pectic polysaccharides: a) branched pectic polysaccharide; b) pectin with high ester content. (Δ Rha, $\blacklozenge$ GalA, $\blacktriangleleft$ Ara)

commercial carrageenan, and commercial carrageenan-pectin mixture), three galactans (arabic gum, arabinogalactan, and galactan), and six pectins having different degrees of methylesterification.

The distribution of the samples along the PC1 axis is as a function of the composition in glucose (PC1 negative) and galactose (PC1 positive). Glucose-rich compounds (starch, amylose, amylopectin, β -glucan, maltose, sucrose and glucose) were all located in PC1 negative, independently of their monomeric or polymeric nature. On the other hand, polysaccharides, such as the carrageenans (except λ -carrageenan) and galactans, and the monosaccharides galactose, fructose and galacturonic acid, were located in PC1 positive. Based on the scores scatter plot, the positive absorption band that can be observed in the loadings plot (Figure 2.5b) in the $1100\text{--}1030\text{ cm}^{-1}$ range, with maximum at 1068 cm^{-1} , can be attributed to Gal, and the band in the range $1030\text{--}944\text{ cm}^{-1}$, with minimum at 998 cm^{-1} , can be ascribed to Glc, in accordance also with other published data (Kačuráková and Mathlouthi, 1996; Kačuráková *et al.*, 2000).

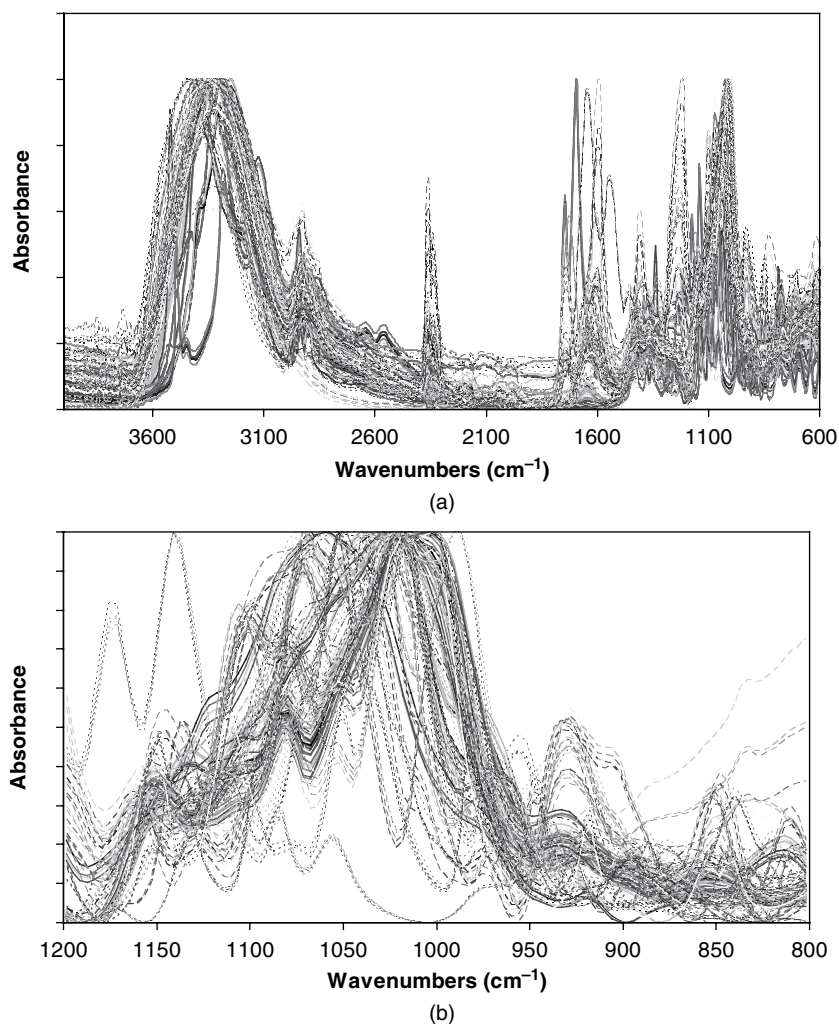


Figure 2.4 FT-IR normalised spectra of replicates of 27 carbohydrate standards: a) 4000–600 cm^{-1} region; b) 1200–800 cm^{-1} region.

PC2 distinguish the spectra of the pectin samples and GalA (PC2 negative) from all the others, especially the carrageenans and sucrose (PC2 positive). Pectins with different degrees of methylesterification were observed together in the same region, not clearly separated using this spectral region. Carrageenans (PC1 and PC2 positive) were placed differently from Gal (PC1 positive and PC2 negative) in the scores plan, except λ -carrageenan, which was located in PC1 negative and PC2 positive. This may be due to the higher sulphate content and the absence of 3,6-anhydro-Gal in this carrageenan when compared to the others. The spectra analysis suggests that the commercial carrageenan is a κ -carrageenan. The distinction of the pectic samples can also be seen in the loadings plot of PC2 by the absorbances at the negative side at 1145, 1100, 1018, and 960 cm^{-1} and by the absorbances at the positive side at 1064 and 1045 cm^{-1} , as has been described for pectic polysaccharides (Coimbra *et al.*, 1998, 1999).

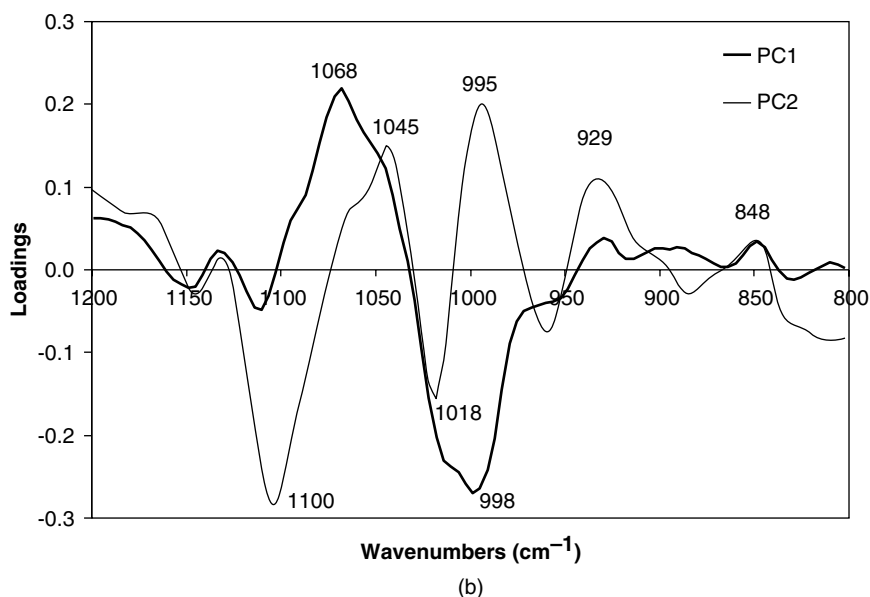
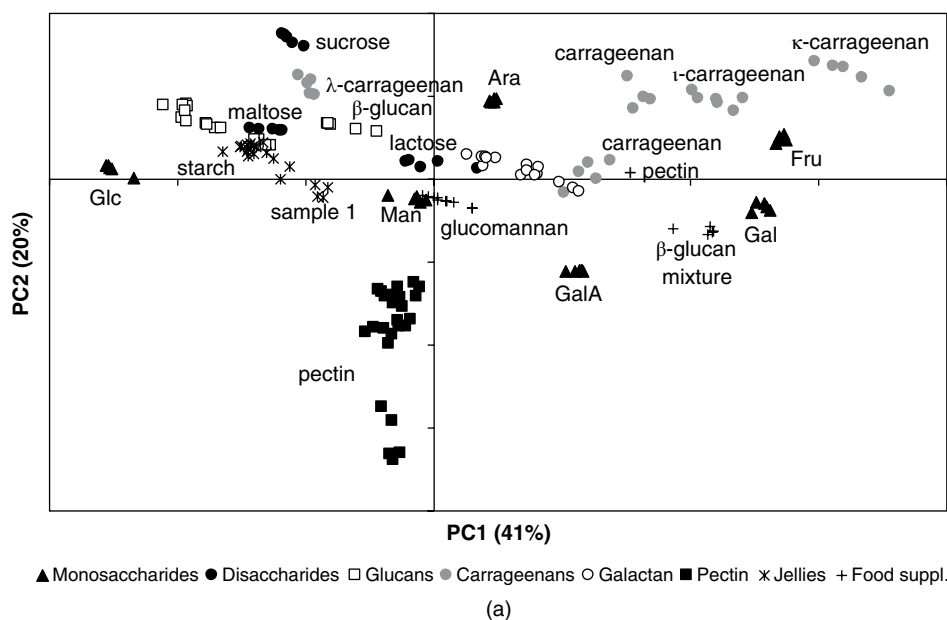


Figure 2.5 PC1 $\times$ PC2 scores scatter plot a) and loadings plot b) of the FT-IR spectra of mono-, di-, and polysaccharide standards, confectionery jelly polysaccharides and food supplements (glucomannan and β -glucan mixture) in the 1200–800 cm^{-1} region. Source: Černá *et al.*, 2003 (Elsevier).

Isolated polysaccharides from confectionery jellies were placed in the PC1 negative side (Figure 2.5a), near starch, albeit this was not compatible with the product labelled by the manufacturers, who claimed that confectionery jellies contained pectin in addition to other sugars (Table 2.1). The occurrence of starch, later confirmed by other

Table 2.1 Characteristics of studied samples, according to the manufacturer labels (Černá *et al.*, 2003).

Sample no.	Sample specification	Sample components
1	pectin jelly with fruit flavour	sugar, glucose syrup, water, pectin, citric acid, acidity regulator (sodium citrate), flavours, colours
2	peach flavoured jellies	sugar, glucose syrup, water, pectin, citric acid, sodium citrate, natural peach flavour, colour
3	fruit flavoured gums with real fruit juices	sugar, glucose syrup, water, pectin, acids (citric acid, lactic acid), acidity regulators (potassium sodium tartrate, trisodium citrate, calcium lactate, sodium polyphosphate), fruit juices
4	fruit jelly	sugar, glucose syrup, water, pectin, citric acid, sodium citrate, natural flavours, natural colours
5	pectin jelly with fruit flavour	sugar, glucose syrup, water, pectin, citric acid, acidity regulator (sodium citrate), moisturiser (sorbitol), flavour, colours

methods (Černá *et al.*, 2003), is an elucidative example of an application of FT-IR spectroscopy in the 1200–800 cm⁻¹ region as a very reliable and quick tool for food authentication of carbohydrate-based food additives.

2.2.2 Influence of hydration on FT-IR spectra of food additive polysaccharides

FT-IR spectroscopy is very sensitive to the carbohydrate changes in conformation, and to the constraints imposed by the hydrogen bonding with water (Kačuráková and Mathlouthi, 1996). However, at least within a certain hydration range, distinction between samples is still possible, as can be seen for amylose in Figure 2.6.

Figure 2.7 shows the PCA scores scatter plot and loadings plot of the FT-IR spectra in the 1200–800 cm⁻¹ region of mono-, di-, and polysaccharide samples, in a dry form and containing 6–40% of water (hydrated). It is possible to see that the distribution of the

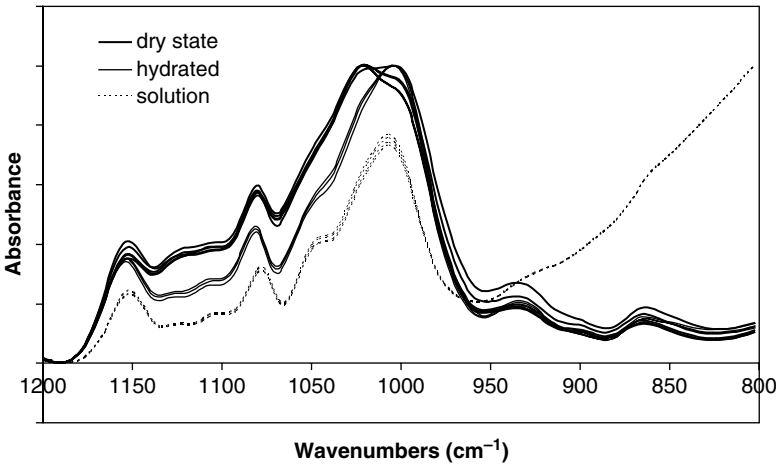


Figure 2.6 FT-IR spectra in the 1200–800 cm⁻¹ region of amylose containing different amounts of water.

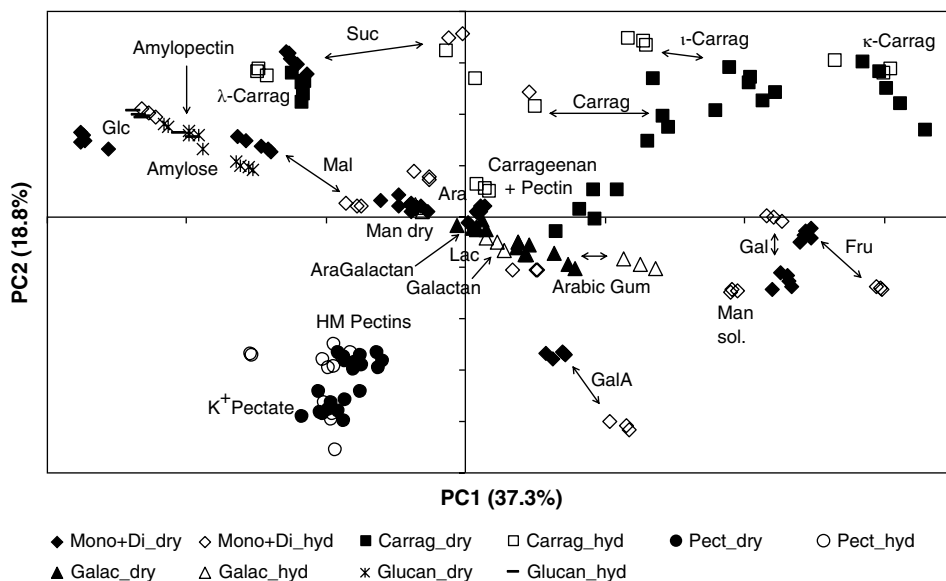


Figure 2.7 Scores scatter plot (PC1 × PC2 – axes cross each other at the origin) of FT-IR spectra in the 1200–800 cm^{-1} region of mono-, di-, and polysaccharide standards in dry and hydrated forms.

Source: Čopíková *et al.*, 2005 (Elsevier).

samples in the PC1 × PC2 plan was invariant from the hydrated status of the sample. The exception was mannose (containing 48% of water), which was shifted from PC1 negative to PC1 positive. Although the hydration of the samples gave broader bands, ascribed to molecular rearrangements and disappearance of the crystalline structures (Kačuráková *et al.*, 1998), the presence of water in the given amount in these samples did not change the overall spectral characteristics that allowed their distinction, as observed by the similar loadings plot to the one shown in Figure 2.5b.

When the samples were measured dissolved in water, the distribution of the samples in the PC1 axis (Figure 2.8a) was found to be a function of the water content in the samples, as all samples measured in dry or hydrated forms (less than 48% of water) were placed in PC1 negative, and the majority of the samples measured in solution were placed in PC1 positive (Čopíková *et al.*, 2005). The smooth curve of the loadings profile of PC1 (Figure 2.8c) is related with the spectra of samples that have been distinguished just for their different amount of sugars (Coimbra *et al.*, 2002).

Figure 2.8b shows that the distinction between the samples can be obtained by the PC2 × PC3 scores scatter plot for all dry and hydrated samples, in a scores scatter plot similar to that obtained for PC1 × PC2 (Figure 2.8a). Also, the loadings plot of PC2 and PC3 was similar to the loadings plots of PC1 and PC2, respectively, of Figure 2.5b, which shows that the variability of the samples not related to the water effects could be recovered in PC2 and PC3 when samples in solution are included. For the majority of the samples analysed in solution, significant shifts can be observed. With the exception of amylose, whose spectra did not change significantly with the water content, the spectra of all other polysaccharides (amylopectin, carrageenans, pectins and pectates, and galactans) were placed near the PC2 and PC3 origin, precluding their distinction.

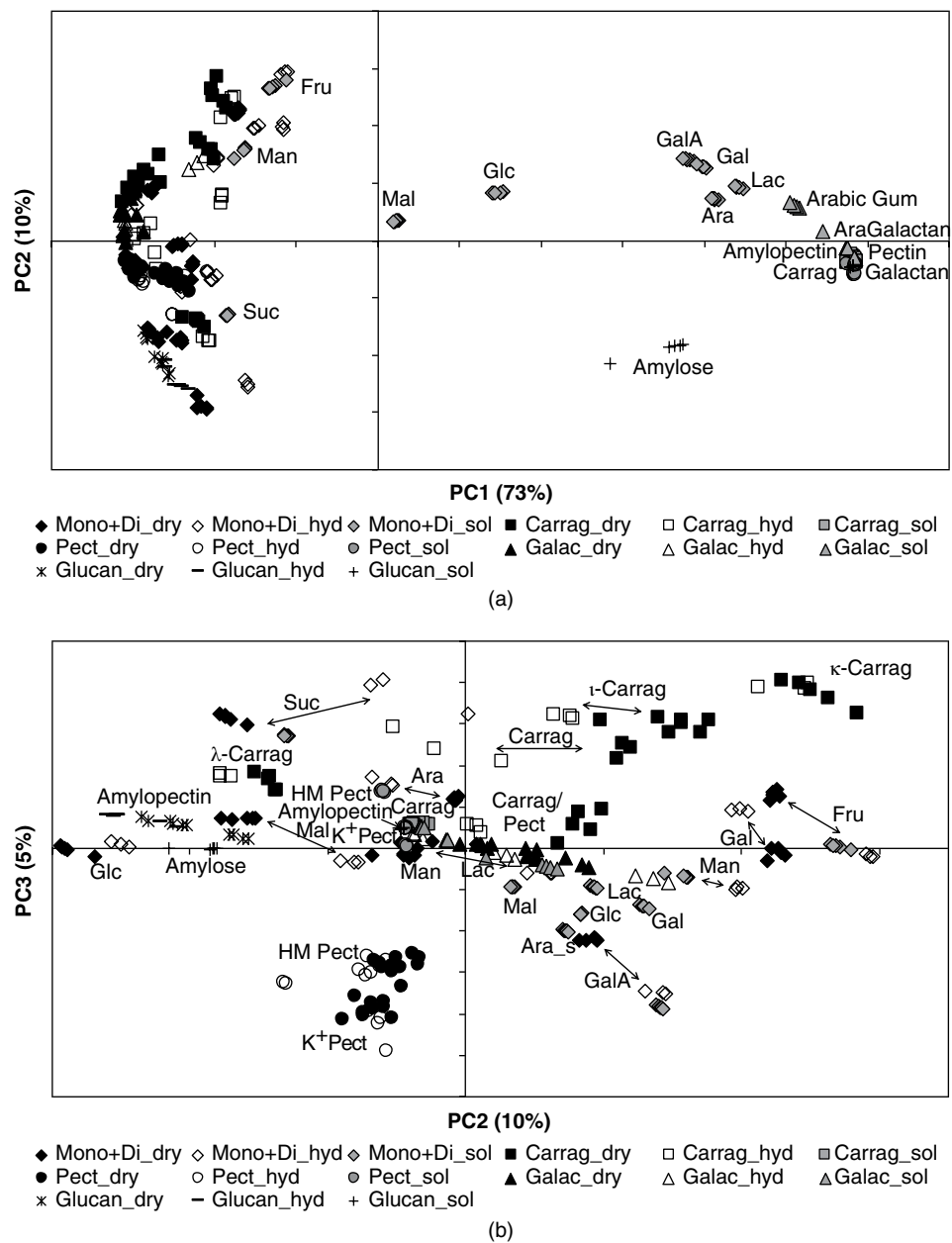


Figure 2.8 PCA of FT-IR spectra in the 1200–800 cm⁻¹ region of mono-, di-, and polysaccharide standards in dry, hydrated and solution forms: a) PC1 × PC2 scores scatter plot; b) PC2 × PC3 scores scatter plot; c) loadings plot. *Source: Čopíková et al., 2005 (Elsevier).*

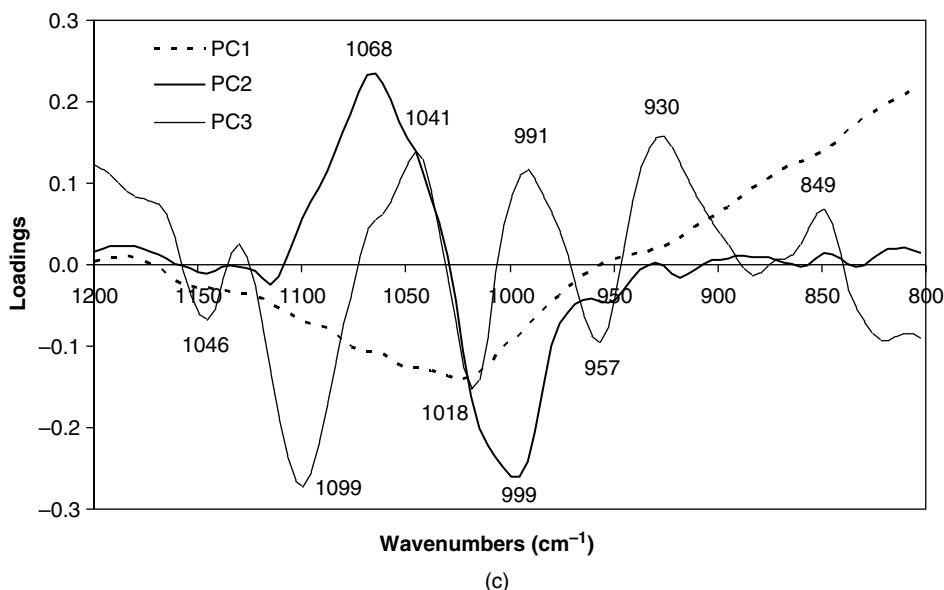


Figure 2.8 (Continued)

2.3 Use of outer product (OP) and orthogonal signal correction (OSC) PLS1 regressions in FT-IR spectroscopy for quantification purposes of complex food sample matrices

2.3.1 Outer product (OP)-PLS1 regression applied to the prediction of the degree of methylesterification of pectic polysaccharides in extracts of olive and pear pulps

Pectic polysaccharides are involved in the complex fibrillar network of plant cell wall structure that defines the mechanical and functional properties of the cell wall (Cosgrove, 2001; Roberts, 2001). As structural components, pectic polysaccharides influence the texture of fruits on ripening (Martin-Cabrejas *et al.*, 1994; Paull *et al.*, 1999; Vierhuis *et al.*, 2000; Jiménez *et al.*, 2001; Mafra *et al.*, 2001), storage (Bartley and Knee, 1982) and processing (Femenia *et al.*, 1998). As already discussed, pectic polysaccharides are also of great importance in the food industry, due to their gelling ability in jams and jellies, as well as fruit preparations for dairies, stabilisers in fruit and milk beverages (Claus *et al.*, 1998) and dietary fibres (Sun *et al.*, 1998; Sun and Hughes, 1999).

Pectic polysaccharides, which have a main backbone constituted mainly of galacturonic acid (GalA) residues (can be partially esterified with methanol – Figure 2.3). The degree of methylesterification (DM) is defined as the percentage of carboxyl groups esterified with methanol (Voragen *et al.*, 1995). The presence of methyl ester groups affects the cross-linking of pectic polysaccharides by Ca^{2+} , which plays an important role in the organisation of polysaccharides in plant cell walls (Brett and Waldron, 1996; Wellner *et al.*, 1998) and, consequently, may influence the texture

properties of fruits during ripening and processing. The gelation mechanisms of pectins are also dependent on the DM (Grant *et al.*, 1973; Walkinshaw and Arnott, 1981; Lopes da Silva *et al.*, 1995).

Several analytical methods have been proposed for the determination of the DM of pectic polysaccharides. These include alkali hydrolysis of the methylester groups and subsequent determination of the DM by titration (Mizote *et al.*, 1975) in galacturonic acid-rich samples. The independent quantification of the total amount of uronic acids colorimetrically, and the methanol released after alkali hydrolysis by gas chromatography (Knee, 1978; McFeeters and Armstrong, 1984; Waldron and Selvendran, 1990) and by HPLC (Voragen *et al.*, 1986), and by enzymatic oxidation (Klavons and Bennett, 1986), are used when the pectic polysaccharides contain also neutral sugars. Another approach is the reduction of pectin methyl ester groups of GalA to galactose (Gal) and the determination of the DM, either by the increase in the amount of Gal or by the change in the amount of GalA, quantified by gas chromatography and colorimetric analysis, respectively (Maness *et al.*, 1990). Instrumental techniques such as ^1H -NMR (Grasdalen *et al.*, 1988; Renard and Jarvis, 1999), ^{13}C -NMR (Pfeffer *et al.*, 1981) and FT-IR (Chatjigakis *et al.*, 1998) spectroscopies have also been proposed.

The use of infrared spectroscopy on pectic substances was previously applied to distinguish between high and low methoxyl contents (Reintjes *et al.*, 1962), and proved to be a useful tool to distinguish and evaluate the methoxyl content of different commercial pectins with high and low levels of esterification (Haas and Jager, 1986). FT-IR spectroscopy, as proposed by Chatjigakis *et al.* (1998), is a simple, quick and non-destructive method of DM evaluation in cell wall material extracts. The estimation of DM is based on a calibration curve using samples of standard pectins with known degree of esterification and the spectral bands at 1749 cm^{-1} and 1630 cm^{-1} , assigned, respectively, to the absorption of the esterified and non-esterified carboxyl groups of pectin molecules. However, this methodology has been shown not to be suitable for the determination of the DM of the pectic polysaccharides when other carboxylates and carbonyl ester groups, such as those from cell wall phenolics, are present.

FT-IR spectroscopy in the wave number region between 1200 and 850 cm^{-1} has been used as a reliable and fast method for the evaluation of polysaccharide composition (Coimbra *et al.*, 1998, 1999; Ferreira *et al.*, 2001). The application of a methodology for the determination of the DM of pectic polysaccharides present in raw cell wall extracts using the combination of the $1800\text{--}1500\text{ cm}^{-1}$ and $1200\text{--}850\text{ cm}^{-1}$ regions of the FT-IR spectra, by means of an Outer Product analysis, has thus been proposed (Barros *et al.*, 2002).

To acquire two sets of signals for the same samples and analyse how they vary simultaneously as a function of some property, one possibility is to apply statistical techniques to the n Outer Product matrices calculated, for each of the n samples. The procedure starts by calculating the products of the intensities in the two signal domains for each sample. All the intensities of one domain are multiplied by all intensities in the other domain, resulting in a data matrix containing all possible combinations of the intensities in the two domains (Figure 2.9a). The Outer Product of two signal-vectors of lengths r and c for the n samples gives n (r rows by c columns) matrices, which are then unfolded to give n ($r \times c$)-long row-vectors (Figure 2.9b). This procedure corresponds to

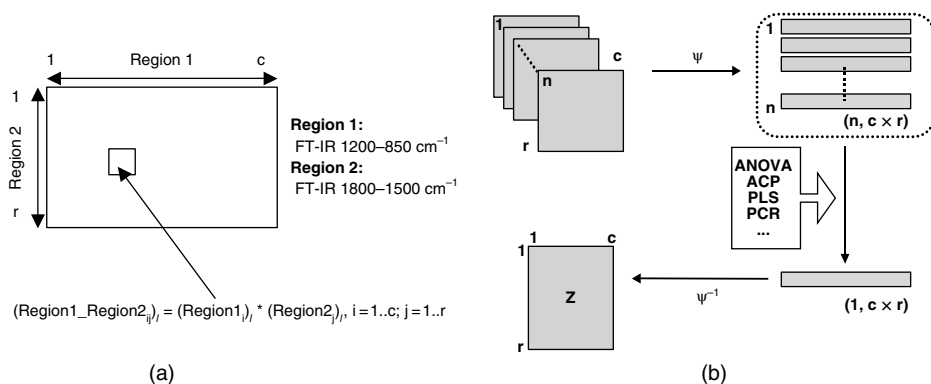


Figure 2.9 a) Calculation of the Outer Product between the two FT-IR regions; b) Unfolding of the Outer Product matrices, concatenation of the vectors, statistical analysis of the resulting matrix and refolding of the vectors of calculated values. *Source: Barros et al., 2002 (Elsevier).*

a mutual weighting of each signal by the other (Barros *et al.*, 1997; Barros, 1999; Rutledge *et al.*, 2001):

- i) if the intensities are high simultaneously in the two domains, the product is higher;
- ii) if the intensities are low simultaneously in the two domains, the product is lower;
- iii) if one is high and the other low, the resulting product tends to an intermediate value.

After analysis of the set of n ($r \times c$)-long row-vectors, each vector of calculated statistical parameters is folded back to give a matrix (r rows by c columns), which may be easily examined to detect the relations between the two domains.

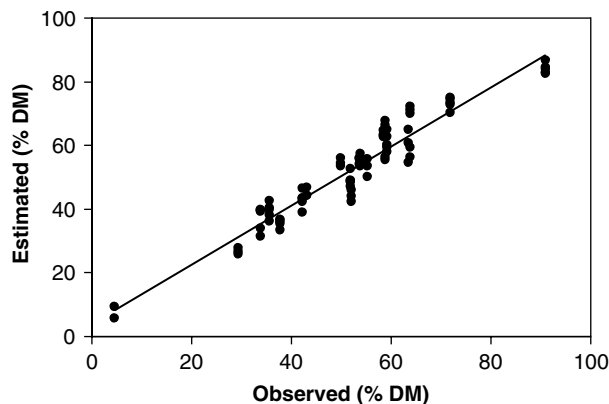
In the present example, the two considered domains belonged to mid-infrared region (homospectral analysis): the first one to the region 1800–1500 cm^{-1} (79 wave numbers); and the second one to the region 1200–850 cm^{-1} (91 wave numbers). The Outer Product of these two regions gave a vector with ($79 \times 91 = 7189$) elements for each sample. All the samples vectors were then concatenated into an **X** matrix, which was then used in partial least squares (PLS1) regression to model the DM. The obtained **b** vector, which established the relationship between the **X** variables and the **y** vector, was therefore composed of 7189 elements. In order to facilitate the interpretation of this vector, it was folded back to give a matrix **B** (79×91), which highlighted the links between the variables (wave numbers) interactions in the two regions.

In this example, pectic polysaccharide-rich samples with a galacturonic acid content greater than 52 mol% were used, obtained from olive pulp and pear matrices after extraction using different aqueous solutions (Barros *et al.*, 2002). The relative amount of polymeric sugars of the samples was 48–85%, and the degree of methylesterification, estimated by gas chromatography from the amount of methanol released after saponification, ranged from 5–91%.

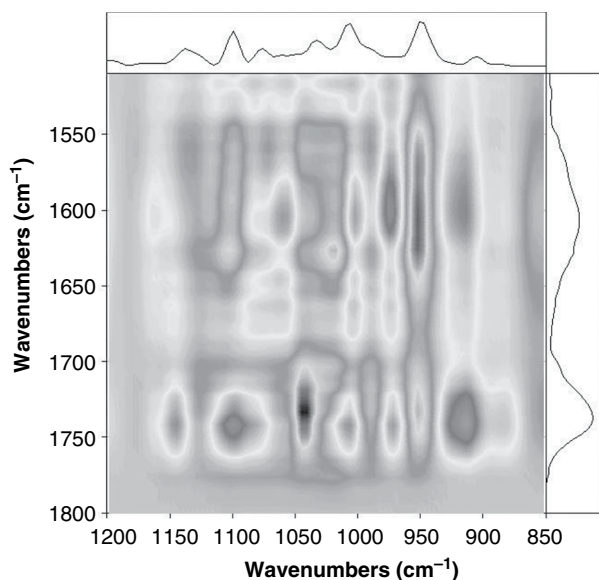
The classical multivariate approach for the determination of the degree of methyl-esterification in the region 1800–1500 cm^{-1} , using the bands located at 1750 and 1630 cm^{-1} , do not allow a regression model for olive pulp and pear pectic polysaccharide extracts with acceptable predictive power (a model with nine Latent Variables (LV) with

a Root Mean Squares Error of Prediction (RMSEP) of 14.7% and a coefficient of determination (R^2) of 0.79). This could be due to the presence of esters and carboxylate groups from phenolics in the samples (presence of UV-absorbing materials and total sugar content less than 85%). To relate more precisely the esters and carboxylate groups with the amount of GalA present in the samples, the combination of the absorbance in the regions 1800–1500 and 1200–850 cm^{-1} can be done by means of an OP matrix.

Figure 2.10a shows the linear relationship between the estimated DM values, obtained by OP-PLS1, and those observed by gas chromatography direct injection of the



(a)



(b)

Figure 2.10 a) OP-PLS1 calibration curve plot for determination of the degree of methylesterification of pectic polysaccharides; b) OP-PLS1 2D **b** vector map for determination of the DM of pectic polysaccharides; the variance profiles of each region are shown in the left and top sides. Source: Barros *et al.*, 2002 (Elsevier).

Table 2.2 **b** vector variables – main relationships as a function of the DM (Barros *et al.*, 2002).

1800–1500 cm ⁻¹ region	1200–850 cm ⁻¹ region
positive relationship	
1746 (ester carbonyl)	913 (s), 971 (m), 1006 (m), 1100 (s), 1145 (m)
1603 (anti-symmetric carboxylate stretching)	913 (m), 975 (s), 1002 (m), 1056 (m)
negative relationship	
1746 (ester carbonyl)	952 (m), 1044 (s)
1603 (anti-symmetrical carboxylate stretching)	952 (s)
1626 (carboxylate)	1018 (m), 1100 (m)

(s) : strong

(m) : medium

saponified solution of the polysaccharide extract. The folded **b** vector is shown in Figure 2.10b as a 2D grey level map. This 2D map allows establishing relationships between the wave numbers of the two FT-IR regions. The most important links between variables in the two domains, represented by darker spots, are shown in Table 2.2. The positive relationships are related to those variables links that are correlated to the DM while, conversely, the negative ones are anti-correlated to the DM.

According to Figure 2.10b and Table 2.2, the most important wave numbers in the 1800–1500 cm⁻¹ region are 1746 cm⁻¹ (assigned to carbonyl esters), and 1626 and 1603 cm⁻¹ (assigned to carboxylates). The **b** vector profiles of these wave numbers in the region 1200–850 cm⁻¹ occurs at 1100 and 1018 cm⁻¹, which correlates positively to the ester vibration and negatively to the carboxylate vibrations. These wave numbers have been assigned to GalA (Coimbra *et al.*, 1998; Kacuráková *et al.*, 2000; Ferreira *et al.*, 2001). The ester band is also positively related to the band at 1145 cm⁻¹, and negatively related to the band at 1041 cm⁻¹. The carboxylate bands are negatively correlated to 1100 and 1018 cm⁻¹ and positively correlated to 1060 cm⁻¹, in accordance with the relative absorbance of GalA and neutral sugars (Coimbra *et al.*, 1998; Ferreira *et al.*, 2001). These results show that the absorbance values of the pectic polysaccharides in the region 1200–850 cm⁻¹ are positively correlated with the absorbance of the ester groups, and anti-correlated with the absorbance of the carboxylate groups in the estimation of their DM.

This OP-PLS1 calibration model, when applied to predict the DM of three commercial citrus pectins of defined DM (93, 67 and 26%), gave very similar values to those present in the samples (Table 2.3), with a RMSEP of 5.6% and R² of 0.99, and also very low coefficients of variation (CV) for the spectra repetitions. These results showed that one could build a model to predict the DM of pectic polysaccharides.

Table 2.3 Prediction of DM values for commercial pectins using OP-PLS1 (9 LV) (Barros *et al.*, 2002).

Sample number	GalA (mol%)	DM (mol%)		CV (%)
		Observed *	Predicted	
41	81	93	94	0.3
42	74	67	72	1.7
43	66	26	23	2.3

*Degree of methylesterification of pectic polysaccharides determined by gas chromatography.

For complex samples, the prediction error (Root Mean Square Error of Prediction – RMSEP) was 21.8%, with a coefficient of determination (R^2) of 0.82 (Figure 2.11a). Despite the observed variability, the OP-PLS1 could predict with some reliability the DM of pectic polysaccharides of samples with low purity and from

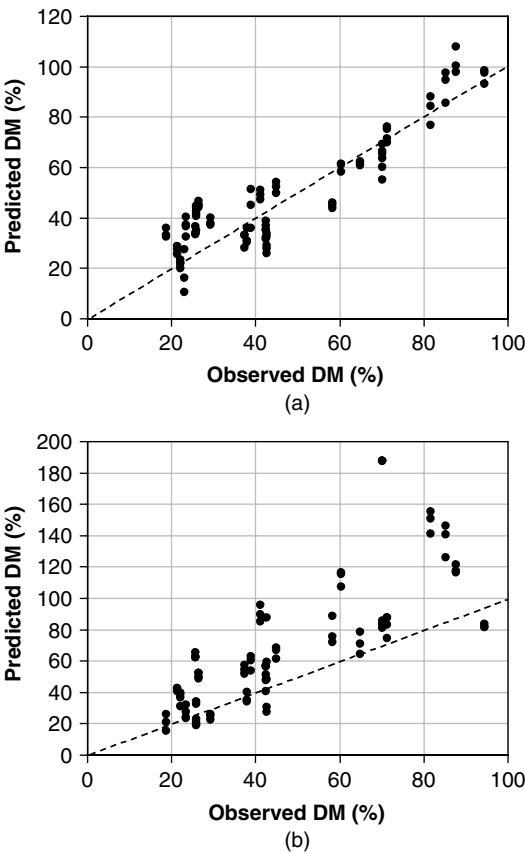


Figure 2.11 Prediction of the DM of pectic polysaccharides for an external data set of olive samples (dotted line represents the optimal prediction): a) Using the OP-PLS1 regression model; b) using PLS1 in the classical 1800–1500 cm^{-1} region. *Source:* Barros *et al.*, 2002 (Elsevier).

different sources. Using the classical $1800\text{--}1500\text{ cm}^{-1}$ region for the prediction of the DM, the obtained prediction error is much higher (RMSEP = 72.5%), with a very low R^2 (0.63). The prediction curve plot for this region (Figure 2.11b.) shows that, using solely this region, it is not possible to quantify the DM in complex pectic samples.

In conclusion, the use of the combination of FT-IR spectra in the region $1200\text{--}850\text{ cm}^{-1}$ with the ester and carboxylate regions ($1800\text{--}1500\text{ cm}^{-1}$) allows the prediction of the DM of pectic polysaccharides in extracts of olive and pear pulps, even in the presence of phenolic compounds.

2.3.2 Orthogonal signal correction (OSC)-PLS1 regression applied to white and red wine polymeric material extracts

Polysaccharides comprise one group of wine macromolecules which, depending on their composition, structure and concentration, is relevant for explaining and controlling wine stability (Segarra *et al.*, 1995) and retention of aroma compounds (Goubet *et al.*, 1998). They are originated both from grape and from microorganisms. Arabinans (Belleville *et al.*, 1993), type II arabinogalactans (Pellerin *et al.*, 1995), rhamnogalacturonans and galacturonans (Pellerin *et al.*, 1996) arise from native cell-wall pectic polysaccharides of grape berry, after degradation by pectic enzymes during grape maturation and during the first steps of wine making. Yeasts produce mannans and mannoproteins during and after fermentation (Waters *et al.*, 1994), whereas glucans are produced by *Botrytis cinerea*, which may infect grape berries (Dubourdieu and Rebereau-Gayon, 1981).

In wine, FT-IR have been proposed and implemented for routine analysis of a large number of parameters, such as ethanol, volatile acidity, pH, tartaric acid, lactic acid, SO_2 , glucose and fructose, acetic acid, citric acid and polyphenols (Dubernet *et al.*, 2000). Also, a rapid method for discrimination of red wine cultivars (Edelmann *et al.*, 2001) and for identification of polysaccharides (Coimbra *et al.*, 2002) has been proposed, based on FT-IR spectra of the phenolic extracts in the $1640\text{--}950\text{ cm}^{-1}$ region. Using a PLS1 regression model based on the FT-IR spectral region between 1200 and 800 cm^{-1} , it has been possible to quantify mannose from mannoproteins in purified white wine extracts (Coimbra *et al.*, 2002). However, this methodology does not allow a predictive ability for a wider range of samples, including samples from different and more complex matrices (such as the whole polymeric material) or from red wines, as the inherent complicated variations can hinder relevant information (Coimbra *et al.*, 2005). Therefore, pre-processing techniques should be used to enhance or sort out the relevant information, making the models simpler and easier to interpret.

Orthogonal Signal Correction (OSC) is one of many pre-processing filters aimed at removing strong systematic variations in a given independent set of variables that are not correlated with the dependent variables. That is, the filter removes, from the dependent variables ($\mathbf{y}$), structures that are orthogonal to the independent set of variables ($\mathbf{X}$) (Wold *et al.*, 1998).

Figure 2.12a shows typical FT-IR spectra of the wine polymeric material extracts. The spectra show high absorbance at wave numbers characteristic of wine polysaccharides – namely, the OH absorbance at 3440 cm^{-1} and the carbohydrate absorbance at $1200\text{--}800\text{ cm}^{-1}$ (Coimbra *et al.*, 2002). Slight differences are observed in the carbohydrate region between the white and red wine (Figure 2.12b).

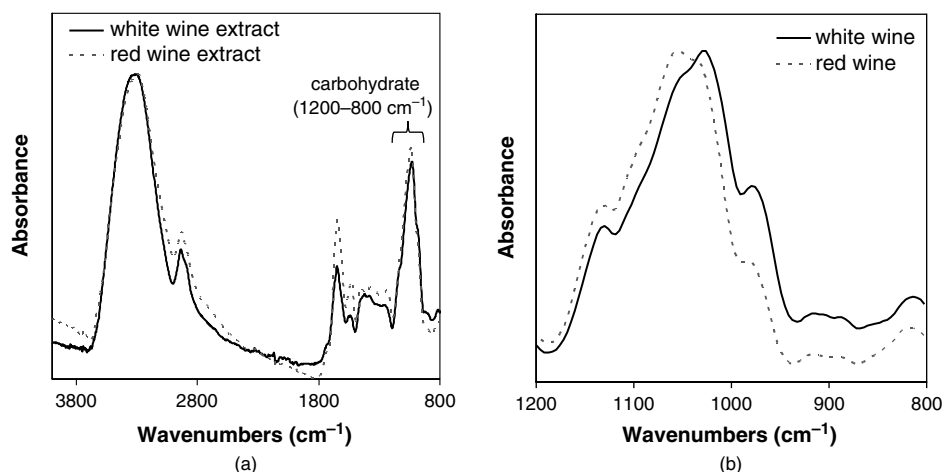


Figure 2.12 FT-IR spectra of the wine polymeric material: a) 4000–800 cm^{-1} region; b) 1200–800 cm^{-1} region. Source: Coimbra *et al.*, 2005 (Elsevier).

Using the 1200–800 cm^{-1} region of the FT-IR spectra, a PLS1 applied to 156 FT-IR spectra of white wine extracts allowed a calibration model with four Latent Variables (Figure 2.13a). The observed Man content, when compared with the amount estimated by gas chromatography of the acetylated mannitol derivatives obtained by acid hydrolysis, reduction and acetylation of mannose residues, showed a linear relationship between 3–88 mol% of Man, with a Root Mean Square Error of Cross-Validation (RMSECV) of 17%, with a R^2 of 0.96. The **b** vector profile of the calibration model (Figure 2.13b) shows that the most important wave numbers related to the variability of Man were the bands located at 1129, 1056, 964, and 817 cm^{-1} , which increase as the Man content increases, and the bands located at 1160, 1079 and 995 cm^{-1} , which increase as the Man content decreases. For the 24 FT-IR spectra of red wines, it was necessary to have a calibration model with five LVs, in order to have a predictive power. The observed versus estimated Man relationships plot is represented in Figure 2.13c, showing a good linear relationship between a wide range (11–77 mol%) of Man. The RMSECV obtained was 14%, with a R^2 of 0.99. The **b** vector profile of the calibration model (Figure 2.13d) shows that the most important wave numbers related to the variability of Man are the bands located at 1145, 1129, 1056, 1025, 964, 921, and 821 cm^{-1} , which increase as the Man content increases, and the bands located at 1176, 1079 and 995 cm^{-1} , which increase as the Man content decreases.

When a PLS1 calibration model for Man using both types of wines (red and white) was built, the optimal dimensionality was found to be a model with two LVs with a RMSECV of 36.2%, and with a plot of the actual vs. estimated values for the polymeric Man as shown in Figure 2.14a, showing that the regression model does not give satisfactory prediction ability. This lower predictive calibration model was possibly related to the heterogeneous nature of the non-carbohydrate material present in the red and white wine extracts. These compounds can originate FT-IR signal complexities that can introduce major sources of variation, which had very small or no predictive ability to the criterion of interest (Man quantification). In these cases, one common approach is to use different data pre-treatments to reduce the effect of variabilities not related to the

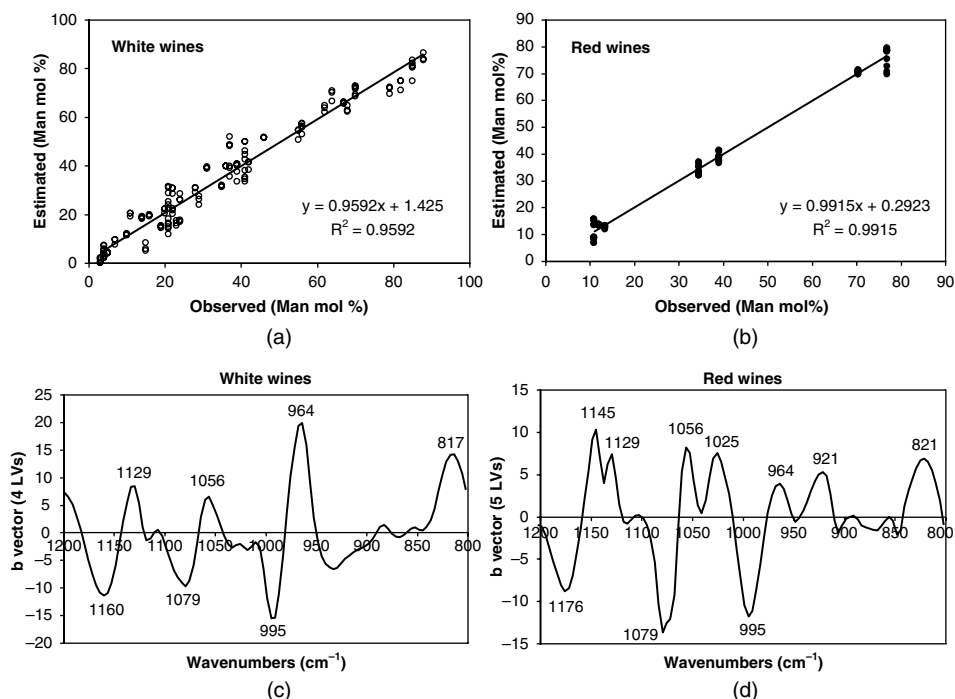


Figure 2.13 PLS1 regression model for the estimation of polymeric mannose in white (four LVs) and in red (five LVs) wine extracts: a) and c) relationship plot between observed vs. estimated amount of mannose; b) and d) **b** vector profiles. Source: Coimbra *et al.*, 2005 (Elsevier).

factor of interest. Among several methodologies that can be used, OSC is one of the most promising, as it can be used to remove from the spectra a certain number of factors orthogonal (not related) to the criterion of interest (Man) (Wold *et al.*, 1998; Fearn, 2000).

The methodology for selection the optimal model dimensionality using the OSC was as follow: a) the OSC procedure was applied to the calibration data set by removing 1, 2, to n factors; b) After each removed factor, a PLS1 regression was applied to the corrected data set, and their predictive ability was assessed by internal cross-validation (leave-3-out). Removing 11 factors from the calibration set left an optimal PLS1 model with two LVs, given a RMSECV of 11.4% (Figure 2.14b). This showed a significant increase in the model predictive power when compared to the PLS1 calibration model, with a better linear trend in the plot of the observed vs. estimated values for the polymeric Man using the OSC-PLS1 model.

The amount of explained variability for each LV for both PLS1 and OSC-PLS1 proposed calibration models is shown in Table 2.4. The OSC-PLS1 explains 97.4% of the total variability contained in the **y** vector (polymeric Man values), whereas PLS1 only explains around 76.3%. Figure 2.14c represents the **b** vectors plot for the PLS1 and OSC-PLS1 with two LVs, where it can be seen that the only difference in the **b** vector plot between PLS1 and OSC-PLS1 is the intensity of the bands, not their positions. The characteristic bands for the quantification of polymeric Man can be identified as the

Table 2.4 Percentage of explained variability for PLS1 and OSC-PLS1 optimal models (Coimbra *et al.*, 2005).

	PLS1		OSC-PLS1	
	X	y	X	y
LV1	32.9%	58.7%	40.5%	69.4%
LV2	55.4%	17.6%	59.4%	28.0%
Total	88.3%	76.3%	99.9%	97.4%

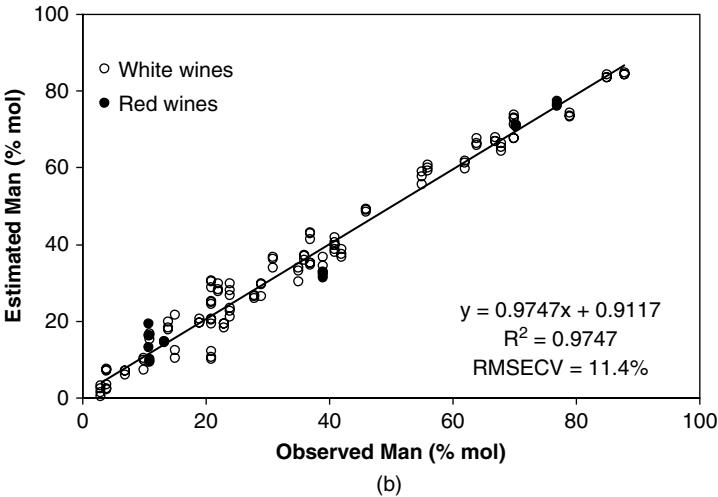
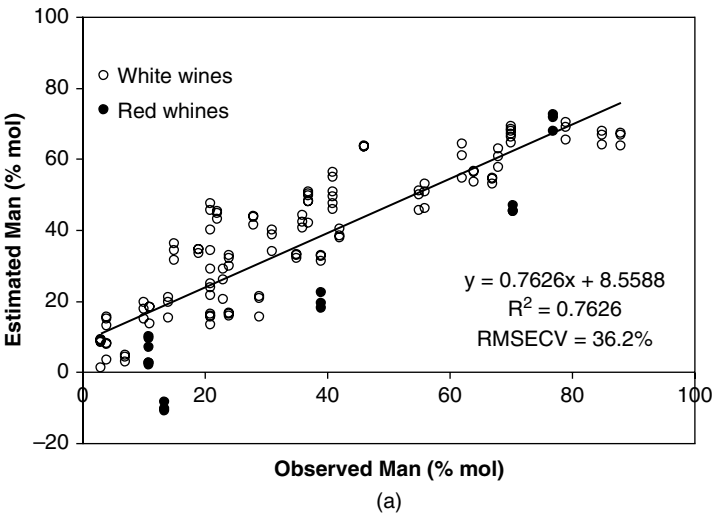


Figure 2.14 Estimation of polymeric mannose in white and red wine extracts (2 LVs): a) PLS1 regression model; b) OSC-PLS1 regression model; c) **b** vector profiles. *Source:* Coimbra *et al.*, 2005 (Elsevier).

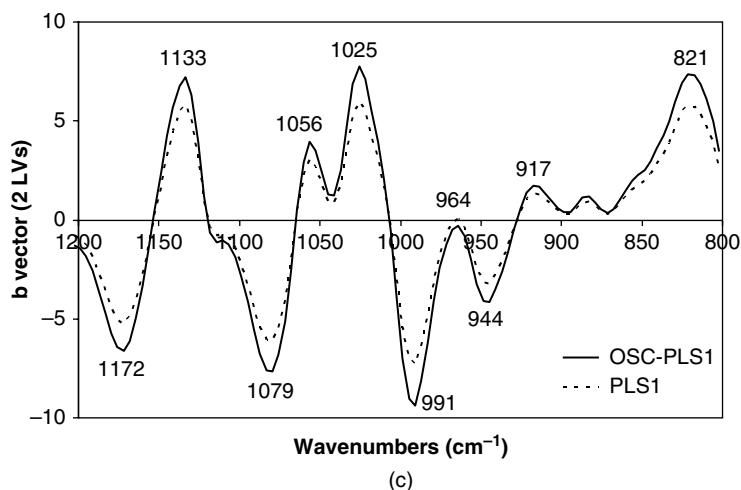


Figure 2.14 (Continued)

bands located at 1133, 1056, 1025, 917, and 821 cm^{-1} . These are positively correlated to Man content, and the bands located at 1172, 1079 and 991 cm^{-1} are negatively correlated to Man content.

These results show that OSC-PLS1 is a procedure that can be used to improve the predictive ability of the model quantification of mannose from mannoproteins, based on the FT-IR spectral region between 1200–800 cm^{-1} , allowing it to be used for a wider range of samples, including white and red wine extracts from complex matrices.

2.4 Screening and distinction of coffee brews based on headspace – solid phase microextraction combined with gas chromatography in tandem with principal component analysis (HS-SPME/GC-PCA)

The two major species of coffee, *Coffea arabica* and *Coffea canephora*, var. Robusta, differ considerably in price, quality and consumer acceptance. The washed Arabica coffees are characterised by some acidity and intense aroma, while natural dry-processed Arabica coffees are less acid and have a less marked aroma but a richer body. Robusta coffees are characterised by their bitterness and a typical earthy and woody flavour. Blending, which can be done before or after roasting, is carried out for the purpose of obtaining coffee brews with a higher quality when compared to their individual counterparts. In espresso, washed coffees bring their fine intense aroma, and natural coffees add body (Illy and Viani, 1995). Torrefacto coffee is widely used in Argentina, Costa Rica and Spain, where its consumption represents 83% of the commercial available coffees in hotel trade. To obtain torrefacto coffee, at the end of the roasting process, sucrose (no more than 15% in weight) is added. At high temperatures, sucrose is converted into caramel, forming a burnt film round the coffee bean, making it bitterer and less odorant (Sanz *et al.*, 2002).

The volatile fraction of roasted coffee has been analysed by many authors (Dart and Nursten, 1985; Flament, 1989, 1991; Maarse and Visscher, 1989; Mondello *et al.*, 2004; Nijssen *et al.*, 1996; Petisca *et al.*, 2013; Tranchida *et al.*, 2009), who have identified about 850 compounds (Belitz *et al.*, 2009; Buffo and Cardelli-Freire, 2004; Flament and Bessi re-Thomas, 2002). Various methods of extraction have been used to study the aroma fraction of coffee brews. As an alternative to injection of an organic solvent extract, the vapour phase surrounding the brew (headspace) can be directly analysed. This alternative approach gives the most accurate composition of flavours that may be perceived by the consumers. However, when a large volume of headspace gas is injected, the carrier gas dilutes the sample. This problem can be solved by injecting the headspace gas directly inside a capillary column, on-column injection (Shimoda and Shibamoto, 1990), using a purge and trap system (Sammelroch and Grosch, 1995), using an adsorbent (Pollien *et al.*, 1997), or using a static headspace sampler (Sanz *et al.*, 2001, 2002). In 1990, the solid phase microextraction (SPME) technique was developed for headspace sampling, extraction and concentration (Arthur and Pawliszyn, 1990). This is a very simple, rapid, solvent-free and not very expensive method (Figure 2.15), which has been shown to be suitable to be used in coffees (Bicchi *et al.*, 1997, 2002; Mondello *et al.*, 2004; Petisca *et al.*, 2013; Ramos *et al.*, 1998; Roberts *et al.*, 2000; Tranchida *et al.*, 2009; Yang and Peppard, 1994).

The sensory characteristics of coffee brew depend on the method of extraction used. Petracco (2001) classified the extraction methods from a qualitative perspective; the grouping criterion chosen takes into account both the mode and the time of coffee/water contact. Among the pressure methods, plunger (cafeti re) coffee (where the suspension of hot water and coffee powder is pressed through a plunger) and espresso coffee are examples. Some studies have been done on the taste and mouthfeel (Maeztu *et al.*, 2001a) and flavour and aroma (Liardon and Ott, 1984; Maeztu *et al.*, 2001b) of espresso coffee. Several compounds have been reported as characteristic odorants of ground and brewed coffee (Shimoda and Shibamoto, 1990; Blank *et al.*, 1991, 1992; Grosch, 1995; Semmelroch and Grosch, 1995, 1996; Mayer *et al.* 2000), and their relative content in each type of matrix explains their particular aroma properties (Blank *et al.*, 1991; Semmelroch and Grosch, 1995).

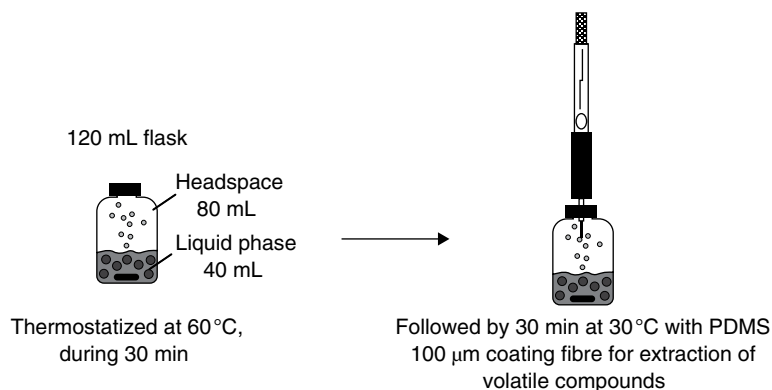


Figure 2.15 Experimental procedure for sample preparation and extraction of volatile compounds from coffee brew by headspace-solid phase microextraction (HS-SPME).

The sensory analysis carried out by a trained panel is a cumbersome and costly process to be introduced as a routine procedure. Assessors of sensory panels cannot always, consistently and objectively, identify the sample – specially a blend. Their perception of the aroma of the coffee will depend on physiological and psychological factors. The coffee industry needs a simple, quick, and objective method to classify, especially, the botanical varieties of coffees (Arabica or Robusta) and/or the type of blends used in coffee brew preparations.

Aiming for screening and distinction of coffee brews, based on the combined technique of headspace (HS) solid phase microextraction (SPME), combined with gas chromatography (GC) and in tandem with principal component analysis (PCA) (HS-SPME-GC-PCA), a methodology based on the definition of the global volatile profiles of coffees brews, that is complementary to that obtained by sensory evaluation, and precluding the identification of the volatile compounds by mass spectrometry, has been proposed (Rocha *et al.*, 2004a). Coffee brews were: a) a blend of natural roasted 80% Robusta and 20% Arabica (R80 : A20); b) a 50% torrefacto of 80% Robusta and 20% Arabica (R80 : A20 torrefacto); and c) a natural roasted 100% Arabica (A100).

Volatile profiles, expressed as relative percentage of GC peak area for the different chemical classes of volatile compounds ensuing from the headspace SPME analysis by GC-MS, are shown in Figure 2.16 (Rocha *et al.*, 2004a). The relative percentage of GC peak area for the different chemical classes of the R80 : A20 natural and torrefacto coffees were similar, for both espresso and plunger coffees, although the natural R80 : A20 coffee had a more intense aroma than torrefacto. The decrease of volatile compounds in torrefacto brew may be due to the fact that, in this blend, a fraction (6%) of the coffee had been replaced with sugar. Also, torrefacto coffee is usually submitted to a lower degree of roast, to avoid flavours produced by the burnt sugar. The presence of sucrose during the roast may also contribute to this decrease, by hindering the volatile compounds in a caramel interface. The relative percentage of GC peak area for furans, in both espresso and plunger coffees, was higher in A100 than in R80 : A20 coffees and, conversely, the relative percentage of GC peak areas for pyrazines were lower. Pyrazines are products obtained of the Maillard reactions, and they are more abundant in Robusta coffee (Sanz *et al.* 2002; Ho *et al.*, 1993), which is consistent with the lower content of free amino acids in Arabica coffees (Illy and Viani, 1995).

Figure 2.17 shows the PCA scores scatter plots of PC1 $\times$ PC2 (Figure 2.17a) and PC1 $\times$ PC3 (Figure 2.17b), which contains 82% of the total variability of SPME volatile profiles of espresso and plunger coffees.

Figure 2.18 represents the corresponding loadings plots that establish the relative importance of each volatile component according to its retention time. PC1 $\times$ PC2 scores plot shows the distinction between the Arabica coffees (PC1 negative and PC2 positive) and Robusta coffees, both R80 : A20 and R80 : A20 torrefacto (PC1 positive and PC2 negative).

According to the corresponding loadings plots (Figure 2.18a,b), the Arabica brews are characterised by the compound with the retention time of 44.9 min (furfurylacetate), and Robusta brews are characterised by the compounds with the retention times of 12.0 min (2-methylbutanal), 38.2 min (2-ethyl-5-methylpyrazine), 39.3 min (trimethylpyrazine), and 41.1 min (3-ethyl-2,5-dimethylpyrazine). These results are in accordance with the distinction between Robusta and Arabica espresso coffee, based on the amount of aldehydes and pyrazines (Petracco, 2001), as the concentration of

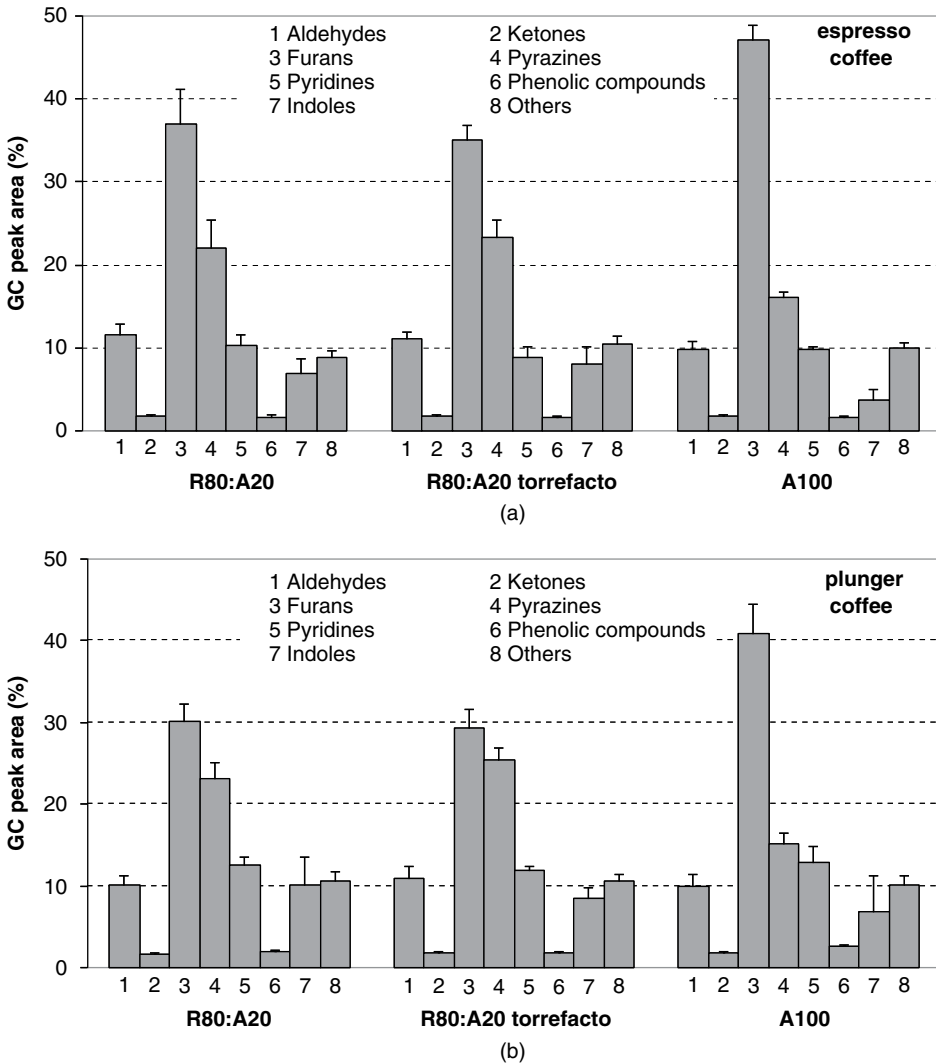


Figure 2.16 Volatile profile, expressed as relative percentage of GC peak area for the different chemical classes of volatile compounds, of: a) espresso; and b) plunger coffees, using a PDMS SPME coating. R80 : A20 – 80% Robusta and 20% Arabica blend; R80 : A20 torrefacto – 80% Robusta torrefacto and 20% Arabica blend; A100 – 100% Arabica coffee. Source: Rocha *et al.*, 2004a (John Wiley & Sons).

furfurylacetate (PC1 negative and PC2 positive) was twice as high in Arabica than in Robusta brews, and levels of 2-methylbutanal, 2-ethyl-5-methylpyrazine, trimethylpyrazine, and 3-ethyl-2,5-dimethylpyrazine (PC1 positive and PC2 negative) were up to three times higher in Robusta than in Arabica brews.

The PC1 × PC3 scores plot (Figure 2.17b) shows the distinction between plunger (PC1 positive and PC3 positive) and espresso (PC1 negative and PC3 negative) coffee

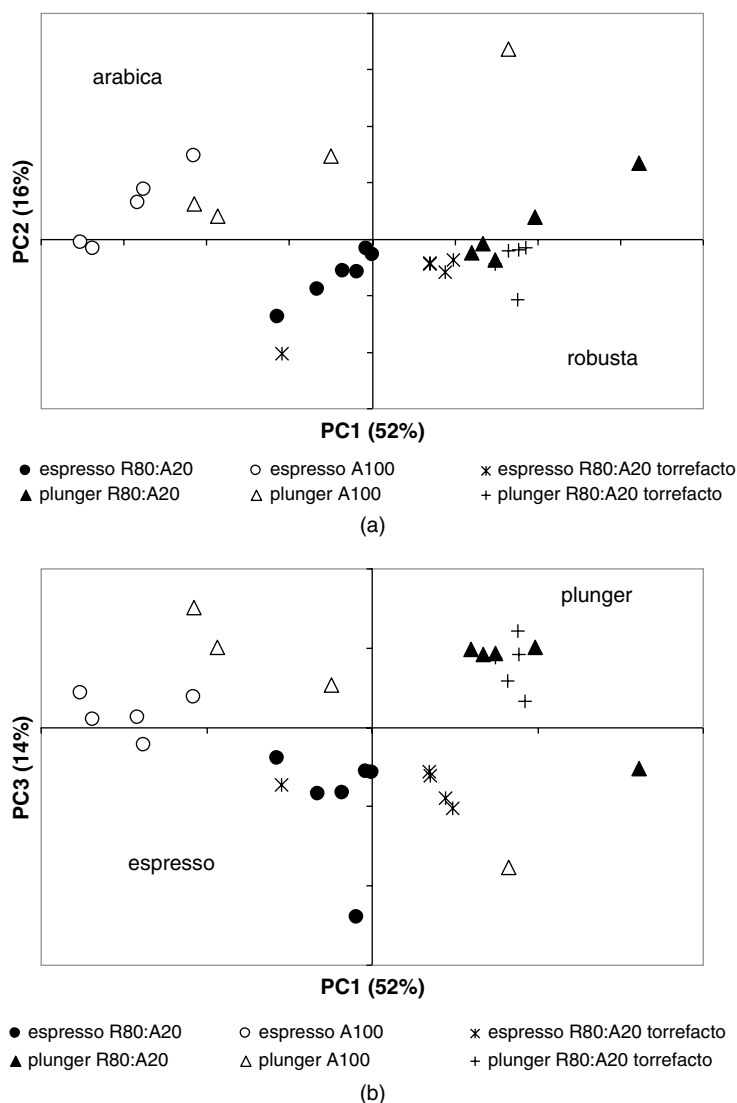


Figure 2.17 PCA scores scatter plot of the chromatographic SPME areas of coffee volatile compounds: a) PC1 $\times$ PC2; and b) PC1 $\times$ PC3 (axes cross each other at the origin). Source: Rocha *et al.*, 2004a (John Wiley & Sons).

brews. According to the corresponding loadings plots (Figure 2.18a,c), the plunger coffee brews are characterised mainly by the compound with the retention time of 27.5 minutes (pyridine), and espresso coffee brews are characterised by the compound with the retention time of 10.1 minutes (2-methylfuran). For the three coffees studied, pyridine (PC1 positive and PC3 positive) was, in fact, 23–43% higher in plunger than in espresso coffee brews, and 2-methylfuran (PC1 negative and PC3 negative) was 10–62% higher in espresso than in plunger coffee brew.

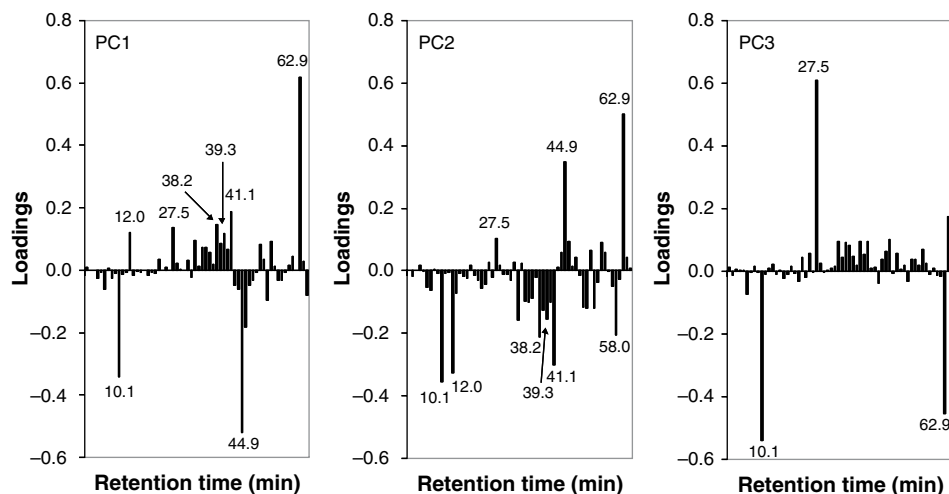


Figure 2.18 PCA loadings plot of the chromatographic SPME areas of coffee volatile compounds: a) PC1; b) PC2; and c) PC3. Source: Rocha *et al.*, 2004a (John Wiley & Sons).

The volatile profile of espresso and plunger coffee brews obtained by SPME-GC-MS seems to be established mostly by the botanical varieties (Arabica or Robusta), rather than by the process of preparation of the brews (espresso or plunger). Furthermore, the use of the variability given just by the GC areas and respective retention times, combined with PCA, allowed for the observed distinction. The combined technique of HS-SPME/GC-PCA, when compared with the conventional techniques based on GC-MS identification of volatile compounds, can be proposed as a lower cost, fast, and reliable technique for screening and distinction of coffee brews (Rocha *et al.* 2004a). This has been used for the characterisation of the coffee flavour and estimation of shelf-life, as well as the analysis of the impact of the roasting degree on the volatile profile, among others.

2.5 Comprehensive two-dimensional gas chromatography (GC × GC) combined with time-of-flight mass spectrometry (ToFMS) as a powerful tool for food products analysis

2.5.1 GC × GC-ToFMS principles and advantages

Food products are complex samples composed of several biomolecules with distinct physicochemical characteristics, being present in concentrations with different orders of magnitude. Thus, the analysis of food products presents some challenges, either on the establishment of global volatile profile or for the quantification of target analytes. In the two last decades, considerable research has been dedicated to the field of chromatographic developments, namely by combining independent techniques with the aim of strengthening resolving power and sensitivity (Cordero *et al.* 2015; Tranchida *et al.* 2004). Comprehensive two-dimensional gas chromatography (GC × GC), combined

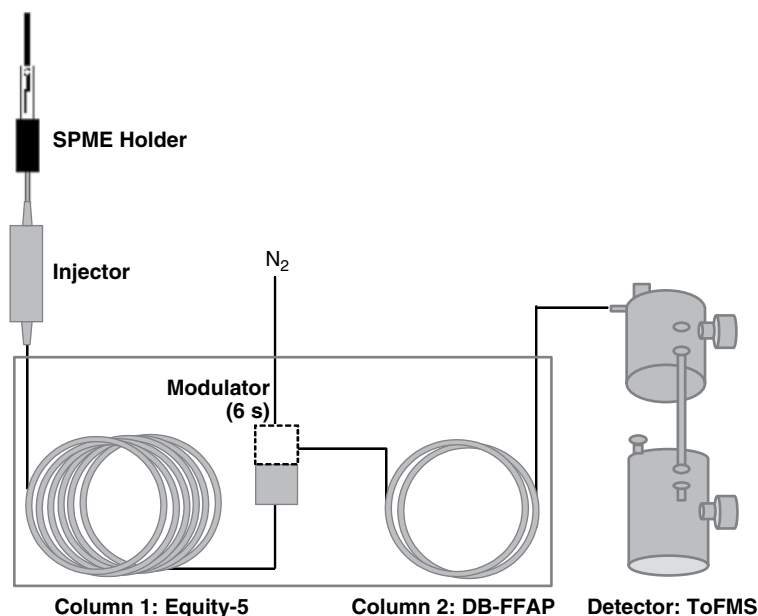


Figure 2.19 Schematic representation of a GC $\times$ GC-ToFMS equipment, with a non-polar (Equity-5) and polar (DB-FFAP) set of columns.

with time-of-flight mass spectrometry (ToFMS), detection represents a successful example of this combination.

GC $\times$ GC employs two orthogonal mechanisms (Figure 2.19) to separate the constituents of the sample within a single analysis, based on the application of two GC columns coated with different stationary phases. In order to achieve this goal, a non-polar (separation ruled by boiling points)/polar (separation by polarity) phase combination, connected in series through a modulator interface, can be used. The interface samples small (several seconds) portions of the first dimension (^1D) eluate by cryofocusing, and re-injects them into the second column (^2D). Each ^1D peak is modulated several times, largely preserving the ^1D separation. The ^2D is very short and narrow and, consequently, each modulated portion is ‘flash’ separated before the next modulation. Using this instrumental approach, compounds co-eluting from ^1D undergo additional separation on ^2D (Górecki *et al.*, 2006). Figure 2.20 shows a practical example of this orthogonal separation in a table wine sample, in which three detected compounds (propyl dodecanoate, α -calacorene, and 4,5,9,10- dehydro-isolongifolene) presented similar volatility (^1t_R – 880 s). Nevertheless, they were separated on ^2D (^2t_R – 0.41, 0.49, and 0.60 s, respectively), due to their different polarities. GC $\times$ GC offers substantial advantages over conventional 1D-GC – namely, enhanced resolution, increased peak capacity, faster run times, enhanced mass selectivity and sensitivity, and improved limits of detection, due to the focusing of the peak in the modulator (Jalali *et al.*, 2012; Seeley and Seeley, 2013; Song *et al.*, 2004; Souza *et al.*, 2009).

Narrow peaks with width at half height of 0.1 s or less are preferably recorded by using a high data acquisition speed of ToFMS to provide sufficient data density required for GC $\times$ GC separations (Shellie *et al.*, 2001). ToFMS brings other advantages, such as full

mass spectra acquisition at trace level sensitivity, and mass spectral continuity, which allows the deconvolution of spectra of co-eluted peaks, enabling the spectral quality when compared with commercial database, as observed for 4,5,9,10-dehydro-isolongifolene, a trace wine sesquiterpenic component (Figure 2.20).

The huge amount of data obtained through this methodology requires an adequate identification of the detected compounds. The most reliable way to confirm the identification of each compound is based on authentic standard co-injection which, in several cases, is economically prohibitive and often unachievable in the time available for analysis, or because standards are not commercially available. Hence, despite the analyte identification by standard co-injection, compounds may be putatively identified, based on a strategy that includes several parameters, namely the MS spectral similarity compared with home-made or commercial spectra databases, and calculation of Retention Index (RI_{cal}) and comparison with values available in the literature (RI_{lit}) for the 1^D column or equivalents. Regarding this latter parameter, it is important to take into account that some differences between RI_{cal} and RI_{lit} should be acceptable ($\approx 5\%$), as the literature data is obtained from a large range of GC stationary phases (several commercial GC columns, equal or equivalent to the 1^D stationary phase). Also, some of the values reported in literature were determined in a 1D-GC separation system, and modulation causes some inaccuracy in t_R (Rocha *et al.*, 2013). Finally, it is important to highlight that the principle of the structured chromatogram is an additional and unique information, crucial for identification purposes. Actually, GC $\times$ GC is an ideal

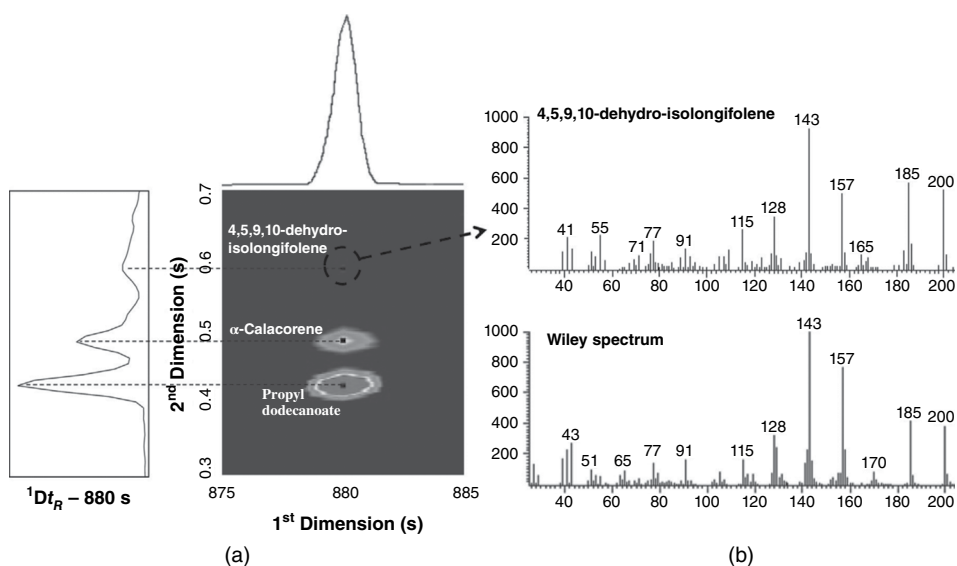


Figure 2.20 (a) Blow-up of a part of total ion GC $\times$ GC chromatogram and corresponding contour plots obtained from a table wine, showing propyl dodecanoate, α -calacorene and 4,5,9,10-dehydro-isolongifolene, which are separated on the 2^{nd} dimension, according to their polarity properties; (b) The 100 ms wide 4,5,9,10-dehydro-isolongifolene (trace wine sesquiterpenic compound) GC $\times$ GC peak is easily defined and identified at a mass spectral acquisition of 100 spectra/s, and its spectral quality allows its identification by comparison with mass spectrum of commercial databases, namely Wiley. Source: Petronilho *et al.*, 2014 (Elsevier).

technique for the analysis of complex mixtures where compounds of similar chemical structure are grouped into distinct patterns in the 2D chromatographic surface, providing useful information on their boiling point and polarity (as a non-polar/polar column set was used), and relationships of structured retentions have proved especially useful for compound identification. This unique characteristic of the structured 2D chromatographic space is a powerful feature selection tool in the identification step since, once extracted, it will reduce the time of analysis (Cardeal and Marriot, 2009; Coelho *et al.*, 2007; Silva *et al.*, 2010; Petronilho *et al.*, 2014).

GC $\times$ GC-ToFMS has been successfully used in food analysis, either on the determination of the volatile profile of different foods, such as table (Santos *et al.*, 2015) and Madeira wines (Perestrelo *et al.*, 2011), or in the determination and quantification of target compounds, namely ethyl carbamate (a potentially toxic compound) in Madeira wine samples (Perestrelo *et al.*, 2010) and furan-derivatives produced during wine high pressure treatments which promote Maillard reaction acceleration (Santos *et al.*, 2013). Also, GC $\times$ GC-ToFMS has been applied as a powerful tool for screening trace components used to trace the origin of natural products, namely grapes (Rocha *et al.*, 2007) or sea salt (Silva *et al.*, 2010). To reveal its utility for food products control and characterisation, a beer volatile profiling study was selected.

2.5.2 Beer volatile profiling by HS-SPME/GC $\times$ GC-ToFMS

The aroma profile of beer is characterised by a complex network of variables, such as raw materials (hop, barley or other cereals, and water), fermentation (yeast strain and temperature) and pasteurisation procedures, as well as packaging and storage conditions after the brewing process (Belitz *et al.*, 2009; Hui *et al.*, 2006). Several approaches have been employed to extract and concentrate beer volatile components, namely the SPME, as it allows a direct beer characterisation. SPME, combined with GC $\times$ GC-ToFMS, allowed a comprehensive study of beer volatile composition (Martins *et al.*, 2015). Figure 2.21 shows a 3D GC $\times$ GC total ion current chromatogram obtained for a beer sample. The developed methodology allowed the detection of, on average, 700–800 components per sample, from a wide range of chemical families, namely, acids, alcohols, aldehydes, aromatic compounds, esters, furan-derivatives, hydrocarbons, ketones, nitrogen-derivatives, norisoprenoids, sulfur, and terpenic compounds.

Several scenarios can be explored to provide in-depth beer volatile molecular composition data. An exploratory approach was performed with six commercial beers (three brands (A, B, and C) commercially available on the Portuguese market, in two package formats: glass bottle (GB) and can (Can)). First, a set of 32 compounds was selected, based on their impact and importance on beer aroma. For a straight and rapid interpretation of the relative abundance of each selected component (maximum normalisation of GC peak area), a heat map was built (Figure 2.22), which corresponds to a graphical representation of the in-depth data, allowing a rapid visual evaluation of similarities and differences between the six samples under study.

According to Figure 2.22, sample A_{GB} had higher content for most of the selected compounds. Some differentiation of the content of acids, alcohols, esters and phenyl-acetaldehyde (compounds that can come from yeast metabolism) can be observed in the analyzed samples, being the most preminent differences related with ethyl

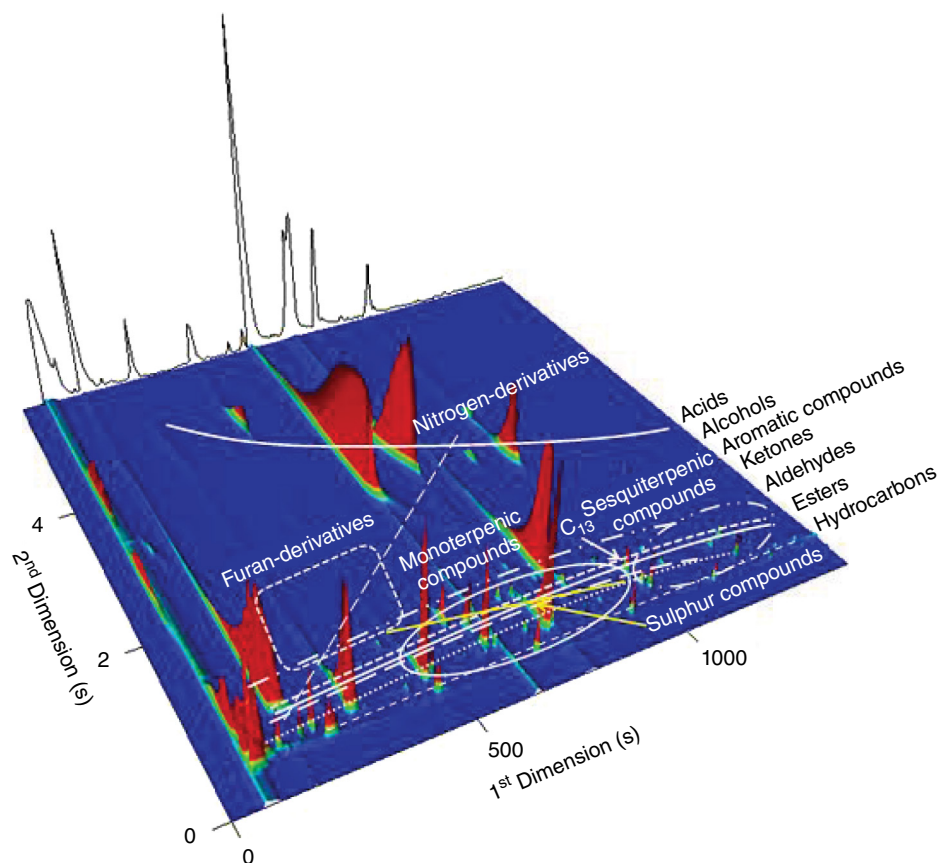


Figure 2.21 3D GC $\times$ GC total ion chromatogram plot of a beer sample highlighting the beer volatile chemical families. Bands and clusters formed by structurally related compounds are indicated.
Source: Martins *et al.*, 2015 (John Wiley & Sons).

octanoate and ethyl decanoate (higher content in cans), and phenylacetaldehyde and isoamyl alcohol (higher content in GB). Brand A showed to be distinguished by ethyl propanoate, once its content was higher in these samples. Regarding brands B and C, the observed differences seem to be more correlated with package format type than with brand. In addition, different types of pasteurisation process can be applied within each company, which can lead to differences among samples from the same brand.

β -damascenone is a norisoprenoid compound related with beer storage/aging, and showed some correlation with can samples age once its content decreased with age ($B_{Can} > C_{Can} > A_{Can}$). A higher content of furan-derivatives components was registered to A_{GB} – namely, furfural and 2-acetylfuran, as well as 3-methylbutanal. Nevertheless, as GB samples are older than Can samples, for each brand, 3-methylbutanal had higher content on GB samples, with the exception of brand C (slightly higher on Can sample). Furfural, 2-acetylfuran, and acetaldehyde (compounds related with beer aging) had higher content in GB samples, with the exception of brand B, which had similar or slightly higher content for Can samples. Once A_{GB} had one of the lowest sample age under study (three months), this higher content of furan derivatives could indicate

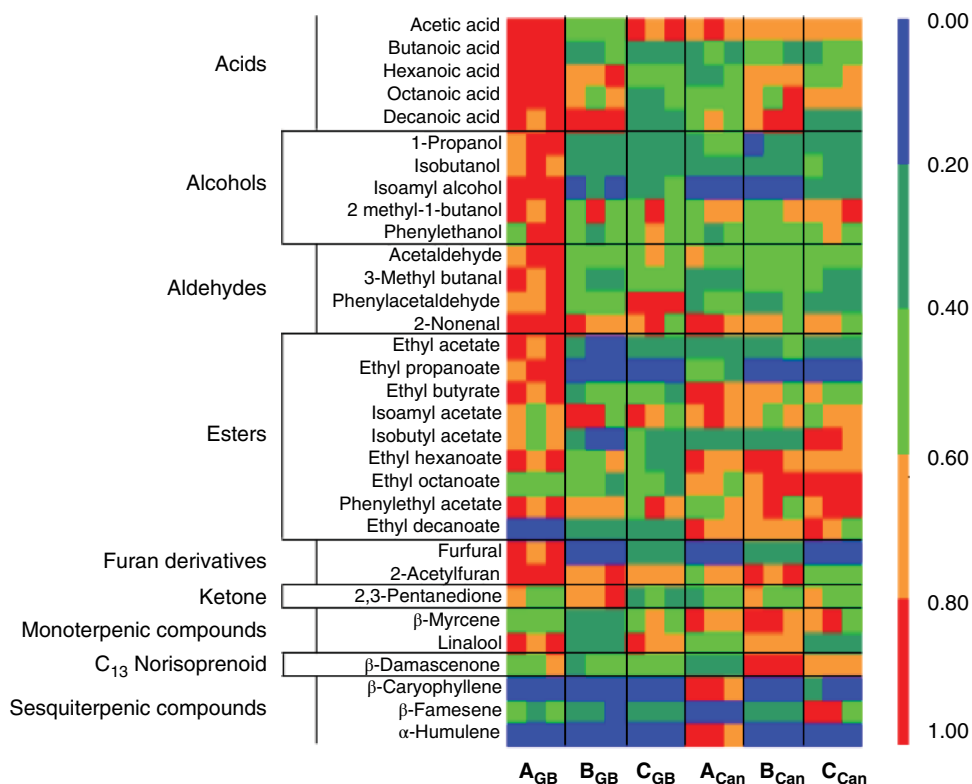


Figure 2.22 Heat map representation of 32 selected volatile beer components from six beers under study: three brands (A, B, and C), each one in two packaging formats – glass bottles (GB) and cans (Can). Different intensities correspond to the normalised (by maximum) GC peak areas of each compound. *Source: Martins et al., 2015 (John Wiley & Sons).*

some excess or different pasteurisation process. A_{Can} was the sample with more content for the most of the selected terpenic compounds, with the exception of linalool (higher for A_{GB}), and β-farnesene (higher for C_{Can}).

Ion extraction chromatography mode (IEC; data processing using specific m/z diagnostic ions) was also applied to increase the specificity and sensitivity, thus minimising the contribution of co-eluted compounds. With this approach, the m/z 93, 161, and 204 (Petronilho *et al.*, 2014) were selected to identify the terpenic (C₁₀ monoterpene and C₁₅ sesquiterpene) and norisoprenoid (C₁₃) compounds, and the ²D chromatographic spaces containing these analytes were defined (Figure 2.23), from ¹t_R 444 to 1326 s, according to ¹D, and from ²t_R 0.490 to 1.250 s, according to ²D. The comparison of Figures 2.21 and 2.23 allows us to infer that this approach is very useful to eliminate the majority of the nonterpenic and non-norisoprenoid compounds, thus simplifying the obtained data and reducing the analysis time.

Structured GC × GC chromatographic behaviour is demonstrated in Figure 2.23, according to the volatility (¹D) and polarity (²D) of beer components identified using m/z 93, 161, and 204. It is possible to observe a volatility decreasing with the increasing of the number of carbons (C₁₀ < C₁₃ < C₁₅) along ¹t_R. A polarity increase along ²t_R was

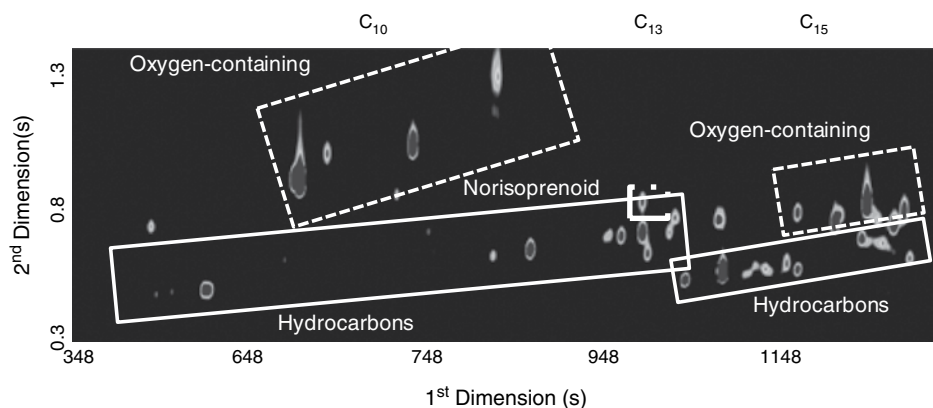


Figure 2.23 GC $\times$ GC–ToFMS extracted ion chromatogram contour plots of m/z 93, 161, and 204, corresponding to a beer sample. The chromatographic spaces corresponding to monoterpene (C_{10}), norisoprenoid (C_{13}), and sesquiterpene compounds (C_{15}) were highlighted.

also observed, where aliphatic components have lower polarity and lower ^2t_R , while oxygen-containing components have higher polarity and, consequently, higher ^2t_R .

In summary, the HS-SPME/GC $\times$ GC–ToFMS developed methodology allowed the comprehensive untargeted beer volatile profiling, through the detection of 700–800 analytes per sample, from several chemical families. GC $\times$ GC seems to be a powerful tool to give distinctive patterns according to beer under study. Otherwise, GC $\times$ GC–ToFMS allowed simultaneous acquisition of data about the beer global volatile profile (full-scan acquisition) plus data about target analytes using diagnostic ions (IEC), which reduces the data complexity and processing time. The obtained results clearly illustrate the potential of the methodology on in-depth study of beer, and a reliable volatile snapshot is only attainable under optimised conditions for instrumental acquisition and data processing.

2.6 Study of cork (from *Quercus suber* L.) – wine model interactions based on voltammetric multivariate analysis

The cork from *Quercus suber* L. is the premium raw material used to produce wine-bottling stoppers. The cork plays an important role in determining wine quality, due to its peculiar features: impermeability to air and liquids (preventing wine oxidation); ability to adhere to glass surface; compressibility; resilience; and chemical inertness (Simpson *et al.*, 1986). If the cork stoppers are in direct contact with the wine, volatile and non-volatile compounds soluble in ethanol/water can migrate, thus contributing to the wine's sensorial properties. However, being a natural product, cork can be attacked and contaminated in different ways that could promote differences in its properties.

Several studies on the off-flavours associated to the cork stoppers have been carried out (Simpson *et al.*, 1986; Rocha *et al.*, 1996; Pollnitz *et al.*, 1996), with 2,4,6-trichloroanisole being reported as the main agent responsible for the cork off-flavours (Capone *et al.*, 2002). New and rapid methodological approaches have been

developed in order to study the volatile fraction associated to the wine cork taint (Boudaoud and Eveleigh, 2003; Juanola *et al.*, 2004; Pizarro *et al.*, 2012; Prat *et al.*, 2011; Ridgway *et al.*, 2010; Zalacain *et al.*, 2004). Conversely, few studies have been carried out concerning the soluble cork fraction that can migrate to wine (Varea *et al.*, 2001), which can have sensorial effects in wines and may form complexes with wine anthocyanins, thus influencing the astringency (Singleton and Trousdale, 1992).

The material unbounded or loosely bounded to the cork cell wall (i.e., the low molecular weight material) may be extracted with ethanol/water. This fraction is composed mainly of phenolic compounds, and exhibits only about 2% of carbohydrates (Rocha, 1997; Rocha *et al.*, 2004b). The major sugar component is xylose (53 mol%), while glucose accounts for 17%, and uronic acids and arabinose represent 13% and 10%, respectively. Only trace amounts of deoxyhexoses can be detected (Rocha *et al.*, 2004b). Low molecular weight polyphenols, such as ellagic acid, gallic acid, protocatechuic acid, caffeic acid, vanillic acid and vanillin, and ellagitannins have been reported as the cork phenols susceptible to migrate into the wine (Varea *et al.*, 2001).

A specific cork contamination is the defect known in the industry as 'Mancha Amarela', or 'Yellow spot' (MA). This shows modifications in the cork's mechanical, structural and optical properties, and is potentially able to cause off-flavours in wine. Studies by scanning electron microscopy carried out on healthy cork (S) and MA cork (Figure 2.24) showed that the cellular structures of the infected and healthy tissues are different, and the attacked tissues were composed of deformed and wrinkly cells, with cell wall separation at the middle lamella level (Rocha *et al.*, 2000). These changes were related to the degradation of lignin and of pectic polysaccharides, as could be inferred by the deposition of calcium in the intercellular space of the attacked cells (Rocha *et al.*, 2000).

In order to evaluate if the cork stoppers were able to contaminate a wine, it would be useful to use a screening technique for monitoring cork prior to its being in contact with wine. As an initial approach, the resulting solutions of the matrix ethanol/water (10% v/v), set in contact both with a standard cork (S) and with a contaminated cork (MA), were studied by voltammetric techniques (Rocha *et al.*, 2005). The need for a fast and reliable methodology for monitoring modifications in a wine model solution promoted by the contact with contaminated cork prompt the application of voltammetric techniques, such as cyclic voltammetry (CV) and/or square wave voltammetry (SWV). Voltammetric methods are relatively simple, rapid, sensitive, and of low cost, requiring minimal preparation of samples and, thus, can be appropriated as screening techniques. Furthermore, voltammetry is suitable for the determination of several redox active organic compounds, namely phenolics, including methoxyphenols, flavonoids and other antioxidant molecules of interest in diverse areas (Wheeler *et al.*, 1990; Evtuguin *et al.*, 2000; Filipiak, 2001; Papanikos *et al.*, 2002). All these compounds can be detected by electrooxidation at glassy carbon electrodes.

Cyclic voltammetry has also been used to identify phenolic compounds in beer (Filipiak, 2001), tea and coffee (Kilmartin and Hsu, 2003; Roginsky *et al.*, 2003), and wines (Zou *et al.*, 2002; Kilmartin *et al.*, 2002). On the other hand, sugars cannot be detected by voltammetry at conventional carbon electrodes, due either to low redox reaction kinetics, more positive potential of oxidation (more positive than the limit of the working potential window), or self-poisoning of the electrode surface (Wittstock *et al.*, 1998). A new methodological approach will be described for the rapid screening

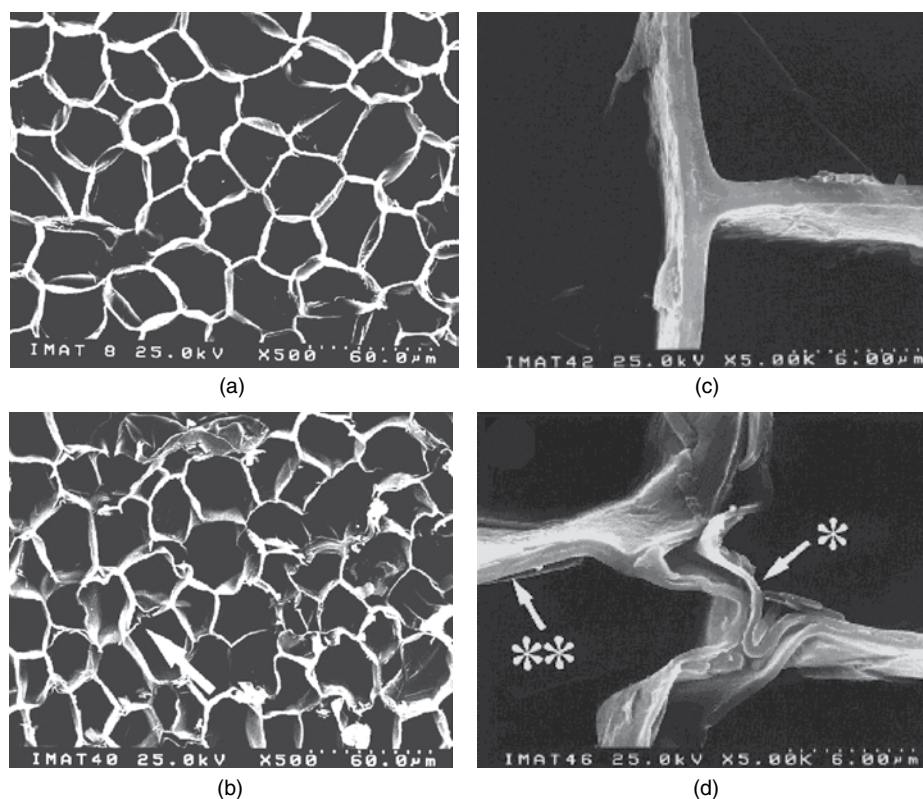


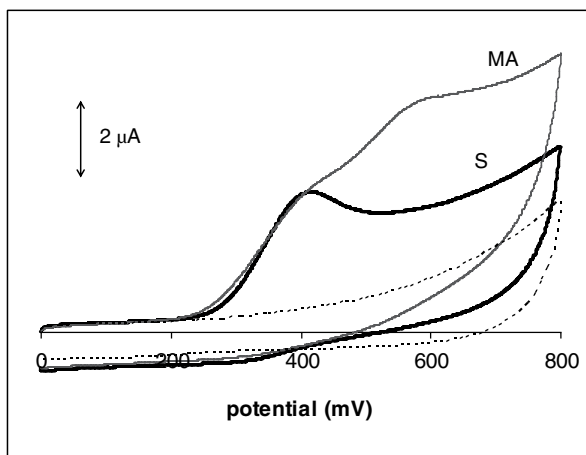
Figure 2.24 SEM photographs of tangential section of reproduction cork showing 'honeycomb' type arrangement of cells of standard cork (a), and of cork with 'mancha amarela', showing the cellular separation (b). Cell wall of standard cork (c) and of cork with 'mancha amarela', showing the cellular separation (*) and thinning of the middle lamella(**) (d). Source: Rocha *et al.*, 2000 (ACS Publishing).

of cork-wine model interactions, in order to determine if the cork stoppers were able to contaminate a wine (Rocha *et al.*, 2005).

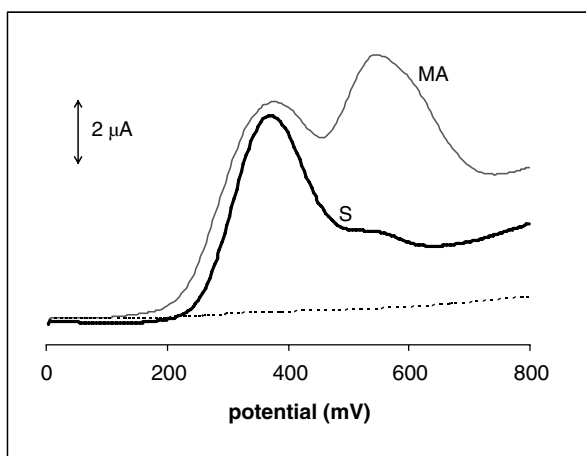
2.6.1 Evaluation of the voltammetric analysis in what concerns the cyclic and square wave technique

Figure 2.25 displays the voltammetric signature of cyclic voltammograms (Figure 2.25a) and square wave voltammograms (Figure 2.25b,c) of the S and MA cork extracts, with 15 days of contact with the wine model matrix, diluted with NaCl electrolyte (no pH adjustment).

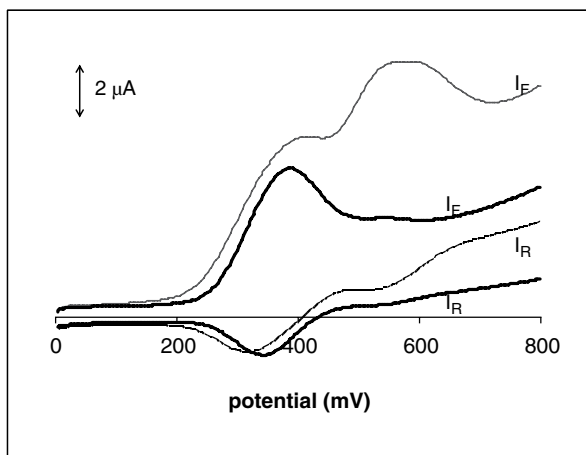
The voltammograms of both samples showed the presence of oxidisable compounds within the potential window +200 to +800 mV; the overall responses will be the sum of the various species present. For the S cork extracts, the cyclic voltammograms exhibited a major oxidation peak, at about +400 mV whereas, for the MA cork samples, a second, more intense, oxidation peak at about +580 mV was also detected. The square wave voltammograms (Figure 2.25b,c) confirm the presence of two oxidisable populations



(a)



(b)



(c)

Figure 2.25 Cyclic voltammogram (a) and square wave voltammograms (b and c) of cork extracts with 15 days of extraction time, diluted in NaCl without pH adjustment, for corks S and MA (pH extracts S = 4.4 and pH extracts MA = 5.1). Dotted curves are the background voltammograms. Voltammograms c) represent the forward (I_F) and reverse (I_R) current components of voltammograms b). Cyclic voltammetry with scan rate of 100 mV/s and square wave voltammetry with frequency 15 Hz. Source: Rocha *et al.*, 2005 (Elsevier).

at about +400 mV and 560 mV. This small deviation of peak potentials is due to the differential nature of the square wave signal. The first oxidisable population appears in both cork extracts, S and MA, but the population at more positive potentials seems to be mostly related to the cork MA. The cyclic voltammograms of both sample extracts showed lack of reversibility, because the cathodic counterparts were absent in the reverse scans. However, the analysis of the individual current components of the square wave voltammograms (Figure 2.25c) allowed the detection of small reverse (reduction) peaks for both oxidation processes.

The first anodic peak observed at 400–410 mV for both the S and MA samples ($\text{pH}_{\text{extracts S}} = 4.4$ and $\text{pH}_{\text{extracts MA}} = 5.1$) may be related to the presence of a population of phenolics containing *ortho*-diphenol groups or triphenol (galloyl) groups (Zou *et al.*, 2002) and, eventually, some flavonol glycosides (Kilmartin *et al.*, 2002). However, the oxidation of *ortho*-diphenols is generally a fully reversible process at glassy carbon electrodes (Zou *et al.*, 2002; Kilmartin *et al.*, 2002). Consequently, if present in the cork extracts, this class of compounds will be a smaller fraction. The major difference between samples S and MA is the occurrence of an important peak at ≈ 580 mV in the MA cyclic voltammograms. This peak, at more positive potential, may be due to the presence of other phenolics with a lower antioxidant strength, such as vanillic (Kilmartin *et al.*, 2002) and coumaric acids (Jorgensen and Skibsted, 1998; Kilmartin *et al.*, 2002), caffeic acid, protocatechuic acid (Filipiak, 2001), vanillyl alcohol (Evtuguin *et al.*, 2000) (all isolated phenols), or *meta*-diphenols on the A-ring of flavonoids (Kilmartin *et al.*, 2002). Considering that some of these compounds are lignin-related compounds (Rocha *et al.*, 1996), this peak can be proposed as a possible marker to follow lignin degradation. The fact that this peak, at ≈ 580 mV, is characteristic of the MA also confirms, as expected, that lignin degradation may occur in MA cork.

PCA was applied to the voltammetric data, in order to assess the differences in the analytical signals, and to recover the main signal features that characterised the S and MA corks. The scores scatterplot of PC1 $\times$ PC2 of the cyclic voltammetry data (Figure 2.26a), containing 94% of the total variability, shows a clear separation between both types of samples along PC1 (which accounts for 77% of the total variability). The signal bands (Figure 2.26b) related to this separation are located at 391 mV (PC1 negative), which characterises the S samples. At the PC1 positive side, the band located at 584 mV is related to MA samples. These results show a very different signal region contribution for the separation of samples.

The scores scatterplot of PC1 $\times$ PC2 of square wave data (Figure 2.26c), which represents 96% of the total variability, shows that the separation is, once more, possible along the PC1 axis (contains 74% of the total variability). The S samples are mainly located at the PC1 negative side, except for one misplaced sample, while MA samples are found at the PC1 positive side. The PC1 loadings profile (Figure 2.26d), shows two intense bands; the one located at 336 mV seems to be characteristic of the S samples, whereas the MA samples are related to the variations linked to the 560 mV band. The slightly different location of the bands is certainly related to the use of different voltammetric techniques – namely, due to the current acquisition regime of the square wave technique, which provides a differential current. Cyclic voltammetry was elected as the analytical technique because it provided slightly better distinction of the characteristic bands by PCA (Rocha *et al.*, 2005).

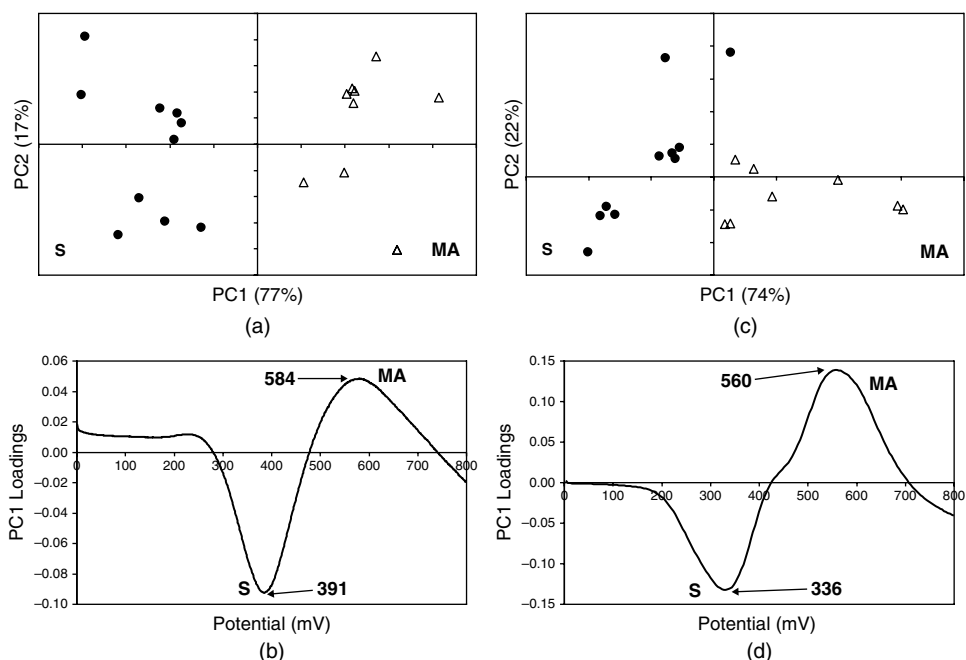


Figure 2.26 Voltammetric PCA for cork extracts without pH adjustment, 15 days of extraction time: a) scores scatter plot from the cyclic voltammetric data ($PC1 \times PC2$); b) the corresponding loadings profiles ($PC1$ and $PC2$); c) scores scatterplot from the square wave voltammetric data ($PC1 \times PC2$); d) the corresponding loadings profiles ($PC1$ and $PC2$).

2.6.2 Cyclic voltammetric analysis for cork classification

The extraction time of 15 days is time-consuming, and is not adequate for prediction purposes. Experiments with just one day of extraction were devised.

Figure 2.27 displays the voltammetric signature of the one-day extracts of S and MA cork. Two main conclusions can be drawn from these results. Firstly, the overall current is lower than the observed for the data with 15 days of extraction (*cf.* Figure 2.25a).

Taking the peak at the more negative potential as a reference (at about +380 mV), the amount of extractable phenolics of high antioxidant strength (e.g. galloyl phenolics or flavonol glycosides) for the one-day extracts may be estimated as approximately one-third of the value for the 15 days of extraction for the cork S, and two-thirds of that value for the cork MA. Therefore, this class of compounds is more rapidly extracted from the cork MA, which may be related to the high degradation of the cell tissues in this cork. Secondly, the voltammetric signature of the cork samples changed, especially for the MA cork (see Figure 2.25a), indicating qualitative changes in the population of extractables. It must be noted that there was a small shift of the peak potentials towards less positive potentials, compared to the data with 15 days of extraction, which is a consequence of the higher pH values (increase of 0.5 pH units) of the extracts with one day of extraction time. The major qualitative differences were seen for the MA cork, namely in the potential region of 560–580 mV, where the characteristic peak that was detected in the 15 days extraction samples was apparently absent. Thus, it appears that the

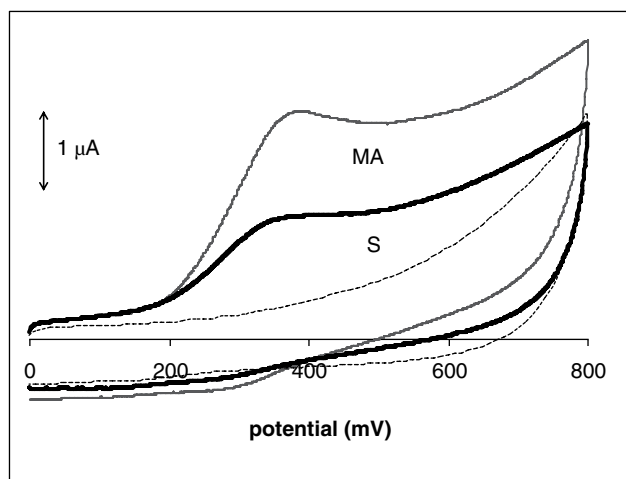


Figure 2.27 Cyclic voltammograms of cork extracts with one day of extraction time, diluted in NaCl, without pH adjustment (pH extracts S = 4.9 and pH extracts MA = 5.6), for corks S and MA. Thin line curves are the background voltammograms. Scan rate of 100 mV/s. Source: Rocha *et al.*, 2005 (Elsevier).

extraction kinetics of that population at more positive potential, which characterise MA cork, is low.

The application of a cluster analysis procedure (PLS_Cluster) to voltammetric data allows to build a discrimination model that was used to classify new samples.

PLS_Cluster (Barros and Rutledge, 2004) is a data clustering method based on the PLS algorithm (Wold *et al.*, 1982; Geladi and Kowalski, 1986). This procedure provides a way to group samples based on the inner variability and/or relationships among samples and/or variables (features) and, at the same time, gives information on the reasons for the groupings. The method is based on a self-organising mechanism that uses the PLS1 procedure to achieve a hierarchical segregation of the samples based on the variability (or relationships) present in the **X** matrix, to progressively build up a feature vector (**y**) which characterises the relationships between the objects of the **X** matrix. PLS properties/entities, such as the regression vectors, loadings **X** (**p**), **W** (**w**) and **B** coefficients (**b**), can be used to characterise the segregation (e.g. the chemical relevance). This method can be used in two different approaches: a) Dichotomic PLS_Cluster (DiPLS_Cluster); and b) Generalised PLS_Cluster (GenPLS_Cluster). The present work uses the DiPLS_Cluster approach for a binary segmentation of the samples.

The application of DiPLS_Cluster method gives the segregation of the samples shown as a dendrogram in Figure 2.28.

One can see from this dendrogram that node 1, apart from one MA and two misplaced S samples, clearly discriminates between the MA and S known groups. The S group is mostly characterised by the variation located around 201 mV, whereas the MA group is mainly related to variations located around 377 and 530 mV. The discrimination promoted by the band at 530 mV, which was unclear in the original voltammograms, is especially important, as it indicates that the population at a more positive potential, clearly seen in the former voltammograms for the 15 days contact samples, is present in the one-day contact. Again, that population is characteristic of the MA

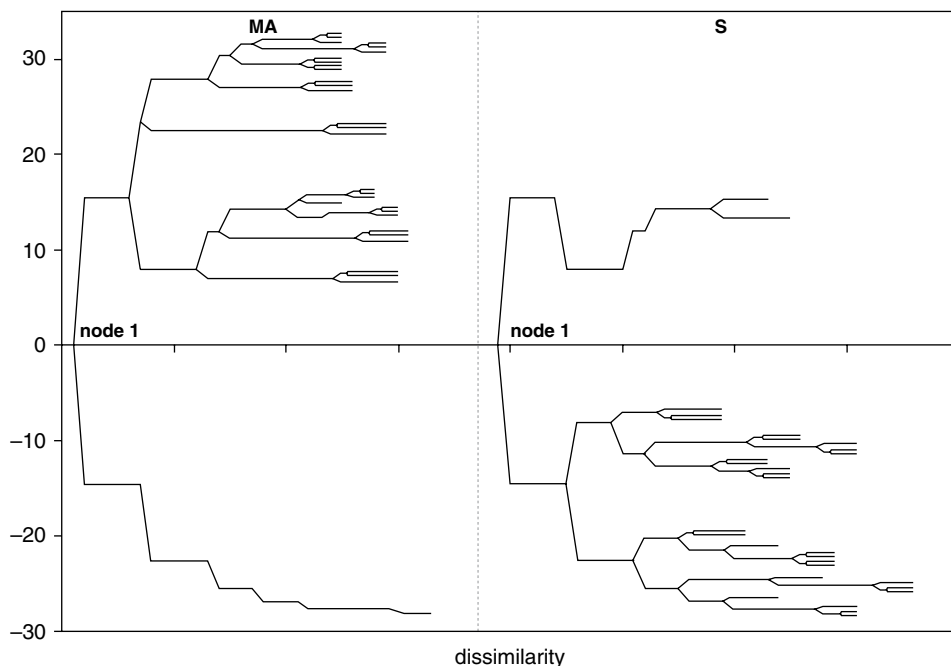


Figure 2.28 DiPLS_Cluster dendrogram plot as a function of MA and S samples (known sample classification shifted along the x axis for clarity). Source: Rocha *et al.*, 2005 (Elsevier).

cork. The variation of the potential from ≈ 580 mV (15 days of extraction time) to 530 mV is certainly related to the higher pH of the one-day extract solutions, as well as to relative changes in the individual compounds contributing the overall oxidation band.

Validation of this model showed that 80% of the data set was correctly classified as S samples, whereas 90% of the data set was correctly classified as MA cork samples. Being a natural product, and due to the various degrees of contamination, the observed classification rates can be accepted as high.

In conclusion, voltammetric methods based on the redox properties of compounds could be successfully used to establish the voltammetric signature of S and MA corks. The major difference between samples S and MA is the occurrence of an important peak at ≈ 580 mV in the MA cyclic voltammograms. This peak, at a more positive potential, may be assigned to lignin related phenolics, so it can be proposed as a possible marker to follow lignin degradation. Furthermore, the comparative analysis of the cyclic voltammograms for extraction times of one day and 15 days points to the existence of a population of easily oxidisable phenolics (oxidation peak at ≈ 380 mV), which seems to be common to both cork samples S and MA. On the other hand, the class of phenolic compounds characteristic of the MA cork presented relatively low kinetics of extraction.

The application of a hierarchical clustering analysis (DiPLS_Cluster) allowed the classification of each type of cork, which allows predicting if the cork stoppers were able to contaminate a wine. Therefore, the cyclic voltammetry associated to multivariate analysis allowed the development of a fast methodology for screening cork type.

2.7 Concluding remarks

This present chapter has shown applications supported by state-of-the-art instrumental techniques for food quality assessment. Due to the food complexity systems, and owing to the fact that the instruments used nowadays allow a deeper signal screening of those systems, it is essential to use mathematical/statistical approaches to extract meaningful information for such bio-complex systems. The results shown had elucidated the advantage and wide spread of the instrumental/chemometric approach for studying a variety of systems in a qualitative and quantitative way. The complementary nature of high throughput and fast methodologies is required to envisage wider application in agro-food industry, aiming the development of low-cost yet robust approaches for answering the ever-increasing challenges to understand and diagnose food systems.

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3

Developments in Electronic Noses for Quality and Safety Control

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3.1 Introduction

'World Health Organization (WHO) defines food-borne illness as "diseases, usually either infectious or toxic in nature, caused by agents that enter the body through the ingestion of food"' (WHO, 2007).

With the rapid growth of technology, people of this century are more prone to food-borne illnesses, which could be due to adulterants, flavouring, pesticides, preservatives, heavy metals, chemical contamination and many more. Also, around 30% of the pathogen attacks are transmitted through food causing food-borne diseases (Kuchenmüller *et al.*, 2009). At present, there are more than 250 different existing food-borne illnesses, including, to name a few (EMSA, 2015):

- Raw or undercooked foods like meat, poultry and eggs which, when infected by *Salmonella* bacteria, cause food poisoning;
- Food items like milk, meat, fish, fruits, vegetables and beverages, which are prone to be contaminated by *Staphylococcus aureus*;
- Pathogens like *Campylobacter jejuni* and *Listeria monocytogenes*, which spread via untreated drinking water, meat, poultry, unpasteurised milk and dairy products;
- *Escherichia coli* (O157:H7; O121; O157) – the other major bacterium that is associated with hemorrhagic colitis, which spreads through beef, unpasteurised milk and contaminated drinking water;
- Shellfish, harvested from polluted water, that spread Hepatitis A, a viral infection;
- Norovirus and Rotavirus, which cause gastroenteritis through contaminated shellfish – around two million lives are lost due to gastrointestinal illness (Fleury *et al.*, 2014);
- Botulism, another infection caused due to *Clostridium Botulinum* bacteria present in canned products with low acid content.

Mostly, the above-mentioned infections may also be spread when already infected people prepare the food items. Over the past years, the world has faced many food-borne illness outbreaks. In the United States, between the periods of 1998–2013, 9.4 million food-borne illnesses (Scallan *et al.*, 2011) due to bacterial and viral pathogens through food were reported annually. Among the bacterial pathogens, *Salmonella* and *Listeria* cause 55% of the illnesses, with the latter being more deadly. Norovirus is predominantly active in causing the illnesses.

Other agents to be mentioned are chemical agents (10% of outbreaks) and parasites (1%) (CDC, 2014). In both the European Union (EU) and the US, the most common serovars are *Enteritidis* and *Salmonella typhimurium*. The fatality rate caused due to *Listeriosis* is high, and is around 17.8% of the reported cases in the EU (European Food Safety Authority, 2014). The developing countries, especially Asia and Africa, have a high profile in food-borne illnesses, due to the lack of clean water for food processing, improper transportation and storage of foods, and unawareness of hygienic food processing procedures. Even in the developed countries, not all the food-borne diseases are being reported, and only 68% of the illness outbreaks are reported to the Centers for Disease Control and Prevention (Angulo *et al.*, 2006). This indicates the actual risk level of food-borne diseases.

After an extensive survey, the Food and Drug Administration (FDA) and the US Department of Agriculture (USDA) pointed out 12 major food items that are easily contaminated. Beef, pork and poultry stand first in the list, as they have higher levels of pesticides than any other plant food, because pesticides are fat-soluble and are accumulated in their fatty tissues. For the same reason milk, cheese and butter also possess a high risk of contamination. Fruits like strawberries, raspberries and cherries are heavily dosed with pesticides in America (Loux, 2011). The FDA has detected neurotoxins in apples and pears (perhaps peeling them help to reduce the toxic effect, but will not eliminate it), while grapes and mangoes have a delicate skin to prevent the penetration of pesticides and artificial ripener effects correspondingly. Also, vegetables like tomatoes, potatoes, spinach, and other greens are exposed to chemicals during the period from cultivation to consumption.

Food-borne illnesses affect the public health and, ultimately, the economy of a country (Helms *et al.*, 2003). There should be effective surveillance and monitoring systems, and emphasis should be laid on educational programs regarding awareness (Navarro, 2009; WHO, 2015). Identification of adulterants or contaminated food products from companies would result in recall of their products, leading to lay-off and economic loss and, thereby, affecting individuals as well as communities. The worldwide cost to society of food-borne diseases is in trillions (Lindqvist *et al.*, 2001; Jahan, 2012; Buzby and Roberts, 2009), even though not all food-borne diseases are reported, due to difficulties in identifying the sources of food that cause illnesses.

Food is the all-time essential commodity for everyone on the planet and we, *Homo sapiens*, make the most of it. Food that is intended for consumption should be up to standards of quality to ensure the health of the consumers, and this will save the economy in millions. Hence, reducing the rate of food-borne illness benefits individuals and society. The first step to prevent food-borne diseases is to identify the causes for the infection.

3.2 Overview of classical techniques for food quality testing

Food quality testing is one of the integral parts of the efficient production of safe food, and to ensure compliance with food safety regulations. Nearly 80% of testing is done in the way most of us learned in high school biology. First, a representative food sample is transported to the laboratories, where cell culture is done using traditional methods (Ricardi, 2010). It takes roughly 24–72 hours to identify the bacteria present in the sample. After that, a trained microbiologist will analyze the bacteria type and its count. Over the last few decades, various innovative and cutting edge technologies have been developed for food quality testing and assessment but, so far, many of them have not been successful in testing food products without affecting their taste and structure. Hence, researchers have been constantly investigating ways to prevent food items from bacteria and other parasites before reaching the consumers. Tables 3.1 and 3.2 summarise the features of the perishable food items that help to assess the quality using various analytical techniques. Subsequently, a brief overview about each technique is presented in the following sections.

Table 3.1 List of analytical methods used to assess the quality of various food items.

Food Items	Techniques	Assessed Quality	References
Milk	Clot-on- boiling	Sourness	Moran, 2013
	Alcohol test	Levels of acid, calcium or magnesium contaminant	
	Lactometer	Fat and Solid Not Fat (SNF) determination	Kleyn <i>et al.</i> , 2001
	Gerber test	Amount of fat content in milk	
	Organoleptic test	Colour, flavour and taste	Bashir, 2013
Meat	Warner–Bratzler shear force test	Tenderness	Timm <i>et al.</i> , 2003
	Slice shear force test		
	Torque Tenderometer		
	Armor Tenderometer		Shackelford <i>et al.</i> , 1999
	Tendertec Penetrometer		Sochor <i>et al.</i> , 2003
	Ultrasonic spectra analysis	Texture, fat content, collagen content	Abouelkaram <i>et al.</i> , 2000
	Magnetic Resonance Elastography	Viscoelastic Properties	Swatland, 2001
	NMR spectroscopy and imaging	Water Activity, Water Holding Capacity, pH	Venturi <i>et al.</i> , 2007
	Electron microscopy	Structural Information	Yarmand and Baumgartner, 2010

(Continued)

Table 3.1 (Continued)

Food Items	Techniques	Assessed Quality	References
Fruits and vegetables	Optical microscopy	Fat and Collagen Organization, Differentiating Fresh and Frozen Products	Oshima <i>et al.</i> , 2007
	Fluorescence spectroscopy	Freshness and Tissue Content	Egelandsdal <i>et al.</i> , 2002
	Optical measurement	Fruit firmness	Khalifa <i>et al.</i> , 2011 Abbott, 1999
	Fluorescence and delayed light emission measurement	Chlorophyll content and photosynthetic activity	Butz <i>et al.</i> , 2005
	X-Ray and CT	Internal disorders measurement, ripeness	Renu and Chidanand, 2013
	MRI	Horticultural applications – Mainly for structural information and chemical environment	Abbott, 1999 Ruiz-Altisent <i>et al.</i> , 2010
	Quasi static force	Deformation tests -Firmness of horticultural products from the obtained Force/deformation curves, the elastic modulus, sensory firmness, hardness and crispness are defined	Chen and Opara, 2013
	Impact test	Effect of handling, packing and transport on the quality of plant foods	Idah <i>et al.</i> , 2007 Altisent, 1991
	Sonic and Ultrasonic Vibration Test	Tissue horticulture	Khalifa <i>et al.</i> , 2011 Abbott, 1999 Nourain, 2012
	Statistical (image processing, Histogram) analysis	Quality assessment, quality categories and Acceptability judgements Edge recognition and texture pattern	Abbott, 1999 Singh-Ackbarali and Maharaj, 2014
	Image analysis reflectance Transmittance and absorption spectroscopy Laser spectroscopy	Physical properties and defects identification	Kim <i>et al.</i> , 2004
	Vibrated Excitation Sonic	Firmness, tenderness and structural information	
	Electromagnetic – impedance MR/MRI	Moisture, sugar and oil contents	

Table 3.1 (Continued)

Food Items	Techniques	Assessed Quality	References
Sea foods	Electrical Measurements using two and four electrodes method	Electrical properties of fish muscle	Nesvadba <i>et al.</i> , 2004
	Visible Spectroscopy	Colour depth profile from the fish muscle.	
	Image Analysis	Physical appearance	
	Texturometer	Muscle texture variation	
	Konica Minolta colorimeter	Discoloration	
Beverages	Soft Drinks and fruit juices: Membrane filter method – Nutrient Pad sets	Microbial detection	Carr <i>et al.</i> , 1976
	Benedict solution, HPLC	Specific sugar content	Azeh <i>et al.</i> , 2015
	HPLC	nitrite, nitrate, bromide, chloride and sulphate ions	Rogers and Brimelow, 2001
	Electromagnetic waves - Near infrared, Microwaves, Radio waves, Drying or titration methods, NMR	Water content	Rogers and Brimelow, 2001
	Fehling solution	Reducing sugar content	Azeh <i>et al.</i> , 2015
	IC (Ion Chromatography)	Sulphite	Rogers and Brimelow, 2001
	Reagents added:	Alcohol in soft drinks	Azeh <i>et al.</i> , 2015
	1) Iodine, potassium iodide, sodium hydroxide are the chemicals used - yellow coloured precipitate finally formed due to the presence of alcohol	Carbon dioxide test	
	2) Calcium hydroxide- lime water from colourless to milky confirmed the presence of dissolved carbon dioxide		
	Acid titration method	Quantification of acid concentration	
	Dipstick method	pH test	
	Reagents added: ammonium molybdate, nitric acid (HNO ₃)	Test for phosphate	
	Method of Wallace, Atomic absorption spectrometry (ASS) analysis	Quantification of heavy metal ions	

(Continued)

Table 3.1 (Continued)

Food Items	Techniques	Assessed Quality	References
	HPLC	Oligosaccharides, preservatives	Smyth and Cozzolino, 2013
	Enzymic methods	The presence of specific components (Eg. Malic acid)	Ashurst, 2016
	Spectrophotometric determination, Electromagnetic Waves determination - Soft ultraviolet, visible, near infrared	Colour	Rogers and Brimelow, 2001
	Imhoff cone test	Pulp content	Ashurst, 2016
	Clevenger or Cocking–Middleton procedures	Oil content	
	Colorimetric method	Yeasts, moulds and bacteria.	
	Radio waves	Structural, morphological properties	Rogers and Brimelow, 2001
	Rheological analysis	Viscosity	
	Texture assessment	Texture properties	
	GLC (Gas-Liquid Chromatography)	Pesticides	
	GC-MS	Freshness or spoilage level assessment	
	Gamma-rays	Density	
	X-rays	Detection of foreign bodies	
	Electromagnetic induction	Metallic foreign body	
	Mid infrared Spectroscopy	Volatiles	
	Acid digestion, distillation, titration and combustion methods , thermal conductivity detection	Nitrogen to protein content	
	Coffee:	Heavy metals ions	Rajković <i>et al.</i> , 2004
	Atomic Absorption spectrophotometry (AAS), SW-846-test, HPLC method		
	Reverse-phased liquid chromatography of high pressure and NIR- infrared spectral content	Caffeine content	

Table 3.1 (Continued)

Food Items	Techniques	Assessed Quality	References
	Method of direct burning, Combustion method	Ash content	Braham and Bressani, 1979
	Method of drying in the vacuum dryer	Moisture	
	Chromatography method	Mycotoxine - aflatoxine in coffee	
	NIR, Microwave technique, Solvent extraction method, NMR	Fat, protein and carbohydrate content	Rajković <i>et al.</i> , 2004 Nogueira <i>et al.</i> , 2011
	Flame atomic absorption spectrometry (FAAS), High-resolution continuumsource flame atomic absorption spectrometry (HRCS-FAAS)	Elemental composition	Madeja <i>et al.</i> , 2012
	Inductively coupled plasma optical emission spectrometry (ICP-OES)		
	Electrothermal atomic absorption spectrometry (ETAAS), Instrumental neutron activation analysis (INNA)		
Tea		Exact color detection	Singh and Kamal, 2013
	Fermenting process, Microscope, Colorimetric method		Yashin <i>et al.</i> , 2015
	Spectrophotometer	Theaflavins (TF) and thearubigins (TR)	Yashin <i>et al.</i> , 2015
	Image processing based electronic vision system	Visual parameters	
	Image acquisition process	Tea grains	
	HPLC/ HPLC-MS	Coloring agents in tea, chemical composition	
	Fluorescence spectroscopy	Tea discrimination and quality assessment	Dong <i>et al.</i> , 2010
	LED-induced fluorescence system	Tea beverages, quality assessment of dried tea leaves and liquid tea samples	
Alcoholic Beverages:		Ethyl alcohol content	Food Safety and Standards Authority of India, 2015a
	Pyknometer method		

(Continued)

Table 3.1 (Continued)

Food Items	Techniques	Assessed Quality	References
	Titrimetric method, Enzymatic and HPLC method	Aldehydes, amyl alcohol	Food Safety and Standards Authority of India, 2015a Restek Corporation, 2002
	Atomic Absorption Spectroscopy (AAS)	Copper	Food Safety and Standards Authority of India, 2015a
	Spectrophotometric method	Methyl alcohol	
	Gas chromatography	Methanol	Food Safety and Standards Authority of India, 2015a Närkki <i>et al.</i> , 2015
	Modified Monier Williams method	Sulphur dioxide (for wines only)	Food Safety and Standards Authority of India, 2015a

3.2.1 Chromatographic techniques

Gas chromatography and high performance liquid chromatography (HPLC) are the two most popular techniques widely used for food quality analysis (Luykx and van Ruth, 2008; Karoui, 2012). Generally, gas chromatography is coupled with a mass spectrometer and is used for structure and analyte identification. High sensitivity, efficient separation and assessment are the key features of this technique. However, this technique is limited, due to the derivatisation process. In other words, the sample preparations including extraction and purification have made this technique laborious and complex. Target compounds which cannot be readily volatilised can be analyzed using HPLC technique. To enhance overall sensitivity of the system, HPLC has been integrated with mass spectrometer.

3.2.2 Spectroscopic techniques

Spectroscopic techniques are fast, cost-effective and, moreover, are non-destructive. All of the spectroscopic techniques use multivariate analysis for the extraction of quantitative, qualitative and structural information from the samples (Karoui, 2012; Lohum *et al.*, 2015; Reid *et al.*, 2006). The spectroscopic techniques include ultraviolet visible spectroscopy (UV-Vis), fluorescence spectroscopy, infrared spectroscopy (IR) and nuclear magnetic resonance spectroscopy (NMR) (Danezis *et al.*, 2016). UV-Vis spectrophotometer is used for the quantification of total carotenes in margarines and quality estimation of saffron spice. The use of principle component analysis (PCA) and pattern recognition techniques are also integrated with UV-Vis spectrophotometer to differentiate the food products.

Table 3.2 List of analytical methods to quantify adulterants/pesticides in various food items.

Food Items	Adulterant / Pesticides	Techniques / Reagents used (Colour Change) to detect	References
Milk	Melamine, urea	Voltammetric studies, ELISA Method, Liquid chromatography, gas chromatography, capillary electrophoresis, Molecular imprinted polymer	Food Safety and Standards Authority of India, 2015b Zhou <i>et al.</i> , 2012 Hilding-Ohlsson <i>et al.</i> , 2012 Yu <i>et al.</i> , 2015
	Hydrogen peroxide	Electrochemical method Potassium iodide – starch (Blue)	
	Starch, detergent	Iodine (Blue)	
	Glucose	Modified Barford's reagent (faint bluish) Phosphomolybdic acid (deep blue colour)	
	Sulphates of ammonium, sodium, zinc and magnesium	Barium Chloride Trichloro acetic acid (Milky white precipitate)	
	Cane sugar (sucrose)	Resorcinol solution (Red) Lane-Eynon method	
	Urea	Reagents: p- Dimethyl amino benzaldehyde (yellow colour)	
	Ammonium compounds	Reagents: Sodium hydroxide, sodium hypochlorite and phenol solution (deep blue)	
	Sodium chloride	Reagents: Silver nitrate and Potassium chromate (yellow colour)	
	Foreign Fat in milk	Geeber butyrometer	
	Nitrates (pond water)	Reagents: Diphenylamine (Deep blue colour)	
	Neutralizers (NaOH, Na ₂ CO ₃ , NaHCO ₃)	Rosalic method Reagents: Rosalic acid powder, ethyl alcohol (rose red colour)	
	Skimmed milk powder	Reagents: Acetic acid, Phosphoric acid (blue)	
	Gelatine	Reagents: Mercury, Conc. HNO ₃ , Saturated picric acid solution	
	Salicylic acid	Reagents: Dilute HCl, ether, neutral ferric chloride solution(violet)	
	Nitrogen content	Kjeldahl method	

(Continued)

Table 3.2 (Continued)

Food Items	Adulterant / Pesticides	Techniques / Reagents used (Colour Change) to detect	References
Fruits and vegetables: Tomato, peach, pear, cucumber, apple, banana, spinach, green bean	Carbendazim, chlorpyrifos, and lambdacyhalothrin	GC, HPLC-MS (MS)	Font <i>et al.</i> , 2016
Leek, pear	Carbofuran, Spinosad	Ultra performance liquid chromatography – quadrupole- time of flight–mass spectrometry	García <i>et al.</i> , 2014
Okra, egg plant, chilli pepper	Allethrin and chlorpyrifos	Gas chromatography (GC)	Blankson <i>et al.</i> , 2016a, 2016b
Honeyed kumquat candied fruit	Dimethoate, chlorpyrifos, malathion, methidathion and triazophos	GC- flame photometric detector	Chen <i>et al.</i> , 2016
welsh onion, radish, pepper, Chinese cabbage, cucumber, eggplant, tomato	<i>'Diazinon, phorate, dimethoate, parathion- methyl, omethoate, dichlorvos, fenitrothion Fenthion, parathion, methamidophos and malathion'</i>	GC- flame photometric detector	Yu <i>et al.</i> , 2016
Mango, pineapple, apple, green pepper, tomato, carrot, lettuce, cabbage	β -HCH, δ -HCH Lindane, Heptachlor, Aldrin, Endosulfan-sulfate, Gamma-chlordane, α -Endosulfan, β -Endosulfan, P, P'-DDE, Dieldrin, Endrin, P,P'-DDT, P,P'-DD	GC-MS	Kofi, <i>et al.</i> , 2016
Persimmons, jujubes	Dimethoate, DDT, quintozene, aldrin bifenthrin, fenpropathrin, cyhalothrin, cypermethrin, Fenvalerate, deltamethrin, triadimefon and buprofezin	Gas chromatography – Flame Photometric Detector	Liu <i>et al.</i> , 2016

Table 3.2 (Continued)

Food Items	Adulterant / Pesticides	Techniques / Reagents used (Colour Change) to detect	References
Cabbage, lettuce, tomato	<i>'Dicofol, chlorpyrifos, DDTs, dimethoate, and λ-cyhalothrin dicofol, chlorpyrifos, DDTs, dimethoate, and λ-cyhalothrin'</i>	HPLC-Diode Array Detector and GC-MS	Diop <i>et al.</i> , 2016
Tomatoes and oranges	<i>'Cis/trans-permethrin, cypermethrin, deltamethrin, esfenvalerate, bifenthrin, cyfluthrin, and cyhalothrin'</i>	Quick Easy Cheap Effective Rugged Safe (QuEChERS) acetonitrile extraction and salting procedures with SPE cartridge combination with LC-MS	Li <i>et al.</i> , 2016a
Cabbage, cauliflower and cucumber	Demeton-S-methyl, Phorate, diazinon, tolclofos-methyl, malathion, fenthion, quinalphos, fenamiphos	<i>'Dynamic microwave-assisted extraction combined with Continuous-flow microextraction'</i>	Wu <i>et al.</i> , 2016
Beverages:			
Wine	Sugar beet or sugarcane	Deuterium Nuclear Magnetic Resonance method	Christoph <i>et al.</i> , 2015
Red wine	Anthocyanins with elderberries extracts	HPLC	Bridle and García-Viguera, 1996
Whiskey	Methylene chloride (Gasoline, phenol, rubber hose, hair)	GC	Rice, 1987
Wheat and rye beers	2,4-dihydroxy-1,4-benzoxazin-3-one, benzoxazolin-2-one, 6-methoxybenzoxazolin-2-one, aglycon	HPLC – DAD & Quadrupole Time-of-Flight Mass Spectrometry	Pihlava and Kurtelius, 2016
Cherry flavoured Beverges	Benzene	Headspace-gas chromatography/MS	Loch <i>et al.</i> , 2016
Orange juice	Carbendazin	Amperometry	Razzino <i>et al.</i> , 2015
Apple juices	Chlorpyrifos	UV-Vis spectrometer	Zhang <i>et al.</i> , 2010
Apple juices	Bendiocarb, Carbaryl, paraxon, malathion	Colorimetry	Luckham <i>et al.</i> , 2009

(Continued)

Table 3.2 (Continued)

Food Items	Adulterant / Pesticides	Techniques / Reagents used (Colour Change) to detect	References
Apple, grape, orange, pineapple Juices	Diazinon, ethoprophos, fenitrothion, nalathion, fenamiphos	Gas chromatography	Ravelo-Pérez <i>et al.</i> , 2008

Note: The availability of commercial measurement techniques primarily needs sophisticated equipment, calibration and maintenance, as well as trained personnel. However, the principal goal of food quality analysis demands more sensitive, non-invasive, real-time and rapid detection, portable and low-cost techniques. In some cases, a large number of samples must be analyzed rapidly. To meet these requirements, researchers have started exploring new and rapid analytical techniques with reduced cost. In this context, eNose has emerged as a possible candidate to satisfy the above stated requirement. The following sections will cover the basics, elements and applications of eNose.

On the other hand, fluorescence spectroscopy has been widely used for the quality determination of milk products (Lawrence and Weber, 1984), cheeses (Herbert *et al.*, 2000; Belletti *et al.*, 2009) detection of adulterants in virgin olive oil (Poulli *et al.*, 2007) and determination of mealiness of apples (Moshou *et al.*, 2003; Ariana *et al.*, 2006). Also, it has been utilised for the quality estimation of fish (Karoui *et al.*, 2006), rice (Fu *et al.*, 2010) and eggs (Herzallah, 2009). Near-infrared spectroscopy (NIR) and medium infrared spectroscopy (MIR) have been used for the quality determination of dairy products, meat products, olive oil, fruits and vegetables (Cen and He, 2007). NMR spectroscopy is also been used for food quality analysis. Formulation of ice cream structure has been investigated using NMR (Lucas *et al.*, 2005), which has also been used for the estimation of the water-holding capacity of fresh beef (ElMasry *et al.*, 2011).

3.2.3 Imaging techniques

Several imaging techniques have been used for the quality assessment of food products (Chen *et al.*, 2013; Mathiassen *et al.*, 2011). Hyperspectral imaging (HSI) technique has been used for the quality determination of white mushrooms (Taghizadeh *et al.*, 2010), and also has been used to determine the quality of chilled pork (Peng *et al.*, 2010) pear, citrus, strawberry and tomatoes (El-Masry *et al.*, 2007). The magnetic resonance imaging (MRI) technique has been utilised to determine the quality estimation of strawberry, apple, peach, meat products and potato (Butz *et al.*, 2005). The quality determination and assurance of apple, sweet onion, chicken, wheat and fish can be done using X-ray Imaging (XRI) technique (Narvankar *et al.*, 2009; Mery *et al.*, 2011). Detection of seal defects in food packages can be done using the ultrasound imaging (UI) technique, as can detection of the addition of foreign bodies in food products.

The thermal imaging (TI) technique has been used for the quality assessment of meat and tomato (Gowen *et al.*, 2010). Confocal scanning microscopy and the laser-induced fluorescence imaging system are the most popular techniques in fluorescence imaging (FI) that are used for the quality assessment of food products (Noh and Lu, 2007). However, the food products showing fluorescence only can be evaluated for quality.

Odour imaging (OI) is the promising technique used for odour evaluation (Suslick *et al.*, 2010). The quality determination of tea and coffee has been done using OI technique. Beer quality analysis has also been investigated with the help of PCA and hierarchical clustering analysis.

3.2.4 Biological techniques

Enzymes-based detection has been applied in quality determination of several food compounds. *'The enzyme, namely pancreatic lipase, is used for the determination of position partition of fatty acids in triglycerides, and freshness determination of fruit juices and dairy products'* (Karoui, 2012; De Pauw and Vanhooren, 1983). Enzyme-linked immunosorbent assays (ELISAs) are mostly used for the quality determination of food products. In addition, it finds its place in the detection of adulterants in honey samples and quality assessment of meat (Asensio *et al.*, 2008). DNA-based methods have been used for the quality determination of meat products (Lockley and Bardsley, 2000). Sea foods have also been investigated using DNA-based techniques for quality and species discrimination (Rasmussen and Morrissey, 2008).

3.3 Electronic Nose

In 1960, investigations were carried out to develop an instrument to detect the odour lead to the invention of a mechanical nose (Cosio *et al.*, 2012; Gardner and Bartlett, 1994). Later, in 1964, the first ever-electronic nose was developed by Wilkens and Hatman, based on a reduction and oxidation reaction at an electrode. Subsequently, in 1965, Buck developed an eNose by modulating the conductivity of the electrodes. Persaud and Dodd established the first notion of a chemical sensor array in 1982. The real-time data classification from the sensor array was demonstrated among 20 different odours and VOCs. Later, in 1984, researchers from North America constructed the portable eNose device using a chemical gas sensors array with real-time detection of VOCs. Then, developments were made to compute the relative concentrations of gas/analyte using simultaneous equations. In 1990s, the perception of an artificial olfaction system using eNose emerged (Cosio *et al.*, 2012; Tang and Goodman, 2001). A simple comparison between the biological and artificial olfactory systems is shown in Figure 3.1.

3.3.1 Various definitions of eNose reported in literature

'The instruments able to mimic the human olfactory systems comprised of an array of sensors with partially overlapping sensitivities and a pattern recognition system, capable of recognizing simple or complex odours.' This was the well-known definition for e-Nose termed by Gardner and Bartlett in 1994 (Di Francesco *et al.*, 2001). Later, several standard definitions for the eNose were coined. Some of the standard definitions of e-Nose are listed below:

- *'In particular, a "bio-electronic nose", that mimics the biological olfactory system, has the capability to identify odorant molecules with high sensitivity and specificity, overcoming the low-selectivity of the well-known electronic noses.'* – defined by Pietrantonio *et al.* (Di Pietrantonio *et al.*, 2015)

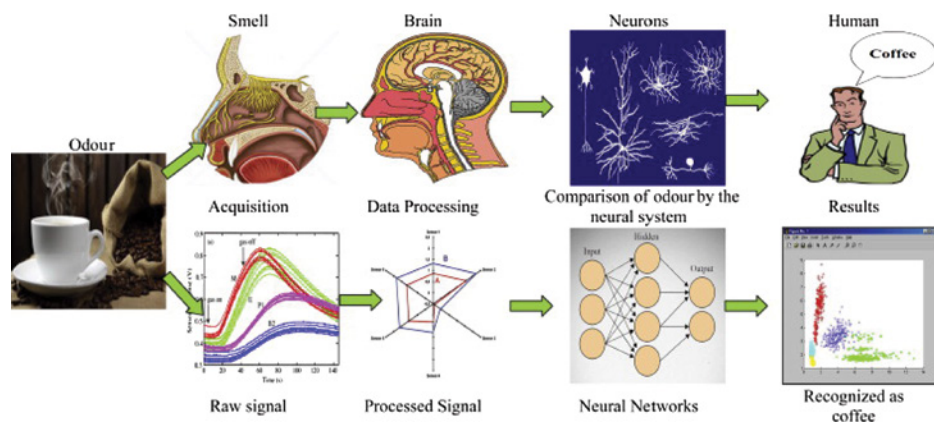


Figure 3.1 A simple comparison between biological and artificial olfactory systems, reported by Ghasemi-Varnamkhasti *et al.* (Ghasemi-Varnamkhasti and Aghbashlo, 2014).

- ‘Electronic nose (eNose) system, which imitates the perceptual mechanisms of biological olfactory using a chemical sensor array, is designed to detect and discriminate complex odours.’ – defined by Zhang *et al.* (Zhang *et al.*, 2012)
- ‘Electronic nose is an electronic instrument that sniffs odor causing volatile organic compounds (VOCs) in target samples and creates chemical fingerprints for identification. Its operational paradigm mimics mammalian smell sensing organ. An electronic nose instrument measures odor responses by using an array of chemical microelectronic sensors and combines multivariate data processing capability of pattern recognition methods’ – defined by Verma *et al.* (Verma and Yadava, 2014)
- ‘Electronic nose, a system that uses the pattern of response of an array of gas sensors to identify an odour.’ – defined by Nagle *et al.* (Nagle *et al.*, 1998)

3.3.2 Aroma as biomarker

Flavour is one of the major criteria used by consumers to accept or reject food items, and the same greatly influences repeat purchases. Many volatile compounds are responsible for the aroma in food items. Natural food spoilage occurs once the shelf life of the food item expires. It is a well known fact that rapid deterioration will occur when perishable food items are kept under inadequate storage conditions. In addition, several aromas and VOCs evolve as a result of microbial spoilage (Shewfelt *et al.*, 1985). The evaluation of the shelf life of 67 chicken meat samples, through sensory examination, chemical and microbial analysis, indicated that the concentration of ammonia constantly increases with storage time. The results obtained by the micro diffusion method for the determination of ammonia in chicken meat are given in Table 3.3.

A positive correlation between the bacterial count and ammonia content made ammonia a potential indicator of meat quality (Kozacinski *et al.*, 2012). Fatty acid profile, aldehydes (acetaldehyde, hexanal, 2-methyl-1-butanol, nonanal, 2, 4-heptadienal), ketones (2-octanone, 2-decanone, 2-propanone), trimethylamine, ammonia, volatile organic compounds (1-butanol, 1-penten-3-ol) sulfur compounds (hydrogen sulfide) are also major compounds identified as biomarkers of meat spoilage levels (Eom *et al.*, 2014; Hui, 2012; Hui *et al.*, 2001; Ray and Bhunia, 2013).

Table 3.3 Percentage of ammonia in chicken meat.

Stage	Ammonia (mg %)
Fresh meat	0.85–3.40
Meat at risk of decomposition	3.50–8.00
Meat affected with initial degradation process	8.16–8.33
Meat with moderate deviation in odour	8.33–9.00
Markedly tainted meat (rotting)	>9

In the case of fish, microbes are responsible for the formation of histamine, a toxin that is produced by converting histidine (an amino acid) into histamine. In addition to the above compounds, volatile sulphur compounds (H_2S , $(CH_3)_2$ and CH_3SH) and esters of lower fatty acids are also produced. However, there is no straightforward technique available for the determination of all volatile compounds in food items. Hence, researchers started exploring various types of sampling systems, sensors and data acquisition systems to evaluate the concentration of volatile compounds.

3.4 Instrumentation of eNose (Loutfi *et al.*, 2015)

In the recent years, the term ‘electronic nose’ has been used in a broader sense to refer to gas sensors that measure the ambient gas atmosphere’ based on the general principle that changes in the gaseous atmosphere alter the sensor properties in a characteristic way. A variety of different sensor types have been developed, to which three types of materials are commonly used: metal oxides; conducting polymer composites; and intrinsically conducting polymers. Apart from conductive sensors, gas detection has also been done using optical sensors, surface acoustic wave sensors, gas-sensitive field effect transistors and quartz microbalance (QMB) sensors. Micro-electro-mechanical systems (MEMS) and nanotechnologies are the most promising emerging technologies in the area. Once the data from the individual sensors from the array is collected, the electronic nose systems require a suitable post-processing procedure to analyze and classify the data.

3.4.1 Sampling system

Adequate isolation and pre-concentration of odour active analytes are required for the chemical characterisation of aromas (Augusto *et al.*, 2003; Peris and Escuder-Gilabert, 2009). Generally, sampling can be done using several methods, including analytical distillation methods, headspace methods and direct extraction methods. The sample-handling systems and techniques need to be designed so that they provide stability of responses by nullifying environmental influences. Generally, the static/dynamic headspace of the samples is transferred to sensor chambers through gas flow. An inert gas environment is provided inside the chamber to clean the odour already present in it. Peris *et al.* (2009) have reported the importance and methods of sampling techniques. Static headspace analysis is widely employed, due to its simplicity of operation.

3.4.1.1 Analytical distillation methods

Analytical distillation can be classified into hydro- and steam distillation (Wilkes *et al.*, 2000). Distillation techniques are used for the isolation of aroma-related compounds from food samples. In combination with GC-MS with NMR techniques, it has been used to detect terpenoid and aliphatic compounds (Sueli *et al.*, 2012) in food items.

3.4.1.2 Headspace analysis methods (HS)

Direct headspace analysis, collection of odorants in headspace using sorbent devices and cold traps are the different methods used for the chemical analysis of food. These can be further classified into static headspace, dynamic headspace and headspace solid phase micro-extraction (Peris and Escuder-Gilabert, 2009; Neta *et al.*, 2010) techniques. Static headspace analysis is the direct method of analyzing the chemical composition of an aroma, and it requires no pre-concentration of analyte. Dynamic headspace analysis provides improved sensitivity compared to the static method, but it requires pre-concentration of the analytes of interest. Headspace solid phase micro-extraction (HS-SPME) is a rapid and simple sample preparation method, widely incorporated in the detection of aromas in alcoholic beverages, raspberry, kiwi, banana and dried plum (Sueli *et al.*, 2012). It is also a pre-concentration method.

3.4.1.3 Direct extraction methods

Direct extraction procedures include liquid-liquid, solid phase supercritical fluid stir bar sorptive and inside needle dynamic extractions and membrane introduced mass spectrometry techniques (Augusto *et al.*, 2003).

3.4.2 Detection system (Loutfi *et al.*, 2015)

The chemical sensor is the core element of the detection system, and works on the principle of the change in electrical conductivity or resistivity of sensing element on exposure to a target gas. In other words, gas molecules interacting with the sensor either act as a donor or acceptor of charge carriers (*receptor function*), or alter the resistivity of the sensing element (*transduction function*). The most important parameters of the chemical sensors are selectivity, stability, sensitivity, baseline of the sensor, response time, recovery time, detection limit, dynamic range and operating temperature.

3.4.2.1 Types of chemical sensors for gaseous environment

Chemical sensors for monitoring gaseous species or aromas are generally classified as catalytic, solid electrolyte, semi-conducting metal oxide, polymer and field effect transistor based sensors (Huang and Wan, 2009; Tomchenko *et al.*, 2003; Guadarrama *et al.*, 2001). Among these sensors, metal oxide semiconductors have been widely used in the electronic nose as sensing elements. The various types of sensors used is schematically represented in Figure 3.2.

Metal oxides are the main materials used in the gas sensor industry. Investigations on metal oxides revolve around studying their properties, and methods of increasing their sensitivity and selectivity towards specific gases at different operating conditions. Specific metal oxides, such as, ' Cr_2O_3 , Mn_2O_3 , Co_3O_4 , NiO , CuO , SrO , In_2O_3 , WO_3 , TiO_2 , V_2O_3 , Fe_2O_3 , GeO_2 , Nb_2O_5 , MoO_3 , Ta_2O_5 , La_2O_3 , CeO_2 , Nd_2O_3 , ZnO , Ga_2O_3 and SnO_2 ', are suitable for sensing because of their electronic structure (Huang and Wan,

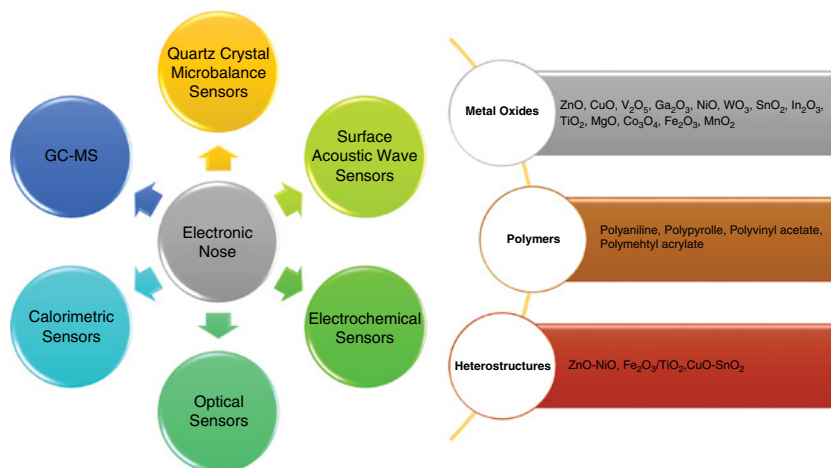


Figure 3.2 Schematic representation of sensors used in eNose.

2009). The metal oxides can be classified as *n*-type and *p*-type, based on the majority charge carriers. The *n*-types ZnO, SnO₂, In₂O₃ and WO₃, and the *p*-types NiO and CuO are the most promising materials used for sensing applications.

3.4.3 Data processing system

Pre-processing of multivariate signals in sensor arrays represents an essential part of the measuring system. Data processing techniques used in post-processing of pattern recognition routines include principal component analysis (PCA), linear discriminate analysis (LDA), partial least squares (PLS), functional discriminate analysis (FDA), cluster analysis (CA), fuzzy logic or artificial neural network (ANN), such as probabilistic neural network (PNN). Among these techniques, PCA, PLS, LDA, FDA and CA are based on a linear approach, while fuzzy logic, ANN and PNN are regarded as nonlinear methods (Scott *et al.*, 2006).

3.5 Recent developments in electronic nose applications for food quality

3.5.1 Meat

Fatty acids are the major sources for the generation of volatile compounds in meat. The volatile compounds have been formed on meat during pyrolysis of peptides and amines, and degradation of sugar and lipids (Wojtasik-Kalinowska *et al.*, 2016). Various factors influence the shelf life of the meat products, including type of slaughtering, mode of packing (individually or collectively), packing materials, and so on. Initially, electronic nose was used to discriminate between spoiled and unspoiled meat. Various other factors, like quality, ageing time, identification of microorganism, diet, storage and processing time of meat have since been analyzed using electronic nose.

In 1993, Winquist *et al.* attempted to determine the quality of beef and pork using NST 3210 emission analyzer, along with the ANN prediction algorithm, which can predict the type of meat and storage time (Winquist *et al.*, 1993). According to previous findings, the hypothesis was that high amounts of polyunsaturated fatty acids in meat muscles promote the formation of polyunsaturated fatty acid-oxidised volatile compounds, and thus inhibited the other volatile compounds (Elmore *et al.*, 2002; Van Ba *et al.*, 2013). Kalinowska *et al.* studied the antioxidant supplementation effects in pork using the Heracles II electronic nose, and found that the amount of sulfur volatile compounds decreased when the pig was supplemented with antioxidants (Vitamin E and Selenium) (Wojtasik-Kalinowska *et al.*, 2016).

Identification of fungal development of dry cured meat products was analyzed by the Artificial Olfactory System (AOS) ISE Nose 2000 by Lippolis *et al.*, and the authors found that seven volatile compounds '(2-methyl-1-butanol, octane, 1R- α -pinene, d-limonene, undecane, tetradecanal, 9-(Z)-octadecenoic acid methyl ester)' were responsible for ochratoxin A (OTA) contamination by *Penicillium* strains (Lippolis *et al.*, 2016). Kun *et al.* investigated the dry cured yak and beef using the PEN 3 portable electronic nose system, and found significant differences in flavour characteristics (Sha Kun *et al.*, 2015). In addition, the PEN3 portable electronic nose has been successfully used to monitor the Total Viable Counts (TVC) and Total Volatile Basic Nitrogen (TVB-N) in packaged pork (Li *et al.*, 2016b). A commercially available Cyranose-320 was used to discriminate the *Salmonella* contaminated beef meat from uncontaminated beef (Ghasemi-Varnamkhasti *et al.*, 2009).

3.5.2 Milk

For the quality assurance of milk, various electronic noses were developed for detecting bacterial contamination, synthetic milk flavours, milk preservatives such as hydrogen peroxide (H_2O_2), and so on. In particular, bacterial contaminations not only create some off-flavours, but can even develop some toxicity. For the early detection of bacteria or yeast, Magan *et al.* developed an electronic nose (BH-114: Bloodhound Sensors Ltd., Leeds, UK) containing 14 conducting polymer sensors. Along with discriminant function analysis (DFA), it proved that it was possible to separate unspoiled milk and milk spoiled by bacteria (Magan *et al.*, 2001). The diagram of the sampling vessel used for analysis is shown in Figure 3.3.

The milk samples were left to equilibrate for 30 minutes before analysis, and the sample connected with an air-filtered sparging system. '*The application of multilayer perceptron neural classifier, combined with multi-dimensional parameter extraction, has proved that the data collected from a 16 sensors-based electronic nose could detect and simultaneously identify a range of microorganisms in cultures*' (Gibson *et al.*, 1997).

The classification of milk from mastitic and healthy cows was identified using hybrid-sensor array system (Applied Sensor 3320, Linköping, Sweden), and dramatic changes in composition of volatile compounds were identified. Moreover, dairy products contain various volatile compounds, depending on the action of enzymes and microbial content. Around 600 volatile compounds were identified in cheese (Curioni and Bosset, 2002).

Arrays of various bulk acoustic wave sensors (BAW) were used to distinguish the varieties of cheese samples produced in the same way, collected from different animals such as cows and goats. According to GC-MS analysis 'acetic acid, 2,3-butanediol,

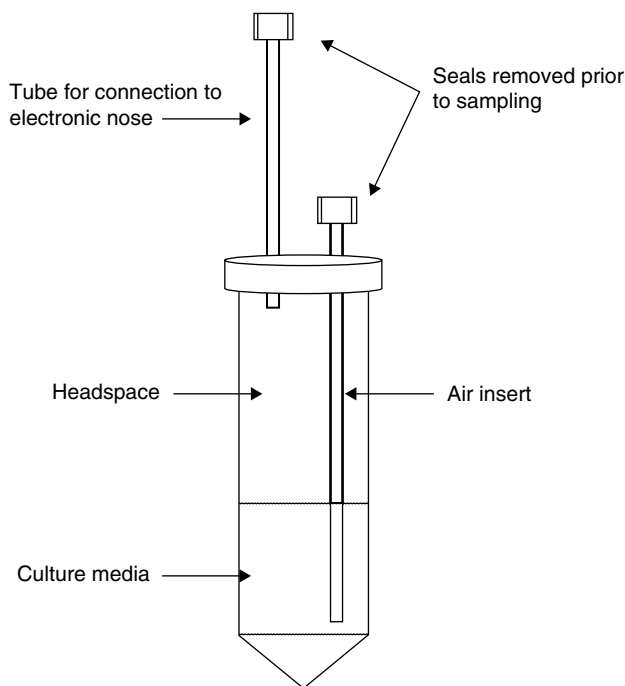


Figure 3.3 Sampling vessel used for milk quality analysis reported by Magan *et al.* (2001).

3-hydroxi-2-butanone, 3-methyl-1-butanol, 3-methylbutyl acetate, phenylethyl alcohol and ethyl acetate' were found in all kind of cheeses. However, ethyl alcohol and furanone were found in ewe cheeses only (Gomes *et al.*, 2014).

Cevoli *et al.* (2011) used EOS 507, an electronic olfactory system consisting of six metal oxide sensors, for the classification of Pecorino cheeses, and compared it with GC-MS volatile analysis. In addition, Idiazabal cheese made from ewe's raw milk and pasteurised milk was analyzed using GC-MS, and it was found that the concentration of esters was higher in raw milk cheeses than pasteurised milk cheeses. No significant concentration changes were observed in alcohol levels (Barron *et al.*, 2007). Rouseff and Cadwallader (2001) investigated the discrimination of three different disinfection resistance bacteria cultures in milk by the NST 3220 electronic nose. The identification of the formation of lactic acid was possible with this system.

3.5.3 Fish and seafood

Fish generally contains proteins, fats, fatty acids, amino acids, flavour, colour and texture in its body. These constituents depend on various parameters, including temperature of the water, farming systems, water chemistry, feeding behaviour, nutrition intake and freshness (Loutfi *et al.*, 2015). Seafood export industries have been concerned with reducing the microbial spoilage factor. The major seafood biomarkers are trimethylamine (TMA), ammonia, hydrogen sulfide, several aldehydes, and volatile organic compounds. Shelf life evaluation, species discrimination and freshness identification are the important areas where eNose has been deployed efficiently.

Zaragoza *et al.* (2015) have developed a colorimetric array of sensors for the quality assessment of squid. An optoelectronic nose, consisting of six sensing elements, was fabricated by combining different dyes, aluminium oxide and silica gels. The spoilage of the squid was monitored by both physico-chemical and microbial analyses. PCA analyses were carried out for the discrimination of squid freshness. The carbon monoxide content and microbial growth were predicted using PLS technique.

Du *et al.* (2015) have reported the freshness determination of shrimp stored at 5°C. By a combination of TVBN and sensory evaluation, the eNose model was developed. With the help of PCA analysis, 86.97% of total variation was obtained using eNose, with better accuracy. Nowadays, irrespective of freshness, an equal amount of importance has been given to fish species determination. Recently, Güney and Atasoy (2015) have reported species discrimination among horse mackerel, anchovy and whiting. In order to reduce the sensor drift, a new method has been incorporated with standard baseline manipulation. In addition to this, a sub-sampling method and a binary decision tree algorithm were also implemented for pre-processing and decision-making. The overall accuracy of species discrimination was achieved as 96.18%.

3.5.4 Fruits and vegetables

eNoses are capable of detecting and distinguishing different fruits and vegetables from bulk production. The aromas of fruits and vegetables generally evolve during the spoilage and ripening stage. Large number of VOCs are released and formed during these stages, because of various enzymes present in the damaged, as well as the fresh, portion of the fruits and vegetables (Baietto and Wilson, 2015).

All fruits and vegetables have a wide variety of aromas, which depend upon the mixture of VOCs present in them. The aromas of fruits and vegetables are major factors for consumer acceptance. Not only quality assessment, but also quality grading, can be manipulated from the sensor array results. At the particular time of ripening stage, different fruits produce different varieties of aromas, because of the chemical constituents/VOCs present in the skin and pulp regions. During the ripening stages, aromas and VOCs level in fruits fluctuate, depending upon the fruit varieties (Baietto and Wilson, 2015). Benady *et al.* stated that eNoses can even differentiate ripening fruits from fresh fruits, with an accuracy of 90.2%. Pruteane *et al.* identified seven different Romanian apples, using the FOX 4000 electronic nose (Baietto and Wilson, 2015; Pruteanu *et al.*, 2016). Around 300 VOCs were identified using the eNose in fresh apples (Baietto and Wilson, 2015). Ethyl butanoate and hexylacetate were the major aromas evolved during the ripening stage.

In grapes, terpenoids have been reported as the major aromatic compound, and the level of this aroma is found to be decreased at the ripening period. Athamneh *et al.* (2008) used the Cyranose 320 eNose for the identification of grape. Mango contains 270 versatile VOCs in total. Monoterpenes have been reported as a dominant aroma, and these contribute to the flavour (Baietto and Wilson, 2015; Caven-Quantrill and Buglass, 2007). Strawberry contains 350 varieties of VOCs (Schwab *et al.*, 2008; Bood and Zabetakis, 2002), among which furaneol, 2,5-dimethyl-4-hydroxy-3(2H)-furanone, hexanal, trans-2-hexenal and cis-3-hexen-1-ol are the major aromas specified for quality detection of strawberries (Baietto and Wilson, 2015). Qiu *et al.* used the PEN 2 eNose for the quality assessment of strawberries (Baietto and Wilson, 2015; Qiu *et al.*, 2014).

Fresh fruits and vegetables are kept at room temperatures for longer periods, or without proper conditioning, so thereby get spoiled by microorganisms. Due to these microbial substances, VOCs are released from the spoiled fruits and vegetables. Consumption of these contaminated fruits and vegetables causes various food-borne diseases, including gastrointestinal infection, pneumonia, diarrhoea, vomiting, fever, and so on. Sohrabi *et al.* (2014) reported disease-causing bacteria such as *Staphylococcus aureus* (gram-positive bacterium), *Salmonella typhimurium* (gram-negative bacterium), and *Escherichia coli*. Biomarkers are produced from bacteria, such as acetonitrile, ethanol, butanol, acetone, acetic acid and indole.

Sperber *et al.* (2009) reported many spoilage-causing bacteria in fruits and vegetables, namely apple, mango, papaya, cucumber, lemon, tomato, chilli, cabbages, onion, spinach, potatoes and luffa. 'Cyranose 320 and AlphaMOS Toulouse' eNoses were used as a major tool for detecting such kind of bacteria (Dutta *et al.*, 2002; Baldwin *et al.*, 2011).

Baietto and Wilson (2015) highlighted the use of eNoses for estimating the quality of fruits at different levels, such as post-harvest stages (FOX 4000) (Bai *et al.*, 2004), prediction of storage time (prototype MOS) (Guohua *et al.*, 2013), aroma profile during spoilage stage (Cyranose 320) (Russo *et al.*, 2013), maturity stage (Cyranose 320, prototype MOS, Aromascan, PEN2, FOX 4000) (Brezmes *et al.*, 2000; Pathange *et al.*, 2006; Oshita *et al.*, 2000; Gómez *et al.*, 2006), cultivar effect (EOS 835, FOX 4000, Enose 4000, Moses II) (Infante *et al.*, 2011; Solis-Solis *et al.*, 2007; Alasalvar *et al.*, 2003, 2012), during fertilisation stage (Aromascan) (Abbey *et al.*, 2004), ecotype effect (ISENose2000) (Russo *et al.*, 2013), shelf life (smart nose, prototype MOS, prototype QMBs, Libra Nose, PEN2, SnO₂, CTO, WO₃ – material with 18 sensors commercialised and SnO₂ – material with 10 sensors commercialised, ripening stage after harvest (EOS835, prototype MOS, SnO₂ material with 10 sensors commercialised, SnO₂ – material with two or five sensors, experimental-based) and dehydration time (enQbe) (Baietto and Wilson, 2015; Berna, 2010).

3.5.5 Adulterants

Hai and Wang detected the presence of corn oil in sesame oil with the aid of a portable electronic nose (PEN 2, Airsense corporation, Germany), and also quantified the presence of maize oil in sesame oil (Hai and Wang, 2006). Mildner-Szkudlarz and Jeleń detected the adulteration in olive oil using an MOS electronic nose (Mildner-Szkudlarz and Jeleń, 2010). Virgin olive oil (VOO) is being adulterated by adding vegetable oils or low quality olive oil. Gonzalez-Martin *et al.* used metal oxide semiconductor gas sensors to determine the level of contamination in VOO (Gonzalez-Martin *et al.*, 2001). Surface Acoustic Wave (SAW) sensor technology was used to identify the adulteration of virgin coconut oil by palm kernel (Marina *et al.*, 2010).

The main adulteration of milk is water, and reconstituted milk powder has been detected using portable electronic nose PEN 2, fabricated by WMA Airsense Analysentechnik, Germany. This is an array of 10 sensors, and it was able to detect mainly ammonia and methane (Yu *et al.*, 2007). The presence of aflatoxins M1 (AFM1) was detected by efficiently employing a commercial sensor array comprising of 12 MOS and 12 MOSFET sensors (Zoecklein *et al.*, 2011). Treated and untreated grapes, and wines with ethanol, were analyzed using conducting polymer and SAW-based gas sensors by Zoecklein *et al.* (2011).

3.5.6 Beverages

3.5.6.1 Non-alcoholic beverages

Different types of bacteria generally contaminate fruits during the harvesting and processing stages. When the contaminated fruits are used for the preparation of beverages, there is an outbreak of food-borne diseases. *Alicyclobacillus acidoterrestris* is the gram-positive bacterium responsible for the contamination of apple fruits/juices. Huang *et al.* detected the same bacterium using a portable eNose (PEN 3) (Huang *et al.*, 2015).

Orthosiphon stamineus is the bacteria responsible for the contamination of herbal tea. Zakaria *et al.* (2010) discriminated the different herbal tea samples (dried and infusion samples) using Cyranose 320 eNose. Cole *et al.* (2011) performed a simple flavour test using chemFET eNose, using conducting polymer layer as a sensing material. The different liquid samples, such as water, milks of different fat content, and orange juice were analyzed for flavour estimation using PCA.

Sharma *et al.* (2013) have used the Alpha MOS FOX 3000 eNose for the classification of odour evolved from orthodox black tea. Spiking Neural Networks (SNNs) are a special class of Artificial Neural Network (ANN), which has been used for classification of aromas from black tea. Civet is one of the most expensive coffees, which is obtained from coffee cherries, and it has been widely seen in the Philippines. The chemometric analysis of coffee was examined by Ongo *et al.* (2012) using EOS835, which contains six metal oxide (MOX) sensors, and the data analysis was done using PCA. Bougrini *et al.* (2014) have designed an eNose setup for measuring the aging time and brand analysis of milk. The hybrid eNose was used and fabricated using a micro-sensor substrate ($\text{SiO}_2/\text{Si}_3\text{N}_4/\text{SiO}_2$) membrane. Pt-doped SnO_2 and Pt-doped WO_3 were also utilised as sensing elements.

'MUST' is one of the freshly prepared fruit juices, and it contains seeds, skin and stem of the grape plant. The classification of white grape MUST from a variety of different categories was carried out by Roussel *et al.* (2003). Especially, sauvignon MUST is a special kind of grape, which produces particular aromas. The device LCA 1000 prototype, built on five SnO_2 gas sensors, was used to analyse its quality. The data analysis was done using genetic algorithms (GA), partial least square discriminant analysis (PLS-DA) and low-level data fusion. Haddi *et al.* (2014) have investigated the quality determination of different brands of commercially available Tunisian fruit juices and multivitamin juices. In total, 46 fruit juices of four different brands were analyzed. The eNose was fabricated with different metal oxide semiconductor sensors, like TGS 8XX, HIH4000-01, LM335Z.

3.5.6.2 Alcoholic beverages

Phenolic contents of red wine were classified using an array of 14 inorganic metal oxide gas sensors by Rodriguez-Mendez *et al.* (2014). These authors also have reported the relationship between the chemical parameters related to oxygen exposure and phenolic compounds in red wines. Prieto *et al.* (2011) have developed the eNose for the evaluation of micro- and nano-oxygenation levels, before and after bottling of wine. This eNose comprises of an array of 15 metal oxide sensors.

In addition, Ibá and Llobet (2011) designed a humid eNose for detecting the VOCs evolving from spoiled wine. Humid eNose consists of different electrodes, namely a

glass electrode, wires of aluminium (Al), graphite and platinum (Pt) electrodes, and the reference electrode as Ag/AgCl. Different varieties of wine with open bottles (exposure to air) were analyzed using the eNoses to help in the quality assessment of wine. Portable eNoses were used for the determination of contamination in whiskey (Wongchoosuk *et al.*, 2010). In this work, the authors used CNT-SnO₂ sensors to detect the levels of ethanol and methanol in whiskeys. This testing was to prove the quality assessment of alcoholic beverages produced by village industries.

3.6 Conclusion

Current economic trends lead to increasing the trade-off between the production and consumption of food products. Hence, it is very much essential to evaluate the quality of food products at each stage of processing. Consumers, retail and wholesale sectors demand a rapid and affordable analytical tool to guarantee the quality of food products in a non-invasive manner. Especially, food freshness is a complex parameter that includes various microbiological, physiological and biochemical reactions.

In this context, eNose technology is a potential tool which satisfies these requirements. Recent reports on development of RFID tag for meat freshness, PERES electronic nose for freshness of beef, pork, poultry and fish prove the success achieved so far. Furthermore, advancements in microfabrication techniques have aided the fabrication of cost-effective electronic nose systems. This, in turn, could help in the development of commercially viable daily use quality checking devices in the near future.

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4

Proteomics and Peptidomics as Tools for Detection of Food Contamination by Bacteria

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4.1 Introduction

Food-borne diseases are a worldwide public problem and also a significant social one. These diseases are caused by consumption of food that has been contaminated by food-borne pathogens – mostly bacteria and fungi – and their toxins. In 2013, in the European Union, 5196 outbreaks of food-borne diseases were reported (EFSA Journal, 2015). These outbreaks resulted in 43 183 infections in humans, 5196 hospitalizations, and 11 deaths. With 9.4 million estimated episodes each year occurring in the United States, resulting in 55 961 hospitalizations and 1351 deaths, the situation is even more serious (Scallan *et al.*, 2011).

There are no exact data about the outbreaks of these diseases in developing countries, but it is obvious that food-borne diseases are even a much bigger problem there. Growing globalization of the food market is an additional factor that makes food safety a universal issue (Akhtar *et al.*, 2012). The outbreak of food poisoning caused by Shiga toxin producing food-borne pathogen *Escherichia coli* O104:H4 in 2011 in both Germany and France was caused by food that was imported from a developing country (King *et al.*, 2012; Frank *et al.*, 2011). In Germany alone, 3816 cases, including 54 deaths, were reported (Frank *et al.*, 2011). These facts clearly show that the prevention of pathogens in food and their detection is, worldwide, of great economic and public health, but also of social importance.

Proteomics and peptidomics are key foodomic techniques that are indispensable for monitoring of pathogen contamination in foods during their production, storage and transportation, up to their consumption (Gallo and Ferranti, 2016). Successful prevention and detection of pathogens in food can prevent an outbreak of food-borne diseases. In this overview, food-borne bacteria and their toxins are discussed, and recent advances in proteomics and peptidomics and foodomic methods for detection of both agents are reviewed.

4.2 Bacteria as food-borne pathogens

Food matrices are known as complex ecosystems that naturally support the growth of microorganisms. This has been exploited for centuries in food processing and storage. In particular, bacteria, moulds, and yeasts have been used for thousands of years in the transformation and preservation of food through fermentation. Even today, naturally fermented foods are often traditionally produced (Bourdichon *et al.*, 2010). Consequently, it is not surprising that a wide array of foodstuffs may support the growth of favourable microflora, as well as spoilage and even the development of food-borne pathogens (Galimberti *et al.*, 2015).

The outcome of microbial growth in food intended for consumption depends on a panel of different basic physical-chemical parameters, including pH, water content and/or availability, food composition (presence and levels of proteins, lipids, carbohydrates, as well as vitamins and other micronutrients) and structure of the food. Moreover, oxido-reductive potential of food, environmental factors such as storage temperature, relative humidity and atmosphere composition, as well as food processing, preserving, distributing and storage approaches and conditions, heavily affect food quality, safety and shelf life.

All these parameters affecting microbial composition and the outburst of pathogens in food have been documented and studied for decades. However, deep molecular analyses of microbial interactions or molecular profiling of food have been made possible only in the few recent decades, upon the introduction of increasingly sophisticated and highly effective analytical techniques and technological platforms. This has tremendously improved the monitoring of whole-food production and the distribution chain, giving new insights on molecular biomarkers of food, food spoilage and identification of microbial agents in specific outbreaks of diseases caused by food-borne pathogens.

Since pathogenic bacteria are ubiquitous, food production is increasingly kept under control not only by means of technology and production/storage conditions, but also by use of specific microbial strains for controlled food processing. For example, the most important microbes in food industry today are starter cultures and probiotic bacteria, such as Gram-positive lactic acid bacteria (LAB) used for production of dairy, meat, wines and health-food supplements. In the past decade, LABs have been intensively studied, due to their inherent ability to produce a wide range of chemicals with antimicrobial activity (Gyawali and Ibrahim, 2014; Wang *et al.*, 2015a). Among them, food-grade bacteriocins, antimicrobials of bacterial origin, have shown enormous application potential as natural food preservatives and therapeutic antibiotics (Perez *et al.*, 2014; Prudêncio *et al.*, 2015).

Indeed, food preservatives are still a highly important topic in the food production sector, as deterioration by spoilage microorganisms may alter the texture, appearance, flavour and odour of the final product, along with reducing the nutritional value of food. Sometimes, the final product which has been subjected to deterioration by spoilage microorganisms is no longer edible, and may even be harmful. Such food decay includes uncontrolled putrefaction, fermentation and rancidity (Remenant *et al.*, 2014), which may be prevented more effectively by deeper understanding of molecular processes caused by microbes and the use of natural preservatives. For example, a natural bacteriocin nisin produced by *Lactococcus lactis* has been proven active against

Gram-positive and spore-forming bacteria in the food industry. More recently, a new bacteriocin-like inhibitory substance, combined with L-lactic acid, proved useful in control of food-borne pathogens *Escherichia coli* O157:H7 and *Salmonella enterica* populations on fresh, ready-to-eat spinach (Cálix-Lara *et al.*, 2014).

Unlike microbial spoilage, bacterial pathogens causing food poisoning do not, usually, deeply alter food appearance or general properties. Here, the problem is in the incubation period needed for symptoms to arise, and that can range from a few hours to a couple of days. This often hampers direct correlation of symptoms with the pathogen causing food poisoning and, as a consequence, many food-borne illnesses remain unreported. According to the European Food Safety Authority (EFSA report, 2015) and European Centre for Disease Prevention and Control (ECDC) food-borne pathogen agents are therefore classified as several groups – namely, viral, bacterial, bacterial toxins, parasites and other causative agents (chemical agents, histamine, lectin, marine and fungi toxins, as well as wax esters from fish) (Figure 4.1).

Unfortunately, the main causative agent can be defined only for about 70% of reported outbreaks of food-borne diseases, and strong correlation between the outbreak and the identified food vehicle, contributory factors and the location where the food was consumed or where the final stages of preparation took place, remains rather low (EFSA report, 2015). Consequently, there is enormous potential for the application of new, sophisticated analytical technologies and platforms. Their ability to monitor molecular processes and biomarkers at high resolution and accuracy will provide a powerful tool for better management of disease outbreaks caused by food-borne pathogens.

The most important food vehicles responsible for bacterial food poisoning are mainly from animal origin, such as meat and mixed food containing minced meat, eggs, milk, seafood and products thereof (Hennekinne *et al.*, 2015; Zeinhom and Abdel-Latef, 2014; Teplitski *et al.*, 2009; Foley *et al.*, 2011). Moreover, so-called RTE food (RTE stands for ready-to-eat food for consumption without prior cooking or washing), such as washed whole or cut fruits and vegetables, bakery items, spices, salads and sandwiches,

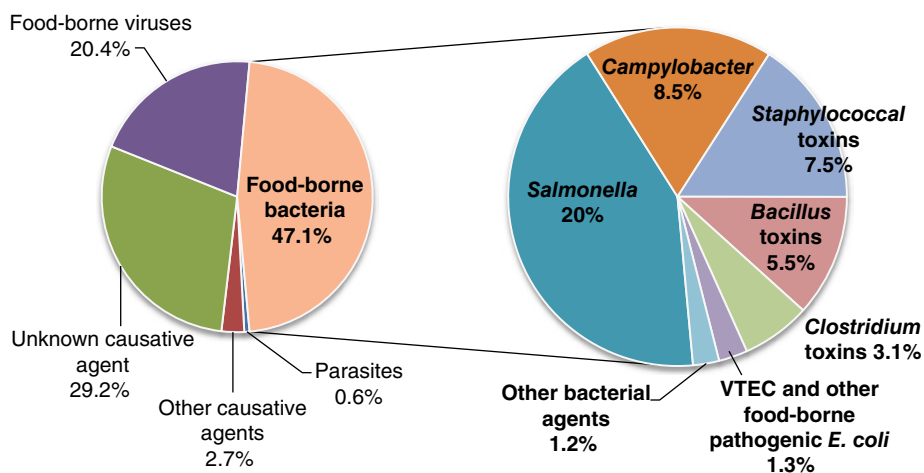


Figure 4.1 Distribution of food-borne outbreaks per causative agent in the EU during 2014 (EFSA report, 2015).

require special measures to preserve adequate levels of safety (Mertz *et al.*, 2014; Ramos *et al.*, 2013; Campos *et al.*, 2015; Proietti *et al.*, 2014; Santos *et al.*, 2012).

In particular, globalization of the food market and changes in dietary habits worldwide, such as intake of raw, unprocessed foods, semi-dry-products, protein and micro-nutrients-rich food and exotic spices, as well as the transportation of food all over the world, are new risk factors, and also a significant change in food management. Additionally, various new (or, until now, seldom-used) food production methods like free, outdoor and organic production or intensive industrial production of meat, milk and eggs are difficult to assess. Diverse minimal and/or mild food processing operations and preserving approaches, such as packaging atmosphere, mildly preserved food, and also lack of understanding of the preservation systems for exotic and traditional ethnic food, next to the climate and microbial resistance changes, are additional factors that have brought novel challenges related to the assessment of food safety and food adulterations (Rešetar *et al.*, 2015; Havelaar *et al.*, 2010; Tirado *et al.*, 2010; Doyle, 2015).

Ubiquitous food-borne bacteria are not entirely destroyed during some food production process and, as a consequence, they can occasionally re-enter the food during the production process under mild conditions (e.g. pH and temperature), and adapt to the food matrix environment. This can happen during processing, preservation or packaging phases, and spores or biofilms may appear. This kind of microbial adaptation serves as a basis for their further growth and the production of toxic, thermostable compounds. These substances can potentially remain biologically active even upon bacterial destruction (Giacometti *et al.*, 2013; Rešetar *et al.*, 2015).

According to the EFSA report, major contributory factors in microbial food contamination include inadequate heat treatment during processing or chilling throughout post-processing time, incorrect packaging and insufficient food handler hygiene. Ineffective purification and sanitizing of the manufacturing equipment and environment contribute to biofilm formation, the formation of microbial spores, cross-contamination or a combination of above-listed factors. Still, food-borne illnesses are even nowadays attributable to improper food handling at home (EFSA report, 2015).

A widely discussed subject in this regard was focused on kitchen surfaces that host remarkably diverse bacterial communities. Therefore, the possibility to transmit food pathogens from raw food items on kitchen surfaces aroused a certain level of fear. However, it has been shown that food pathogenic bacteria broadly distributed throughout the kitchens are present in low abundance, and represent a rather low risk factor for food-borne outbursts (Flores *et al.*, 2013). As a conclusion, human error in food handling and storage remains a major contributory factor for food poisoning outbreaks.

An interesting fact is that the number of food-borne outbreaks caused by viruses (adenovirus, calicivirus, hepatitis A virus (HAV), flavivirus, rotavirus and other unspecified viruses) has been constantly increasing since 2011. The highest level ever reported in EU was in 2014, representing an increase of 105.4% since 2011 (EFSA report, 2015). Still, overall food poisoning caused by bacterial food agents remains high, and is responsible for almost 50% of all food-borne outbreaks reported in the EU (Figure 4.1).

Bacterial food-borne pathogens *Campylobacter*, *Salmonella* and other bacterial agents *Yersinia*, *Escherichia*, *Shigella*, *Listeria*, *Brucella*, *Vibrio*, *Francisella* and *Leptospira*, induce food poisoning upon consumption of bacteria-contaminated food (EFSA report, 2015). Moreover, several bacteria from the *Bacillus*, *Clostridium* and *Staphylococcus* genera produce extremely potent bacterial toxins. A less common

bacterial food poisoning form, but equally important from a food safety point of view, results from ingestion of pre-formed bacterial toxins or toxin-mediated infections poisoning caused by bacteria producing toxins that grow in the host gut (Simpson, 1986).

Of particular concern are biofilm-producing pathogens, such as *Salmonella enterica*, *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus* (Tan *et al.*, 2014). A biofilm can be characterized as a very complex, but well-structured community of one or multiple species, adhered to a solid surface where a self-produced polymeric matrix surrounds the cells and protects them from dehydration, antimicrobials and sanitizing agents, enabling them to survive even harsh decontamination processes from food contact surfaces (Bridier *et al.*, 2015; Whitehead and Verran, 2015; Josic and Kovac, 2008).

Dispersal and colonization on food-processing surfaces, as well as final food products, compromise food processing and safety, and new detection, control and prevention strategies should be directed towards this issue. Certainly, a better understanding of structures as competitive and cooperative inter-bacterial interaction might provide new evidence for better understanding of biofilm formation (Giaouris *et al.*, 2015). In addition, another important food contamination group of viable but non-culturable (VBNC) and therefore extremely difficultly detectable bacteria are the *Vibrio cholerae*, enterohaemorrhagic *E. coli*, *Shigella flexneri*, and *S. enterica* (Senoh *et al.*, 2012). This topic will require innovative solutions for fast and reliable detection in the years to come.

Due to the National Control Programmes, European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC), during 2014, more than 5000 food-borne outbreaks (including water-borne outbreaks) were reported only in the EU, with almost 46 000 documented human cases and 27 deaths that were associated with *Salmonella*, *Listeria*, *Clostridium perfringens* and mushroom toxins (EFSA report, 2015). Age-related alterations in the gastrointestinal tract, changes in diet, and host immune system reactivity predictably affects the predisposition of certain groups of people to food-borne diseases. Therefore, elderly population, pregnant women, immunocompromised patients and new-borns represent the highest risk groups. All of these data urge a continuous development of technologies and procedures for assessment of food safety microbiological aspects, as the most common prevalent worldwide food hazard. It should be also noted that modern food-related problems, such as food allergies, obesity and unhealthy diets, still retain a lower impact in comparison with food poisoning on public health and economy.

4.3 Gram-positive bacteria

The thick peptidoglycan layer that stains during the standard Gram-stain test (a rapid method used to differentiate bacterial species), and that ensures the exposure of purple Gram-positive bacterial cells under the microscope, actually protects Gram-positive bacteria from environmental factors and provides certain survival advantages. Structurally, this layer consists of peptidoglycans composed of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues connected with β -(1-4)-glycosidic bond linked to the S-layer (with the exception of *B. subtilis* and some strains of *S. aureus*) on the cell wall containing anionic polymers (teichoic acid), the latter being another specific feature of Gram-positive bacteria. This extremely

complex structure of the Gram-positive bacterial cell makes it resistant to different surrounding conditions, in that it allows for a persistent resistance to some antibiotics, including the first-line antibacterial drug vancomycin – a glycopeptide antibiotic that prevents incorporation of building blocks into the peptidoglycan layer (Friedman, 2015).

In food production, *S. aureus* still poses many problems as a non-spore forming bacterium, frequently inhabiting human and animal skin and the mucous membranes of the upper respiratory tract in healthy organisms and in disease (Kluytmans and Wertheim, 2005; Kadariya *et al.* 2014). Since it has the ability to act with several virulence factors (Sandel and McKillip, 2004), for example with carotenoid staphyloxanthin (Liu *et al.*, 2008), this flexible pathogen provokes a wide spectrum of infections; minor, uncomplicated skin and respiratory infections, but also life-threatening ones, such as fatal endocarditis (Tong *et al.* 2015).

Transmission of strains of animal origin via direct contact is usually limited to veterinarians, farmers and slaughterhouse workers (Ho *et al.*, 2014). However, infections of dairy animals' mammary glands caused by *S. aureus* remain a wider problem (Chaffer *et al.*, 1999). In particular, this problem, and other bacterial infections in animals grown for food production, has been approached by increased use of antimicrobials. This usage has, however, been insufficiently controlled, and has contributed to the development of antibiotic resistant *S. aureus* strains.

Although *B. cereus* and *C. perfringens* may also develop resistance to standard antibiotic treatments, the majority of concerns are directed towards methicillin-resistant *S. aureus* (MRSA), a recognized worldwide problem in many hospitals. In addition, MRSA sequence type 398 (ST398) has been recognized as a new reservoir of MRSA in food production animals and people involved in animal production (Kluytmans, 2010). Although MRSA does not pose a major risk for food safety, since ST398 rarely possesses toxin-coding genes (Köck *et al.*, 2009), antimicrobial resistance of *S. aureus* strains continuously expands also on other food contamination agents (Chambers, 2001) through the acquisition of genetic material from other resistant organisms due to transformation, transduction and conjugation processes. Also, its ability to survive in potentially dry and stressful environments increases the survival rate of *S. aureus* during mild food processing and storage conditions (Chaibenjwong and Foster, 2011; Gustafson *et al.*, 2014; Hennekinne *et al.*, 2012).

In addition, *Staphylococcus* can grow in high salt concentrations, up to 15% of NaCl salinity, in a wide temperature range from 7°C to 48.5°C, and a pH range between 4.2 and 9.3. This bacterium is the most tolerant pathogen to low water content (Qi and Miller, 2000). However, the biggest concern from the food safety aspect in staphylococcal food intoxication is ingestion of pre-formed thermostable staphylococcal enterotoxins (SE), produced by some *S. aureus* strains (Hennekinne *et al.*, 2010; Argudín *et al.*, 2010). The onset and severity of symptoms depend on individual susceptibility to SE and the amount of ingested SE, and it should be noted that hospitalization is usually not required.

In the majority of strong evidence EU outbreaks in 2014, staphylococcal food poisoning has been associated with consummation of mixed food, pig and broiler meat and products thereof (EFSA report, 2015). Rare cases of staphylococcal food poisoning after consumption of raw milk and soft cheese (Johler *et al.*, 2015), egg products, RTE salads and cream-filled pastries, as well as cakes, sandwiches and ice-cream, have been reported only occasionally (Fetsch *et al.*, 2014). In general, food handlers' nasal or hand

transmission of exotoxin-producing strains remains an important source of food contamination associated with post-process contamination (Castro *et al.*, 2015). Equipment and environmental surfaces (e.g. poor hygiene practices and improper food handling) may cause cross-contamination with *S. aureus*, since this versatile bacterium is capable of biofilm development (Abee *et al.*, 2011). Additionally, recent genomic studies on catalase-negative *Staphylococci* (CNS) confirmed sequences homologous to *S. aureus* enterotoxins, bringing evidence on a more important role of CNS strains in food processing and human infection outbreaks (Podkowik *et al.*, 2013).

Bacterial exotoxin production is not only confined to *S. aureus*, as widely spread endospore-forming bacteria *Clostridium* and *Bacillus* have the same ability, and their endospores withstand industrial pasteurisation, food transformation conditions, DNA-damaging and hydrolytic agents (Hutchison *et al.*, 2014). Indeed, when appropriate conditions appear, *B. cereus* and *C. perfringens* spores start to germinate, repair, and continue vegetative growth, which results in the production of potent exotoxins (Warda *et al.*, 2015).

Spore-forming bacteria are known to cause food spoilage. However, inadequate heat treatment, storage time or temperature abuse on the day before serving, and spore passage through the gastro-intestinal tract, accentuates their role in toxico-infectious and intoxication food poisoning. The latter issue is an object of high priority concern for the food industry. This is due to the lack of adequate monitoring and control measures, or simple and precise detection methods, where only partial information about their frequency, diversity, morphological and biochemical elements and mechanisms are established. Therefore, after contamination, it is almost impossible to reduce their presence in raw, dehydrated or even processed food (Postollec *et al.*, 2012). For example, it is well known that improper storage temperatures after cooking have a positive effect on the growth of *C. perfringens*. Similarly, due to an inherent ability to produce five toxin types with specific actions and roles in illness development, a genetically dynamic population of *C. perfringens* is a causative agent of several diseases in humans.

The food industry remains, however, mainly focused on *C. perfringens* type A enterotoxin. Meat-containing dishes, such as bovine meat, mixed red meat and products thereof, are among the most reported food vehicles responsible for clostridium toxin outbreaks, which is not surprising, knowing that *C. perfringens* itself is incapable of synthesising 13 amino acids.

Outbreaks associated with canned food products, vegetable and juices can be associated with *C. botulinum*, which causes botulism, a rare but severe neuromuscular disease (Peck *et al.*, 2011). Food botulism is a consequence of ingestion of preformed toxin produced by *C. botulinum* Groups I (proteolytic) and II (non-proteolytic). Spores formed by strains from Group II are of moderate resistance while Group I forms highly heat-resistant spores. Therefore, to control food processing technologies, 'Botulinum cook' measure (121 °C/3 minutes) has been introduced to inactivate these spores (Carter and Peck, 2015).

The *Bacillus* genera exert food-degrading enzyme activities, and are capable of propagating at refrigerated temperatures. They are highly tolerant to an extensive pH range, and are known to produce food-poisoning toxins. For the food industry, the biggest concern are the *B. cereus* group, *B. licheniformis* and *B. subtilis* (Pirttijärvi *et al.*, 2000), the most critical among these being *B. cereus*. It is also known that some *B. cereus* strains compete with pathogenic *Salmonella* and *Campylobacter* in the host's gut, and

its presence may reduce the number of mentioned pathogens. However, one should bear in mind that pathogenic *B. cereus* strains produce an enterotoxic protein called cereulide, and an emetic toxin responsible for intoxication and toxico-infection food poisoning.

B. cereus is responsible for two types of food poisoning with different incubation times. Unlike other food toxin-producing bacteria, *B. cereus* must be present at high levels ($1 \times 10^5 - 1 \times 10^8$ CFU/g) to produce sufficient toxins leading to food poisoning (Schoeni and Wong, 2005). In the majority of strong evidence outbreaks by *B. cereus* in EU during 2014, mixed food was the most frequently reported vehicle, followed by muesli products and broiler meat (EFSA report, 2015). However, according to literature data, *B. cereus* food poisoning is frequently associated with the consumption of farinaceous, rice, meat sauces, unpasteurized milk and milk products (pudding) and vegetables as well (Ankolekar and Labbé, 2009; Pirhonen *et al.*, 2005). Moreover, even spices delivered from developing countries (Hariram and Labbé, 2015) and ready-to-reheat foods have been associated with *B. cereus* outbreaks. Commercial food establishments, such as restaurants, cafés, pubs, hotels or canteens and catering were the most reported settings (Daelman *et al.*, 2013; Rajković *et al.*, 2013).

In summary, Gram-positive bacteria have extremely well-established defence mechanisms toward stress, and remain among major concerns in food industry. Of particular interest is their ability to form various biopolymers (e.g. polysaccharides, proteins, lipids and nucleic acids) for adhesion to surface and among cells, as well as the ability to create mechanically stable three-dimensional scaffolds for biofilm generation. For example, poly-N-acetyl glucosamine (PNAG) in *S. aureus* (O'Gara, 2007) or protein BapL in *L. monocytogenes* biofilm have been described as matrix-generating molecules (Jordan *et al.* 2008). Moreover, these molecules contribute to extracellular DNA in adhesion and biofilm formation, which was confirmed for *B. cereus*, *S. aureus* and *L. monocytogenes* species (Abee *et al.*, 2011).

However, the chemical composition of extracellular matrix may vary, depending on the medium in/on which the biofilm is grown (Whitehead and Verran, 2015). The ability to develop biofilm in food-processing surfaces may also depend on abiotic and biotic factors, as well as on the resident biofilm (Renier *et al.*, 2011). In addition, for some Gram-positive strains, such as *L. monocytogenes* and *B. cereus*, flagellar-based motility has great impact on cell attachment and biofilm formation (Abee *et al.*, 2011). This adaptation is of particular importance for food production and safety control, since such biofilms enable both protection and survival of bacterial and other agents during severe decontamination procedures of food contact surfaces. Biofilms also provide an optimal environment for sporulation of bacilli in general (Bridier *et al.*, 2015; Whitehead and Verran, 2015).

Listeria monocytogenes is a facultative intracellular pathogenic, non-sporogenic bacterium and is the causative agent of a rare, but life-threatening food-borne disease – listeriosis. Acquisition of listeriosis is mainly due to consumption of contaminated food. Although the infection cycle and key virulence factors have been well characterized (Renier *et al.*, 2011), a statistically significant increasing trend of listeriosis was noted in the EU during 2014, when the highest annual number of deaths due to the consumption of contaminated food in Europe since 2009 was reported (EFSA Report, 2015).

Invasive listeriosis infections are frequently diagnosed within high-risk groups, such as the elderly population (Munoz *et al.*, 2012), as well as in immunocompromised

patients (EFSA Report, 2015). This bacterium can cross the intestinal, foeto-placental and blood-brain barriers in mammals, and enter (and multiply in) the cytosol of most human cell types, allowing its spreading throughout the organism (Cossart and Lebreton, 2014). This is particularly critical for pregnant women, causing pre-term delivery, stillbirth or, frequently, lethal neonatal infections (Mateus *et al.*, 2014). For the period of dispersal through the host's organism, virulence factors target a variety of cellular components, and consequently subvert host cell functions (Allerberger and Wagner, 2010; Cossart and Lebreton, 2014). However, only particular serovars of *L. monocytogenes*, namely 1/2a, 1/2b, and 4b, are indicated as human pathogens, and are responsible for a large part of clinical cases of listeriosis worldwide (Lomonaco *et al.*, 2015).

Listeria monocytogenes enters the food processing facility via the environment or contaminated raw materials (Muhterem-Uyar *et al.*, 2015). This bacterium is extremely potent and adaptable, due to the ability to grow in a fairly wide range of temperatures between -0.4°C and 45°C , a pH range between 4.3 and 9.6, and NaCl content up to 10%, as well as low water content. It can, indeed, spread from its original habitat across food-processing and preservation areas and equipment (Renier *et al.*, 2011). Moreover, different isolates grow under refrigerated conditions, and form genetically defined and structurally diverse biofilms on surfaces within the food production chain, which augments the likelihood of post-processing recurrent cross-contamination. In addition, mixed-species biofilms, including those of *S. aureus* strains with *L. monocytogenes*, exert increased resistance against commonly used sanitizers if compared to single species biofilm (Abee *et al.*, 2011; Rieu *et al.*, 2008).

Prevention of listeriosis is a subject of stringent commercial food establishments controls – in particular, those that provide RTE fish products, smoked fish, soft and semi-soft cheeses, RTE meat products (sausages) and hard cheeses (EFSA report, 2015). In particular, post-processing, as the weakest link, requires frequent controls and environmental sampling. Also, in-package pasteurization approaches, and/or use of preservatives such as nisin, may be used to slow down the growth of *L. monocytogenes*. In recent years, considerable effort has therefore been made to improve and develop new post-processing approaches, by the use of technology in combination with natural products as antimicrobial agents (natural preservatives in food applications), with the purpose of controlling food-borne pathogens, preserving quality and prolonging product shelf-life.

Increased demand for natural products is a trend accepted by the market and the consumers, and a variety of natural sources, including plants and animals, as well as bacteria, algae and fungi, have been subject to research for active compounds that might serve as natural preservatives. However, despite their potential, the use of natural antimicrobials in food systems remains limited, due to the side-effects of undesirable flavour or aroma.

In conclusion, ubiquitous Gram-positive food pathogens easily contaminate and withstand a variety of food production processes and conditions, including disinfection procedures. The food vehicle implicated in staphylococcal food poisoning may vary from country to country, depending on established consumer food habits (Loir *et al.*, 2003). Still, human error remains a major contributory factor to food-borne outbreaks caused by Gram-positive bacteria. Further understanding of molecular mechanisms that allow endospore formation and survival of the digestive tract, as well as growth and production of exotoxins, is still among major topics in the food research field. Bacterial

exotoxin-mediated outbreaks remain the second most reported causative agent for food poisoning in EU (Figure 4.1).

4.4 Gram-negative bacteria

Gram-negative bacterial cells do not stain by crystal violet during the standard Gram-stain test. Gram-negative bacteria *Salmonella*, *Campylobacter* and *E. coli* are ordinary inhabitants of animal and human gastrointestinal tract. However, pathogenic strains may enter the food processing environment via contaminated raw materials, or by cross-contamination through animal faeces (causative agents of less common Gram-negative bacteria food-borne infections are listed in Table 4.1). However, food poisoning outbreaks by these types of pathogens may be avoided by complete cooking and adequate storing procedures.

The World Health Organization (WHO) estimates that Gram-negative *Campylobacter* bacteria, together with norovirus, are the most frequent causes of diarrhoeal disease associated with consumption of unsafe food. Furthermore, other Gram-negative bacteria, such as the non-typhoidal *Salmonella enterica* (NTS), are causative agents of food-borne diarrhoeal illness, with the highest mortality rate in the world (WHO, 2015a). Similarly to the previously discussed global Gram-positive bacteria resistance problem, multidrug-resistant (MDR) *Campylobacter*, *Samonella*, *Typhi*, nontyphoidal salmonellae and *Shigella* (Doyle, 2015) have also been recognized.

These bacteria employ several survival mechanisms, including significant decrease of metabolic activity and 'dormant' state, cellular structural changes or production of inactivation enzymes. For example, *S. typhimurium* has 10 different types of efflux pumps, while *Campylobacter* present a characteristic CmeABC system (Olah *et al.*, 2006). Although Gram-negative bacteria have a thinner peptidoglycan layer, another structural component, a polysaccharide capsule, facilitates their survival and adherence to the host gastrointestinal epithelial cells. In addition, it has been suggested that a N-linked glycosylation structure in the Gram-negative pathogen *C. jejuni* facilitates immune avoidance, protection from gut proteases and increases colonization chances (Bolton, 2015). Moreover, immunogenic lipoproteins on the cell surface of Gram-negative bacteria such as *C. jejuni* and *Vibrio cholera* have been recently reported. Identification and understanding of surface proteins is of great interest due to the possibility of usage as vaccine targets (Willson and Bernstein, 2015).

Among the Gram-negative bacteria, *S. enterica* and *E. coli* may attach to a variety of surfaces and create biofilms. Several extracellular matrix components have been identified in *Salmonella* biofilms – for example, curli fimbrial adhesion and cellulose. It has been confirmed that these molecules contribute to biofilm formation only in specific environmental conditions, such as low nutrient conditions at ambient temperature (Castelijn *et al.*, 2012). Curli that are found in *E. coli* strains are responsible for cell adhesion and invasion, and may induce the host inflammatory response (Barnhart and Chapman, 2006).

In addition, several other fimbrial adhesins have been identified, and some of these are highly species dependent, such as SadA (*S. typhimurium*) or BapA (*S. enteritidis*) (Giaouris *et al.*, 2015). Several studies have revealed that coexistence with other bacteria promotes Gram-negative biofilm production. For example, *E. coli* O157:H7 biofilm

Table 4.1 The most common Gram-negative food-borne bacteria.

Gram-negative bacteria	Food-borne disease
Kingdom: <i>Bacteria</i>	
Phylum: <i>Proteobacteria</i>	
Class: <i>Gammaproteobacteria</i>	
Order: <i>Enterobacteriales</i>	
Family: <i>Enterobacteriaceae</i>	
Genus: <i>Escherichia</i>	watery diarrhoea or 'traveller's diarrhoea'
Species: <i>E. coli</i>	
Genus: <i>Salmonella</i>	salmonellosis
Species: <i>S. enterica</i>	
Genus: <i>Yersinia</i>	yersiniosis
Species: <i>Y. enterocolitica</i>	
Genus: <i>Shigella</i>	shigellosis or 'bacillary dysentery'
Species: <i>S. sonnei</i>	
Class: <i>Epsilonproteobacteria</i>	campylobacteriosis
Order: <i>Campylobacterales</i>	
Family: <i>Campylobacteraceae</i>	
Genus: <i>Campylobacter</i>	
Species: <i>C. jejuni</i>	
Class: <i>Gammaproteobacteria</i>	watery diarrhoea (known as 'rice-water stool')
Order: <i>Vibrionales</i>	
Family: <i>Vibrionaceae</i>	
Genus: <i>Vibrio</i>	
Species: <i>V. cholerae</i>	
Order: <i>Thiotrichales</i>	gastrointestinal tularemia
Family: <i>Francisellaceae</i>	
Genus: <i>Francisella</i>	
Species: <i>F. tularensis</i>	
Class: <i>Alphaproteobacteria</i>	brucellosis
Order: <i>Rhizobiales</i>	
Family: <i>Brucellaceae</i>	
Genus: <i>Brucella</i>	
Species: <i>B. melitensis</i>	
Phylum: <i>Spirochaetes</i>	leptospirosis
Class: <i>Spirochaetes</i>	
Order: <i>Spirochaetales</i>	
Family: <i>Leptospiraceae</i>	
Genus: <i>Leptospira</i>	
Species: <i>L. interrogans</i>	

biomass increased 180% during synergistic life with *Burkholderia caryophylli* strains. Interestingly, even adherence-deficient strains of *E. coli* can form biofilms (Whitehead and Verran, 2015).

Gram-negative, motile enterobacterium *Salmonella enterica* still remains the most commonly reported bacterial causative agent (Figure 4.1), despite well-established anti-infective strategies, guidelines and control measures for preventing salmonellosis. Typhoidal *S. enterica* serovars *Typhi* and *Paratyphi* A, B and C cause typhoidal fever and severe systemic illness, whereas non-typhoidal serovars (NTS) *Typhimurium* and *Enteridis* strains are responsible for self-limiting gastroenteritis worldwide. However, in immunocompromised patients, serious bacteraemia, with high mortality rates, may appear. Therefore, food-borne salmonellosis is often called non-typhoidal salmonellosis. Symptoms usually occur 12–36 hours after ingestion of food contaminated by pathogen bacteria. NTS infection across the intestinal epithelium induces major inflammatory response, with influx of neutrophils into the intestinal lumen (McCormick *et al.*, 1995). Again, immunocompromised adults (for example, older individuals or people infected with HIV) are highly susceptible to NTS infections.

In a recent study, it has been also suggested that NTS strains induce inflammation aimed to outcompete fermenting gut microbiota by using specific nitrate and tetrathionate by-products (Winter *et al.*, 2010). Due to extremely wide adaptation mechanisms, non-typhoidal *S. enterica* serovars are efficient in intestinal colonization, cell invasion and pathogenicity inside the broad host range. Several major pathogenicity islands (i.e. gene clusters), distributed throughout the *Salmonella* genome, as well as virulence plasmids, code for virulence factors. *Salmonella* has also the ability to produce endo- and exotoxins (cytotoxins and enterotoxins), with a wide range of biological activities (Foley *et al.*, 2013). Biofilm formation additionally contributes to interaction and infection, since extracellular matrix components, such as BapA protein, act as virulence factors (Simm *et al.*, 2014).

Non-typhoidal infections in humans are often linked to the intake of contaminated poultry meat (but less often to fresh pig and bovine meat). The most important sources of food-borne *Salmonella* outbreaks are still eggs and egg products (EFSA report, 2015). Increasing concern should be directed to newly recognized food pathogen vehicles, such as RTE foods. In addition, in EU member states, increasing occurrence of other serotypes implicated in human salmonellosis infections have been perceived, namely serotypes *S. infantis*, *S. stanley*, *S. kentucky* (EFSA report, 2015). In the USA, *S. heidelberg* serotypes have been identified as one of the most common human and poultry serotypes, with several clones.

Campylobacter is a commonly reported gastrointestinal Gram-negative, non-spore-forming bacterial pathogen in humans. This food-borne pathogen is a causative agent of human campylobacteriosis that causes moderate to severe acute diarrhoea. *Campylobacter* outbreaks have statistically increased over the seven-year period from 2008–2014 in the EU but, luckily, the mortality rate remains low (EFSA report, 2015). This spirally curved, microaerophilic bacteria is a commensal organisms in the gastrointestinal tract of many domestic animals so, therefore, direct human-animal contact and inadequate hygiene often result in contamination. Due to its ability to survive at 4°C during refrigeration, contaminated broiler meat is considered to be the most important

single source of human campylobacteriosis infections. However, *Campylobacter* is also detected in raw or low heat-treated cow's milk.

First symptoms of campylobacteriosis are headaches, fever and vomiting, which are followed by abdominal pain, and watery or bloody diarrhoea in more serious cases. Sometimes, this type of food-borne illness is even associated with the development of secondary complications, such as Guilliane-Barré and/or Millere-Fisher Syndrome. In both cases, the immune system attacks a part of the peripheral nervous cells, causing muscle weakness and respiratory failure (Lunn and Willson, 2009). *Campylobacter jejuni*, and to a lesser extent *Campylobacter coli* and *Campylobacter fetus*, are causative agents for the majority of reported human campylobacteriosis (Bolton, 2015; Wagenaar *et al.*, 2014).

Unlike other Gram-negative bacteria, such as *Salmonella* and *E. coli*, the survival and infection mechanisms in *Campylobacter* are still poorly investigated or understood. Adherence to the gastrointestinal epithelial cells is an essential step for colonisation of these bacteria in the host's digestive tract and subsequent inflammation. Several adhesins (CadE, CapA, PEB1, FlpA) on the surface of the bacterial cell, flagellar secretion system for delivery of non-flagellar proteins FlaC and Cia into the host's cytoplasm, and cytotoxins, induce cell internalisation, swelling and cell death (Bolton, 2015).

Based on studies on *C. jejuni*, it can be concluded that, despite highly restricted catabolic capacity, efficient colonization and survival in the host organism depends on a number of adaptive responses, including adherence, invasion, protein secretion and replication (Epps *et al.*, 2013). Due to the highly proficient mechanisms, about 800 *C. jejuni* bacterial cells are sufficient to arouse campylobacteriosis infection in the small intestine. This is a considerably lower infective dose than the dose needed for *S. typhimurium* or pathogenic *E. coli* strains outbreaks (Kothary and Babu, 2001). Increased resistances to the majority of antimicrobial agents are, here, also due to the observed high frequency of horizontal gene transfer. Moreover, intragenomic recombinations increase the ability of these bacteria for survival and virulence in a variety of stress conditions in the food chain (Ridley *et al.*, 2008).

Due to the ability to produce virulence factors such as exotoxins, diarrhoeagenic strains of enterobacterium *E. coli*, closely related to *S. enterica*, are well-known human food-borne pathogens. As a result of irreversible cytopathic effect on cultured Vero cells, such toxin-producing *E. coli* strains are called verocytotoxigenic (VTEC). VTEC encompasses over 100 different serotypes. However, O157:H7 is the most dominant and, if an additional virulence factor that causes lesions on human intestinal epithelium during bacterium attachment is confirmed, this enterohaemorrhagic VTEC is also the most virulent one. Enterohaemorrhagic VTEC pathotype produces toxins homologous to the shiga toxin (Stx) of *Shigella dysenteriae*. Therefore, it is often called shiga toxin-producing *E. coli* (STEC).

It is interesting to mention that VTEC O157:H7 is the main natural host in ruminants. However, EHEC serogroups O26, O111 and O103 are pathogenic for both animals and humans. Therefore, direct contact between humans and VTEC from the animal faeces, either due to improper hygiene, faecal contamination of milk and products thereof, or contamination of meat with intestinal content during slaughter and contaminated water, remain weak food chain points.

4.5 Bacterial toxins

Bacteria invade and/or damage the host's organism, often using an intertwined variety of action mechanisms. Invasive bacteria grow in the extracellular or intracellular environment, where they produce different enzymes that injure the host tissue. For example, several well-characterized mechanisms underlie *L. monocytogenes* intracellular parasitism. Adhesion to the surface of eukaryotic cells is accomplished through cell wall proteins internalin A and B. Phagocytosis enables entrance into the host cell, while listeriolysin O and phospholipases C PlcA and PlcB facilitate the exit of bacteria from the phagocytic vacuole. Actin-based motility enables cell-to cell spreading *via* membrane-anchored ActA (Renier et al., 2011).

Contrary to this physical intrusion, the majority of bacterial food pathogens act through toxins, upon autolysis or external lysis, and by extracellular toxin secretion by vegetative cells. Bacterial toxins are structurally bioactive lipopolysaccharides or proteins/polypeptides, and some of them are among the most powerful natural poisons ever known, such as the botulinum heat-labile toxin (produced by *C. botulinum*, groups I and II). Different food-borne bacterial toxins cause irreversible modifications of the host cellular targets, resulting in extensive losses in their function. As similar immunological responses and symptoms are induced by different bacterial toxins, the causative agent remains often unknown, and new technological platforms enable significant improvements in toxin identification approaches (Martinović et al., 2016).

4.5.1 Endotoxins

During the attachment and internalization processes of bacteria in the host intestine, the immune system is usually activated through the recognition of pathogen-associated molecular patterns, such as flagella and lipopolysaccharide molecules on the cell surface of Gram-negative pathogens. Indeed, pathogenicity and virulence of Gram-negative bacteria is often associated with highly acylated saccharolipid (e.g. lipopolysaccharide (LPS)). This endotoxin is a structural component of the outer bacterial membrane, also known as endotoxin.

The majority of research into the endotoxin chemical structure has been done on model organisms *S. typhimurium* and *E. coli*. From these studies, it has been found that LPS prevents the entry of small hydrophobic compounds such as antibiotics, bile salts and detergents into the bacteria, which facilitates survival and colonization in the host gut (Zhang et al., 2013). Moreover, LPS contributes to the survival of *S. enteritidis* in the egg albumen (Gantois et al., 2006).

The chromosomally encoded structure of LPS consists of three regions: highly conserved core oligosaccharide; lipid A moiety; and O-specific polysaccharide. Sialylation of LPS increases invasive potential and reduces immunogenicity; whereas lipid A acts as the toxic component. Polysaccharide side chains are the nontoxic and immunogenic components of the Gram-negative bacterial endotoxins; and polysaccharide side chains determine their virulence. Endotoxins are rarely released during bacterial growth *in vivo*, but are always present upon the cell's autolysis and external lysis (by the host's immunological mechanism or antibiotics) or phagocytic digestion of bacterial cells.

In conclusion, LPS plays an important role in immune evasion, host cell adhesion and invasion, even though recent studies on *Acinetobacter baumannii* revealed that LPS is not an essential part of the outer membrane (Zhang *et al.*, 2013).

4.5.2 Exotoxins

Exotoxins are protein or polypeptide bacterial toxins, secreted in the extracellular space both by Gram-positive and Gram-negative bacteria during exponential growth in food or on the surface of food (Table 4.2). Exotoxin production may be performed by endospore-forming bacteria, even after ingestion of food into the host intestine. Once secreted, bacterial exotoxin acts at the site of bacterial growth or invasion and, alternatively, may also spread through the host body towards target organs or cells. Target organs are correlated with the site, specificity bacterial exotoxins that are, therefore, divided into sub-groups of:

- a) enterotoxins
- b) neurotoxins
- c) leukocidins or
- d) haemolysins (Henkel *et al.*, 2010).

For example, the botulinum toxin targets only motor neurons, while some toxins produced by staphylococci and clostridia have a broad nonspecific cytotoxic activity on a different tissue. Moreover, intertwined mechanisms of action have been documented for different strains of bacterial enterotoxins. These are exotoxins that target specific receptors on the intestine cells, where it should be noted that the same pathogen can have multiple mechanisms of action with similar outcomes. For example, *E. coli* food pathogenic strains are responsible for acute watery ('traveller's') diarrhoea in developing countries, and express two conserved heat-stable enterotoxin peptides – STa and STb. Structurally different STa and STb bind to different receptors on the surface of epithelial cells within the small intestine and colon, where different cellular cascades are initiated, leading to the same outcome – accumulation of fluid within the lumen of intestine. Another *E. coli* exotoxin, the pore forming alpha-toxin (α -PFT), inserts itself into the membrane as an α -helix and causes membrane permeability (Henkel *et al.*, 2010).

In food production, special concern is placed on staphylococcal enterotoxins, as they cannot be inactivated by thermal processing and extreme pH. In contrast, *B. cereus* non-haemolytic enterotoxin is heat labile and is thus easily inactivated by standard thermal procedures. Individual susceptibility to toxins is in correlation with a number of factors – for instance, elderly, infants, and immunologically debilitated people are more sensitive and more often hospitalized. Moreover, quantity is an important factor in symptoms development and clinical presentation. For example, only 0.5 ng/mL of staphylococcal enterotoxin A is needed to cause nausea, vomiting, diarrhoea, abdominal pain and cramps (Pinchuk *et al.*, 2010). On the other hand, 30–100 ng of neurotoxin is sufficient to cause human botulism (Carter and Peck., 2015).

At last, researchers in the field are interested in studying the effects resulting from ingestion of low or moderate levels of toxins, as almost no data on this subject has been published so far (Rajković, 2014). For example, *S. aureus* exotoxins are responsible for

Table 4.2 The most common exotoxin producing food-borne bacteria.

		Biofilm formation	Spore formation	Type of food-borne illness	Bacterial exotoxins
Gram-positive bacteria					
Kingdom: <i>Bacteria</i> Phylum: <i>Firmicutes</i> Class: <i>Clostridia</i> Order: <i>Clostridiales</i> Family: <i>Clostridiaceae</i> Genus: <i>Clostridium</i>					
	Species: <i>C. perfringens</i>	YES	YES	Toxico-infectious food poisoning	<i>C. perfringens</i> type A enterotoxin
	Species: <i>C. botulinum</i>	YES	YES	Food intoxication	heat-stable botulinum neurotoxin
Class: <i>Bacilli</i> Order: <i>Bacillales</i> Family: <i>Bacillaceae</i> Genus: <i>Bacillus</i>					
	Species: <i>B. cereus</i>	YES	YES	Food intoxication	heat-stable enterotoxigenic protein cereulide
				Toxico-infectious food poisoning	thermo-labile nonhemolytic enterotoxin Nhe, haemolytic enterotoxin Hbl and cytokine K
Family: <i>Staphylococcaceae</i> Genus: <i>Staphylococcus</i>					
	Species: <i>S. aureus</i>	YES	YES	Food intoxication	heat-stable enterotoxins
Gram-negative bacteria					
Kingdom: <i>Bacteria</i> Phylum: <i>Proteobacteria</i> Class: <i>Gammaproteobacteria</i> Order: <i>Enterobacteriales</i> Family: <i>Enterobacteriaceae</i> Genus: <i>Escherichia</i>					
	Species <i>E. coli</i>	YES	NO	Food intoxication	Shiga-like toxins Stx1 and Stx2
	Species <i>S. enterica</i>	YES	NO	Food intoxication	pore-forming alpha toxin heat-stable enterotoxins STa AND STb and enterotoxin Stn

gastroenteritis, nausea, vomiting, abdominal pain/cramps and, rarely, diarrhoea upon ingestion of contaminated food but, so far, among 23 single-chain, chromosomally encoded, gastrointestinal protease-tolerant enterotoxins identified for *S. aureus*, only half of them are responsible for the development of symptoms. These toxins are superantigens (Gustafson *et al.*, 2014), and they stimulate immune activation through binding to the major histocompatibility complex II (MHCII) and peptide-independent MHCII/T cell receptor (Henkel *et al.*, 2010). According to recent reports, staphylococcal enterotoxins serotypes A and B are responsible for the majority of staphylococcal food poisoning.

The same emetic symptoms can also indicate food poisoning intoxications by emetic thermostable toxin cereulide, produced by *B. cereus* (Ceuppens *et al.*, 2012). Due to improper cooking, this cyclic 1.2 kDa dodecapeptide remains active in food, and causes intoxication symptoms (Rajković *et al.*, 2008). Cereulide is encoded by plasmid *ces* genes (Stenfors Arnesen *et al.*, 2008). In addition, *B. cereus* may produce enterotoxins in the small intestine of the host organism as well, and may again be responsible for toxicoinfectious food poisoning (Rajković *et al.*, 2013). Until now, it has been unclear whether spores germinate, grow and produce toxins as vegetative cells, or whether toxins are realised as a consequence of mother cell lysis and release of mature endospores. In particular, chromosomally encoded, thermo-labile and vulnerable to acid/protease degradation, non-haemolytic enterotoxin (Nhe) is known to cause diarrhoea, abdominal pain and cramps.

Two additional toxins have also been identified as food poisoning problems – namely, haemolytic enterotoxin (Hbl) and cytokine K (CytK). Hbl and Nhe toxins are pore-forming toxins, as they are inserted into cellular membranes, where they cause pore formation with hydrophilic interiors. This process results in the loss of cellular membrane potential and ultimately causes cell death. In addition, it is now increasingly evident that other *Bacillus* species also produce toxins responsible for food poisoning – for example, *B. pumilus* and *B. licheniformis*. However, so far, these toxins have been rarely isolated and described.

Another spore-forming bacterium, *C. perfringens*, produces various extracellular enzymes and enterotoxins (CPE), classified into five types (A to E). These toxins degrade macromolecules in the host and use degradation products as their own essential nutrients. This process results in food poisoning. In contrast to *B. cereus*, *C. perfringens* expression of the enterotoxin coding gene *cpe* in the type A strain (responsible for food poisoning) depends on sporulation in the intestine. In addition, production of usually chromosomally-encoded CPE in food poisoning strains is primary dependent upon three positive sporulation-specific sigma factors – namely, SigF, SigE and SigK.

Moreover, during early sporulation, gene expression is negatively regulated by *virX* small RNA (Freedman *et al.*, 2015). The toxin is accumulated intracellularly until the end of sporulation processes, and is released with mature endospore during mother cell lysis. 35 kDa CPE single polypeptides bind to claudin receptor on the apical surface of the small intestine or colonic cells. This binding process initiates toxin oligomerization and pore formation, activation of the Ca^{2+} influx, consequent cytotoxicity and luminal fluid accumulation (Huang *et al.*, 2004). It has been estimated that 30% of *C. perfringens* food poisoning strains carry *cpe* gene on a plasmid. This means that high-frequency transfer of plasmids in the intestine might induce changes in normal *C. perfringens* microbiota, towards prevalence of virulent strains (Freedman *et al.*, 2015).

The botulinum neurotoxin (BoNT) is, for humans, one of the most toxic food-borne contaminants. It is a 150 kDa complex protein, consisting of a heavy and a light chain. It is the causative agent of botulism, and is encoded by several *Clostridia* spp. Symptoms of botulism intoxication develop 18–36 hours after toxin ingestion. First symptoms resemble staphylococcal or *B. cereus* emetic food poisoning but, later on, paralysis occurs. Indeed, botulinum is a cleaving zinc metalloprotease that stops neural signal transmission within synaptic vesicles, leading to paralysis (Adams *et al.*, 2014).

Eight botulinum neurotoxins serotypes, A to H, have been identified so far, with more than 30 different subtypes (Hill *et al.*, 2013). Food botulism is caused by toxin serotypes A, B and E, produced by *C. botulinum* groups I and II strains (Carter and Peck, 2015). Intoxication occurs upon synthesis, when the single chain botulinum neurotoxin of little proteolytic activity forms complexes with protective non-toxinogenic proteins (e.g. non-hemagglutinin protein) to facilitate its absorption into the body. Therefore, all encoding genes for these proteins are situated together in *ha* or *orf-X* conserved neurotoxin clusters. After structural reorganization into di-chain with AB structure-function properties, botulinum toxins acquire their full activity (Henkel *et al.*, 2010).

Further studies of influence of quorum-sensing systems on sporulation regulation, the structure of conjugative plasmids, and their role in the mechanisms of food-borne diseases and antimicrobial resistance, would improve food safety mechanism. In addition, many Gram-negative bacteria produce different cytotoxins – for example, *Salmonella* serovars produce heat-labile, trypsin-sensitive cytotoxins with different molecular masses, and an enterotoxin known as Stn. Moreover, *E. coli* produces Shiga-like toxins (Stx), members of the AB5 bacterial toxin family (Foley *et al.*, 2013). The Stx1 differs by one amino acid from the original *S. dysenteriae* Shiga toxin, and is 400 times more potent (in mice). Stx2 shares 56% sequence identity with the Stx2, and is exclusive to *E. coli* (Bryan *et al.* 2015; and see also above).

Until improved food processes and control mechanisms for food processes are further developed and implemented, strict preventive measures should be maintained. For that purpose, continuous education and training for all participants of the food production chain (manufacturers, distributors, grocery stores, regulatory and control agencies, consumers) is required in order to enforce responsibility and importance of applying effective hygienic practices. Further and constant evaluation of food industry Good Manufacturing Practices (GMP) as an important part of the Hazard Analysis and Critical Control Point (HACCP) safety system in a food business remains a necessity. In particular, investment in the development of sensitive, fast and highly precise laboratory techniques and methods for the detection and identification of food pathogen organisms and their toxic compounds metabolites are urgently needed. This should dramatically increase food safety status during preparation, storage and transportation.

4.6 Detection of bacterial contamination in food

Control of bacterial contamination in food, as a part of risk management in the modern society, is undertaken at several levels, starting from raw material to the final product. Positive legislation of developed countries shares the task of control between reference

laboratories and food producers and, to a lesser extent, with consumer associations and food distributors. Authorities are making efforts to force food producers, with the introduction of mandatory food safety management systems (e.g. HACCP – Hazard analysis and critical control points), to identify physical, chemical and biological sources of food contamination, in order to prevent, eliminate or reduce the hazards to safe levels (Nyachuba, 2010).

However, different tendencies, caused by the worldwide globalization of food production and trade and new customs in food consumption, promote the occurrence of bacterial food-borne illnesses: large-scale production; new technologies in food production; wide distribution of food; worldwide transport of raw material and food supply, eating outside of home, microbial genomic diversification/emergence of new pathogens; and a growing population of at-risk consumers (Nyachuba, 2010; Newell *et al.*, 2010; Garcia-Cañas *et al.*, 2012). The United Nations World Health Organization operates surveillance through a global environment monitoring system – a food contamination monitoring and assessment program. Surveillance systems are of special importance for strengthening efforts for reduction of food-borne diseases.

Analytical tools that concerned parties have at their disposal in the struggle for food safety are standardized reference methods. Most of the current standardized reference methods for the detection of bacteria in food are based on traditional culture methods (the US Food and Drug Administration Bacteriological Analytical Manual, or the International Organization for Standardization [ISO] in Geneva). Despite reliability and robustness, traditional culture methods may display different limitations which should be overcome, in order to make food safety management systems more efficient and eligible to purpose. These methods can be laborious and time-consuming, lack quantitative information, and are unable to detect viable but non-culturable bacteria (Josefsen *et al.*, 2015; Senoh *et al.*, 2012; López-Campos *et al.*, 2012).

The development of new analytical methods for the detection of bacterial contamination in food is a challenging task, which requires a multidisciplinary approach. A special concern about newly developed methods is that they have to be validated against standard reference methods. It is important to note that a gold standard method does not mean that it is perfect. These methods can also produce false positive or false negative results. By definition, a gold standard method provides the correct results and, hence, a false positive (negative) result obtained with the gold standard method would be interpreted as a false negative (positive) result with the new method being evaluated (Wiedmann *et al.*, 2014). Thus, standard methods should be reviewed, from the sample preparation procedure, through subsequent steps, in order to perform comparison and validation of a new method (López-Campos *et al.*, 2012).

Increased sensitivity of new methods can induce confusion in regarding legal issues, since ‘safe levels’ of bacteria in a food sample are hard to define. Most countries demand ‘absence’ for most pathogens; but ‘absence’ (‘zero tolerance’) usually means ‘below detection limit’ by use of a particular method, and confusion arises if a new method is of higher sensitivity (Hoorfar, 2011).

Here, we provide a short overview on what contemporary proteomic/peptidomic tools are providing in detection and quantification of bacteria in food samples.

4.6.1 Omics methods for detection of bacteria

Different PCR/qPCR-based techniques (Law *et al.*, 2015), second and third generation sequencing techniques (Diaz-Sanchez *et al.*, 2013) and other nucleic acid-based methods (e.g. microarrays) are powerful techniques for bacterial detection. Serotyping and quantitative determination of food- and waterborne pathogen bacteria by polymerase chain reaction (Salazar *et al.*, 2015) was also performed in a high-throughput manner by the use of microfluidic quantitative PCR (Ishii *et al.*, 2013). However, the application of polymerase-based techniques in the analysis of food-borne pathogens has limitations, which may lead to false negative results. As a very complex matrix, food may contain compounds that inhibit a polymerase reaction completely or partially. Inhibiting compounds may be also introduced during sample preparation.

Strategies are being developed for specific design of internal positive control that would overcome these shortcomings (Wiedmann *et al.*, 2014; Murphy *et al.*, 2007). However, these inhibitors may not interfere with proteomic-based approaches (e.g. do not influence antibody-antigen reaction or mass spectrometry based identification), and vice versa. This is why proteomic tools are compatible with DNA-based approaches in analysis of food-borne bacterial pathogens, and assays should be validated to one another before implementation.

A proteomic tool exploited for the design of a PCR-based probe for identification and serotyping of the pandemic O3:K6 clone of *Vibrio parahaemolyticus* in seafood was described by Williams *et al.* (2004). In this investigation, a mass spectrometry-based proteomic approach was used for identification of a histone-like DNA-binding protein, whose C-terminal amino acid sequence was found to be specific for a pandemic clone of *V. parahaemolyticus*. Based on the determined amino acid sequence of this unique protein, a specific PCR probe for its open reading frame was designed. This approach, which includes comparative analysis of bacterial proteomes to search for clone specific proteins, whose sequences are then used for the design of PCR probes, is of special value, since the presence of some genetic element does not imply that this element would be actually expressed (Rosselló-Móra and Amann, 2015).

Such improved understanding of behaviour and metabolic activity of microorganisms during production processes is crucial for preserving and improving food quality, safety and nutritional value (Rantsiou *et al.*, 2011). Recent topics in this area include analysis of signalling molecules that underlie communication between microorganisms. Until now, four types of signalling molecules have been identified in bacteria, namely the autoinducer-2 family, autoinducer peptides, acylhomoserine lactones and autoinducer-3. Signalling processes based on quorum sensing in bacteria are highly dependent on population density (Rul and Monnet, 2015).

Another hot topic refers to the analyses of molecular processes underlying interactions/competitions between microbes that are naturally present, or purposely added during food processing with pathogenic ones (Gaggia *et al.*, 2011).

4.6.1.1 Proteomic and peptidomic methods

Proteomic/peptidomic tools are designed for analysis of proteins/peptides. The definition of proteomics has changed in parallel with technical and computational advances. Nowadays, with mass spectrometry-based approaches, the definition of proteomics approaches has become: 'the ensemble of all conceivable polypeptides in a species and

their modifications and organization into macromolecular assemblies' in a given time interval (Ahrens *et al.*, 2010; Mann *et al.*, 2013; Weekes *et al.*, 2014).

However, with current technologies, the full complexity of human cell/tissue proteome still cannot be accessed in a single study. It is questionable whether it can be reached with current mass spectrometry approaches. One possible way towards a complete proteome map proposed defining of a proteome on three or more levels, which are not mutually exclusive, with each level of different resolution and degree of annotation (Ahrens *et al.*, 2010). Nevertheless, a huge amount of accurate, quantitative, and biologically significant information about the proteome could be obtained by targeted (Picotti *et al.*, 2009) and shotgun (Mann *et al.*, 2013) bottom-up MS approaches. Modern mass spectrometric instrumentation brings > 5000 proteins in five hours of nanoHPLC-MS/MS analysis, with a false discovery rate for peptides as low as < 1% from 1 µl of sample volume. Deeper analysis brings ≤ 20 new proteins per hour of chromatographic gradient (Zubarev, 2013).

Although bacterial proteomes contain far fewer proteins than human ones, the dynamic range still covers over seven orders of magnitude. Moreover, concerning analysis of bacteria in food, proteome complexity could be increased during sample preparation, due to the possible modifications of these proteins by food components (e.g. some enzymes), and due to the existence of mixed culture contaminations, or contaminations with several bacterial strains of the same species. Food samples are of a different complexity, covering the whole range from no or low protein content, to tissues or whole organisms. Complex samples may contain different compounds that may interfere with the enrichment during sample preparation or with detection.

However, for identification, quantification and serotyping of particular bacterium, one specific protein, or a few of them, can be satisfactory. The question is how we can assure that this specific protein and its amount is preserved during sample preparation, to be delivered to the analytical device in a way that accurately represents the presence of a particular bacterial strain and its amount in the sampled food. Knowing that affinity-based and MS-based proteomic tools can detect femto- to atto- molar amounts present in as low volumes as 1 µl of adequately prepared sample, complicated sample preparation methods can cause a poor reproducibility among different laboratories. Moreover, methods suitable for laboratories with PhD-level operators may not necessarily perform at the same level when applied under field conditions, or performed by operators with limited expertise, training and education. Wiedmann *et al.* (2014) thoroughly reviewed assessment criteria and approaches that should be used by food industry in order to evaluate a new detection method.

As shown in Table 4.3, proteomic approaches currently in use for the detection of bacterial contamination in food are: affinity recognition combined with MS or LC-MS/MS, and combined culture based with MALDI-TOF MS detection [Martinović *et al.*, 2016].

4.6.1.2 Affinity-based methods

Affinity recognition is the most often used antibody-based approach, and benefits from high selectivity and affinity of antibodies developed against proteins specific for bacterial species of interest. Affinity recognition can be based on other highly specific protein-protein interactions, such as affinity recognition of components of a bacteriophage with bacterial cell wall components. Different technical solutions have been developed

Table 4.3 Most common bacterial toxins and proteomic methods most frequently used for their detection.

	Toxin	Detection method
Gram-positive bacteria		
Kingdom: <i>Bacteria</i>		
Phylum: <i>Firmicutes</i>		
Class: <i>Clostridia</i>		
Order: <i>Clostridiales</i>		
Family: <i>Clostridiaceae</i>		
Genus: <i>Clostridium</i>		
Species: <i>C. perfringens</i>	Epsilon toxin (ETX)	PSAQ MS/MS (Dupré <i>et al.</i> , 2015)
Species: <i>C. botulinum</i>	Botulinum neurotoxin (BoNT)	Mouse bioassay (Pellett, 2013), Endopep MS (Kalb <i>et al.</i> , 2015; Wang <i>et al.</i> , 2014; Wang <i>et al.</i> , 2015b)
Species: <i>C. difficile</i>	Toxin A (TcdA), Toxin B (TcdB)	LC MS/MS (Hensbergen <i>et al.</i> , 2014; Cafardi <i>et al.</i> , 2013)
Class: <i>Coccus</i>		
Order: <i>Bacillales</i>		
Family: <i>Staphylococcaceae</i>		
Genus: <i>Staphylococcus</i>		
Species: <i>S. aureus</i>	Staphylococcal enterotoxin (SE)	MALDI TOF MS (Sospedra <i>et al.</i> , 2011), MALDI TOF/TOF MS (Alam <i>et al.</i> , 2012), LC MS (Sospedra <i>et al.</i> , 2012a), LC MS/MS (Muratovic <i>et al.</i> , 2015), PSAQ MS (Adrait <i>et al.</i> , 2012), PSAQ MS/MS (Dupré <i>et al.</i> , 2015), IC ELISA (Liang <i>et al.</i> , 2015), Immuno capture PCR ELISA (Reddy <i>et al.</i> , 2014)
Class: <i>Bacilli</i>		
Order: <i>Bacillales</i>		
Family: <i>Bacillaceae</i>		
Genus: <i>Bacillus</i>		
Species: <i>B. cereus</i>	Cereulide	LC MS/MS (Zuberovic Muratovic <i>et al.</i> , 2014)
Species: <i>B. anthracis</i>	Anthrax lethal factor	ID MALDI TOF MS (Kuklenyik <i>et al.</i> , 2011), LC MS/MS (Kuklenyik <i>et al.</i> , 2011)
Species: <i>B. subtilis</i>	Enterotoxin	MALDI TOF/TOF (Reddy <i>et al.</i> , 2015), LC MS/MS (Šrajer Gajdošik <i>et al.</i> , 2013)

Table 4.3 (Continued)

	Toxin	Detection method
Family: <i>Listeriaceae</i>		
Genus: <i>Listeria</i>		
Species: <i>L. monocytogenes</i>	Listeriolysin O	LC MS/MS (Miyamoto <i>et al.</i> , 2015)
Gram-negative bacteria		
Kingdom: <i>Bacteria</i>		
Phylum: <i>Proteobacteria</i>		
Class: <i>Gammaproteobacteria</i>		
Order: <i>Enterobacteriales</i>		
Family: <i>Enterobacteriaceae</i>		
Genus: <i>Escherichia</i>		
Species: <i>E. coli</i>	Shiga-like toxin (Stx) Heat labile enterotoxin	MALDI TOF/TOF MS (Fagerquist <i>et al.</i> , 2014), MALDI TOF MS (Sospedra <i>et al.</i> , 2012b), LC MS (Sospedra <i>et al.</i> , 2012b)
Genus: <i>Salmonella</i>		
Species: <i>S. enterica</i>	Enterotoxin	LC MS/MS (Imami <i>et al.</i> , 2013)
Genus: <i>Yersinia</i>		
Species: <i>Y. enterocolitica</i>	Yersinia stable toxin (Yst)	MALDI TOF MS (Kanauja <i>et al.</i> , 2015)
Class: <i>Epsilonproteobacteria</i>		
Order: <i>Campylobacteriales</i>		
Family: <i>Campylobacteraceae</i>		
Genus: <i>Campylobacter</i>		
Species: <i>C. jejuni</i>	Cytolethal distending toxin (CDT)	LC MS/MS (Elmi <i>et al.</i> , 2012)

to exploit affinity recognition, including immune-diffusion discs, ELISA, lateral flow assays and biosensors (Zhao *et al.*, 2014; Hoorfar, 2011).

A serious drawback of all affinity-based methods is the cross-reactivity between similar proteins. Antibodies developed against cell wall proteins may cross-react between bacteria within the same genera, but all species of the same genera are not necessarily pathogens. In this case, cross-reactivity would lead to false positive identification. This does not necessarily need to be a drawback if the fast and low-cost screening method is used to reduce the large number of samples to a low number of positive ones, which will subsequently be analyzed by a more stringent method. Antibody microarrays incorporating several antibodies have been designed to overcome the problem of cross-reactivity. However, complex food matrices may contain substances (e.g. polyphenols, tannins) which interact or covalently modify target proteins or antibodies and induce or reduce specific binding, leading to over- or under-estimation (Zhao *et al.*, 2014; Hoorfar, 2011).

Rapid response of bacteria to their environment typically involves altered gene expression, in order to favour growth or survival under new conditions. These changes in their physiological state, typical for food processing (e.g. high/low pH, pressure, temperature, availability of O₂, exposure to UV light or disinfectants), can interfere with detection system (Denes and Wiedmann, 2014) – of particular importance for affinity-based detection systems that involve reactions with the surface of the pathogen.

4.6.1.3 Mass spectrometry-based methods

Phenotypic tests, such as colony characteristics, growth on selective agar plates, biochemical pattern characterization and Gram staining, are the methods currently used for the identification of bacteria. However, they are time-consuming and are less practical if a fast analysis is needed. Known for its sensitivity, accuracy and reproducibility, MALDI TOF MS is a method most commonly used for bacterial identification (Tevell Åberg *et al.*, 2013). This approach is very rapid, since it does not involve a sample preparation step but, rather, relies upon the introduction of a bacterial colony onto a MALDI plate. The result is a unique intact, or trypsin-digested ribosomal or intracellular protein and peptide profile of whole bacterial cells – a bacterial ‘fingerprint’ which allows for an accurate identification of bacterial contamination.

Acquired bacterial MALDI TOF MS fingerprints are matched against spectral libraries previously collected under identical MALDI conditions without further identification. Consequently, identification success remains highly dependent on the number of well-characterized food pathogen biomarker sequences available in reference databases which, in the majority of cases, are still not publicly available (Böhme *et al.*, 2012).

MALDI TOF MS can be successfully used for the discrimination between bacterial subtypes is the detection of *Mycobacterium avium* subspecies *paratuberculosis* (MAP). This pathogenic bacterium, which affects cattle and causes paratuberculosis (PTB), is heat-resistant, making its contamination of milk a major cause of MAP transmission to humans (Piras *et al.*, 2015). Enzyme Linked Immunosorbent Assay (ELISA) is the diagnostic method for detection of MAP in cattle, but the use of this technique is limited because of low sensitivity, it cannot be used for detection of early infections, and cross-reaction with environmental mycobacteria is known to occur, causing false positive results (Whitlock *et al.*, 2000).

A 2D gel electrophoresis, coupled with MS as a more sensitive method for early detection of MAP, has been recently developed (Piras *et al.*, 2015). Lin *et al.* (2015) have also shown that MALDI TOF MS is applicable for rapid discrimination of *M. avium* from other *Mycobacterium* species. These results also demonstrate the serious potential of this method for clinical application and for early detection of MAP, as well as for other food-borne pathogen bacteria and their toxins.

The isolation of microorganisms and their culturing still remains a major bottleneck for further implementation of MALDI TOF MS for early and fast detection of food-borne pathogens (Biswas and Rolain, 2013). For that reason, sample preparation protocols for viable but non-culturable (VBNC) or difficult-to-culture food poisoning bacterial strains such as *Vibrio cholerae*, enterohaemorrhagic *E. coli*, *Shigella flexneri*, and *Salmonella enterica* should be developed (Senoh *et al.*, 2012). It is important to note that not all bacteria pose a health threat themselves, but proteins that they secrete can be severely toxic to humans. The disadvantage of MALDI TOF MS is that this technique can only provide information on occurrence or absence of food-contaminating

bacteria, and it does not give any data about the expression of toxin encoding genes or toxin levels in food.

4.7 Analysis of bacterial toxins

Bacterial endotoxins are lipopolysaccharides (LPS), located in the outer membrane of Gram-negative bacteria. These agents are seldom secreted during bacterial growth, but can be released after lysis of bacteria resulting from either autolysis or external lysis – for example, as a result of the effects of antibiotics or phagocytic digestion by the host's immune system. Thus, endotoxins most often act close to bacterial growth. They are moderately toxic and heat stable (Rešetar *et al.*, 2015).

Exotoxins, on the other hand, are proteins secreted by both Gram-positive and Gram-negative bacteria. Compared to endotoxins, they are more potent and more specific, because they act enzymatically. Since they are mostly secreted, they act at a site that can be distant from the original place of bacterial growth. Some exotoxins are released only upon bacterial lysis (Rajković, 2014). About 16% of all food-borne outbreaks in 2013 in the EU were caused by bacterial exotoxins produced mainly by *Bacillus cereus*, *Clostridium botulinum*, *Clostridium perfringens* and *Staphylococcus aureus* (Scallan *et al.*, 2011).

Staphylococcus aureus-secreted enterotoxins (SE) are bacterial exotoxins that are gastrointestinal protease-tolerant. There are over 20 defined serotypes of these proteins, but staphylococcal enterotoxin A (SEA) causes 80% of staphylococcal food-borne illnesses, while the next one, staphylococcal enterotoxin B (SEB) is responsible for just 10%, and only 10% of residual poisonings are caused by other SE exotoxins (Piras *et al.*, 2015). SE contamination in food is mainly due to poor hygiene during production (Lin *et al.*, 2015). These proteins have a remarkable ability to resist food treatments by heat, acidity and pH changes, which makes them persist in food substrates during processing. MALDI TOF MS is used for detection of SE.

Prior to sample analysis, separation of proteins is usually done by liquid chromatography, but gel-based techniques are still used. After protein separation by SDS PAGE protein, subsequent band excision, and protein extraction followed by tryptic digestion, SEA was determined. When SEA extracted from milk was analyzed, 13 peptides were revealed with 58% sequence coverage, while 19 peptides were identified as products of trypsin cleavage of the SEA standard, with 73% coverage of the protein sequence (Sospedra *et al.*, 2011). Among other toxins, *Clostridium epsilon* toxin (ETX) and SEA, were also analyzed with MALDI TOF TOF MS, and ETX was detected in aerosol at 0.2 ppb concentration (Alam *et al.*, 2012). Furthermore, MALDI TOF TOF MS has been used for the identification of Shiga toxin 2 (Stx2) subtypes from 26 Shiga toxin-producing *E. coli* strains (Fagerquist *et al.*, 2014).

Although most heat-stable exotoxins cannot be inactivated by thermal processing of food during production, heat labile deadly botulinum neurotoxin (BoNT) is easily denatured (Kalb *et al.*, 2015). This extremely potent toxin causes botulism, a severe illness with a very low survival rate. Since BoNT has proteolytic activity, the toxicity of some strains of *Clostridium botulinum* is dependent upon the existence of exotoxins that are secreted, and not the actual bacteria (Kalb *et al.*, 2015).

The quantitative and widely accepted assay for BoNT detection is the *in vivo* mouse bioassay. In this assay, mice are injected with different amount of BoNT, and toxicity

levels are observed (Pellett, 2013). This bioassay has been the gold standard for BoNT detection for years, but large experimental error, high costs and also ethical concerns are the main reasons for development of further, alternative *in vitro* methods for identification and quantitative determination of this highly toxic protein. Immunological methods, such as Enzyme Linked Immunosorbent Assay (ELISA), are more sensitive than the mouse bioassay, but they have the limitation of false positive results due to cross-reactions, as well as detection of both active and inactive forms of this enterotoxin (Tevell Åberg *et al.*, 2013). Further assays are based on the endopeptidase activity of BoNT's intrinsic enzymatic function, and a mass spectrometer is coupled to the reaction chamber for rapid detection of substrate cleavage location. Kalb *et al.* (2015) used this approach for differentiation of the serotype of this protein.

MALDI-TOF MS and HPLC ESI MS/MS have been directly compared for an endopeptidase activity assay-based quantification of the *Bacillus anthracis* toxin, a protein with intrinsic enzymatic activity called Anthrax lethal factor (Kuklenyik *et al.*, 2011). Since the quantitative data resulting from both platforms were very similar, isotope dilution MALDI TOF MS is a faster and more robust and precise quantitative MS technique. Wang *et al.* (2015b) developed novel peptide substrates for an Endopep-MS approach to detect all seven BoNT serotypes, but opted for MALDI TOF MS because of the short sample analysis times and high throughput capability. The same group also demonstrated the use of this method for quantitative detection of four BoNT serotypes (Wang *et al.*, 2014b).

Although MALDI TOF is a powerful mass spectrometric technique, most analyses of staphylococcal enterotoxins, are still based on immunoaffinity assays (Fagerquist *et al.*, 2014; Kalb *et al.*, 2015; Pellett, 2013). However, these assays require a specific antibody against a certain enterotoxin and, so far, there are only few available: staphylococcal enterotoxin A to E, G, H; and staphylococcal enterotoxin like Q (Kalb *et al.*, 2015). Development of antibodies against enterotoxins is difficult and expensive, and unspecific cross-reactivity between the antibody and molecules with similar properties as enterotoxins is quite common. Therefore, antibody-free methods are growing more popular.

A label-free bottom-up proteomic approach for the detection of SEA and SEB in milk and shrimp has been reported. After tryptic digestion, ¹³C-labeled internal standard peptides were used for isotope dilution LC ESI MS/MS using Multiple Reaction Monitoring (MRM) on a triple quadrupole mass spectrometer (Kalb *et al.*, 2015). The toxins were identified with detection levels of 2.5 and 5 ng/g levels of SEA and SEB, respectively. Sospedra *et al.* (2012a) looked for the same toxins in milk and fruit juices using single, instead of tandem, MS on a triple quadrupole, and achieved detection levels lower by a factor of 10. Such differences in detection levels could be explained by variable recoveries due to analyte loss during sample preparation.

To overcome this problem, the Protein Standard for Absolute Quantification (PSAQ) strategy was recently proposed, which uses an isotope-labelled whole-protein analogue of the protein target. The labelled protein is added to the matrix, resulting in the same handling of the target and the standard protein, which leads to robust quantification. Using such assay, developed for the quantification of SEA in serum, the toxin was detected by a hybrid quadrupole/linear ion trap (LTQ) mass spectrometer with a detection limit of 352 pg/mL (Adrait *et al.*, 2012).

Dupré *et al.* (2015) used the same method on a LTQ Orbitrap to quantify SEB, ricin and ETX in complex human biofluids and food matrices, with lower limits of detection close to $1 \text{ ng}\cdot\text{mL}^{-1}$. Finally, levels of *B. cereus* toxin cereulide in rice and pasta were determined by LC-MS/MS MRM with 13C6-cereulide toxin as internal standard, making this a superior method to previous ones that used the antibiotic valinomycin as a standard (Zuberovic Muratovic *et al.*, 2014).

Bacterial secretome analysis allows for clarification of the role of secreted, membrane and cell wall proteins in pathogenicity. Different strategies are employed to pre-fractionate the whole proteome in order to enrich the low abundance proteins, which are often not detected during the initial analysis (Yang *et al.*, 2012). Enany *et al.* (2012) used sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and strong cation exchange fractionation, both combined with tandem mass spectrometry, to analyze the secretomes of a methicillin resistant *Staphylococcus aureus* strain (MRSA). A total of 174 distinct proteins were identified, with the expression of some proteins such as enterotoxins U and B being identified for the first time in this strain. The same group combined SDS PAGE with LC MS/MS to examine the proteomes of MRSA and methicillin susceptible *S. aureus* (MSSA). They identified 261 extracellular proteins in MRSA and 168 in MSSA, respectively. Out of 144 proteins unique to MRSA, some are suggested to be the most probable virulence factors of this strain (Enany *et al.*, 2014).

Quiblier *et al.* (2013) provided new insights on the relevance of SecDE, an accessory protein in the Sec secretion pathway, in *S. aureus* pathogenicity. Using LC-MS/MS, these authors showed that deletion of *secDF* leads to an altered exoproteome, resulting in *S. aureus* having reduced adhesion, invasion and cytotoxicity.

The same protein, SecDE, was studied with respect to the secretion of *Bacillus cereus* toxins. A *secDF* knock-out mutant showed slower growth rate, reduced virulence and motility. It was demonstrated by use of label-free mass spectrometry that three most extensively studied diarrheal enterotoxins, non-haemolytic enterotoxin (Nhe), haemolysin BL (Hbl), and cytotoxin K (CytK), were less abundant in the secretome of the ΔsecDF mutant than in the wild type bacteria (Vörös *et al.*, 2014). Secretome of *B. cereus* was further analyzed using SDS PAGE prefractionation, combined with a LTQ Orbitrap XL mass spectrometer (Clair *et al.*, 2010).

In the natural environment, *Bacillus cereus* grows in the oxygen-limited small intestine. In the following experiments, it was cultured under three redox conditions (low oxiredution potential (ORP) anoxic, high (ORP) anoxic and fully oxic), after which the secretomes were compared. A total of 57 secreted virulence-related proteins were detected, out of which 31 were found for the first time in the secretome of this bacteria (Clair *et al.*, 2010). Further analysis of the identified proteins suggested that the redox dependent regulatory pathway may modulate the expression of several virulence factors.

The same instrument, LTQ Orbitrap XL, was used for the evaluation of the role of EntD, a *B. cereus* exoprotein, in the production of numerous virulence factors. Proteomics were used for in-depth characterization of an *entD* knockout mutant and for investigation of the profiles and functions of cellular and extracellular proteins controlled by EntD (Omer *et al.*, 2015). Furthermore, exoproteome analysis of a novel strain of *B. cereus*, using a 2DE MS approach, demonstrated that this strain is implicated in a disease similar to cutaneous anthrax (Ghosh *et al.*, 2014).

A proteomic investigation of the food-borne pathogen *Bacillus subtilis*, and the change of its proteome under inhibition of growth, was performed by quantitative

label-free LC MS/MS and a subsequent bioinformatic analysis (Šrajer Gajdošik *et al.*, 2013), and several differently expressed proteins were detected, not only in this micro-organism but also in a couple of other investigated food-borne pathogenic bacteria. Those proteins were mostly cellular enzymes and cofactors involved in protein synthesis and energy metabolism, as well as chaperones, including heat shock proteins (Šrajer Gajdošik *et al.*, 2013).

A multipronged quantitative proteomics approach was used to investigate changes in *B. subtilis* after treatment with antimicrobial agent totarol. A total of 139 proteins were found to be differently expressed using two DE, DIGE and iTRAQ analyses. Several central metabolic primary dehydrogenases, such as glyceraldehyde 3 phosphate dehydrogenase and succinate dehydrogenase, were significantly downregulated. Upregulated proteins were those involved in anaerobic respiration (nitrate reductase and lactate dehydrogenase are the most important ones), as well as enzymes involved in heme biosynthesis and cell homeostasis (Reddy *et al.*, 2015).

In order to identify novel extracellular factors of *Clostridium difficile*, a Gram-positive spore-forming bacterium that infects the human colon, causing diarrheal infections (Ternan *et al.*, 2014), proteomic techniques were used. The secretomes of the three *C. difficile* strains were examined and compared, and 158 differently expressed proteins were identified, most of them of cytoplasmatic origin (Boetzkes *et al.*, 2012). Ternan *et al.* (2014) used LC/MS, based on an exponentially modified Protein Abundance Index (emPAI) spectral counting method, to determine proteomic changes in response to heat stress of this bacterium, and 65 proteins (37%) were modulated by 1-5 fold or more when this bacterium was grown at 41°C, compared to the growth at 37°C. Moreover, two novel zinc metalloproteases implicated in *C. difficile* toxicity were identified by LC MS/MS (Hensbergen *et al.*, 2014; Cafardi *et al.*, 2013).

Secretome of another highly pathogenic *Clostridium* species, *C. perfringens*, has been widely studied (Sengupta *et al.*, 2010; Alam *et al.*, 2009). Comparative proteomic analysis of proteins secreted by *C. perfringens* type A and type C, using 2D gel electrophoresis followed by MALDI TOF TOF, revealed differently expressed proteins that have not been reported in the secretome of any other Gram-positive bacterium (Sengupta *et al.*, 2010). SagA protein, DnaK-type molecular chaperone hsp70 and endo-beta-N-acetylglucosaminidase were among the most abundant extracellular in *C. perfringens* ATCC 13124. These proteins can be used as markers for contamination of food samples by *C. perfringens*, since they are conserved among *C. perfringens* strains. These proteins share very low homology (<50%) with the nearest similar ones expressed in other bacteria.

The genus *Salmonella*, especially two species *S. enteritica* and *S. bongori*, are a major cause of food poisoning throughout the world (WHO, 2015b). Different serotypes of *S. enteritica* are facultative intracellular pathogens. These bacteria replicate within the host cells, and are localized in a membrane-bound compartment, the Salmonella containing vacuoles (SCVs). Different typhoid exotoxins are secreted by *S. typhi* and other non typhoidal serovars like *S. javiana*. This so-called cytolethal distending toxin (CDT) in *Salmonella* consists of a CdtB (with DNase I like catalytic activity) subunit and two subunits PltA (with ADP ribosylating activity) and PltB subunits that are for excretion of CdtB (Spano and Gallan, 2008). The finding that this typhoid toxin binds to the cell surface glycans containing the N-acetyl neuraminic acid provides insight into the molecular base for *S. typhi* toxicity and host specificity (Deng *et al.*, 2014).

Salmonella spp. uses two distinct type III secretion systems (T3SS). The first one is a syringe-like macromolecular assembly that functions to inject exotoxins into the host cell. The SPI1 protein delivers effectors required for internalization by intestinal epithelial cells, while the SPI2 T3SS protein promotes intracellular replication and survival, leading to a systemic infection with this pathogen (Spano and Gallan, 2008). Phosphoproteomic study of phosphoinositide phosphatase (SopB), one of SPI1 effectors, on phosphorylation events important in the initial stages of infection, was presented by Rogers *et al.* (2011). Furthermore, Tahoun *et al.* (2012) demonstrated that SopB induces transformation of enterocytes and promotes host colonization and invasion.

Impact of SPI2 effectors onto host phosphoproteome and complete proteome was analyzed by Imami *et al.* (2013). It was demonstrated that these effectors differentially modulate the host phosphoproteome and some cellular processes, such as protein trafficking, cytoskeletal regulation, and immune signalling. Host targets of SPI2 T3SS-secreted SteC Ser/Thr protein kinases were identified, and an actin network rearrangement that enhances the *Salmonella* replication in SCV was demonstrated. These analyses were performed with phosphopeptide enrichment on TiO₂ column and LC fractionation, followed by nanoHPLC LTQ Orbitrap MS. Additionally, the *Salmonella* interactome was reviewed by Schleker *et al.* (2012).

Escherichia coli strains have been frequently used as industrial and model microorganisms, and their proteomes have been subjects of numerous investigations. However, some *Escherichia coli* strains, especially the Shiga toxin-producing *E. coli* O104:H4, can cause severe food poisoning with sometime fatal consequences (Frank *et al.*, 2011). As mentioned above, other *E. coli* strains have been frequently used as industrial and model microorganisms, and their proteomes have been subjects of numerous investigations. Some of these include the analyses of protein changes of both bacteria under different growth conditions, and for studying the effect of different anti-microbial agents (Frank *et al.*, 2011; King *et al.*, 2012; Reddy *et al.*, 2015; Sengupta *et al.*, 2010; Šrajer Gajdošik *et al.* 2013).

Detection of the food pathogen *E. coli* O104:H4 and characterization of the Shiga-toxin by use of genomic, proteomic and immunochemical methods have been topics of extensive investigations (Frank *et al.*, 2011; King *et al.*, 2012). Characterization of *E. coli* heat-labile enterotoxin was achieved by the use of LC/ESI MS and MALDI TOF MS (Martinović *et al.*, 2016).

The Gram-positive bacteria *Listeria monocytogenes* and the Gram-negative *Yersinia enterocolitica* came relatively late into focus as food pathogens (Gray and Killinger, 1966; Xanthopoulos *et al.*, 2010). The enterobacterium *Y. enterocolitica* is frequently involved in human enterocolitis. Outbreaks of infections are mostly associated with consumption of raw fruits and vegetables. Both the bacterium and its enterotoxin Yst are capable of surviving under refrigeration temperatures (Xanthopoulos *et al.*, 2010; Delor and Cornelis, 1992).

To investigate this microorganism and toxin production during bacterial growth, genomic and proteomic investigations were performed (Kanauja *et al.*, 2015). Differences in the proteome of *Y. enterocolitica* were investigated by 2D electrophoresis, followed by proteolytic digestion of excised proteins and MALDI TOF. Quantitative LC-MS/MS analysis of different bacterial lysates after treatment with inhibitors was performed by Šrajer Gajdošik *et al.* (2013). Use of the latter method resulted in the identification of

more than 1000 bacterial proteins, and detection of up- and downregulated proteins during the inhibition of bacterial growth.

Listeria monocytogenes exotoxin Haemolysin Listeriolysin O was the first focus of investigation in connection with growth and virulence of this food pathogen (Gray and Killinger, 1966; Gaillard *et al.*, 1986). The analyses of bacterial proteome and its changes under influence of biologically active peptides and synthetic inhibitors of growth were by use of 2D electrophoresis, followed by proteolytic digestion and LC MS for detection of proteins that were excised from the gel (Miyamoto *et al.*, 2015). Results of a quantitative label free LC MS/MS analysis of changes in listeria proteome were also recently published (Reddy *et al.*, 2015).

Some strains of *Campylobacter spp.* are pathogens. The most prominent food pathogenic strain is *C. jejuni*, a typical bacteria that is responsible of acute infective diarrhoea in humans. In many developed countries, this bacterium is causing more food-borne illnesses than *Salmonella spp.* (WHO, 2000). *Campylobacter jejuni* is unique among bacterial species, in that it possesses both N-linked glycosylation and O-linked glycosylation systems (Song *et al.*, 2015). In some other bacterial species, only one of these pathways can be present (Nothaft and Szymanski, 2010). Glycoproteins of *C. jejuni* may contain bacteria-specific monosaccharides, such as pseudaminic acid and diacetamidobacillosamin (Gilbreath *et al.*, 2011). These glycans are important for specific detection of this bacterium in food.

Current proteomic and glycomic studies are aimed at understanding the mechanisms of *C. jejuni* biogenesis and pathogenicity. Elmi *et al.* (2012) analyzed the role of outer membrane vesicles (OMV) in the interaction of this bacterium with host cells and delivery of virulence factors. For these investigations, nano HPLC ESI 3D IT MS was applied, and more than 150 proteins were identified in OMV (Elmi *et al.*, 2012).

4.8 Conclusions

In addition to already used techniques, the use of foodomics, especially proteomics, peptidomics and metabolomics, will result in better, faster and more efficient identification of food-borne bacterial pathogens and their toxins, and more effective monitoring of food during its production, storage, and transportation, and will prevent the outbreak of food-borne diseases.

Use of high-throughput methods for sample preparation and further, targeted development of MALDI TOF MS will promote the use of these technologies for routine analyses of food samples.

Technological advances will also lead to further optimization of Orbitrap and Fourier transform ion cyclotron MS technology, bringing ultra-high resolution mass spectrometry (UHRMS), with a resolving power higher than 100 000, to a wider range of users, and it will further promote the use of LC-MS/MS in the analysis of bacterial toxins and identification of biomarkers for food-borne diseases.

Immunochemical and other methods that are already used for detection of food-borne bacteria and their toxins still have a perspective for routine use, especially in the near future. Miniaturization and introduction of new high-throughput technologies and robotics will further yield lower limits of detection, reduction of sample and solvent amount, and significantly shorter analysis time.

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5

Metabolomics in Assessment of Nutritional Status*Kati Hanhineva**Univ Eastern Finland, Institute of Public Health & Clinical Nutrition, Kuopio, Finland***5.1 Introduction**

Food and nutrition research has undergone massive transformation during past 10–15 years, by changing from assessing the deficiencies of single nutrients to focusing on balancing complex whole, chemically rich diets to individual needs. Earlier, the nutritional value of food was determined based on its content of proteins, carbohydrates, fats, vitamins and minerals, and the adequate intake of each category was aimed at ensuring efficient nutrition. Now, instead, whole diets that maximise optimal health and prevent disease, rather than simply sustaining life are in focus, taking into account the wide range of biologically active components in diets (Zivkovic and German, 2009; Jacobs, *et al.*, 2009; McEvoy, *et al.*, 2014).

Likewise, rather than treating symptoms of diseases, diet is seen as a protective strategy to reduce the risk of chronic diseases. Currently, the complex link involving the interaction between genes, lifestyle and diet with health status is well demonstrated, and one of the key goals in modern nutrition is to comprehensively characterise these connections at the molecular level, to identify the diet-derived factors (including bioactive compounds) and examine their bioavailability and metabolism in humans, as well as to locate those areas of metabolism that are affected by certain diets or dietary compounds (Jones *et al.*, 2012; Bordonni and Capozzi, 2014; Kim *et al.*, 2016).

5.2 Usability of metabolomics in nutrition sciences

The aim of deciphering the relationship between nutrition and health requires understanding of a wide array of biologically active compounds and examination of various cellular metabolic pathways, which likely is not achievable with traditional ‘one hypothesis – one measurement’ approaches. Metabolomics and other wide-scale profiling technologies, including genomics, transcriptomics and proteomics, have entered the analytical suite of food and nutrition research as they similarly have in other bio- and medical sciences. They are particularly well suited for this purpose, as they enable concomitant examination of hundreds to thousands chemical entities, representing a large

fraction of the metabolites within any biofluid or tissue (Brennan, 2013; Scalbert *et al.*, 2014; Zivkovic and German, 2009; Manach *et al.*, 2009; McGhie, 2012).

The most important advantage of metabolomics studies, in particular non-targeted metabolite profiling approaches, is the ability to discover novel, unsuspected metabolic alterations that can provide valuable information and raise novel hypotheses in regard of the physiological, health-related effects of whole diets or nutritional components. Furthermore, utilisation of metabolomics approaches in nutrition research enables assessing inter-individual variation and tailoring dietary scheme to individual needs, better enabling advice on how diet can improve health on individual level, as a preventive strategy to reduce risk of disease (German *et al.*, 2003, 2011; Claus, 2014).

Metabolomics or metabolite profiling approaches aim to measure and identify all the compounds present in a taken sample analyzable with the technology in question (Patti *et al.*, 2012; Weckwerth, 2003). Typically, non-targeted profiling approaches result in collection of thousands of chemical signals that are evaluated for occurrence between the different study groups, after which those signals showing significant difference are taken into deeper inspection in order to resolve their identity.

The most typically applied analytical approaches within metabolomics are nuclear magnetic resonance (NMR) or mass spectrometry-based analyses, combined with either gas or liquid chromatographic separation (GC-or LC-MS). The metabolomics workflow constitutes of study setup, metabolite extraction, measurement of the samples in the analytical platform, data collection, data pre-processing, statistical analysis, identification of differential metabolites, and interpretation of the results within the scientific context. Detailed description of the various steps is the subject of many extensive reviews, and is not discussed in detail here (Dunn *et al.*, 2011; Want *et al.*, 2013; De Vos *et al.*, 2007).

5.3 The metabolite complement in human studies

Metabolites are substrates, intermediates and end products of cellular metabolism, and fall into various different chemical classes, including amino acids, lipids, steroids, carbohydrates, and phytochemicals such as flavonoids, phenolic acids, terpenoids and tannins. Extensive databases listing currently known compounds include the Human Metabolome Database (<http://www.hmdb.ca/>), Metlin (<https://metlin.scripps.edu/index.php>), Food Database FooDB (<http://foodb.ca/>), Phenol-Explorer (<http://phenol-explorer.eu/>), and the Dictionary of Natural Products (<http://dnp.chemnetbase.com>), which all list compounds with varying information regarding their chemical and analytical properties and occurrence in humans and foodstuff.

The metabolite complement of an individual can be roughly divided into three sections, all of them can be studied with metabolomics techniques. Firstly, human metabolism produces an endogenous variety of metabolites that are essential to the normal growth and development of life, and which are typically present in the majority of cells (for example, certain lipids and amino acids). These are often referred to as primary metabolites, in particular within plant sciences, or endogenous metabolites in the case of human studies.

Secondly, in addition to the endogenous metabolite complement, there is a plethora, several (tens) of thousands of compounds that we ingest from our diet. Within food and

nutrition sciences, the composition of these compounds is typically in focus either directly from the food, or after ingestion (Cevallos-Cevallos *et al.*, 2009; Wishart, 2008). Often, foodstuffs that are particularly rich in components that confer beneficial health effects are termed 'functional foods'. Metabolomics is an essential technique in the research on functional foods, as it can help in characterising the composition of potentially bioactive compounds present in functional foods, as well as monitoring bioavailability and bioactivity from human-based samples after intervention (Claus, 2014).

The third major contributor to the metabolite content essential for nutrition research are those compounds produced by the colonic microbiota, typically from dietary chemicals (Duffy *et al.*, 2015; Carmody and Turnbaugh, 2014; Wikoff *et al.*, 2009). Microbial colonisation, in particular in the gut, is responsible for numerous metabolic modifications of dietary compounds in various classes – for example, production of short-chain fatty acids from complex dietary carbohydrates (Bach Knudsen, 2015), or structural modification of dietary phenolic compounds (Aura, 2008).

The metabolite content originating from diet or microbiota may be referred to as 'secondary' metabolites, that fine-tune our metabolism and, together with inherited traits, largely contribute to our health by determining how we respond to environmental stimuli, such as nutrition or other lifestyle choices. Currently, one of the most intensively studied topics is the bidirectional interaction between diet and microbiome, and its contribution to overall metabolism and disease risk thereafter. In this task, hypothesis-free, large-scale profiling approaches are inevitable to monitor these issues in wide aspect (Turnbaugh and Gordon, 2008; Wikoff *et al.*, 2009).

5.4 Metabolomics within the analysis of relationship between diet and health

Health care in the West is no longer focused solely on diseases caused by microbial pathogens or deficiencies in basic nutrients carbohydrates, fats, and proteins. Nowadays, it is well acknowledged that diet is the most influential and complex exogenous factor in modulating our metabolic phenotype and disease risk. In particular, diets rich in plant-based foods, such as fruits, vegetables, legumes and whole-grain products, provide a wealth of plant-borne secondary metabolites (phytochemicals) and their microbiota-modulated metabolites, which have been suggested to be key players in the ability of plant-based food to enhance health beyond simply providing energy and nutrients (Liu, 2013; Jacobs *et al.*, 2009). The main focus in modern nutrition science is on optimising the balance within the primary nutrients, basic or general nutrition and, in addition, optimal intake of additional nutritional compounds (secondary metabolites) that possess bioactive characteristics, often from plant origin. This will enable metabolic functions, so that we maintain our optimal health under the prevalent inborn and external conditions.

Chronic imbalances in nutrition, including disturbed energy metabolism, contributes markedly to increased risk of disease via dysfunction in metabolism. It has been estimated that nutrition may account for as much as 50% of the risk of major non-communicable, lifestyle related diseases, such as cardiovascular diseases, certain cancers, obesity and metabolic syndrome (Zivkovic and German, 2009; Zeisel *et al.*, 2005; Odriozola and Corrales, 2015).

Several biological functions (antihypertensive, anti-inflammatory, antioxidant, anti-atherogenic, and anti-thrombotic) have been associated with plant-rich diets or individual phytochemicals (Buitrago-Lopez *et al.*, 2011; Van Hung, 2014). However, a wide set of chemicals is derived from diet – often hundreds to thousand within each meal, and in particular from various plant sources. Their interactions with numerous cellular processes and simultaneous modulation of several metabolic pathways hinder comprehensive understanding of the molecular events. Therefore, further identification of the involved metabolites associated are required in order to understand better how dietary compounds like phytochemicals participate in sustaining healthy metabolic homeostasis. Metabolomics approaches within the interplay between diet and health are crucial, as they enable increasing the number of metabolites measured, and understanding how environmental perturbations affect the metabolite levels. Furthermore, they allow us to understand how the changes in their concentration affect health and, in particular, how the relative abundancies between different metabolites affect metabolic pathways and health (German *et al.*, 2005; Jones *et al.*, 2012).

Furthermore, the metabolomics approaches in nutritional assessment enable the examination of tissue-specific metabolic responses, in particular when applied on animal feeding trials that enable sampling of internal organs as well (Hanhineva *et al.*, 2013; Pekkinen *et al.*, 2013; Want *et al.*, 2013). The combination of this information and monitoring against relevant human intervention trials will enable drawing a picture of whole-body metabolic homeostasis under specific dietary challenges and habitual nutritions, in a wider and more accurate way than before.

5.5 Individual differences in metabolic and nutritional phenotype

The metabolic phenotype of an individual is a net result from various dynamic, mutual interactions between the endogenous traits governed by genetic features, postgenomic and post-transcriptional events, and the extrinsic factors related to lifestyle, including diet, as well as physiological condition and age (Holmes *et al.*, 2008; Kinross *et al.*, 2014). The heterogeneity in these phenotypic differences has prompted the development of the concept of personalised nutrition, which has evolved alongside personalised medicine, namely, to assess individual needs, taking into account the inter-individual variation in the metabolic response to medical or nutritional exposure (German *et al.*, 2003, 2011; Zeisel *et al.*, 2005).

The overarching objective in personalised nutrition is to push the individual's metabolic phenotype by nutrition towards a more favourable metabolism in regard of health. Although nutrition is not the single cause of lifestyle-related diseases, it is a key factor and most influential and, therefore, focal in the prevention and treatment of lifestyle-related diseases. The analytical assessment of metabolic and nutritional phenotype involves the complement of endogenous metabolites, as well as dietary compounds and, furthermore, the microbiota-produced/metabolised compounds and metabolomics analytics is therefore required to make comprehensive readout of individual metabolic status (Zeisel *et al.*, 2005). Typically, such results are correlated with clinically validated chemical or physiological parameters, such as lipid profile, blood pressure and so on, to find out the eventual bioefficacy of the nutritional scheme (Rubio-Aliaga *et al.*, 2012).

Although this is still in the level of basic science and not in use in practice, it is foreseen that the personalised nutrition approaches fostered/enabled by metabolomics approaches will aid in nutritional recommendations within the near future (Fogelholm, 2016).

5.6 Assessment of nutritional status, example studies

The majority of nutritional research utilising metabolomics approaches is focused on profiling the metabolite content of food (Cevallos-Cevallos *et al.*, 2009; Wishart, 2008), the investigation of dietary intervention challenges with specific diets (Hanhineva, 2015; Medina *et al.*, 2013), dietary assessment to find plasma/urine biomarkers for intake (Scalbert *et al.*, 2014; Perez-Jimenez *et al.*, 2010), or analyzing epidemiological samples representing differential habitual diets (Zamora-Ros *et al.*, 2014; Guertin *et al.*, 2014). Additionally, investigations of metabolic effect of excess energy load and obesity are many (Xie *et al.*, 2012; Park *et al.*, 2015; Palau-Rodriguez *et al.*, 2015), but there are fewer studies where metabolomics has been applied on analysis of malnutrition and adequate nutritional status, or particular deficiencies. Example studies exist describing approaches with *in vitro* approach, with animal trials, or human populations, as reviewed below.

5.6.1 Malnutrition

The most important nutritional assessment is to monitor the adequacy of nutrients, since millions of people – in particular, children – suffer from malnutrition. The pathogenesis involved in the development from normal physiological condition to severe acute malnutrition, and its complications, remains poorly understood. Hormonal and metabolite levels were measured from Ugandan children aged between six months and five years when they were hospitalised due to malnutrition, and after they received therapeutic food and supplemental macronutrients (Freemark, 2015). In the beginning of the study, high levels of non-esterified fatty acids, ketones, and even-chain acylcarnitines were measured, indicating active lipolysis and fatty acid oxidation. Albumin, amino acids and C3 carnitine were low and, additionally, several hormonal levels were also low, including insulin, insulin-like growth factor 1 (IGF-1), adiponectin, and leptin. On the contrary, ghrelin, growth hormone, cortisol, interleukin 6 (IL-6), peptide YY (PYY) and glucagon-like peptide 1 (GLP-1) were high. Upon formula feeding, the levels of metabolites and hormones were reversed. The authors also assessed whether any of the biomarkers measured would predict higher risk of mortality, and found out that, in particular, low leptin levels were associated with higher risk of death among malnourished children (Freemark, 2015).

Several studies with animal models have been used to study physiological changes associated with malnutrition. For example, four-week old piglets were fed nutritionally optimal or nutrient-deficient feed for seven weeks (Jiang *et al.*, 2015). The differences in metabolite content were assessed in weekly collected plasma samples by non-targeted LC-MS metabolite profiling. The pigs receiving nutrient-deficient feed had markedly reduced body-weight gain and lean-mass proportion, and clear differences in plasma amino acid content were observed. Additionally, choline metabolites and compounds produced by gut microbiota were in higher abundance in malnourished pigs (Jiang *et al.*, 2015).

Rodent models have also been used in the study of metabolic changes related to malnutrition, including rat (Wu *et al.*, 2010) and mouse (Marino *et al.*, 2014; Alasandro *et al.*, 1984). A combined approach with gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) was taken on urine samples from protein-energy malnourished rats (Wu *et al.*, 2010). The urine metabolite profile of the malnourished rats indicated perturbations in all branches of energy metabolism, namely amino acid, carbohydrate, fatty acid and purine metabolism. Individual metabolic changes included higher levels of creatine, threitol, pyroglutamic acid, gluconic acid and kynurenic acid, as well as decreased levels of succinic acid, cis-aconitic acid, citric acid, isocitric acid, threonic acid, trimethylglycine, N-methylnicotinic acid and uric acid in the malnourished group.

In addition, more rare experimental models have also been used, including mosquitoes (Price *et al.*, 2015) and rainbow trout (Baumgarner and Cooper, 2012). The effect of environmental changes, including starvation during larval stage on the adult mosquito energy metabolism, were studied with transcriptomics and metabolomics approaches (Price *et al.*, 2015). Changes detected by both technologies indicated, for example, a difference in amino acid metabolism, as the mosquitoes that were grown under starvation had higher levels of free amino acids (Price *et al.*, 2015).

Comprehensive metabolite profiling analysis was conducted on serum, liver and muscle tissue from rainbow trout that were grown under starvation and compared with normal fish (Baumgarner and Cooper, 2012). Marked differences were observed between the two groups, but also sample type specific differences – for example, protein catabolism was evident in liver as the levels of methionine, proline and lysine metabolite 2-piperidine carboxylic acid increased, but in plasma the levels of several amino acids were reduced (Baumgarner and Cooper, 2012).

The effect of maternal diet on the development of foetus, and metabolic consequences thereafter later in life, are a focal area of research in nutritional assessment, and have been addressed with profiling technologies, including metabolomics (Marincola *et al.*, 2015). Malnutrition, both excessive (Koletzko *et al.*, 2012) and insufficient, is detrimental for metabolic development of the foetus, and is linked with increased risk of future chronic diseases, as reviewed by Dessi *et al.* (2013). For example, urinary samples were analyzed by NMR-metabolomics approach over the first week of life from newborns that were classified to three different groups based on fetal nutrition: adequate for gestational age (AGA); with intrauterine growth retardation (IUGR); and large for gestational age (LGA). There were time-related metabolic changes occurring in all the groups, and also differences between the groups (citrate and myo-inositol were higher in the IUGR and LGA, whereas higher contents of betaine and succinate were found in AGA) (Marincola *et al.*, 2015).

Malnutrition of elderly people has been assessed by targeted metabolite profiling on carnitines and amino acids (Dror *et al.*, 2013). The study aimed at elucidating metabolic changes related to re-feeding of malnourished, frail elderly people, which often leads to increased risk of death. Patients were fed with enteral nutrition, and the control group comprised elderly people who had been on re-feeding for more than three months. During deprivation, the concentrations of all even monocarboxylic carnitine fatty acids were higher and, during re-feeding, remarkable decreases in all carnitine fatty acids were observed. Furthermore, there was large daily fluctuation in the metabolite levels, indicating metabolic instability, and this was most evident in participants who died

shortly after the re-feeding. Metabolomics was proved useful in assessing metabolic fluctuations, which may be helpful in optimising personalised treatment for elderly malnutrition patients (Dror *et al.*, 2013).

Utilisation of metabolomics analytics in the assessment of nutritional adequacy, and the evaluation of the pathogenesis of physiological complications involved in malnutrition, has not yet been fully exploited. However, the individual studies conducted utilising both NMR- and LC or GC-MS profiling have all shown remarkable differences in various metabolic branches – in particular, those involved in energy homeostasis, including amino acid, carbohydrate and lipid metabolism. Therefore, further utilisation of these technologies is advisable, as they will facilitate both the understanding of the metabolic events related, as well as aiding in the design of targeted, personalised treatment for different conditions of malnourishment, while taking into account inter-individual differences in inborn metabolism. Examples of nutritional assessment studies on malnutrition and deficiencies utilising metabolomics analytics are compiled in Table 5.1.

5.6.2 Deficiencies in particular nutrients

Within the context of deficiencies of particular nutrients, the studies focusing on various vitamins are the most frequently reported. Metabolomics investigations have been employed in studies with human subjects (Wang *et al.*, 2014; Johnson *et al.*, 2012; Gregory *et al.*, 2013; Rios-Avila *et al.*, 2015) and animal trials (Kirkwood *et al.*, 2012; Duggan *et al.*, 2011; Lamarre *et al.*, 2012) as well as *in vitro* cell culture assays (da Silva *et al.*, 2014). The importance of metabolomics analytics alongside other ‘postgenome’ approaches is also acknowledged for the detailed examination of trace element inadequacies – in particular, in order to understand the intra-individual differences in the requirement of different trace elements, including copper, zinc and iron (Young, 2003).

One of the most intensively studied areas of vitamin deficiencies is the low vitamin B-6 nutritional status. Pyridoxal 5'-phosphate (PLP), the active coenzyme form of vitamin B-6, participates in numerous cellular metabolic processes, including the metabolism of amino acids, carbohydrates, organic acids, and neurotransmitters. Therefore, its deficiencies are associated with several diseases, including cardiovascular disease and certain cancers. A study conducted with HepG2 cells that were cultured in various concentrations of vitamin B6 and analyzed for metabolite content revealed wide metabolic impacts; in cells cultured in low vitamin B6, a higher concentration of betaine and lower concentrations of creatine 5,10-methenyltetrahydrofolate were observed. Additionally, cystathionine, cysteine, glutathione, and cysteinylglycine were higher at the two lower vitamin B6 concentrations, when compared to the higher concentrations (da Silva *et al.*, 2014).

In a human study, an NMR-metabolomics and targeted MS analysis was done on fasting plasma samples from healthy men and women who were either receiving adequate vitamin B6 or a low vitamin B6 diet. Vitamin B6 restriction increased the ratios of glutamine/glutamate and 2-oxoglutarate/glutamate, as well as the concentrations of acetate, pyruvate, and trimethylamine-N-oxide, whereas the restriction of vitamin B6 caused increase in plasma proline, but not other amino acids or acylcarnitines (Gregory *et al.*, 2013).

In another human study, the target group was women who use oral contraceptives (OCs). Vitamin B-6 supplementation on the *in vivo* kinetics of one-carbon metabolism,

Table 5.1 Nutritional assessment studies on malnutrition and deficiencies utilizing metabolomics analytics.

Title	Authors	Focus of the study	Subject of the study	Journal	Pub Year
Metabolomic profiling can predict which humans will develop liver dysfunction when deprived of dietary choline	Sha <i>et al.</i>	choline deficiency	human (adult)	The FASEB Journal	2010
(1)H NMR-based urine metabolic profile of IUGR, LGA, and AGA newborns in the first week of life	Marincola <i>et al.</i>	malnutrition	human (newborn)	Clinica Chimica Acta	2015
Metabolomics in nutrition research: biomarkers predicting mortality in children with severe acute malnutrition	Freemark	malnutrition	human (children)	Food and Nutrition Bulletin	2015
Progressive changes in the plasma metabolome during malnutrition in juvenile pigs	Jiang <i>et al.</i>	malnutrition	pig	Journal of Proteome Research	2015
Urinary metabolomics study in a rat model in response to protein-energy malnutrition by using gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry	Wu <i>et al.</i>	protein-energy malnutrition	rat	Molecular BioSystems	2010
The impact of refeeding on blood fatty acids and amino acid profiles in elderly patients: a metabolomic analysis.	Dror <i>et al.</i>	refeeding after food deprivation	human (elderly)	Journal of Parenteral and Enteral Nutrition	2013
Effects of starvation and refeeding on the excretion of urinary steroid metabolites in mice with different genetic background	Alasandro <i>et al.</i>	starvation and refeedign	mouse	Journal of Chromatography	1984
Evaluation of a tandem gas chromatography/time-of-flight mass spectrometry metabolomics platform as a single method to investigate the effect of starvation on whole-animal metabolism in rainbow trout (<i>Oncorhynchus mykiss</i>)	Baumgarner and Cooper	starvation	rainbow trout	Journal of Experimental Biology	2012
Small mosquitoes, large implications: crowding and starvation affects gene expression and nutrient accumulation in <i>Aedes aegypti</i>	Price <i>et al.</i>	starvation	mosquito	Parasites & Vectors	2015

Regulation of autophagy by cytosolic acetyl-coenzyme A	Marino <i>et al.</i>	starvation	mouse, cultured human cells	Molecular Cell	2014
Formate can differentiate between hyperhomocysteinemia due to impaired remethylation and impaired transsulfuration	Lamarre <i>et al.</i>	vitamin B12 deficiency	rat	American Journal of Physiology – Endocrinology and Metabolism	2012
Targeted metabolomics and mathematical modeling demonstrate that vitamin B-6 restriction alters one-carbon metabolism in cultured HepG2 cells.	da Silva <i>et al.</i>	vitamin B6 deficiency	HepG2 cells	American Journal of Physiology – Endocrinology and Metabolism	2014
Metabolomic analysis reveals extended metabolic consequences of marginal vitamin B-6 deficiency in healthy human subjects	Gregory <i>et al.</i>	vitamin B6 deficiency	human (adult)	PLoS One	2013
Pyridoxine supplementation does not alter in vivo kinetics of one-carbon metabolism but modifies patterns of one-carbon and tryptophan metabolites in vitamin B-6-insufficient oral contraceptive users	Rios-Avila <i>et al.</i>	vitamin B6 deficiency	human (adult)	American Journal of Clinical Nutrition	2015
Vitamin C deficiency activates the purine nucleotide cycle in zebrafish	Kirkwood <i>et al.</i>	vitamin C and vitamin E deficiency	zebrafish	Journal of Biological Chemistry	2012
Metabolic profiling of vitamin C deficiency in Gulo-/- mice using proton NMR spectroscopy	Duggan <i>et al.</i>	vitamin C deficiency	mouse	Journal of Biomolecular NMR	2011
Biomarkers identified by urinary metabolomics for noninvasive diagnosis of nutritional rickets	Wang <i>et al.</i>	vitamin D deficiency (rickets)	human (children)	Journal of Proteome Research	2014
Novel metabolites and roles for alpha-tocopherol in humans and mice discovered by mass spectrometry-based metabolomics	Johnson <i>et al.</i>	vitamin E deficiency	human, mouse	American Journal of Clinical Nutrition	2012

and the concentration of one-carbon and tryptophan metabolites in vitamin B6-deficient OC users. In addition to an increase in plasma PLP concentrations, the vitamin B6 supplementation decreased plasma leucine and glycine concentration and caused alterations in the ratios between metabolites pairs, including (for example) 3-hydroxykynurenine:kynurenine, 3-hydroxykynurenine:3-hydroxyanthranilic acid, and 3-hydroxykynurenine:anthranilic acid (Rios-Avila *et al.*, 2015). In addition, an NMR-metabolomics study focusing on vitamin B6-deficient rats showed not only increases in the homocysteine and methylmalonate levels, but also unexpectedly high elevation in the formate levels, both in plasma and urine (Lamarre *et al.*, 2012).

Additional vitamin deficiencies that have been addressed with metabolomics technologies include deficiency of vitamin D and subsequent rickets (Wang *et al.*, 2014), vitamin E inadequacy in both humans and mouse model (Johnson *et al.*, 2012), vitamins C and E in zebrafish model (Kirkwood *et al.*, 2012), and vitamin C deficiency in mouse (Duggan *et al.*, 2011). There are marked differences in the outcomes of the studies that most likely result from the differences of the analytical platforms employed, as the metabolite complement measurable with NMR is very different from that encompassed in MS-based analytics. Furthermore, the study population within the studies conducted so far (for example, in the assessment of vitamin B6 deficiencies) is largely variable, which likewise causes alteration in the outcomes of the analyses. However, the obvious use of the metabolomics technology within this is also evident, as it has brought changes in the metabolite content that have been anticipated. These include the differences within metabolites from the vitamin B6 metabolic route, but also, in addition, unexpected findings that can only be discovered with the non-targeted profiling approach.

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6

Rapid Microbiological Methods in Food Diagnostics

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6.1 Introduction

6.1.1 Why the need for rapid methods – their benefits and potential limitations

Rapid technologies for use in the biological and clinical sciences have now existed for the better part of 30 years or more, and newer generations of scientists and researchers have ‘grown up’ with these technologies, while older generations of scientists have marvelled at the rapid changes in the field since their days at the bench. It is true to say that the applications of rapid methods in diagnostics have probably played a greater role in the clinical/medical side of the business, where a lot of these methods first began, than in the food industry. In some cases, one could say that the need to save lives may have played a greater role in pushing the pace of the technology to rapidly identify a disease or to diagnose an illness from the clinical perspective.

Regardless, the past 20–30 years has also seen an explosion in the development of and reapplication of these technologies for use in the food industry. Without a doubt, the impact of massive outbreaks of illnesses, lives and work days lost, product withdrawals and recalls, lawsuits and brand loyalty loss (goodwill), as a consequence of consumption of contaminated food, has probably been a driving force in changing how we view foods, their distribution, their consumption and the consequences, in terms of safety for the consumer. For these reasons, the use of, and development of, technologies for rapid diagnostics is likely to be a mainstay of the food industry for many years to come. In writing this chapter, we would like to acknowledge our colleague Dr. Daniel Fung, who wrote the previous version of this chapter in the first edition of this book (2007), and had provided his top 10 predictions for the future. Interestingly, many of his predictions have come to fruition and still hold true today.

There are several advantages of rapid diagnostics: shorter times to result; less labour-intensive work; and, in some cases, decreased cost. Gone are the days of long incubations and continuous culture, to find out if a sample is positive or negative for an organism of interest, followed by extensive testing to type and subtype the organism. Such tests are becoming less of a mainstay, and are only necessary when further

follow-up, requiring an isolate, is needed. In the food industry, faster results facilitate informed decisions about whether a product is safe for market release, should be diverted for re-processing using the same processing method, or a different type, or should simply be withdrawn from market. In this chapter, we will discuss some of the current and emerging technologies of rapid method diagnostics for foods and, where possible, we have included some food industry perspective.

6.2 Quantitative vs qualitative

Perhaps the single most important question in any rapid method is, what is the objective? What does the user hope to achieve from a rapid test or diagnostic? If the answer is a yes/no for detection of a pathogen, then the diagnostic test must be qualitative. Here, one of the things to consider within the realm of a qualitative test is the minimum level of detection – in other words, how low a level of analyte or DNA or immunogen needs to be present for a result? In general, most rapid detection technologies have minimum detection levels in the 10^4 organisms per volume range, for a test to accurately read for a sample. This either needs to be achieved through a pre-concentration step (such as immunomagnetic separation), or the method requires enrichment to increase the level of the target to detectable levels.

Regardless of how the target population is increased, the current trend and pressure for good rapid diagnostics is to find a way to reduce this detection level. In the food industry, qualitative testing is usually employed for the detection of the presence of pathogens such as *Salmonella* spp., *Listeria monocytogenes* and pathogenic *Escherichia coli*. On the other hand, quantitative testing is typically used to determine the levels of quality and spoilage indicators such as aerobic plate count, yeast, mould, lactic acid bacteria, and the levels of hygiene indicators such as coliform and total *Enterobacteriaceae* counts.

The second aspect of detection is the specificity of the test. For the most part, sensitivity and specificity of an assay are complementary, in that the test should be able to detect low levels of the analyte but should also be accurate for the target of interest. Often, target assays focus on the detection of a species in general, rather than a specific serotype or sub-species type. In many current lateral kit assays on the market, the detection is based on antigen-antibody reaction, which focuses on binding to a target analyte that is present in many species or organisms. The specificity of the assay is important in ensuring that the detection of the pathogen is correct, and not a false negative or false positive result.

6.3 Culture dependent vs independent

Perhaps the most important factor in any detection assay is determining how to detect the pathogen or target of interest in a feasible way. For example, most standard microbiological assays depend on detection of a pathogen once it has reached a detectable threshold. In previous reports, we have identified minimum detection levels for some assays that require a minimum concentration of 10^4 organisms to be present before the assay can detect the pathogen of interest (Logue and Nolan, 2012). In this case,

detection may be dependent on a culture-based approach that increases the population to detectable levels. Such assays include PCR-based (including real time) lateral flow immunoassays and, often, microscopic-based assays.

Culture-independent assays are assays that do not require an actual strain or biological culture of the target of interest to use. In general, such tests do not result in any bacterial or viral isolate, but focus on telling the user if the target is present or not. For example, culture-independent assays can detect the presence of EHEC or STEC in a sample, based on detection of *stx1* and *stx2* targets, which will indicate if the pathogen is present or not, and if it has toxin genes. Often, these types of assays are used as an indicator of the presence of an organism, with follow-up by culture if the isolate is needed for further study (Zuraw, 2014). In the food industry, such an approach allows for decisions to be made, based on the assay, before cultural confirmation is performed. Positive assay results are regarded as presumptive, and require further confirmation using culture-based analysis.

Perhaps most important is the fact that culture-independent assays can be used as a screening tool for large numbers of samples where, most often, the result is expected to be negative. This approach has been used with PCR-based assays (Krause *et al.*, 2006; Abdulmawjood *et al.*, 2004). The downside to culture-independent-based assays can be the prevalence of false negative results, in which case a contaminated product may not be implicated until an outbreak of illness occurs.

6.4 Automation and multi-pathogen detection

Automation in the detection of pathogens has become a significant important approach in the application of rapid technologies for pathogen detection. This has resulted in the automation of most phases of detection, from automation of the enrichment to application of the detecting agents, to reading of the result and output of the report. In the clinical environment, automation has existed for a considerable number of years, but such systems have also made the transition to the food industry, with technologies such as bacT/Alert and Rapid microbiology analyzers (BioMerieux) as examples.

Such approaches involve inoculation of a media-containing bottle with the sample, and then the automation takes over, with incubation and monitoring at set intervals until a result is detected – then the report is automatically generated. This approach then allows for sub-culture to be carried out from the culture bottles, should a culture be required. In the food industry, large numbers of environmental samples are usually tested as part of routine monitoring for pathogens in food production environments. Finished product pathogen testing is also performed as a verification of food safety programs. Automation is, therefore, critical in ensuring that the time to results for this testing is significantly shorter, compared to testing using conventional culture procedures.

Multi-pathogen detection is usually focused on detection of target pathogens, using multiple technologies or rapid methods at the same time, or using the same technology but designed for different targets. In the case of using multiple technologies at the same time, their use depends on the development of an enrichment system that allows for all targets to grow and reproduce at the same time. This can be a challenge, as conditions for one pathogen may not favour another, or competition of microflora may negatively

influence detection strategies. One approach may include the use of different types of lateral flow kits that target different antigens of the various targets simultaneously. The detection is, however, limited by appropriate conditions that result in growth of target pathogens to detectable levels for each assay. Alternatively, use of molecular based methods such as real-time PCR, using primers targeting different pathogens, may be more appropriate, and such an approach would allow detection of multiple targets simultaneously.

A recent paper by Woubit *et al.* (2012) reported on the simultaneous detection of six specific pathogens, including *E. coli*, *Salmonella*, *Shigella*, *Vibrio*, *Yersinia* and *Francisella*, using a real-time assay with detection limits as low as 6–60 cfu/ml; similar studies by Fukushima *et al.* (2010) also used eight multiplex real-time SYBR Green PCR panels to simultaneously detect three target primers and (internal control) for detection of 24 target genes in 23 food-borne pathogens, with detection levels of 10^3 – 10^4 cfu/g. Pathogens detected by the method included *Clostridium perfringens*, *Providencia*, *Campylobacter*, *Vibrio*, *Listeria*, *B. cereus*, EHEC, ETEC, EAEC, DAEC, *S. aureus*, *Yersinia spp.*, *Plesiomonas*, *Shigella*, and *Aeromonas*. Detection time was less than three hours when assayed from human stools. The assay was demonstrated to detect the causative pathogen in 33 of 35 food-borne outbreaks using the multiplex designed.

6.5 Separation and concentration

For any rapid diagnostic assay to have any strength, separation of the pathogen of interest from background flora is of significant importance.

6.5.1 Filtration

In general, filtration-based approaches are used in the detection of pathogens in liquids, where volumes of liquids are filtered through a membrane designed to catch bacteria. The membranes can then be incubated on selective media, or media-soaked pads, that will allow the growth of the target organisms. Membrane filtration is most often used in water analysis, as a means to concentrate and determine levels of the organism present per volume of liquid analyzed. The advantage of the technique is that it allows larger volumes to be concentrated on a membrane; however, this can also be a disadvantage where contamination levels are high, or where the liquid being tested contains particulates which may interfere with filtration and detection. The method can be made selective for particular organisms, such as *Clostridium perfringens* or *E. coli*, by use of selective media to selectively support the growth of these pathogens. Typical pore sizes of membranes used in membrane filtration are 0.45 μm (FDA – BAM).

6.5.2 Stomacher

The stomacher was designed as a tool to separate bacteria from solid samples such as foods. The stomacher was designed out of a need to blend a sample in a single unit, without the need for sterilisation between samples. In previous years, lab blenders (Waring blender) were used, which consisted of a jar with rotating blades in the base that blended the liquid and the sample. The stomacher is said to mimic the action of a

stomach, in that paddle blenders pummel the food sample in liquid, thus releasing the bacteria from the food into the liquid. There are multiple types of stomachers on the market, but all work along the same principle.

More current blenders use a circulating motion when pummelling, to achieve better release of bacteria into the liquid. Modification of the stomacher bags from a square shape to a round bottom also support the easier release of the bacteria by better circulation of liquid. Also of value is the use of filtered stomacher bags, which have a built-in mesh that reduces the interference of particulates when removing liquid for sampling or testing.

6.5.3 Pulsifier

Following the success of the paddle blenders (stomacher), there was a need to reduce the interference of particulates in the analysis of foods. The pulsifier was designed to use ultrasonic vibration to vibrate bacteria from a sample without major destruction of the sample. The method results in a cleaner sample, with the liquid not containing as much debris or contaminants that would impede sample analysis. The sample is weighed into diluent in a plastic bag and placed inside a vibrating ring. As the ring hits the bag, the force becomes translated into shock waves that are transmitted through the liquid, which results in tumbling of particles in the liquid and, in essence, removes them from the sample being tested by a washing action. Workers have demonstrated that the plate counts from samples prepared by conventional paddle blending (stomacher) versus pulsifier preparation are equivalent (Fung *et al.*, 1998).

6.6 Rapid methods that are currently in the market

Application of rapid methods in diagnostics take a number of forms, and are dependent on what the investigator wants to know. These are assays broken down by method of detection, and these include microscopic analysis, metabolism-based detection, luminescence-based detection, immunological/serological-based detection, nucleic acid-based and sensor-based. Each technique has its own applications and limitations. This section will try to cover these, as well as describing some of the more common methods we see and use. This list and techniques is by no means inclusive of every technique and method currently available, and we apologise for any that have been overlooked.

6.6.1 Microscopic-based

Microscopic-based techniques usually involve staining and observation using microscopy, however this is not the only method and in some cases staining is used. However, detection uses alternative methods that do not involve the use of a microscope.

6.6.1.1 DEFT – direct epifluorescent filter technique

The direct epifluorescent filter technique, or DEFT, is a method developed as a direct counting technique for microscopy. It was originally devised to determine bacterial counts in milk, but has since been expanded for use in meat and fish microbiology. DEFT involves pre-treatment of sample by homogenising in fluid, and then treating the

homogenate with detergents and enzymes to remove protein, followed by filtration of the liquid through a polycarbonate membrane to capture cells, and fluorescent staining to enable visualisation. A common stain used to detect bacteria include acridine orange; alternative stains include DAPI (4', 6'-diamidino-2-phenylindol). There are also dyes that can be used to detect live and dead cells (BacLight). Detection limits for DEFT are in the range of 10^4 – 10^6 cfu/ml. One of the drawbacks of DEFT is the lack of specificity when specific stains are sought. However, specific detection can be achieved using an antibody-based approach to target specific bacterial types, which are then conjugated with a fluorescent dye for detection (Sheridan *et al.*, 1997, 1998; Cloak *et al.*, 1999; Uttendaele and Debevere, 2006). Other microscopic-based techniques include the following:

6.6.1.2 FISH – fluorescent *in situ* hybridisation

FISH gained popularity as a tool for detection of pathogens and other microorganisms in the early 1990s (Rhode *et al.*, 2015). It has been used for identification of microorganisms, based on targeting of abundant RNAs within bacterial cells. It has application in human medicine for targeting pathogens in blood or faeces, and for mutation detection, as well as for studying the ecology of microbial communities in environmental microbiology. The principal of FISH involves fixation of a sample, permeabilisation to allow entry fluorescent probes into cells, followed by hybridisation of the probe to the target. A rinse step removes excess stain, and visualisation of the target uses a microscope or other methods such as flow cytometry.

The application of FISH in food microbiology requires some alteration of the methods to include enrichment of the target to detectable levels and potential separation of the target from materials associated with the food or contaminants. All such methods require optimisation for the target, and protocols to concentrate the target for adequate detection, as well as removing contaminants that would interfere with the assay. FISH assays have been developed for a range of pathogens, including, *E. coli*/*E. coli* O157 in meat, beverages and milk, *Listeria spp.* in milk, meats salmon and cheese, *Salmonella spp.* in meats, milk, cheese and vegetables, and *Campylobacter* on poultry meat and meat products.

Baysal (2014) used a FISH-based approach for detection of *Listeria* in a range of meat and poultry products in Turkey, with an accuracy of 98.15% and specificity of 99.47% when samples were examined. *Listeria* was detected in 37.4% of samples by culture, versus 24.7 by FISH using pre-enriched samples. A multiplex FISH (mFISH) is able to detect multiple pathogens simultaneously. Here, multiple fluorescent probes were designed for different targets. The mFISH is, however, limited by the detection technology, which is restricted to the different wavelengths of light needed for each probe that can be detected simultaneously. Another limitation of mFISH is the hybridisation temperatures necessary for the probes to be complementary, to ensure multiple target detection. With these concerns, some mFISH protocols have been developed for simultaneous detection of *Salmonella* and *Listeria* in milk (Oliveira *et al.*, 2004). A recent paper by Rhode *et al.* (2015) provides an excellent overview of the use of FISH in food microbiology diagnostics.

6.6.1.3 Live dead assay

The live dead assay is a dye-based assay that is designed to identify and differentiate live bacterial cells in a population from dead cells. The common commercial kits most often

used include BacLight, which consist of different probe colours that can bind to live and dead cells thus providing an indication of the fractions of each present in a sample. Two dyes – Propidium iodide and SYTO9 dye – are used in the kit, with live cells staining green and dead cells red.

The success of the stain is based on its ability to bind to the various structures of the cells; SYTO9 dye is able to bind to all cells, with both intact and damaged cell membranes, while the second dye, propidium iodide, can only bind to cells with damaged membranes. This reduces the fluorescence of the cells stained with propidium iodide, and reduces fluorescence from the SYTO9 dye. Thus, the bacteria with intact membranes will stain green (live), while cells with damaged cell membranes stain fluorescent red (dead). The two-dye combination can be used in light microscopy and flow cytometry application, as well as multi-well plate analysis. Liu and Mustapha (2014) used a live-dead cell approach to detect viable *E. coli* O157 in ground beef, while Flekna *et al.* (2007) suggested that the live dead cell assay may not be accurate enough and compromise detection of *Listeria* and *Campylobacter* using a real time assay.

6.6.1.4 Enzyme-linked immunosorbent assay (ELISA)

ELISA is an immunological-based method for pathogen detection that relies on binding of a bacterial antigen using monoclonal or polyclonal antibodies, targeted against the organism of interest. Usually, the ELISA assay a sandwich-type format that consists of an immobilised antibody on a solid surface (such as a microtitre plate), followed by exposure of the antibody to the sample containing the target of interest (in this case, a bacterial target or cells). The stationary antibodies bind or capture the target cells and, once captured, a washing step is used to remove unbound sample. A second antibody is then applied that is usually conjugated to an enzyme, which is activated by the addition of an enzyme substrate that the enzyme will act upon, resulting in substrate digestion.

A positive result is detected as a colour change, due to the release or production of product from the reaction between the enzyme and the substrate. Law *et al.* (2015) highlighted some applications of ELISA for pathogen detection in foods for *Vibrio* and *Salmonella*, and toxins such as those associated with *Clostridium*, *E. coli* O157 and staphylococci. An automated version of the ELISA assay has been commercially developed (e.g. VIDAS and EIA) that can detect pathogens such as *Salmonella*, *Campylobacter*, staphylococci and *E. coli* O157 (Law *et al.*, 2015).

6.6.1.5 MALDI-TOF MS

Matrix-assisted laser desorption ionisation-time of flight mass spectroscopy (MALDI-TOF MS) is a soft ionisation technique used in mass spectroscopy. It uses lasers in excitation of biomolecules that are detected by mass spectroscopy and which can then be characterised, based on scatter and patterns to identify an isolate. More often, the technique is used in the rapid identification of bacteria, rather than waiting for the results of conventional ID systems, which require culture incubation and analysis of results based on biochemical reactions.

The novelty of and rapidness of MALDI-TOF MS is that diagnostics can be performed from isolated colonies selected directly from a sample plate. What has really changed the landscape is the application of MS-based technologies for application in other aspects of bacterial identification, toxin identification, antimicrobial susceptibility and diagnostic development. A very recent article by Cheng *et al.* (2016) has highlighted the

expanded application of mass spectrometry (MS)-based application beyond simple bacterial species identification.

MALDI-TOF MS works by ionisation of extracted molecules found in a whole-cell culture, typically without protease treatment (however, some protocols may require pre-treatment). Usually, a suspension of the cells is mixed with a chemical matrix that encourages ionisation of the sample, which is loaded on a MALDI plate inserted into a MALDI-TOF MS machine. A vacuum is drawn, and the suspended cells are then subjected to laser light energy, which results in changes in the nature of the sample as molecules are ionised, protonated and deprotonated. The active molecules are usually detected by a mass spectrometer. Signals generated from a spectrum are compared against a database of known patterns, and a match (identification) for the signal is generated.

Recently, new applications for MALDI-TOF MS have been identified. These include fatty acid profiles, as well as detection of antimicrobial resistance activities, based on activity of the culture of interest in the presence of the drug. A recent approach has used MALDI-TOF MS to type the flagella of *E. coli* (H typing) (Cheng *et al.*, 2013). Other applications include shiga toxin 2 (*stx2*) detection in *E. coli* (Fagerquist *et al.*, 2014), sub-typing of *Campylobacter jejuni* species (Zautner *et al.*, 2013), *C. botulinum* identification and toxin typing (Grosse-Herrenthey *et al.*, 2008; Kalb *et al.*, 2015; Wang *et al.*, 2015), and also for detection of *C. difficile* toxins (Moura *et al.*, 2013). MALDI-TOF MS was also used in sub-typing *Listeria spp.* (Barbuddhe *et al.*, 2008).

Some of the significant advantages of using a MALDI-TOF MS-based approach include speed of results obtained, relatively few reagents required and the relative cost of sample testing, as well as the repeatability and reliability of the results obtained and a reduced risk of human error associated with biochemical reaction interpretation. One of the disadvantages of the approach is the significant cost associated with purchase of the instrument and associated service. Another limitation is the database of information from which spectra are compared and, in some situations, the machine needs to 'learn' from samples that are tested and added to the database. Other reports suggest that strain differentiation between closely related strains may be difficult, while other studies suggest that the culture method can affect identification. Ways to help solve these problems in the future will require further building of databases of information, based on profiles, including information for new strains not already available in the database, and designing optimised protocols for culture and testing.

MALDI-TOF MS currently has application in clinical, food and environmental analysis, and it has received FDA approval for clinical application. Two machines receiving this approval are the Bruker MALDI-TOF MS (Bruker) and the VITEK MS (BioMerieux). Clinical applications include detection of blood infections, urinary tract infections, diarrhoeal illness, meningitis, and respiratory tract infections. In food applications, MALDI-TOF MS has been used in water analysis, organism identification associated with spoilage of foods, and food-borne illness. In environmental applications, it has been used to identify and characterise isolates from various environments/ecosystems. Other applications of MALDI-TOF MS include detection of biological warfare agents and antimicrobial resistance (Singhal *et al.*, 2015).

6.6.1.6 Flow cytometry

Flow cytometry is an optical-based method that uses a liquid-based flow system to analyze individual cells in a sample. The system uses a liquid suspension of the

target cells of interest, which is introduced into a flow cytometer, where the cells are analyzed on the basis of light scatter patterns following exposure to light. Typically, these cells are labelled with a fluorescent dye to make detection easier. Once the light hitting the cells is scattered, the pattern of scatter provides information as to the size, quantity and shape of cells present in the sample. Flow cytometry has had greater application in the medical field, and can also have application in the food industry but it has limitations in terms of a minimum detection limit which, for bacterial cells, is in the region of 10^2 – 10^3 cfu/ml of liquid. It also suffers from limitations associated with the samples being tested, and interference from particulates in the sample that may interfere with the analysis and, as a consequence, require pre-treatment (such as an enzymatic clearance) of the sample to remove potential interference.

Applications of flow cytometry in foods can include total viable counts in a sample to live/dead cell counts if the appropriate live/dead cell stain is incorporated, or more targeted assay if a specific antibody and fluorescent tags are applied. Newer-based flow cytometers often incorporate separation techniques that enable separation of specific cell types, if required for further analysis. Although flow cytometry is a rapid field for analysis, it does require a specialised machine and trained personnel to design the analysis and interpret the data. Comas-Riu and Rius (2009) highlighted some of the applications of flow cytometry in the food industry from assessment of viability of cells to shelf life studies to detection. A recent review by Ibrahim and van den Engh (2007) provided a great overview of how flow cytometry and cell sorting works.

6.6.1.7 Solid phase cytometry

Solid phase cytometry is a modification of liquid flow cytometry where the bacteria of interest are first captured on a solid surface. Once captured, the cells are subjected to staining using appropriate stains. This type of approach is somewhat similar to a membrane filtration approach, and appropriate cell stains can be used. A limitation of this system is that the samples under investigation must be filterable, and there are risks where the samples contain large amounts of debris that may interfere with the sample analysis.

One such commercial example is the MuScan marketed by Lumibyte (<http://lumibyte.eu/>), which offers quantitative detection of cells within an hour. A similar system, marketed by BioMerieux (ScanRDI), uses filtration with laser scanning to enumerate and detect cells; however, this system is more associated with biopharma than clinical or food systems. The application of solid phase cytometry in the food diagnostics appears to have limited application, although a paper by Pyle *et al.* (1999) explored the detection of *E. coli* O157 by a combination of immunomagnetic separation coupled with solid phase cytometry.

6.6.2 Metabolism-based detection

Most methods described below are metabolism-based. In other words, detection relies on metabolic activity of the target of interest – that is, the target must be growing or changing or utilising a substrate, in order for it to be detected. Most metabolism-based assays do, however, have higher thresholds of detection before a response or signal can

be detected, and these have been estimated to be in the region of 10^4 – 10^5 cfu/ml. Usually, detection of the signal is limited to a small timeframe.

6.6.2.1 Head space analysis

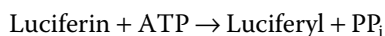
Another such method that could be included in this section, but is detailed later, is the application of electronic nose technology that relies on bacterial metabolism to use as an indicator of spoilage – (see section 6.6.7).

6.6.3 Luminescence-based

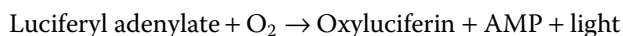
6.6.3.1 Bioluminescence/ATP detection

ATP bioluminescence works by measuring the adenosine triphosphate (ATP) levels in living cells as a means to estimate the number of viable cells present. Often, bioluminescence is used as a means to assess the effectiveness of cleaning or disinfection of surfaces and utensils.

Detection of ATP for assessing viability of a cleaning procedure involves analysis of a sample swab of an area. The swab is then combined with a mixture of the enzyme luciferase and its substrate luciferin. The reaction of luciferin and luciferase is dependent on the presence of adenosine triphosphate (ATP) from bacterial contamination of the poorly cleaned surface (or other viable material, such as blood, fats, etc.) to catalyze the reaction, which is only found in living cells. The ATP combines with luciferin to form luciferyl adenylate and pyrophosphate.



The luciferyl adenylate reacts with oxygen to form oxyluciferin and adenosine monophosphate, with the release of light:



The enzymatic process results in the release of light, which is measured as relative light units (RLU) and is proportional to the amount of ATP (or bacterial cells) present. Testing time takes about 10–15 minutes. A number of commercial hand-held devices are available for this type of testing. Typical levels of detection are 1–200 cfu.

Several drawbacks exist for this method. ATP is an energy source in *all* living cells; as such, food samples are likely to contain large amounts of ATP from fat cells, blood or muscle cells. This means that the amount of ATP measured will not only include ATP from bacterial cells, but will also include ATP from these other cell types. In addition, ATP assays cannot detect specific pathogens, or identify the source of ATP. ATP assays may also have limitations, and may have errors associated with the sampling technique, which can result in false positive and negative results (Carrick *et al.*, 2001).

Despite these drawbacks, ATP detection is still being widely used in the food industry as verification of cleaning and sanitation effectiveness, because results are obtained in an extremely short time, compared with culture methods. This allows corrective actions to be implemented in a timely manner, to minimise the risk of product contamination. Recent modifications to ATP detection have incorporated fluorescence for detection of fluoroimmuno stained cells and bioluminescence with immunomagnetic separation (Squirrel *et al.*, 2002; Takahashi *et al.*, 2000; Tu *et al.*, 2000).

6.6.4 Immunological/ serological based

6.6.4.1 Antibody-based latex agglutination assay

Latex agglutination assays are designed to provide a result for the user in a matter of minutes. The most common latex agglutination assays use antibody-coated coloured latex beads, or colloidal gold particles, that clump together in the presence of a target antigen. In bacterial-based assays, insoluble bacterial cells will clump in the presence of antibody. In general, latex agglutination assays require a pure culture of the target antigen to be accurate, as the sensitivity of the antibodies can be relatively low, thus leading to potential cross-reactions and false positive results.

Latex agglutination assays are cheap, easy to perform and read, and available for a range of food-borne bacterial pathogens, including *E. coli* O157:H7 and other STEC members, *Salmonella* spp., *Campylobacter* spp., *Listeria*, *Shigella*, *S. aureus*, *Vibrio* and *Yersinia*. One of the potential limitations of latex agglutination assays is the ability of the latex beads/particles to cross-react with other members of the same genus because of structural similarities in the lipopolysaccharide layer (Feng, 2007).

6.6.4.2 Immunoprecipitation

The lateral flow kit/dipstick method is based on the use of immunochromatography for pathogen detection. In the assay, a sample of interest (usually in liquid form) is applied to the open well of the dipstick or kit, and the liquid is wicked into the kit through a strip, until it reaches an area of the kit containing antibodies conjugated to a precipitable material, such as colloidal gold. If the target pathogen is present in the sample, then an antigen-antibody complex forms and moves to a second region, where binding to a second antibody occurs, resulting in an antibody-antigen-antibody complex, which usually is usually associated with a coloured immunoprecipitate. The immunoprecipitate results in a double line in the kit, indicating a positive reaction; one of the lines is usually the positive control line, to indicate that the kit is working correctly with control sample, while the second line indicates the true positive as a result of the antigen antibody reaction from the test sample.

Lateral flow/dipstick-based kits are fast and relatively common in food diagnostic situations, and have been developed for most of the major pathogens of interest, including *Salmonella*, *Campylobacter*, *Escherichia*, and *Listeria*. Advantages of the kit are the ease of use and rapid time to result. However, the kit has limitations, and needs a high level of the target analyte in the sample to ensure detection. These kits may also suffer limitations in terms of their specificity, which is usually linked to the sensitivity of the antibodies used in the kit. As a consequence, there are liable to be both false negatives (where the test fails to detect the target) and false positives (where the test detects a similar target but not the correct one – cross reaction). Fakhr *et al.* (2006) noted some of these limitations in detecting *Salmonella* from naturally contaminated turkey.

6.6.4.3 Immunomagnetic separation (IMS)

Immunomagnetic separation (IMS) is an immunologic-based detection system that uses antibody-coated immunomagnetic beads to isolate and separate the pathogen of interest from an enrichment sample. The magnetic beads used in the assay are paramagnetic, which means that they are only magnetic in the presence of a magnetic field. The surfaces of the beads are coated with an antibody specific to the target of interest.

When an enrichment sample is incubated with the beads for a period of time, the antibody will react with any target cells that are present in the enrichment sample, forming a bond. A magnetic field is applied to the sample to concentrate the magnetic beads to a specific location (usually the side of the tube), along with the bound target. The beads containing the bound cells are then washed and suspended in a small volume of diluent, after which the cells can be removed from the beads, resulting in a concentrated product. Alternatively, the beads and bound target can be plated directly on selective media to detect the target organism, or further processed into a PCR reaction or other assay (immunofluorescence), to detect the target pathogen.

One of the drawbacks with IMS is the fact that it is immuno-based, and there is potential for cross-reactivity with non-target or non-specific antigens, leading to false positive results. There is also the potential for false negative results, where levels of the target organism are too low for adequate detection and binding by the IMS beads, as most antibody-based assays have a minimum detection limit of about 10^4 cfu/ml.

Literature on IMS assays suggest that they can detect one organism in a sample, provided that adequate opportunity for replication occurs and the target is not out-competed by other background or resident flora. False binding can also occur against antigenically similar organisms to the target, which may interfere with detection. A false negative rate of 2–10% can occur with IMS, due to limitations from inoculum level, background flora and potential interference of the sample matrix (Bennett *et al.*, 1996; Chapman *et al.*, 1994; Wright *et al.*, 1994). False positive results are non-existent, as presumptive positive samples can be further confirmed using verification assays. IMS beads have been developed for detection of pathogens such as *Salmonella*, *Listeria*, *E. coli* O157 and EPEC (O145, O111, O103, O26) from enrichment. The system has also become more automated, which enhances multiple sample handling.

6.6.5 Nucleic acid-based (molecular)

6.6.5.1 DNA microarrays

DNA microarrays consist of gene probes arrayed on a substrate. These arrays are designed to detect genes of interest, or can be multi-genome-wide in scope. They can be used for comparative genomic hybridisation (CGH) (also called genomotyping), to study genome-wide gene expression, or for the detection of multiple pathogens or resistance genes in a sample, and they have quickly become a powerful tool in genomic analysis. The microarray itself (often called a gene chip or biochip) consists of a series of DNA molecules of known sequence, called probes, that are fixed to a substrate (usually a special type slide). The probes consist of partial gene sequences, generated from PCR, full length cDNA, or oligonucleotides (Pagotto *et al.*, 2005) of the pathogens of interest.

Microarrays can be used to detect a large number of pathogens in a sample or, alternatively, perform expression studies of a whole genome or select genes. For the purposes of this chapter, we will focus on microarrays as a detection technology. Ojha and Kostrzynska (2008) highlight the application of microarray technology in the field of veterinary research for pathogen infection investigations, diagnostics and studies of host pathogen interactions. Similar research by Jin *et al.* (2005) used microarray technology to investigate *E. coli* O157:H7.

Microarrays can also be used to assess similarities between strains, characterise strains or subtype strains. Boyd *et al.* (2003), Hain *et al.* (2006), Parker *et al.* (2007) and Raengpradub *et al.* (2008) have described the use of microarray technology to analyze strains at the genetic level for comparative purposes. Anjum *et al.* (2007), Batchelor *et al.* (2008), Chen *et al.* (2005), Reen *et al.* (2005), Volokhov *et al.* (2003) and Yoshida *et al.* (2007) have used microarrays to identify various bacteria to species or subspecies level, detection of virulence or antimicrobial resistance genes. Call *et al.* (2001), Chandler *et al.* (2001), Keramas *et al.* (2004), Kostic *et al.* (2007), Kostrzynska and Bachand (2006) and Quinones *et al.* (2007) have used microarrays for detection, identification and characterisation of pathogens in a range of samples. Moreover, Wang *et al.* (2007) described a microarray assay capable of detection and identification of 22 food-borne pathogens simultaneously.

Given the unlimited amount of information available, microarrays can be designed and built for a range of purposes, including determining expression of specific virulence or antimicrobial resistance genes, invasion studies, flagellar production, growth processes or biofilm production. DNA microarrays also offer promise for future studies in understanding pathogens, hosts and production systems by modelling interactions, and it is likely that this technology will continue to provide insight into the mechanisms of pathogenesis used by food-borne pathogens that could be exploited to make food safer. Also of importance is the ability to automate the system. Significant drawbacks of microarrays include the cost of developing the array slides or obtaining them commercially, as well as the technology for assaying and reading results.

6.6.5.2 DNA colony hybridisation

Colony hybridisation is a low cost method for pathogen detection, where detection is based on hybridising a DNA-based probe to the DNA of the target. Colony-based hybridisation involves testing colonies of a potential target by selection from a culture plate, and transferring to a solid support such as a membrane. The cells of the colonies are then lysed, using an enzyme to release DNA. The DNA is denatured to allow linkage to the support membrane, and then hybridised to a gene probe with complementary sequence to the target DNA. Often, the DNA gene probe is labelled with a fluorescent tag or other colour marker, to allow easier detection of the pathogen. Recently, the solid support used for detection of target DNA has been modified and a dipstick-based approach has become common, where the DNA is hybridised to probes embedded on a solid surface, and hybridisation can be achieved in solution.

One commercial system, the Gene Trak kit (Neogen), can detect pathogens such as *Listeria monocytogenes* and *Salmonella spp.* Neogen has also developed the next generation of Gene Trak, called GeneQuence, which uses a microwell format for the assay. Here, potential target cells are added to a lysis tube, where the cells are lysed, and a portion is transferred to a microwell plate, where probes and reagents are added. The probe consists of an oligonucleotide *capture probe* that is specific to the target rRNA and labelled at the 3' end. A second *detector probe* is present and labelled at the 5' end with the enzyme horseradish peroxidase (HRP). Hybridisation occurs for about one hour and, if the target rRNA is present, both probes will hybridise. The capture probe has an extension that allows it to bind to a complementary portion on the surface of the microwell, resulting in capture of the rRNA. Detection of the hybridised detector probe usually occurs by a colour reaction indicating the presence of the target of interest (http://www.neogen.com/FoodSafety/pdf/DNA_H_Diagram.pdf).

6.6.5.3 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) has significantly revolutionised molecular studies and our approaches for rapid detection and diagnostics. The basic principle of PCR is that a thermostable DNA polymerase is used to amplify a specific region of DNA on a target organism to detectable levels from the template DNA of the target. In conventional PCR, the amplicons generated are then separated out by gel electrophoresis, in an effort to concentrate same-sized fragments together, which allows the DNA to be stained, and the size of the DNA bands are determined by comparison to standard DNA and control DNA.

Currently, uses of PCR have changed considerably over recent years. PCR is no longer just about detection of a target pathogen, but also has uses in pathogen identification in foods and other substrates, gene detection associated with pathogenesis, virulence or toxins, and virus or fungus detection (Auvray *et al.*, 2007; Baert *et al.*, 2008; Kim *et al.*, 2007; Niessen, 2008). The application of PCR has taken a significant foothold in diagnostic facilities (such as diagnostic labs – clinical and otherwise), simply because of the rapid turnaround, in a matter of hours.

Rapid PCR techniques have also emerged, making PCR-based approaches more desirable and useful for implementation in a diagnostic setting. Newer PCR reactions are often configured to amplify multiple gene targets simultaneously, thus allowing a potential user to detect multiple pathogens or genes simultaneously. Multiplex PCR (mPCR) reactions have been used for the simultaneous detection of multiple pathogens in a mixed sample or matrix, including the detection of pathogens such as *Salmonella*, *Shigella*, *Listeria*, *E. coli* O157:H7 and so on in foods (Alarcon *et al.*, 2004; Kawasaki *et al.*, 2010; Kim *et al.*, 2005; Yuan *et al.*, 2009; Lee *et al.*, 2014) or the detection of multiple virulence or resistance or toxin (STEC) genes in *E. coli* and *Salmonella* (Johnson *et al.*, 2007; Li *et al.*, 2006; Nde and Logue, 2008; Skyberg *et al.*, 2003, 2006; Verstraete *et al.*, 2012).

A further improvement of mPCR includes modification of GenomeLab's Gene Expression Profiler (GeXP) genetic analysis system, to allow high-throughput detection of multiple pathogens in a single reaction (GeXP-PCR), as described by Zhou *et al.* (2013) for the simultaneous detection of six food-borne bacterial pathogens: *Salmonella enterica*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* O157:H7, *Shigella* spp. and *Campylobacter jejuni*.

When PCR is used for detection of targets in food samples, the quality of the DNA that serves as template for amplification is significant; natural flora and the food matrix can contain PCR-inhibiting substances, such as carbohydrates, fats, polysaccharides and proteins (Fakhr *et al.*, 2006). Commercial PCR clean-up kits are invaluable for reducing interference, and should be considered where inhibition of the target is suspected. The efficiency of the PCR reaction can be enhanced by incorporation of different enzymes, labelling agents and specialty *Taq* polymerases (Fratamico and Bayles, 2005), while fluorescent-labelled primers can lower detection limits.

Chen *et al.* (1998) used fluorescent-labelled primers to detect shiga toxin-producing *E. coli* (STEC) toxin genes *stx1*, *stx2*, and *stxe*, with detection limits of 1–5 CFU per PCR mixture (pure culture), and 3 CFU per 25 g of food. Fanning *et al.* (1995) used a colour-amplified PCR to detect the heat-stable toxin gene (ST) in enterotoxigenic *E. coli* (ETEC), with a lower detection limit of 10 fg of purified DNA and 270 CFU.

Other applications of PCR have included diagnosis and quantification of mycotoxin-producing fungi in foods and other commodities (Niessen, 2008), and detection of shiga

toxins using an immuno-PCR assay (Zhang *et al.*, 2008). Another modification of PCR combined restriction fragment length polymorphism (RFLP) with PCR (Atanassova *et al.*, 2001) for the detection of *S. aureus* and staphylococcal enterotoxins in pork and pork products.

6.6.5.4 Nested PCR

Nested PCR is a refinement of standard PCR, designed to enhance detection of specific targets within targets. Nested PCR uses a primary set of primers to amplify a specific region of DNA, then a secondary set of primers is used to amplify a product which lies, or is 'nested', within the initially amplified product. The role of the nested reaction is to increase the accuracy of the PCR, in that a dual reaction ensures so that, if the wrong target is amplified in the first PCR reaction, then the odds of a second successful amplification becomes significantly lower as the second primer pair are designed only to amplify in the region of the first PCR product. Nested PCR has been used in the detection of *Shigella* and enterotoxigenic *Cl. perfringens* (Miwa *et al.*, 1996; Lindqvist, 1999; Warren *et al.*, 2006).

6.6.5.5 Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification technique that works along similar principles applied in PCR, and has significant application in diagnostics. LAMP was originally reported by Notomi *et al.* (2000). It works by an autocycling strand displacement DNA synthesis reaction, along with two inner and two outer primers that recognise six separate regions on the target DNA strand. LAMP has high specificity, as amplification only occurs when all six regions within the DNA are recognised by all of the primers. Amplification occurs under isothermal conditions between 59–65°C for 60 minutes.

DNA structures after amplification have a cauliflower-like appearance, because of the multiple DNA loops that occur, and higher levels of product are made in the timeframe of the amplification compared with standard PCR. Products of this amplification can be detected by gel electrophoresis, but alternative end-point detection strategies have included turbidity and fluorescence measurement (Mori and Notomi, 2009; Wang *et al.*, 2008; Zhao *et al.*, 2014).

LAMP application in food-borne pathogen detection has included detection of six genes in *E. coli* O157 (Maruyama *et al.*, 2003), and commercial kits for detection of pathogens such as *Listeria*, *Salmonella*, *Campylobacter*, verotoxin producing *E. coli* and *Shigella* have been reported (Mori and Notomi, 2009). Other applications for *Vibrio* detection have been developed (Di *et al.*, 2015), and for enterotoxin A in *S. aureus* (Suwanampai *et al.*, 2011). Different LAMP assays have also been developed, such as multiplex LAMP, real-time LAMP and *in situ* LAMP (Chen *et al.*, 2008; Han and Ge, 2010; Shao *et al.*, 2011; Ye *et al.*, 2011).

6.6.5.6 Real-time PCR

Real-time PCR is a modification of conventional PCR that eliminates gel electrophoresis, and provides users data in a shorter timeframe – in other words, in real time. A review by Mackay (2004) provides good information into the detail of the technology. Real-time PCR is based on standard PCR, but a fluorescent tag is attached to the primers, so that the amplicons produced can be detected by monitoring fluorescence

produced during the amplification step in a specifically designed thermocycler. Real-time PCR protocols can also be designed to detect multiple products simultaneously, using the same approach of standard multiplex PCR – typically, four or five fluorescent tags can be detected simultaneously by using different coloured fluorophores.

One significant advantage of real time multiplex PCR is that products of a similar size can be amplified without the interference issues that occur with standard gel electrophoresis, as each amplicon uses a different fluorescent tag. Some other advantages of real-time PCR over conventional PCR include: easy resolution of product; a faster time to result (negating the need for gel electrophoresis); and increased sensitivity. Real-time PCR sensitivity is greater than conventional PCR, as the technology requires detection of a light signal that can be quantified, with the release of light being in proportion to amplified product formed. Fluorescent dyes such as HEX, FAM, ROX, and SYBR green are some of the more common fluorescent dyes used in multiplex reactions (Huang *et al.*, 2007; Nde and Logue, 2008; Nde *et al.*, 2008; Wang *et al.*, 2007; Velasco *et al.*, 2014).

Multiple commercial thermocyclers are available for use in performing real-time PCR, offering capabilities from single tubes to 96 and 384 well formats, and they have the capability to detect up to five different fluorescent targets simultaneously. Despite the practical advantages of this method, the expense of the requisite thermocyclers and custom primers is high. Potential applications of real-time PCR include detection of genes or strains in a range of media (Bohaychuk *et al.*, 2007; Fakhr *et al.*, 2006; O'Grady *et al.*, 2008; Rodriguez-Lazaro *et al.*, 2004; Wang *et al.*, 2007), as well as diarrhoeagenic *E. coli* (Vidal *et al.*, 2005), and toxin gene detection in staphylococci, *Vibrio* and *E. coli* (Horsmon *et al.*, 2006; Fykse *et al.*, 2007; Grant *et al.*, 2006).

A primary limitation of PCR has been that it could not distinguish between live and dead cells. However, newly designed PCR protocols can target viable cells by detection of mRNA which is a marker of viability (Klein and Juneja, 1997; McIngvale *et al.*, 2002; Morin *et al.*, 2004).

Overall, PCR has tremendous power as a molecular detection and profiling tool. especially as a means to detect a pathogen as well as determine a pathogen's traits or to define its pathotype (Rodriguez-Siek *et al.*, 2005a, 2005b), and commercial kits for application in real time assay are commonplace (Maurer, 2011).

6.6.5.7 Quantitative PCR (qPCR)

Quantitative PCR (qPCR) is a modification of real-time PCR that can take advantage of the amplification nature of real-time PCR to quantify concentrations of the target present. In practical terms, the approach allows the user to quantify the copy number of a sequence present, relative to the amount of light produced during amplification (Omiccioli *et al.*, 2009; Zhao *et al.*, 2014).

6.6.5.8 Digital PCR

Digital PCR is a new concept in PCR that focuses on absolute measurements of nucleic acids by examining the amplification of single molecules within a PCR reaction. By optimising the template dilution, it is possible to only target one molecule per reaction partition. Early generation digital PCR reactions were based on a chip containing microfluidic channels, or plates that were either hydrophobic or hydrophilic. The target molecule is monitored in real time (like a real-time PCR reaction), and false positive reactions can be interpreted from amplification curves.

A number of companies have developed digital PCR systems, including BioRad, RainDance Technologies and others. RainDance Technologies claim to be able to detect one mutant in 250 000 wild-type molecules using their system (<http://raindancetech.com/>). The advantage of digital PCR is in its ability to employ thousands to millions of reactions simultaneously, allowing accurate detection of DNA on a scale far more sensitive than other standard laboratory technologies. Its application in food systems may not be too far away. Hudecova (2015) provides an overview of digital PCR and its application in clinical settings. General applications of digital PCR include use in detection of mutations in a system, copy numbers of samples, and a means to measure gene expression.

6.6.5.9 Droplet digital PCR

Droplet digital PCR is a variant of digital PCR with a slight variation in its application – for digital PCR, a tile-based approach can be used with multiple wells for PCR reactions and readout. Droplet digital PCR takes advantage of the creation of multiple droplets, each incorporating the reagents and DNA, and each drop becomes its own single PCR reaction. The droplet contains a water-oil emulsion in which the PCR reaction occurs. A droplet generator is employed to create the droplets in which the oil-water emulsions are made, containing the reagents and DNA. Following PCR amplification, a special reader is used to determine the PCR positive and negative droplets, which are quantified. Recent studies have reported on the use of droplet digital PCR for detection of *Cryptosporidium* oocysts in human faecal samples (Yang *et al.*, 2014), and to quantify genetically modified organisms in complex food and feed matrices (Morriset *et al.*, 2013).

6.6.5.10 16S Riboprinting

Riboprinting is a molecular tool for rapidly and accurately identifying and differentiating microorganisms. The technology, in essence, makes genetic strain-level fingerprints of a bacterium and compares the patterns against a database of patterns. Riboprinting depends on the selection of colonies which are picked from culture plates and then heat-inactivated; the DNA is extracted and restricted using a restriction enzyme. DNA fragments are separated by molecular size and transferred to a membrane and, after hybridisation of the fragments to a labelled DNA probe, a chemiluminescent agent is added and the light emitted from the fragments measured using a camera. Data captured is input into a system that uses algorithms to assign a fragment pattern, which can be compared to patterns in a database.

Riboprinting is an alternative rapid method PFGE, as it generates a fingerprint pattern in a significantly shorter timeframe than PFGE. Riboprinting has application in situations where comparison of the DNA fingerprints from multiple contamination events is used to determine sources of contamination in the food production environment. One of the limitations of the system, however, is the number of samples it can handle in a day, which is only eight. Riboprinting has most often been used in the typing of *Clostridium difficile* isolates and *Campylobacter*, as well as *Listeria* (Wassenaar and Newell, 2000; Matloob and Griffiths, 2014).

6.6.6 Next-generation technologies

The application of sensitive and rapid detection technologies has become of paramount interest in advancing methods for rapid detection and identification of pathogens of

concern. In this section, we review some of the newer emerging technologies and their potential for enhancing detection and diagnostics in food-borne pathogens. As we write this section, however, the reader should be aware that the technology is rapidly changing. Some reviews in relation to the use of biosensors include articles by Kaittani *et al.* (2010), Tallury *et al.* (2010), Velusamy *et al.* (2010) and Law *et al.* (2015). Two important factors in sensor detection methodologies are the ability to obtain a result in a short timeframe, and the ability to automate the system (Anderson and Taitt, 2005).

Biosensors are detection devices that use biological molecules to recognise and quantify analytes of interest. These analytes include antibodies, receptors, enzymes, nucleic acids, oligosaccharides, peptides, and so on. A recognition event between a biomolecule and an agent (e.g. nucleic acid, receptor, etc.) results in an output that can be measured either optically, chemically or electronically. Biosensors should be able to monitor a sample continuously for analyte and provide a quantitative readout (such as measure of concentration).

6.6.7 Immunosensors or biosensors

Biosensors are analytical devices that convert a biological response into an electrical signal. They work on the principle of a biological component that is coupled to a physicochemical type transducer, which collects signal and converts it to an electronic readout. Bioreceptors currently recognised for use in pathogen detection include antibodies, enzymes, nucleic acids, cellular, biomimetic, and bacteriophages (Velusamy *et al.*, 2010).

6.6.7.1 Electronic nose sensors

Electronic nose technology for pathogen detection works on the principal of detection of volatiles produced by active organisms in a sample. Detection is based on adsorption of volatiles to a series of conducting organic polymers, which cause a change in resistance of the sensors that can be equated to a population of specific organisms. Balasubramanian *et al.* (2005) used a commercial electronic nose system to detect *Salmonella* in inoculated beef samples, and was able to differentiate contaminated from control samples. Similar systems have also been used to differentiate *E. coli* O157:H7 from non O157 strains (Younts *et al.*, 2002, 2003). Others have used commercial systems to detect *Pseudomonas*, and *Bacillus* species in milk (Magan *et al.*, 2001) and *Salmonella*, *B. cereus* and *V. parahaemolyticus*, and was able to differentiate each based on its signal (Arshak *et al.*, 2009). Detection of *Salmonella* in alfalfa sprouts (Siripatrawan and Harte, 2015).

6.6.7.2 Mass-sensitive biosensors

Mass-sensitive biosensors work on the principal of piezoelectric crystals that vibrate at a specific frequency as a result of electrical input at the same frequency. If the mass of the crystal increases, due to binding of agents, the weight of the crystal and its oscillation frequency will change, and the difference can be measured electronically. Quartz is the most common piezoelectric material, and the main types of sensors are quartz crystal microbalance (QCM) or surface acoustic wave (SAW). When the surface of the sensor is coated with an antibody and exposed to liquid containing the target pathogen, the pathogens will bind to the antibody, resulting in a change in the weight of the crystal and a shift in frequency oscillation.

QCM have been used in the detection of *Salmonella* (Su and Li, 2004), *Listeria monocytogenes* (Vaughn *et al.*, 2001), *E. coli* O157:H7 (Berkenpas *et al.*, 2006) and *Campylobacter* (Masdor *et al.*, 2016). Modifications of the microbalance principle include piezoelectric excited millimetre-sized cantilever and magnetoelastic sensors (Mutharasan and Campbell, 2008; Ruan *et al.*, 2004), and nanoparticles or aptamers (Masdor *et al.*, 2016; Ozalp *et al.*, 2015).

6.6.7.3 Surface plasmon resonance (SPR)

Surface plasmon resonance (SPR) uses reflectance spectroscopy to detect pathogens, and is able to detect minor changes in refractive index that occur as cells of the target bind to receptors immobilised on a transducer surface. Changes in the angle of reflected light are measured as a function of changes in density of the medium versus time. SPR use antibodies that are immobilised on a gold electrode surface that can measure miniscule changes in resonance frequency, as a result of antibody antigen binding occurring (Feng, 2007).

Su and Li (2004) incorporated SPR with a piezoelectric system for the detection of *E. coli* O157:H7; a similar approach by Oh *et al.* (2005) could detect *Salmonella typhimurium*, and Leonard *et al.* (2004, 2005) reported detection of *Listeria monocytogenes*. Oh *et al.* (2005) reported the development of an SPR chip capable of detecting multiple organisms simultaneously, including *E. coli* O157:H7, *Salmonella typhimurium*, *Legionella pneumophila* and *Yersinia enterocolitica*. SPR sensors have also been used to detect *Listeria monocytogenes*, *Salmonella*, *E. coli* O157:H7, and *Campylobacter* (Bhunia *et al.*, 2004; Bokken *et al.*, 2003; Koubova *et al.*, 2001; Meeusen *et al.*, 2005; Subramanian *et al.*, 2006; Taylor *et al.*, 2006; Waswa *et al.*, 2007; Singh *et al.*, 2011).

Two commercially available biosensors (SPR) are the SPREETA biosensor and BIACORE 3000 biosensor, both of which have been used to detect food-borne pathogens, including *E. coli* O157, *Salmonella* and *Listeria* (Waswa *et al.*, 2007; Son *et al.*, 2007; Lan *et al.*, 2008; Leonard *et al.*, 2004; Bokken *et al.*, 2003; Waswa *et al.*, 2006; Wang *et al.*, 2011).

6.6.7.4 Raman and Fourier transform spectroscopy

This technology is frequently used in whole-organism fingerprinting, but also has an alternate application that depends on increasing the population, so that there is sufficient amount for biomass analysis. Raman spectroscopy is an optical technique that uses light scattering to detect pathogens (Schmilovitch *et al.*, 2005). It has been used to detect *E. coli* O157 in beef (Cho *et al.*, 2015) and *Listeria* in milk (Wang *et al.*, 2015). Modifications of Raman spectroscopy using surface-enhanced technologies (SERS) have also been developed (see below) (Grow *et al.*, 2003; Kalasinsky *et al.*, 2007).

6.6.7.5 Fourier transform infrared spectroscopy (FTIR)

FTIR is a non-destructive technique for pathogen detection that measures infrared loss after passing through a sample, with post-analysis of the sample data using Fourier transformation and an output that has a spectrum identical to the conventional infrared spectroscopy. FTIR has been used to differentiate and quantify *Salmonella*, *E. coli* O157:H7 and *Listeria* in various media or substrates (Al-Holy *et al.*, 2006; Lin *et al.*, 2004; Yu *et al.*, 2004). Ellis *et al.* (2002) reported the use of FTIR directly on food to

detect unique biochemical fingerprints of pathogens, and provided a measure of bacterial loads, while Wang *et al.* (2010) used FTIR to assess internalisation of *E. coli* in spinach leaves.

6.6.7.6 Fibre optic biosensor

Optical fibres for the detection of pathogens work on the principal of a fibre optic taper that sends excitation laser light to a detection surface and receives emitted light, and it has been used in the detection of food-borne pathogens. Optical biosensors measure changes in refractive index, fluorescence emission or quenching, chemiluminescence, and fluorescence energy transfer. Geng *et al.* (2004) described the use of a fibre optic-based detection system to detect *Listeria* in mixed cultures, and meat sample enrichments with a detection level as low as $10-10^3$ cells/ml. Kramer and Lim (2004) developed a rapid automated fibre-based biosensor to detect *Salmonella* in sprout rinse water, with a minimum detection limit of 50 cfu/g in inoculated seeds. Fibre optics have been used in *Listeria*, *Salmonella*, *E. coli* O157:H7, and *Clostridium botulinum* toxin detection (Ko and Grant, 2006; Ogert *et al.*, 1992; Simpson and Lim, 2005; Strachan and Gray, 1995; Zhang *et al.*, 2014), and for the detection of *S. aureus* (Chang *et al.*, 1996). Mendonça *et al.* (2012) incorporated immunomagnetic separation with fibre optics for detection of *Listeria spp.*

6.6.7.7 Aptamer-based biosensors

Aptamers are oligosaccharide- or peptide-based molecules that are used in the detection of pathogens or other analytes of interest. Usually, DNA- or RNA-based aptamers consist of short strands of oligonucleotides, while peptide-based aptamers are based in short variable peptide domains that are bound to a protein scaffold. Applications of aptamer-based technology have been incorporated into lateral flow assays for detection of *E. coli* O157 and other food-borne pathogens (Wu *et al.*, 2015; Bruno, 2014), as well as for use in immobilised slide assays and sandwich or ELISA-based assays (Maeng *et al.*, 2012; Wu *et al.*, 2014; Bruno *et al.*, 2009). Work in this arena is still in its infancy, but the technology offers alternative means for rapid detection.

6.6.7.8 Nanotechnology for pathogen detection

Nanotechnology presents a great opportunity for rapid detection, diagnosis and identification of pathogens. Nanoparticles – in particular, gold and silver – have electronic and optical properties that make them useful in next generation detection technology. When the particles are coupled to affinity ligands, they can be useful in pathogen detection. For example, gold nanoparticles, coupled with specific oligonucleotides, can detect complementary DNA, while other types of nanoparticles, including quantum dots and carbon nanotubes, have been used in assays for detection of pathogens, toxins, DNA and in immunoassay development (Kaittanis *et al.*, 2010).

Articles by Kaittanis *et al.* (2010) and Tallury *et al.* (2010) provide some interesting overviews of nanotechnology and its application in pathogen detection. Recent work by Masdor *et al.* (2016) has identified the use of nanoparticles for detection of *Campylobacter* using a quartz microbalance, while Wang and Alocija (2015) used gold nanoparticles in association with immunomagnetic separation to detect pathogens. A recent review by Verma *et al.* (2015) suggest that gold nanoparticles have application in point of care diagnostics, as well as application in the detection of nucleic acids.

6.7 Conclusion

One rapid method not included in this review, but which is, perhaps, worth considering for the future, is the rapid emergence of whole genome sequencing (WGS) to better understand pathogens, their genetics and the traits they carry. Increasingly often, the literature demonstrates the use and application of WGS as a part of routine diagnostics, and WGS appears to be the next wave. Its ever-increasing presence and application for comparative analysis, and as a potential means for sub-typing outbreak strains (Mellmann *et al.*, 2011), makes it an invaluable resource in the lab (Bertelli and Greub, 2013).

We have already seen that whole genomes can be sequenced in less than a day, providing a huge wealth of information that may other typing tools alone would never succeed in providing in this timeframe. Perhaps the power of the whole genome will give rise to faster methods for target identification, and potentially reduce the time to control an outbreak or treat a patient, enhance current sub-typing tools or even enhance high-resolution typing tools such as the CRISPR system (Barrangou and Dudley, 2016). The future for rapid diagnostics is a rapidly changing landscape.

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7

Molecular Technologies for the Detection and Characterisation of Food-Borne Pathogens

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7.1 Introduction

Food-borne microbial infections continue to cause a substantial public health and economic burden across the world. There is a need for improved detection methods to assure food safety and protect consumer health, and a need to reduce detection times to allow food to be monitored along the complete 'farm to fork' food chain, as part of food safety management systems such as Hazard Analysis Critical Control Points (HACCP). Faster methods are needed for testing of foods, to ensure that they conform to microbiological criteria which may be set by regulators or customers, and to ensure that process controls and performance criteria have been achieved during food production. In this respect, rapid methods are particularly important as, in many cases, they combine the specificity to detect key pathogens with short analysis time.

There are many challenges in the detection of food pathogens. A primary one is that they are generally present in very low numbers in the food (often < 100 cfu g⁻¹), are sometimes in the midst of up to a million other micro-organisms, and they may be on the surface or embedded in a complex food matrix. As many foods contain agents inhibitory to microorganisms, and may be stored at chill or frozen temperatures, the microorganisms on the food are often in an injured/stressed condition and, indeed, may be in a viable but not culturable state. Under the right conditions, these sub-lethally injured organisms have the potential to recover and cause illness, and therefore their detection is of public health importance.

Traditional methods for the detection of bacterial pathogens from foods rely on culturing of the organisms on agar plates (1–2 days), and usually necessitates an initial liquid enrichment step (1–2 days) to increase the numbers of the target organism and to enable recovery and detection of the organism from the food. The suspect colony on the agar plate can then be identified by morphological, immunological or biochemical means (De Boer and Beumer, 1999). These methods are, thus, very time-consuming, taking 5–7 days to detect specific pathogenic micro-organisms. In food microbiological analysis, while rapid methods are becoming more widely available and accepted, many still lack sufficient sensitivity, robustness and specificity for use by the food industry.

Major advances in omic technologies now allow the detection and identification of infectious agents for which routine growth-based culture and microscopy methods are not adequate, such as viruses, parasites, and bacteria which are difficult to culture. Although molecular biological assays are more specific, sensitive and faster than conventional microbiological methods, the complexities of food matrices continue to pose unique challenges that may preclude the application of molecular biological methods in the agri-food sector.

Molecular diagnostic techniques are based on the detection of a fragment of genetic material (nucleic acids – i.e. DNA or RNA) which is unique to the target organism and, as such, they are highly specific. DNA is a large molecule composed of a series of sub-units called nucleotides. Each nucleotide consists of a phosphate group, a five-carbon sugar, deoxyribose, and one of four different nitrogenous bases (adenine, thymine, guanine and cytosine, or A, T, G, C). These bases bond specifically, according to the number of hydrogen bonds that they form and, as a result, A always pairs with T and G with C.

The nucleotides are linked together to form a polynucleotide chain, and two strands of polynucleotides are twisted together to form a double helix. The base pairs can be arranged along the length of the helix in any order, and it is the sequence of base pairs along the length of the molecule which is important, and which forms the genetic 'fingerprint'. RNA is similar to DNA in that it is made up of nucleotides, the only difference being that the five-carbon sugar is ribose instead of deoxyribose, and the base uracil (U) is found instead of thymine. A single gene might be a segment of DNA or RNA, consisting of hundreds or thousands of nucleotides. Depending on the level of specificity required (genus, species, serovar, virulence or other phenotypic related factor), different regions and lengths of the genome can be used as targets in molecular assays (De Boer and Beumer, 1999).

Polynucleotides with a sequence complementary to the single-stranded DNA or RNA can be used as probes. Nucleic acid-based assays can be either performed directly, or after amplification of the target sequence.

7.2 Hybridisation-based methods

Hybridisation is the process by which a region of single-stranded DNA or RNA is bound to a probe with a complementary nucleotide sequence.

7.2.1 DNA hybridisation methods

DNA hybridisation tests may be performed in many ways. The southern blot method, originally described by Southern (1975), is based on the transfer and immobilisation of DNA fragments and separation by gel electrophoresis, all conducted on a solid support, such as nylon or nitrocellulose membrane. Solid-phase hybridisation can also be performed as colony blots or dot-blot. In colony blots, an aliquot of the test sample is spread-plated onto an appropriate agar. After incubation, an imprint of the colony/colonies is transferred to a solid support, such as a membrane or paper filter, by pressing the support onto the agar surface. Next, the cells are lysed, DNA-denatured and affixed to the support, and then detected by a labelled probe.

In the dot-blot technique, micro-organisms grown in liquid or solid culture are transferred, with a loop or other device, as a 'dot' onto a localised area of a membrane. The 'dot-blot' is particularly useful for identification of specific bacteria directly from agar plates. Dot-blot techniques have been described for the detection of pathogens including *Campylobacter* from poultry (Fontanot *et al.* 2014).

GeneQuence (Neogen) is a commercial kit which utilises DNA hybridisation technology in a microwell format for detection of pathogens including *Salmonella*, *Listeria spp.*, *Listeria monocytogenes*, and *Campylobacter* from food (Alles and Mozola, 2009).

7.2.2 RNA hybridisation methods

In Northern hybridisation analysis, RNA molecules are fractioned by size, using denatured agarose gel electrophoresis. The fractionated RNA is transferred to a solid support, such as nylon or nitrocellulose membrane, and then, similarly, to Southern blot analysis, is hybridised with a specific labelled probe.

7.2.2.1 Fluorescent *in situ* hybridisation (FISH)

Fluorescence *in situ* hybridisation (FISH) is a technique that uses a fluorescently labelled probe to hybridise specifically with a complementary ribosomal RNA target sequence (usually 16s rRNA or 23s rRNA) within the intact cell. Labelling of the probe with a fluorescent dye enables the organisms to be visualised using fluorescent microscopy. The use of FISH to detect a range of different food pathogens was reviewed by Rohde *et al.* (2015). A novel FISH technique, using peptide nucleic acids probes (PNAs) (synthetic DNA analogues), was described by Almedia *et al.* (2013) for detection of *Salmonella* in various foods (milk, eggs and mayonnaise). This PNA FISH method was shown to be 100% in agreement with the ISO method. However, FISH is not yet routinely used to analyse and monitor pathogens in food products, in part due to its dependence on microscopic detection, which requires considerable operator training and, generally, cannot be fully automated.

7.2.3 DNA microarrays

The underlying principle of a microarray analysis is also the hybridisation of complementary nucleic acid strands. Oligonucleotide probes with specific short sequences, ranging from 25 to 80 bp, can be applied to different microarray platforms including glass slides or silicon chips. The nucleic acid (DNA, mRNA or cDNA) from the test samples is labelled with fluorescent dye, and then denatured to single-stranded fragments. These fragments will then hybridise to their corresponding oligonucleotide probes on the array surface, giving a visual fluorescence signal. Unlike other hybridisation formats, DNA microarrays allow significant miniaturisation and also speed, automation and high throughput.

Microarrays were originally used for the study of gene expression, but oligonucleotide DNA microarrays have been developed and applied to the detection of a number of food-borne pathogens including *E. coli* O157:H7, *Listeria monocytogenes*, and *Campylobacter* (Suo *et al.*, 2010; Huang *et al.*, 2014). There are various commercially available DNA microarrays, manufactured by Affymetrix, Roche NimbleGen and Agilent

Technologies, but there are challenges in application to routine food microbial analysis, as they require complicated equipment and a complex data management system.

7.3 Nucleic acid amplification methods

Many methods include an amplification step for the target DNA/RNA. Such methods increase the target nucleic acid material by up to a million-fold, and are particularly important in the arena of food microbiology, where one of the major hurdles is the recovery and detection of very low numbers of a particular pathogen. However, even with the incorporation of an amplification step, a liquid enrichment of the food sample (24–48 hours) is generally still required to yield sufficient nucleic acid material from the target organism for the reaction.

7.3.1 Polymerase chain reaction

The most popular method of amplification is the polymerase chain reaction (PCR) technique. In this technique, the DNA is extracted from the organism and the double strands are denatured into single-stranded DNA. Short-sequence DNA primers are annealed to the complementary DNA target in the organism. The primers are then extended across the target sequence, using a heat-stable DNA polymerase (usually *Taq* polymerase, a thermostable and thermoactive enzyme from *Thermus aquaticus*), in the presence of free deoxynucleoside triphosphates (dNTPs), resulting in a double replication of the starting target material. Multiple repeats of the denaturation, annealing and extension steps results in an exponential increase in the levels of the initial target DNA, thus greatly increasing the sensitivity of the method. Theoretically, a single gene target can be amplified a million-fold, to allow detection in only a few hours.

The sensitivity of PCR is limited, in part, by the small sample volumes used in PCR, and may also be inhibited by the presence of inhibitory substances, such as proteinases and collagen present in foods (Powell *et al.*, 1994; Kim *et al.*, 2001). Sample preparation, including an enrichment step to increase the number of target cells, effective extraction of the pathogen from the enriched food, extraction of DNA, and the use of the appropriate DNA polymerases and primers, are critical to achieve the desired sensitivity in PCR reactions.

In conventional PCR, the steps in the PCR cycle are carried out in an automated, programmable block heater. The PCR products are separated by gel electrophoresis, stained with ethidium bromide and visualised using ultraviolet light. The target gene used in the reaction defines the specificity of the test, and may be focused on a particular bacterial species, serovar, virulence factor, antibiotic resistance factor, and so on. The PCR can be used to identify or to characterise bacteria in a colony taken from an agar plate, or can be applied directly to an aliquot from enriched food samples. One of the main disadvantages of conventional PCR is that it is generally time-consuming and labour-intensive.

7.3.1.1 Real-time PCR

Real-time PCR allows continuous monitoring of amplification through the use of fluorescent double-stranded (ds) DNA intercalating dyes or sequence specific probes

(Wittner *et al.*, 1997). Real-time PCR assays offer many advantages over traditional PCR methods. They are much quicker to perform, with the enhancements in speed attributed to reduced amplification time and the elimination of an additional step(s) needed for product detection. Moreover, the use of a closed system for amplification and detection minimises the potential for amplicon carryover contamination (Bankowski and Anderson, 2004). Although more expensive in capital terms, real-time PCR based strategies are popular in research and public health.

Real-time PCR methods can be divided into those that are not sequence-specific, such as DNA minor groove binding dyes. A method for non-specific real-time detection of PCR amplicons employs fluorescent double-stranded DNA intercalating dyes, such as SYBR Green I (McKillip and Drake, 2004). SYBR Green I binds to the minor groove of ds DNA during the extension step of the PCR, and falls off during the denaturation step (Bustin, 2000). The specificity and sensitivity of SYBR Green is limited, as it binds to all ds DNA, including non-specific PCR products and PCR primer dimers (Wittner *et al.*, 1997; Bustin, 2000).

Alternatively, a diverse array of fluorescently labelled probes can be used for sequence-specific detection of target DNA or RNA (McKillip and Drake, 2004). They involve fluorescence resonance energy transfer (FRET) between fluorogenic labels, or between one fluorophore and a quencher group (Didenko, 2001).

FRET is a process by which energy is passed between molecules separated by 10–100 Å that have overlapping emission and absorption spectra (Stryer and Haugland, 1967; Clegg, 1992). Regardless of the specific means in which the fluorophore-quenching pair is applied, these methods have the advantage of sequence specificity that ds DNA intercalating dyes do not offer (McKillip and Drake, 2004). Sequence-specific detection can be performed using linear oligoprobes, such as hybridisation probes (HybProbes), dual-labelled oligoprobes (TaqMan probes), or hairpin oligonucleotides (molecular beacons).

Real-time PCR has been applied to detect a wide range of food pathogens, including Verocytotoxicogenic *E. coli*, *Listeria*, *Salmonella*, *Campylobacter* and others. (Oravcová *et al.*, 2005; McCabe *et al.*, 2011; Luedtke *et al.*, 2014; de Boer *et al.*, 2015). A number of commercial systems, including BAX[®] System (Qualicon) are available for detection of a range of food pathogens.

A limiting factor in the uptake of PCR methods and, indeed, many other rapid pathogen methods by the food industry, relates to a lack of full validation against the accepted cultural method, in accordance with the International Standards Organisation (ISO). It is generally essential, when testing against microbial criteria set by regulatory authorities, to use a recognised ISO method, or an equivalent rapid method. In the EU, a procedure for determining equivalence is outlined in EN/ISO 16140: 2003. The lack of validation and ring trials is now being recognised as a shortfall, and research to fully validate methods is now being undertaken. However, this issue has advanced considerably in recent years, and an ISO method (ISO 13136) for detection of Verocytotoxigenic *E. coli* is based on a real-time PCR method which targets the verotoxin genes as well as serogroup-specific genes.

When a microbial pathogen is detected in a food, a cultured isolate is generally still required to support actions to be taken by risk managers, so PCR methods in the food industry are generally used as a screening test, which is followed by cultural examination and isolation when a positive PCR result occurs. A difficulty arises in the application of PCR to agri-food samples, when a PCR positive, but no cultural isolate, is

obtained (Noll *et al.*, 2015). This may indicate that the cultural method is not performing well, or that the bacteria is viable but not culturable. The reason for discrepancy in the methods requires further research.

7.3.1.2 Quantitative PCR

Increasingly, to support risk assessment for pathogens in foods, quantitative data, as well as qualitative data, on pathogens in foods is needed. However, the direct enumeration of food pathogens is difficult. It can be done by directly plating an aliquot of the test food onto agar plate and counting the colonies that develop, but this method has a low sensitivity (generally $\geq \text{Log}_{10} 2.00 \text{ cfu g}^{-1}$). A most probable number (MPN) approach involves preparation of a dilution series of the faecal sample and enrichment in broth, followed by plating on selective agar plates and subsequent biochemical assays for identification of species (Duggan *et al.*, 2010). While it does enable detection of the target pathogen, even at low concentration, it is laborious and requires several days for confirmation of results.

An approach used to overcome the issue of low sensitivity is the employment of an initial enrichment step, which is combined with the application of real-time PCR and the use of a standard calibration curve which relates the PCR crossing point value (C_T) with the initial number of pathogens in the sample. This approach was shown by Kramer *et al.* (2011) to be suitable for enumeration of *Salmonella* on pork carcasses, with a limit of quantification (LOQ) of 1.4 CFU 20 cm^{-2} per sample. Further studies have used this approach for enumeration of VTEC O157 and O26 in milk and bovine rectal swabs (Mancusi and Trevisani, 2014; Lawal *et al.*, 2015). De Oliveira *et al.* (2010) also used this approach for quantification of *Listeria monocytogenes* in minimally processed leafy vegetables.

7.3.1.2.1 Digital PCR

Digital PCR (dPCR) is a technique which provides absolute quantification of genes without the need for a standard curve. This is achieved by partitioning the sample, on micro-fluidic chips or micro-droplets, into hundreds of individual reactions, so that each reaction contains one or no copies of the nucleic acid target. The dPCR signal is measured after completing amplification, and the absolute number of target nucleic acid molecules in the sample is calculated, based upon counting positive versus negative amplification results, according to Poisson distribution. The technique has been successfully applied to quantify Hepatitis A and Norovirus in lettuce and water samples (Courday-Meunier *et al.*, 2015), but the application of this technology to detection of food pathogens needs future research and development.

7.3.1.3 Multiplex PCR

In multiplex PCR (mPCR), several specific primer sets are combined to give rapid detection of multiple target genes in a single reaction. The primer sets are designed to have similar annealing temperature, while still distinguishing between amplicons following thermal cycling. The concentration of primer is also important as, when multiple primers are included in a test, interactions may arise between the multiple primer sets, resulting in primer dimers

This approach could thus be very useful to screen food samples for multiple pathogens in a single test. Singh and Mustapha (2015) have described multiplex real-time

PCR assays for detection of eight Shiga toxin-producing *Escherichia coli* in food samples, by melting curve analysis, while Niu *et al.* (2014) described the detection and identification of six different food-borne bacteria by two-tube multiplex real-time PCR and melting curve analysis.

7.3.2 RNA-based amplification assays

While DNA is generally selected as a target molecule in designing PCR assays for food pathogens, a limitation of this approach is that it is not possible to distinguish between viable and non-viable bacteria, though this is somewhat overcome by sample enrichment, which increases the numbers of viable cells and target DNA. mRNA, which has a short half life, is a better target for the determination of viability (Sheridan *et al.*, 1998). The isolation of RNA is an essential step in the analysis of patterns and mechanisms of gene expression (Surzycki, 2000). Moreover, the viruses of importance in food-borne illness have RNA, rather than DNA, as genomic material.

7.3.2.1 Reverse transcriptase polymerase chain reaction

Real-time reverse-transcriptase polymerase chain reaction (rt-RT-PCR), detects the presence of mRNA (shorter half-life than DNA), and has shown great potential for detecting viable pathogens. The reaction employs the enzyme reverse transcriptase to convert messenger RNA (mRNA) into complementary DNA (cDNA), which is subsequently amplified by DNA PCR (Sambrook and Russell, 2001). Thus, this technique allows an exponential increase in the amount of mRNA in the form of cDNA copies.

The benefits of this procedure include its sensitivity, large dynamic range, and the potential for high throughput, as well as accurate quantification. To achieve this, however, appropriate normalisation strategies are required to control for experimental error introduced during the multistage process required to extract and process the RNA (Huggett *et al.*, 2005). A number of methods to detect food pathogens have been developed using the reverse transcriptase approach, including a *sigDE*-based real-time reverse-transcriptase PCR for the detection of viable *Salmonella enterica* (Zhou *et al.*, 2014). McCabe *et al.* (2011) described the validation of an RNA real-time assays targeting the *hilA* gene of *Salmonella enterica* serovars in food analysis.

A difficulty with the rt-RT-PCR technique is that isolation of RNA is technically more difficult than DNA, and is also less stable. RNA samples also can be contaminated with residual DNA, which makes it impossible to correctly determine RNA concentration or perform RT-PCR; thus, removal of DNA is critical in this technique. Overall, the use of this technology is currently more used for gene expression studies than as a detection system for food-borne pathogens.

7.3.2.2 Viability dyes in RT-PCR

A further option to detect only viable cells utilises a viability dye in a real-time PCR to detect DNA only in intact cell/wall membranes. The strategy relies on the use of DNA-binding molecules, such as propidium monoazide (PMA) or ethidium monoazide (EMA) as a sample pre-treatment prior to the PCR. These molecules permeate only membrane-compromised cells, and have been applied successfully for different types of food-borne pathogens, including bacteria and viruses (Elizaquivel *et al.*, 2014).

The use of propidium monoazide in real-time PCR has been described for detection of live *Salmonella* and *E. coli* O157 in foods (Li and Chen, 2013; Liu and Mustapha 2014). However, Barbau-Piednoir *et al.* (2014) reported some difficulties in the use of PMA in a viability-qPCR to assess viable and dead cells of *Salmonella enterica* subsp. *enterica* serovar Enteritidis. They found that some dead cells were still giving a signal, and that the use of PMA could also decrease the qPCR signal observed on living *S. Enteritidis* at low concentration. This highlights that further research is required on this technology.

7.3.3 Isothermal amplification

While PCR requires thermocycling to separate the double strands of DNA, more recently methods have been developed to amplify nucleic acids under isothermal conditions. These assays have the advantage of not requiring the use of a thermocycler.

7.3.3.1 Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is a gene amplification technique that is carried out at a constant temperature of 60–65°C, and is completed in less than one hour. Multiple sets of primers are used in the reaction, together with a polymerase (*Bst* DNA), which has high strand displacement activity in addition to replication activity. The amplicons are mixtures of many different sizes of stem loop DNAs, with several inverted repeats of the target sequence and cauliflower-like structures with multiple loops. The amplification products can be detected by agarose gel electrophoresis, or by real-time monitoring of SYBR Green I dye. As LAMP uses up to four primers that recognise six separate regions within a target DNA, the amplification reaction occurs only when all these six regions are correctly recognised by the primers, leading to a higher specificity than classical PCR.

LAMP assays have been successfully applied to detect a range of different food pathogens. Zhuang *et al.* (2014) reported detection of *Salmonella* spp. by a loop-mediated isothermal amplification (LAMP) method targeting the *bcfD* gene with potential for use as on-site diagnostic tool in the food and agricultural industries. A multiplex loop-mediated isothermal amplification (mLAMP) assay was developed for simultaneous detection of the *stx1* and *stx2* genes and applied for detection of *E. coli* (STEC) in cattle farm samples, and was shown to be accurate and rapid (Dong *et al.* 2014a). These authors also developed a LAMP method for detection of *Campylobacter* in farm samples (Dong *et al.*, 2014b).

7.3.3.2 Nucleic acid sequence-based amplification (NASBA)

NASBA is an isothermal amplification reaction for the detection of RNA or DNA. The method selectively amplifies RNA through the concerted action of three enzymes: reverse transcriptase, RNAseH and RNA polymerase. These act in tandem to amplify sequences from an original single-stranded RNA template. The reaction is performed at a single temperature (Cook, 2003), generates the same number of copies in a shorter time than RT-PCR, and incubation times are short at ≈ 90 –150 minutes. NASBA was first used for the amplification of blood-borne viruses (Kievits *et al.*, 1991), but it has been optimised for detection of pathogenic bacteria (Cook, 2003), including viable *Listeria monocytogenes* in food products (Nadal *et al.*, 2007) and viable *Escherichia coli* in water (Heijnen and Medema, 2009).

7.4 Molecular characterisation methods

Accurate typing and sub-typing of pathogenic bacteria is essential if human cases of infection are to be linked within epidemiological investigations, and sources of microbial contamination traced and controlled in food safety management systems. Typing methods can be classified as phenotypic (detecting characteristics expressed by the organism), or genotypic (directly examining the organism's genetic content). Phenotypic methods include phage typing, serotyping and biotyping. A range of genotypic techniques for the detection of bacteria in food have been developed, as described below.

7.4.1 Pulse field gel electrophoresis (PFGE)

PFGE employs restriction enzymes (endonucleases) to make a limited number of cuts in bacterial chromosomes to provide chromosomal restriction patterns that form a 'fingerprint' for each organism (Méndez-Álvarez *et al.*, 1995). In this technique, DNA is first prepared and encapsulated in agarose plugs, digested with appropriate restriction enzymes (depending on the target organism), and subjected to electrophoresis, in which an electric field periodically changes direction and/or intensity relative to the agarose gel. The periodic re-orientation of the electric field allows the separation of large DNA fragments, which would not be adequately separated by conventional agarose gel electrophoresis using a constant (direction) electric field (Maslow and Mulligan, 1996).

Each time the field is switched, larger molecules take longer to change direction and have less time to move during each pulse, so they migrate more slowly than smaller molecules, leading to overall separation. In this way, while conventional agarose gel electrophoresis may achieve a maximum resolution of 50 kb, PFGE is able to separate molecules as large as 12 Mb.

The concept of contour-clamped homogeneous electric field to separate large DNA molecules was introduced by Chu *et al.* (1986). PFGE requires highly technically skilled personnel, but is very reproducible, and has the advantage of generating genetic data which can be statistically analysed. Using standardised protocols, PFGE is used to share genetic information on food pathogens in national and international networks such as Pulse Net (a US national network of public health and food regulatory agency laboratories, coordinated by the Centers for Disease Control and Prevention).

7.4.2 Amplified fragment length polymorphism (AFLP)

The AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. The technique involves the digestion of DNA with restriction enzymes, and then the ligation of the fragments' ends to nucleotide adapters, which are designed in such a way that the initial restriction site is not restored after ligation. This allows simultaneous restriction and ligation, while re-ligated original fragments are cleaved again. Finally, PCR amplification of restriction fragments is achieved by using the adapter and restriction site sequence as target sites for primer annealing.

Visualisation of the amplified fragments is performed by gel electrophoresis. Two restriction enzymes are used, one with an average cutting frequency (like *EcoRI*), and a second one with a higher cutting frequency (like *MseI*). The vast majority of bands

detected on AFLP gels are fragments flanked by both enzyme recognition sites. AFLP analysis is applicable to DNA of any origin and complexity, without prior sequence information, and combines the reliability of the RFLP technique to the power of the PCR technique (Vos *et al.*, 1995). AFLP is of particular value for studies of closely related strains, such as analysis of variation within a serovar of *Salmonella enterica* (Lan and Reeves, 2007).

7.4.3 Restriction fragment length polymorphism (RFLP)

RFLP analysis investigates certain types of sequence polymorphisms – so-called point mutations – which can be base exchanges, base deletions or insertions. The basic mechanism of RFLP analysis relies on the ability of restriction enzymes, the ‘endonucleases’, to cut double-stranded DNA according to a certain succession of bases, in a process called digestion. RFLP can be performed by digestion of DNA samples, followed by analysis using standard gel-transfer hybridisation procedures.

Another method is the restriction digestion of a PCR-amplified DNA segment that contains a variably present restriction site. The technique thus requires some knowledge of the DNA sequence flanking that restriction site (Dietrich *et al.*, 1999). A three-gene PCR-RFLP typing method with the use of the 2100 Agilent Bioanalyzer was described by Soler-Garcia *et al.*, (2014) for the differentiation of *Salmonella* species, subspecies and serovars.

7.4.4 Multi-locus variable-number tandem repeat analysis (MLVA)

Most bacterial genomes contain tandem duplications of short DNA sequences, termed ‘variable-number tandem repeats’ (VNTR). Repeat unit sizes and repeat sequences can vary when multiple loci (sites on a chromosome where a gene is located) are examined in a number of different isolates of an individual microbial species. It has been documented on many occasions that the number of repeat units per locus is a strain-defining parameter. Consequently, there is isolate specificity in the number of repeats per locus when different strains of a given bacterial species are compared.

A sub-typing method targeting these repeats, multiple-locus VNTR analysis (MLVA), is a useful tool for characterisation of bacterial pathogens (van Belkum, 2007). MLVA has been used effectively for tracing *Salmonella* and VTEC and is a modern, timely and versatile bacterial typing methodology (Hyytiä-Trees *et al.*, 2006; Torpdahl *et al.*, 2007). In an outbreak related to *E. coli* O103 in fermented sausage in Norway, MLVA gave more discriminatory results than PFGE (Schimmer *et al.*, 2008). However, as yet there is no common allelic or profile nomenclature to enable laboratories to compare data easily, though Holmes *et al.* (2015) reported on an inter-laboratory comparison of an eight-loci MLVA scheme, using a set of 67 isolates of VTEC O157.

7.4.5 Multi-locus sequence typing (MLST)

Multi-locus sequence typing (MLST) exploits the unique nucleotide sequences in micro-organisms by amplifying and sequencing segments of a number of housekeeping genes. Between isolates, there will be so-called point mutations, which can be base (A, T, G, C) exchanges, base deletions or insertions, and the sequences from different

isolates can be compared to establish relatedness. This tool has been applied to a wide range of food-borne pathogenic bacteria (Urwin and Maiden, 2003), and there is a database (<http://www.mlst.net>) of profiles which also contains specific protocols. This technique has been shown to be particularly useful for sub-typing *Campylobacter* spp., with sequences from seven housekeeping genes used to define sequence types and clonal complexes (Miller *et al.*, 2005; Griekspoor *et al.*, 2010).

7.4.6 Whole genome sequencing (WGS)

Whole genome sequencing (WGS) is a process that determines the complete DNA sequence of an organism's genome. The earliest method for genome sequencing was Sanger sequencing (Sanger *et al.*, 1977), also referred to as dideoxy sequencing or chain termination, and is based on the use of dideoxynucleotides (ddNTPs) during *in vitro* DNA replication. This was followed by shotgun sequencing (Staden, 1979), a method in which DNA is broken up randomly into numerous small segments, and then sequenced using the 'Sanger' chain termination method. Next generation sequencing (NGS) technologies were then developed, which generally involve parallel sequencing, producing vast quantities of data that require modern computation methods to assemble the sequence reads. These technologies have vastly enhanced the speed, increased the throughput and reduced the cost of DNA sequencing.

The earlier platforms for NGS sequencing included pyrosequencing (Roche Diagnostics), which is based on the detection of released pyrophosphate during DNA synthesis, and SOLiD Sequencing (Life Technologies), which uses a ligation-based approach. More recent platforms include Ion semiconductor sequencing (Life Technologies, Ion Torrent), which is based on the detection of hydrogen ions that are released during the polymerisation of DNA, and Illumina Sequencing (Illumina), which uses a sequencing-by-synthesis method, detecting release of fluorescent labels from incorporated nucleotides to determine sequence. Illumina Sequencing includes MiSeq, NextSeq and HiSeq, which have moderate to high throughput, respectively. Single molecule real-time sequencing (Pacific Biosciences) observes natural synthesis of unmodified DNA by DNA polymerase, using nucleotides with fluorescent labels attached to the terminal phosphate.

The choice of a specific platform is guided by application, since available systems have variable characteristics (e.g. cost, read-length and number of reads). Numerous analysis pipelines and publicly available websites are being developed to enhance the assembly, annotation and interpretation of these data, and will facilitate the transition to use in diagnostic laboratories. Bioinformatics tools for comparative genome analysis are reviewed by Kisand and Lettieri (2013). Thus, WGS is becoming more accessible for use in studying the evolution and emergence of food pathogens, epidemiology and investigations of outbreaks and sporadic cases and in diagnostics and characterisation of isolates.

The use of WGS in clinical and public health microbiology has been reviewed by Kwong *et al.* (2015), and Franz *et al.* (2014) published a position paper, arising from an OECD (Organisation for Economic Co-operation and Development) workshop, which gives an overview on how WGS could be used to tackle STEC in global food production systems. However, WGS still remains most applicable for implementation in clinical and public health microbiology laboratories and, indeed, is now the choice

for sub-typing, in use in many national reference laboratories (Dallman *et al.*, 2015; Kwong *et al.*, 2016). It has been shown to be highly valuable in recent food pathogen outbreak investigations (Jenkins *et al.*, 2015; Ashton *et al.*, 2015; Schmit *et al.*, 2014; Hoffmann *et al.*, 2016). However, even in this specialised arena, uptake and effective use is limited by the need for highly specialised staff, effective semi-automated pipelines, standardised quality control and data interpretation, bioinformatics expertise, and infrastructure.

7.5 Conclusion

The food industry has seen significant scientific and technological advancements, but food-borne contamination and outbreaks continue to occur. An increasingly global food market, and changes in food production systems and in consumer eating habits, will continue to place a greater burden on global food safety systems. Thus, a greater emphasis needs to be placed on addressing these global food safety concerns, and on counteracting the increasing risk for contamination along the food chain. The incorporation of technological developments by the food industry will be essential to facilitate the rapid and sensitive detection and tracing of pathogens along the farm-to-fork chain.

Apart from improving laboratory-based detection technologies, there is a particular need for hand-held mobile devices for use in the 'field' in primary production and 'on site' in food production and processing facilities. Developing omic technologies, linked with ICT and big data handling, has the potential, over the next decade, to provide such solutions. However, as methods to detect contamination become ever more sensitive, there is a corresponding need for harmonised risk assessments among regulators and risk managers. New methods must be fully validated, with national and international standardisation of new methods required in a timely manner. In the food industry, molecular characterisation and typing of isolates can give scientific evidence about where a pathogenic or spoilage micro-organisms is entering the chain, where cross-contamination may be occurring, and can demonstrate if particular strains are endemic in a factory environment, allowing focused and effective risk-based management decisions.

As molecular tools continue to advance and to be adapted in the food sector, the concept of molecular risk assessment will likely become routine, with strains recovered from food assessed for virulence potential on the basis of their gene profile and relatedness to human clinical isolates, and risk management decisions made based on the outcome. Such data will enable focusing of resources most effectively on high-risk vectors and vehicles of contamination, to reduce the overall public health risk posed by pathogens.

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8

DNA-based Detection of GM Ingredients

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8.1 Introduction

Deoxyribonucleic acid (DNA) is a ubiquitously occurring cell constituent that provides highly specific information on organisms at every taxonomic level. Therefore, it is an ideal target for diagnostic approaches. The major breakthrough for DNA analysis was the development of the polymerase chain reaction (PCR) (Mullis and Faloona, 1987, Saiki *et al.*, 1985). This technique, enabling the exponential amplification of selected DNA sequences with high degrees of sensitivity and specificity, paved the way for the incorporation of DNA analysis as a standard tool in the portfolio of modern analytical methods. Meanwhile, this technique has been widely applied in many fields, such as clinical diagnostics, forensic sciences and histopathology, and has become the method of choice for the detection of genetically modified (GM) plants. Since commercialisation in 1996, GM crops gained importance worldwide, underlined by a yearly acreage increase (James, 2014). As a consequence, the need for methods to detect and to quantify DNA from genetically modified organisms (GMO) has been a major drive for the development and optimisation of PCR-based techniques. In this review, the basic principles, challenges and current developments in DNA-based detection of GM crops will be outlined.

8.2 Analysis of GMO

Many countries have established regulatory frameworks for the use of recombinant DNA techniques in the course of the production of foods and food ingredients (Broeders *et al.*, 2012). In addition to safety aspects, labelling of foods derived from GMO is a central issue of public debate. In the European Union (EU), legal requirements for labelling of GMO-derived foods was first provided by the so-called Novel Foods Regulation (European Commission, 1997) and its amendments (European Commission, 1998, 2000a, 2000b). The need for labelling was triggered by the detection of either protein or

DNA from the GMO. This initiated the first wave of analytical approaches, mainly focusing on the detection of DNA from GMO via qualitative conventional PCR.

Regulations have been progressively enforced, containing harmonised provisions for the risk assessment and authorisation of GMO, as well as traceability, labelling and post-marketing surveillance of the use of GMO in the food and feed chain (European Commission, 2003a, 2003b). Regulation (EC) No. 1830/2003 defined the establishment of a traceability system, allowing the documentation and the monitoring of the flow of GMO and GMO-derived products at all stages along the food and feed chain. According to Regulation (EC) No. 65/2004, information on traceability includes a unique code identifier for the respective transformation event, in the case of products consisting of or containing GMO (European Commission, 2004).

With this novel strategy, labelling requirements have been extended to highly processed products, and are no longer dependent on a positive testing of recombinant DNA or protein. However, it is acknowledged that, in agriculture, adventitious contaminations with traces of GMO-derived material cannot be excluded. Therefore, the current regulations determine certain thresholds for adventitious or technically unavoidable presence of GMO in food and feed. Labelling of products in the EU is not required if the proportion of material containing an authorised GMO is not higher than 0.9 % (considered individually for each ingredient) and the presence is considered to be adventitious or technically unavoidable. In food, no levels are tolerated for material derived from unauthorised GMO (zero tolerance policy).

To be able to control compliance with these legal provisions, appropriate methods for detection, as well as quantification of GMO, are required. The European Commission's Reference Laboratory (EU-RL GMFF) provides official methods for the specific detection and quantification of material from different GMOs. Despite these valuable methods, major analytical challenges arise from the increasing number of authorised and unauthorised GMO, the lack of reference materials, and the need to determine GMO contents in composite and processed foods.

8.2.1 Sampling and DNA extraction

In an analogy to other analytical approaches, appropriate sampling is the first requirement to be met when subjecting foods to DNA analysis. Inhomogeneous distribution of GMO in bulk materials is the main contributor to overall analytical variance. Errors may occur at various production stages (sampling, sub-sampling, preparation of aliquots for analytical steps, etc.). Sampling strategies are especially important if low GMO concentrations are to be analyzed (Anklam *et al.*, 2002a; Miraglia *et al.*, 2004). One of the objectives of the European Network of GMO Laboratories (ENGL) is to identify, develop and harmonise sampling strategies to support EU legislation (ENGL, 2016).

The next essential step is the extraction of sufficient amounts of DNA from the food matrix, exhibiting the quality required for successful PCR analysis. A broad spectrum of DNA extraction methods is available (Elsanhoty *et al.*, 2011; Holden *et al.*, 2003; Mafra *et al.*, 2008; Peano *et al.*, 2004). In general, plant tissue is ground, a detergent is applied to disrupt the cell membranes and to inactivate endogenous nucleases, different agents are used to remove proteins (Proteinase K), polysaccharides (CTAB) and lipids (chloroform) and, finally, the DNA is isolated by alcohol/salt precipitation (Anklam *et al.*, 2002a).

Various extraction kits are available, in which the isolation and purification of DNA is achieved by chaotropic salts on silica columns or by binding the DNA on magnetic particles.

At present, the CTAB-method and DNA-binding silica materials are most commonly used for isolation of DNA from food and feed samples. However, due to the increasing amount of samples undergoing DNA analysis, more and more laboratories use DNA extraction robots to meet the high workload (Guertler *et al.*, 2013). In addition to a higher reproducibility compared to manual DNA extraction, DNA extraction robots also reduce the likelihood of cross-contamination (Liu *et al.*, 2012), and the applied extraction approaches can also cope with difficult matrices, such as honey (Guertler *et al.*, 2014a).

Various matrix components or chemicals applied during DNA extraction may influence the purity of DNA and inhibit the subsequent PCR reaction (Wilson, 1997). To overcome false negative results, and to verify the absence of inhibitory effects, the use of homologous or heterologous internal positive controls in PCR amplification presents a powerful tool (Bahrtdt *et al.*, 2010; Cottenet *et al.*, 2013).

8.2.2 Choice of target sequences

A detection cascade (Figure 8.1) comprising three categories is commonly used in GMO detection. As a first step, screening methods are applied, which target genetic elements that are commonly incorporated into GM crops, such as the Cauliflower Mosaic Virus (CaMV) 35S promoter (P-35S), the *Rhizobium radiobacter* (former *Agrobacterium tumefaciens*) *nopaline synthase* terminator (T-*nos*), or the *epsps* gene coding for the 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase. The detection of such sequences indicates the presence of GMO-derived DNA. However, positive signals may also be obtained due to other factors, such as naturally occurring CaMV (Wolf *et al.*, 2000). As many GMOs contain these genetic elements, the GMO detection specificity is rather low, and does not allow a distinct identification of a certain GM event.

An increase in specificity is achieved by applying construct-specific detection methods. These methods target the junctions between two incorporated genetic elements. However, the complete gene construct may have been transformed into different GM events. To overcome this problem, event-specific detection methods are used, targeting

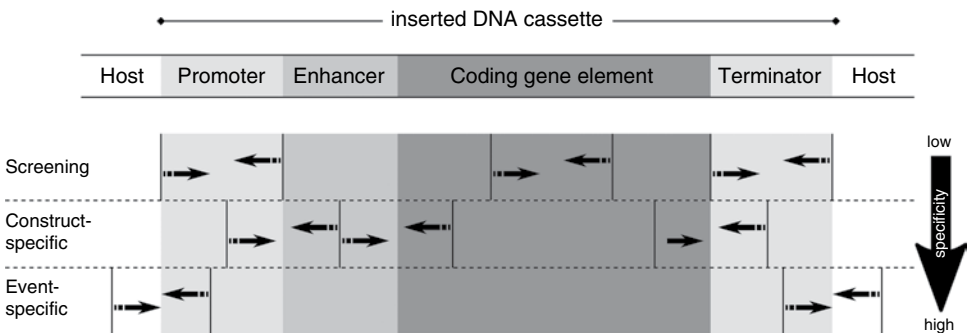


Figure 8.1 Cascade for DNA-based detection of genetically modified organisms.

the junction at the integration site, which is unique for each single GM event. Thus, event-specific detection methods provide the highest level of specificity and allow a differentiation between authorised and non-authorised GMO. The EU-RL GMFF publishes PCR-based methods for screening, identification and quantification of GMO on their website (<http://gmo-crl.jrc.eu.europa.eu>).

8.2.3 Conventional end-point PCR

PCR permits the detection of minute amounts of specific DNA sequences. These sequences are exponentially amplified *in vitro* using short oligonucleotides (primers), flanking the sequence of interest, and a thermostable DNA polymerase. In general, one PCR cycle is subdivided into three phases: double-stranded DNA is melted in a denaturation step, followed by an annealing step, in which primers bind to the target sequence. Finally, an enzymatic elongation takes place during the extension phase. Multiple cycles of this exponential amplification lead to a detectable quantity of the desired DNA fragment. Detailed descriptions and applications of this widely used methodology have been published (Baumforth *et al.*, 1999). Every gene of interest (e.g. a transgene) can be amplified in this way.

Following the PCR, the amplicons are generally separated according to their length, via gel electrophoresis or capillary electrophoresis. Verification of their identities is achieved by cleavage with restriction enzymes and subsequent separation of the digestions products via gel electrophoresis, capillary electrophoresis, southern blotting or by direct sequencing.

8.2.4 Real-time PCR

Real-time PCR (qPCR) is the state-of-the-art technique to detect and quantify DNA. This technique permits the direct measurement of PCR product amounts at every stage of the reaction by using fluorescence techniques. The fluorescence signals are proportional to the amounts of PCR products amplified, and can be generated by different approaches. As shown in Figure 8.2a, double-stranded (ds) DNA-intercalating dyes (e.g. SYBR green or EVA green) can be used to generate a fluorescent signal. DNA intercalating dyes bind to any kind of dsDNA, which is also the main disadvantage of this technology. Besides specific amplicons, non-specific PCR products, such as primer dimers, are also detected. However, the possibility of separating the desired amplicon from unspecific background by melting curve analysis, and an easy application at relatively low costs, are benefits of this detection format.

A higher DNA detection specificity is achieved by applying hydrolysis probes (also called *TaqMan* probes). A probe, complementary to the target sequence, is labelled with a reporter fluorophor at the 5'-end, and a quencher dye at the 3'-end. After excitation, the reporter fluorophor transfers the energy to the nearby quencher dye, which emits the energy as light of a distinct wavelength, or as thermal energy (dark quencher). During the extension phase of the PCR, hybridised probes are hydrolyzed by the 5'-3' exonuclease activity of the *Taq* DNA polymerase (Figure 8.2b). The distance between the reporter and the quencher dye is enlarged, impeding the energy transfer from reporter to quencher. Hence, the reporter itself emits light of a distinct wavelength, which can then be measured. The generated signal is proportional to the exponential amplification of the templates.

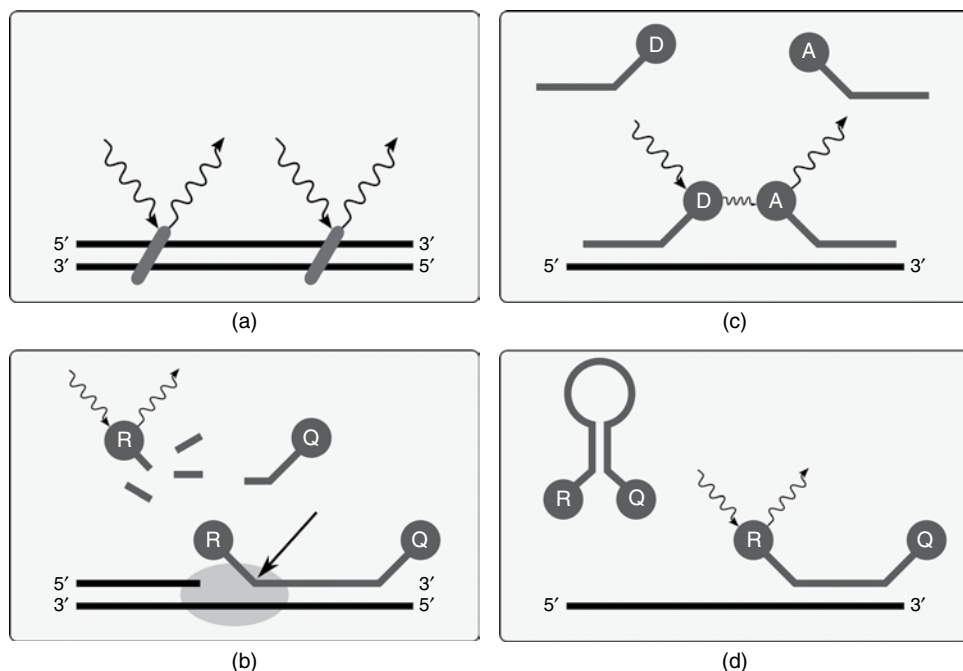


Figure 8.2 Commonly used detection formats in real-time PCR: (a) DNA-intercalating dye (b) hydrolysis probes (c) HybProbes (d) molecular beacon. A = acceptor, D = donor, Q = quencher, R = reporter.

A variation of this approach is the use of hybridisation probes (HybProbes), two sequence-specific probes hybridising adjacent to each other on the target, within a distance of 1–5 bases. Donor and acceptor probes are 3'- and 5'-terminally labelled, respectively (Figure 8.2c). In case of successful hybridisation of both HybProbes, the excitation of the donor is transferred to the acceptor, and the emitted fluorescence can be detected. The molecular beacon, another hybridisation format, is labelled on both ends with reporter and quencher fluorophor, respectively (Figure 8.2d). The central part of the probe is complementary to the target, whereas the terminal part is self-hybridised, forming a stem-loop structure. The probe binds to the template during the annealing phase, while the reporter dye is no longer quenched and a fluorescence signal is obtained.

The main advantage of internally hybridising probes is the additional sequence-specificity, as fluorescence signals of a distinct wavelength are only generated when probes hybridise and the target sequence is successfully amplified.

Quantitative statements can be made on the basis of the cycle of quantification (C_q) value (former: threshold cycle (C_t) or crossing point (C_p) (Bustin *et al.*, 2009)). The C_q value is defined as the intersection between the amplification curve and a threshold line (Figure 8.3a). Besides samples with an unknown concentration, quantification standards of known target concentrations are amplified to generate a standard curve, which describes the logarithmic plotting of starting copy numbers and the determined C_q -values. The template copy number of target sequences in an unknown sample can then be extrapolated on the basis of this standard curve (Figure 3B). An exact quantification of unknown samples is only assured if the amplification efficiency is equal to that of the

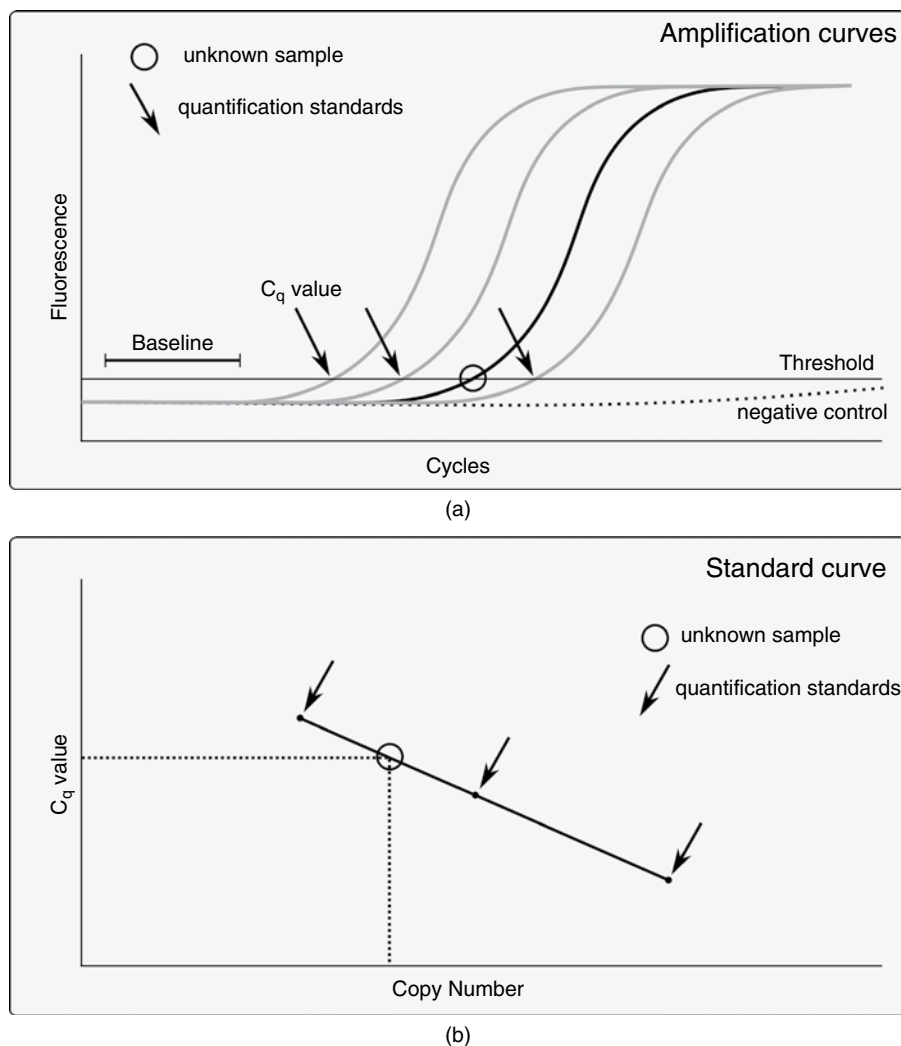


Figure 8.3 Principle of DNA quantification via real-time PCR. The threshold intersects the amplification curves in the exponential phase of PCR. The respective C_q-values of the quantification standards are correlated with the starting copy numbers in the standard curve.

standards used. PCR efficiency can be determined by examining the slope of the linear trend-line that should be between -3.1 and -3.6 (European Network of GMO Laboratories, 2015). Cloned plasmid fragments (Taverniers *et al.*, 2004), synthetic hybrid amplicons (Moreano *et al.*, 2005a; Pardigol *et al.*, 2003) and certified reference materials (CRM standards) have been described as quantification standards (Trapmann *et al.*, 2002).

8.2.5 Digital PCR

In 1992, Sykes *et al.* (1992) described the measurement of DNA concentrations via limited dilution, end-point PCR and Poisson statistics. This led to a third generation of

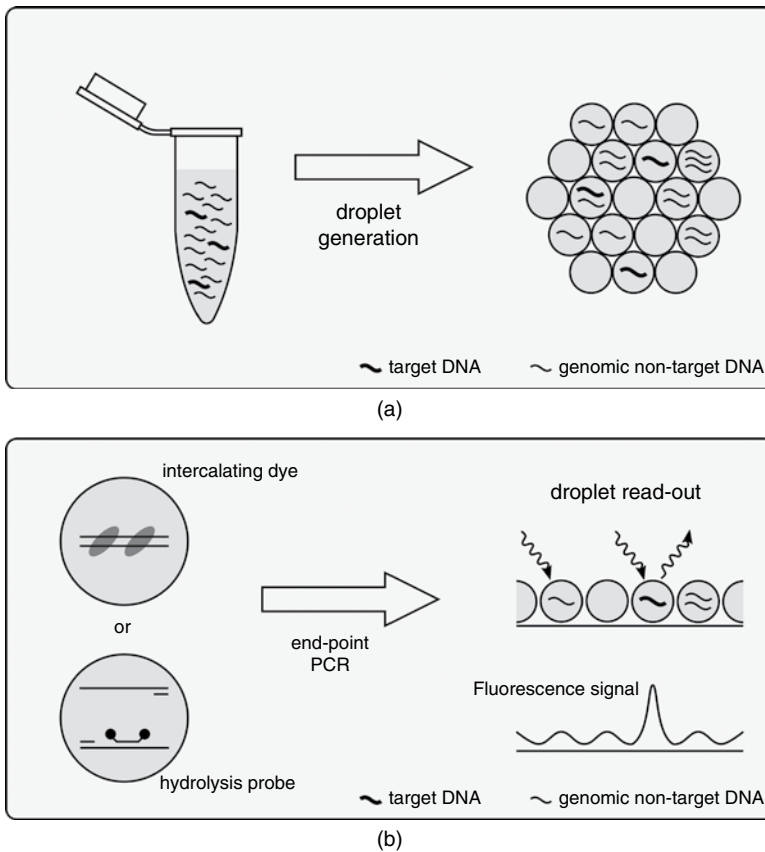


Figure 8.4 Basic principle of the digital droplet PCR (ddPCR). a) droplet generation and partitioning of the sample. b) end-point PCR and fluorescence detection.

PCR-based methods, which became known as digital PCR (dPCR) (Vogelstein *et al.*, 1999). DPCR consists of three steps: dilution and partitioning of the sample, end-point PCR using intercalating dyes or hydrolysis probes, and detection of positive partitions by measuring the fluorescence signal.

The diluted sample is randomly distributed into many partitions so that some partitions do not contain copies of the target sequence, whereas other partitions contain one or more copies of the target sequence (Figure 8.4a). After amplification of the target sequence by end-point PCR, the fluorescence signal is detected, resulting in positive (1) and negative (0) partitions. These 0/1 results led to the term ‘digital PCR’. Detection of the target sequence can be accomplished either by using an intercalating dye or a hydrolysis probe. Copy numbers are calculated from the fraction of positive partitions using Poisson statistics (Hindson *et al.*, 2011).

Currently, there are two common approaches in dPCR, depending on the partitioning of the sample. In the first approach, partitioning is accomplished by using chips with a fixed number of partitions (digital chamber PCR; dcPCR). In a newer approach, water-in-oil droplets with a fixed volume are generated (digital droplet PCR; ddPCR).

A hydrolysis probe- or intercalating dye-based end-point PCR is performed in each droplet, followed by fluorescence signal detection of each droplet (Figure 8.4b).

Quantification in dPCR is possible without the use of an external calibrator (e.g. a standard curve), which is one of the major advantages of this method, compared with qPCR. Further, dPCR is less prone to PCR inhibition, as detection of the fluorescence signal is performed after an end-point PCR (Dingle *et al.*, 2013).

In contrast, the fluorescence signal in qPCR is detected at every cycle. PCR inhibition leads to a shift of the amplification curve and, as a consequence, to a higher C_q value. In this case, a decrease in qPCR efficiency influences, to a vast extent, the result of the experiment (Cankar *et al.*, 2006; Demeke and Jenkins, 2010).

8.2.6 Multiplex approaches

In order to ensure compliance with labelling requirements, appropriate analytical approaches are required for the detection, identification and quantification of the steadily increasing number of GMO. Multiplex assays, allowing the simultaneous detection of several GMO or a GMO and a reference gene in a single PCR reaction, have been developed to meet this challenge (Germini *et al.*, 2004; Hernández *et al.*, 2005; Huber *et al.*, 2013; Onishi *et al.*, 2005). Digital droplet PCR, as a follow-up technology of the qPCR, is also capable of detecting two different fluorescent dyes and can therefore also be used for multiplex detection of GMO.

Morisset *et al.* (2013) transferred two qPCR-based detection assays (GM event MON810 and *hmg* as a maize taxon-specific reference gene) to ddPCR, and compared the results with previous qPCR experiments. Different parameters, like limit of detection (LOD), limit of quantification (LOQ) or repeatability, were comparable, or even superior, to those of qPCR. The applicability of this technology for multiplexing was further demonstrated by Dobnik *et al.* (Dobnik *et al.*, 2015), who quantified 12 different GM events by multiplex ddPCR. Additional examples of multiplex approaches for the detection of GMO are given in Table 8.1.

The simultaneous detection of screening elements in combination with construct- or event-specific targets provides a profiling-like strategy for GMO analysis, including unauthorised events (James *et al.*, 2003; Permingeat *et al.*, 2002; Forte *et al.*, 2005). Other approaches partly use similar primers for the detection of different targets, to overcome the difficulties of simultaneous amplification with multiple oligonucleotides in one single reaction (Germini *et al.*, 2004; Hernández *et al.*, 2005). Disadvantages of these approaches using gel-electrophoresis for separation are the long amplicon lengths and length differences of the targets, which pose a problem when applying the method to the analysis of processed foods (Matsuoka *et al.*, 2001), where DNA degradation may occur. At present, simultaneous amplification using nine primer pairs for the detection of different GM maize events represents the most comprehensive conventional multiplex PCR method (Onishi *et al.*, 2005).

Additionally, qualitative applications for identification of GMO in food have been introduced, using detection via microarray technology (Germini *et al.*, 2004, 2005; Xu *et al.*, 2005). Peptide nucleic acids (PNA) have been described as useful microarray probes for the analysis of Roundup Ready soybean and different GM maize lines (Germini *et al.*, 2004, 2005). PNA are analogues of DNA, with peptides, rather than pentose sugar phosphates, forming the backbone. This results in a higher affinity of

Table 8.1 Examples of multiplex approaches for the detection of GMO.

Targeted transgenes	Targeted endogenous genes	Technique	Detection	Reference
Maize Bt11, Bt176, MON810, GA21, RR-soybean	Maize <i>zein</i> , Soybean <i>le1</i>	Multiplex conventional PCR + ligation detection reaction (LDR)	Microarray	Bordoni <i>et al.</i> , 2005, 2004; Peano <i>et al.</i> , 2005
35S promoter, NOS terminator, FMV 34S promoter, SAMS, LY		Multiplex qPCR	qPCR	Bahrtdt <i>et al.</i> , 2010
35S promoter, NOS terminator, CP4 epsps, pat, bar		Multiplex qPCR	qPCR	Huber <i>et al.</i> , 2013
Maize Bt11, Bt176, MON810, T25, GA21	Maize <i>zein</i>	Multiplex conventional PCR	CGE-LIF	García-Cañas <i>et al.</i> , 2004
Maize Bt11, Bt176, MON810, GA21, RR-soybean	Maize <i>zein</i> , Soybean <i>le1</i>	Multiplex conventional PCR	Agarose gel electrophoresis, EtBr staining	Germini <i>et al.</i> , 2004
Maize Bt11, Bt176, MON810, GA21, RR-soybean	Maize <i>zein</i> , Soybean <i>le1</i>	Multiplex conventional PCR + asymmetric PCR with labelled primer	PNA-Microarray	Germini <i>et al.</i> , 2004, 2005
Maize Bt11, MON810, T25, GA21		Multiplex conventional PCR	Agarose gel electrophoresis, EtBr staining	Hernández <i>et al.</i> , 2005
P35S, NOS, cp4-epsps, nptII, cryIA(b), pat, bar, gox, oxy, barnase	Maize <i>ivr</i> , Soy <i>le1</i> , β -actin Rapeseed <i>cruciferin</i>	Multiplex conventional PCR	Agarose gel electrophoresis, EtBr staining	James <i>et al.</i> , 2003
P35S, T35S, NOS, <i>cp4-epsps</i> , <i>nptII</i> , <i>cryIA(b)</i> , <i>pat</i> , <i>bar</i> , <i>gox</i> , Pr-act		Multiplex conventional PCR	Agarose gel electrophoresis, EtBr staining	Matsuoka <i>et al.</i> , 2001, 2002
Maize Bt11, Bt176, MON810, T25, GA21, NK603, MON863, TC1507	Maize <i>ssIIb</i>	Multiplex conventional PCR	Agarose gel electrophoresis, EtBr staining Capillary electrophoresis	Onishi <i>et al.</i> , 2005
NOS, 35S-epsps, cryIA(b), pat,	Maize <i>zein</i>	Multiplex conventional PCR	Agarose gel electrophoresis, EtBr staining	Permingeat <i>et al.</i> , 2002
P35S, NOS, Amp, Maize Bt11, Bt176, MON810, T25, GA21, DBT418, CBH351	Maize <i>hmga</i>	Two-step PCR, labelled probe hybridization	Microarray	Rudi <i>et al.</i> , 2003

(Continued)

Table 8.1 (Continued)

Targeted transgenes	Targeted endogenous genes	Technique	Detection	Reference
GT73, MS8, RF3, Falcon GS40/90, T45	Rapeseed <i>cruciferin A</i>	Multiplex ligation-dependent probe amplification	Capillary electrophoresis	Guertler <i>et al.</i> , 2014b
18S rRNA, NOS, NOS/ <i>cp4-epsps</i> , CP4/CTP, 35S/CTP, 35S/plant, <i>cp4-epsps</i> , <i>nptII</i> , <i>cryIA(b)</i> , <i>bar</i> , PG	Maize <i>ivr</i> , Soybean <i>le1</i> , Rapeseed napin	Asymmetric PCR, labelled primer	Microarray	Xu <i>et al.</i> , 2005
MON810	Maize <i>hmga</i>	Duplex digital PCR	Digital PCR	Morisset <i>et al.</i> , 2013
Bt11, NK603, DAS59122, TC1507, GA21, MIR604, MIR162, MON810, MON863, MON88017, MON89034, T25	Maize <i>hmga</i>	Multiplex digital PCR	Digital PCR	Dobnik <i>et al.</i> , 2015
TC1507, MON810, NK603, MON863, Bt176, T25, GA21, Bt11	Maize <i>hmga</i>	Multiplex ligation-dependent probe amplification	Capillary electrophoresis	Holck <i>et al.</i> , 2009

PNA oligomers to hybridise with DNA; they are more sensitive to single mismatches and thus provide higher sequence-specificity (Jensen *et al.*, 1997).

A combined assay of multiplex PCR and ligation detection reaction, coupled with microarray, has also been developed (Gerry *et al.*, 1999) and applied to the analysis of traces of GMO in foods (Bordoni *et al.*, 2005, 2004; Peano *et al.*, 2005). This approach involves an additional confirmation step of the PCR products through ligation of sequence-specific probes, prior to microarray hybridisation. Only the ligated products generate a fluorescence signal when hybridised to the array with their unique 'zip code' sequence (Bordoni *et al.*, 2004; Gerry *et al.*, 1999). A specific, multiplex quantitative DNA array-based PCR has also been presented for the quantification of transgenic maize in food and feed (Rudi *et al.*, 2003).

Another innovative multiplex approach for quantitative analysis is the technique of ligation-dependent probe amplification (LPA). Originally, ligation-dependent PCR was applied to the relative quantification of DNA in the field of medical diagnostics (Schouten *et al.*, 2002). The suitability of this method for the detection, and relative quantification of GMO in food samples, has been demonstrated using commercially available maize standards (Moreano *et al.*, 2006). A synthetic probe set is used for the detection of target sequences, to avoid complex cloning and preparation steps required for the isolation of single stranded DNA probes.

These sequence-specific probes contain a target-specific hybridisation site and primer binding sites (PBS, identical for all probes) at their 5'- and 3'-ends, respectively.

In case of successful hybridisation to adjacent sites of the target sequence, the probes are ligated by a thermostable ligase. The use of stuffer sequences between hybridisation sites and PBS assures ligation products with distinct lengths, characteristic for each target DNA.

In the second step of the reaction, the ligation products are amplified competitively, using one labelled pair of primers. Labelled PCR amplicons are finally separated by capillary electrophoresis and detected via laser-induced fluorescence. The schematic workflow is depicted in Figure 8.5.

The use of one pair of universal primers in the LPA method avoids one of the major difficulties of multiplex PCR applications, namely the complexity of amplification reaction arising from the use of multiple primer pairs. In contrast to multiplex PCR reactions, the application of this novel approach offers great flexibility, due to its modular system, which can be complemented with further probes to broaden the range of target sequences. The use of synthetic oligonucleotides as probes and the employment of classical thermocycler and detection methods enable the implementation of the technique in commonly equipped laboratories.

Detection using capillary gel electrophoresis via laser-induced fluorescence (CGE-LIF) represents a very sensitive and rapid separation and detection approach in an automated manner. Different amplicons can be distinguished by size, using either labelled PCR primers or DNA-intercalating dyes. The better sensitivity and resolution of CGE-LIF, compared with agarose gel electrophoresis, has been demonstrated (García-Cañas *et al.*, 2004). Capillary electrophoresis is a useful tool for optimisation of multiplex PCR reactions, offering the possibility to obtain quantitative data in form of peak areas/heights (Butler *et al.*, 2001). Compared to real-time PCR, quantitative results can be achieved in a cheaper way.

8.3 Quantification of GMOs

The need to quantify the content of GMO in a product varies among different countries, depending on tolerance thresholds and labelling policies (Holst-Jensen, 2009; Gruère and Rao, 2007). The quantification in composed and processed products requires simultaneous assessment of the recombinant DNA and of a species- or taxon-specific reference gene. Thus, determination of ingredient-related GMO contents, as legally required, is possible. Two possible quantification strategies are commonly used in the field of GMO analysis: the standard curve method and the $\Delta\Delta C_q$ approach (Pfaffl, 2004, 2006). When using the $\Delta\Delta C_q$ approach, C_q values of two measurements are directly compared, whereas the standard curve method uses the relation of C_q values to a standard curve obtained by measuring quantification standards with predefined copy number concentrations (Milavec *et al.*, 2014).

Principles for the development, quantification and validation of methods for GMO detection are described in technical standards ISO 21570 and ISO 24276, enabling the use of comparable analytical methods by different laboratories. In Europe, GMO testing laboratories have to be accredited according to ISO 17025. In addition to the standards, the Joint Research Centre (JRC) has provided a technical guidance document to all laboratories, quantifying GMO with flexible scope accreditation (Trapmann *et al.*, 2013).

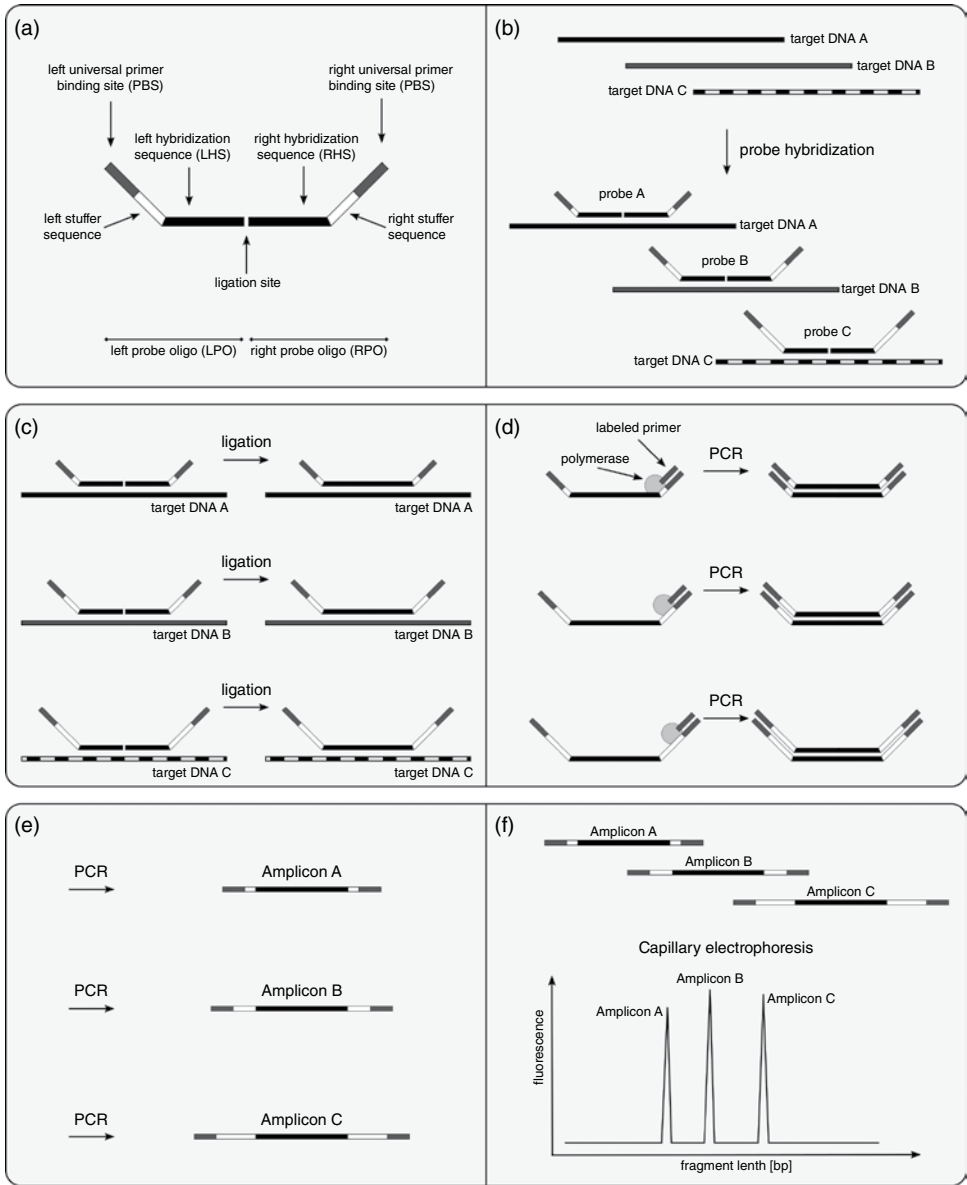


Figure 8.5 Schematic workflow of the multiplex ligation-dependent probe amplification (MLPA).

The first application of qPCR to quantify a GMO content in foodstuff was described by Wurz *et al.* (Wurz *et al.*, 1999). The method was developed to detect a recombinant region in the genome of Roundup Ready soybean, and a plant-specific sequence within the *lectin (le1)* gene. Special attention was paid to avoiding significant differences in amplicon lengths, meeting basic requirements for its application in processed foods. The approach was afterwards tested with certified reference materials containing

0.1–2.0 % by weight transgenic soybean, yielding results that were in agreement with the expected data.

Approaches for the quantitative detection of Bt176 maize and Roundup Ready soybean, targeting the transgenes *cryIA(b)* and *CP4 epsps*, respectively, have also been introduced (Vaitilingom *et al.*, 1999). The maize-specific *zein (ze1)* and the soya-specific *lectin (le1)* genes were used as endogenous reference targets. For the first time, PCR conditions were optimised to allow the quantification of transgenic and isogenic targets in one reaction vessel, thus eliminating tube-to-tube variations.

To date, various methods for the quantification of GMO proportions in raw food materials have been presented and validated in international interlaboratory trials (Wurz *et al.*, 1999; Vaitilingom *et al.*, 1999; Berdal and Holst-Jensen, 2001; Broll *et al.*, 2002; Höhne *et al.*, 2002; Pietsch and Waiblinger, 2001; Taverniers *et al.*, 2001; Terry and Harris, 2001; Waiblinger *et al.*, 2001). Commercial kits are also available for the quantification of different transgenic soy, maize and canola species. Interlaboratory testing of available kits for the quantification of Roundup Ready soybean and Bt176 maize have additionally been performed (Broll *et al.*, 2002).

Latest developments show the application of dPCR for the quantification of GMO (Corbisier *et al.*, 2010; Köppel and Bucher, 2015). Köppel and Bucher (2015) demonstrated comparable results of the duplex ddPCR quantification of the most abundant GMO employing real-time qPCR with certified reference material.

Another sensitive detection method, which might also be applicable for on-field testing, is the loop-mediated isothermal amplification (LAMP). LAMP is carried out at a constant temperature and, thus, does not require a thermocycler. A set of four primer pairs is used to amplify six regions of the target (Notomi *et al.*, 2000), using two inner primers and two outer primers, which undergo strand displacement DNA synthesis. Two loop primers are also added, leading to stem-loop DNAs, with several inverted repeats of the target (Ahmed *et al.*, 2009) after isothermal PCR. Detection of amplicons can be achieved by applying gel-electrophoresis. A quantitative approach, also named qLAMP, can be performed (e.g. by using intercalating dyes) (Nixon *et al.*, 2014). LAMP is faster compared to qPCR and, due to the fact that six different regions are targeted, it is proposed to be even more specific. However, validation and ring trials still need to demonstrate the suitability of both techniques in the field of GMO analysis.

Even though DNA-based methods are the key methods in GMO quantification, protein-based methods can also be successfully applied. For example, Chang *et al.* (Chang *et al.*, 2014) showed the application of matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) to quantify GM soya by detecting the protein CP4 EPSPS.

8.4 Validation

Analogous to other analytical methods, PCR-based approaches for detection and quantification of DNA also have to be validated (Anklam *et al.*, 2002b). The LOD and LOQ are method-specific, but also depend on the sample being analyzed. Three types of detection and quantification limits have been distinguished:

- i) the absolute limits – that is, the lowest number of copies required at the first PCR cycle to obtain a probability of at least 95% of detecting/quantifying correctly;

- ii) the relative limits – that is, the lowest relative percentage of GM material that can be detected/quantified under optimal conditions; and
- iii) the practical limits – that is, limits considering factors such as the actual contents of the DNA sample and the absolute limits of the method (Berdal and Holst-Jensen, 2001).

Both the LOD/LOQ of the method and the practical LOD/LOQ of the test sample should be reported, together with the results (Berdal and Holst-Jensen, 2001; Holst-Jensen *et al.*, 2003; ISO/DIS 21570, 2005).

Validation of quantitative assays for GMO in foods cannot be limited to unprocessed reference materials. Validation procedures must demonstrate that neither food composition, nor processing, will result in distortions of relative quantification results (Hübner *et al.*, 2001; Waiblinger and Grohmann, 2014). To date, standard protocols – for example, target recombinant and taxon-specific sequences of nearly equal efficiencies – are employed to ensure correct quantification (Bustin *et al.*, 2009). In Europe, the technical guidance standard ISO 24276:2006 (ISO, 2006) provides assistance for PCR method validation.

8.5 Challenges in GMO detection

8.5.1 Influences of food composition and processing

Industrially produced foods usually contain various ingredients. Such ingredients may be derived from the same (GM) crop, but may differ significantly in techno-functional properties. For instance, mixtures of corn milling fractions with different particle sizes are industrially applied to influence the characteristics of bakery products. It has been demonstrated that unequal efficiencies in the extraction of DNA from fractions differing in particle size distributions may contribute to distortions of GMO quantification (Holden *et al.*, 2003; Prokisch *et al.*, 2001). For corn milling fractions, a strong correlation between the degree of comminution and the DNA yields in the extracts was observed. Real-time PCR quantification of the GMO content in mixtures containing conventional and transgenic corn of different particle size distributions resulted in significant over- or underestimations of GMO contents (Moreano *et al.*, 2005b).

As expected from the phenomenon described previously for qualitative PCR, the length of the targeted DNA fragment is also crucial for the quantitative analysis of DNA in processed foods. Application of a method validated in an interlaboratory ring trial for the quantification of Bt176 maize delivered accurate data for unprocessed reference materials, but resulted in a significant underestimation of the GMO contents in heat-treated samples (Broll *et al.*, 2002; ISO/DIS 21570, 2003). The differences in amplicon lengths of the targeted reference gene (79 bp) and the transgene (129 bp) permitted the assumption that distortions in the results obtained by relative quantification resulted from the increased probability of fragmentation of the longer sequence. This could be confirmed by following the heat-induced DNA-degradation in mixtures of conventional and transgenic corn (1%) (Moreano *et al.*, 2005b; Moreano, 2005).

Two established quantitative assays, differing in the lengths of the recombinant and reference target sequences (A: $\Delta l_A = -25$ bp; B: $\Delta l_B = +16$ bp; values related to the

amplicon length of the reference gene), were applied. Method A resulted in underestimated recoveries of the GMO contents in heat-treated products, reflecting the favoured degradation of the longer target sequence used for the detection of the transgene. In contrast, method B resulted in increasing overestimation of the recoveries of the GMO contents in the course of the heat-treatment.

8.5.2 Copy numbers

The relative quantification strategy applied in GMO analysis determines the ratio of transgene copies to the respective copies of a reference gene assuming a 1 : 1 relationship between the two genes. In diploid homozygous lines (for example, achieved by means of self-pollination), this prerequisite is normally met (Miraglia *et al.*, 2004; Lipp *et al.*, 2005). However, the generation of genetically enhanced lines for commercial purposes involves cross-breeding with optimised conventional varieties. The resulting hybrids, with altered levels of ploidy have lost the original correlation between transgene and reference gene. Additionally, unequal levels of ploidy can be found in separate tissues of one organism as, for example, in the diploid embryo and the triploid endosperm of maize kernels (Lipp *et al.*, 2005; Trifa and Zhang, 2004).

The relative quantification of GMO-derived material requires special attention to be paid to the selection of suitable reference genes. Preferably, the reference target is a stable gene of known copy number in all varieties, and unique to the species. A systematic evaluation of plant species-specific reference genes has shown that these criteria are not fulfilled by every candidate gene (Hernández *et al.*, 2004; Papazova *et al.*, 2010; Scholdberg *et al.*, 2009; Wang *et al.*, 2010).

8.5.3 Certified reference material

The availability of reference materials plays an essential role in the course of the development and the validation of detection and quantitation systems for GMO, as well as for the implementation of surveillance testing. Commercially available reference standards can be purchased from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium) or the American Oil Chemists' Society (AOCS, Urbana, IL, USA). There is only very limited access to reference material for the analysis of non-authorised GM crops.

Approaches using DNA fragments cloned in a plasmid as external calibration standards were first described for the determination of Roundup Ready soybean (Taverniers *et al.*, 2004), and are now being applied to the analysis of various GMO (Taverniers *et al.*, 2005; Hernández *et al.*, 2003; Kuribara *et al.* 2002; Weighardt *et al.*, 2004). The synthesis of hybrid amplicon molecules by a novel two-step PCR amplification represents another strategy to obtain quantification standards. The reaction starts with separate amplification of the targeted recombinant and taxon-specific sequences, with bipartite primers generating complementary overhangs.

In the second PCR, the mixed purified amplicons are able to self-prime due to the overhang-sequences, and generate the complete hybrid molecules containing both targets (Moreano *et al.*, 2005a; Pardigol *et al.*, 2003). The application of this approach for a quantitative screening of genetically modified rapeseed lines with a number of transformation events has been described (Moreano *et al.*, 2005a). Two duplex real-time PCR

assays allow the simultaneous detection of the construct-specific junction between the 35S promoter and the *pat* gene in LibertyLink™ lines, between the *bar* gene and the g7 terminator in SeedLink™ lines, as well as the detection of a rapeseed-specific *acetyl-CoA carboxylase* gene. The moderate level of specificity avoids false positive results, and presents a valuable tool for GMO surveillance in food and feed products.

8.5.4 Sequence information

In order to be able to develop and validate new detection assays for GM crops, detailed sequence information of the genetic modification and the insertion site of the transgene needs to be on hand. As part of the EU authorisation process, the applicant is obliged to provide sequencing information on the genetic modification. However, in general, detailed information on unauthorised GM crops is not available, which hinders the development of event-specific detection methods. PCR and Southern blot analysis are common strategies to gain information on the number of insertions. Genome walking (Fraiture *et al.*, 2014) and Sanger Sequencing (Zhang *et al.*, 2008) can be used to characterise the transgene and to identify the flanking regions of the inserted DNA cassette. For these approaches, customised assays need to be developed, which can be very time-consuming, especially when dealing with complex mixtures.

As a new technology in the field of GMO analysis, Next Generation Sequencing (NGS) can make a contribution in revealing sequence information of unauthorised GM crops. One major advantage of NGS is the possibility to characterise a novel DNA fragment with limited or no knowledge of the transgene sequence. The applicability of this technology in the field of GM crop analysis was shown by Kovalic *et al.* (2012), who describe the use of NGS for characterisation of the flanking regions, based on the availability of reference genome sequences. Such reference genome sequences have already been determined for the most important GM crops: maize (Schnable *et al.*, 2009), soybean (Schmutz *et al.*, 2010), rapeseed (Chalhoub *et al.*, 2014) and cotton (Li *et al.*, 2014).

Li *et al.* (2014) proposed a combined approach of genome walking and NGS analysis for characterisation of GM crops, which outperforms the conventional Sanger sequencing strategy. Generally, due to the fact that NGS is a relatively new technology, the computing infrastructure and the laboratory equipment are not readily available in most GM crop surveillance units. By developing a statistical framework for the GM crop detection by NGS, Willems *et al.* (2016) demonstrated the practicability of this technology. NGS library generation can easily be accomplished without the need for special equipment, due to the widespread availability of commercial NGS kits. However, data analysis and interpretation still remain major tasks to be mastered.

8.5.5 Stacked events

In recent years, so-called stacked GM events have gained importance worldwide. Stacked events are GM crops that were produced through conventional breeding of single GM events. The result is a biotech crop that combines new traits of its respective parental lines in one organism. In the EU, stacked events must be authorised, regardless of the authorisation status of their parental lines. Therefore, in the GM surveillance process, stacked events need to be distinguished from their parental single events.

When testing DNA from a stacked event using parental event-specific detection methods, positive results will be obtained, as both the transgenic inserts and the integration sites of the parental lines will be found unchanged in the stacked event after conventional breeding. This also applies for screening and construct-specific methods. As a consequence, by using screening, construct- or event-specific methods, it is not possible to distinguish between a stacked event ($A \times B$) and a mixture of its respective parental lines ($A + B$) (Taverniers *et al.*, 2008).

Several studies have been published that proclaim the application of suitable methods to discriminate between a stacked event and the respective single events (Hernández *et al.*, 2005; Huber *et al.*, 2013; Onishi *et al.*, 2005; Morisset *et al.*, 2013; Dobnik *et al.*, 2015). All articles follow the same strategy, by detecting all insertion sites in a stacked event by using a panel of primer sets. A positive result for all primer sets would imply the presence of a stacked event. However, this approach only gives an indication, and does not prove the presence of a stacked event. This would only be possible in cases when genomic DNA from a single organism (e.g. DNA from a single kernel or a single leaf) is used. For complex matrices, like processed food or feed, this does not apply.

So far, there is no suitable method available allowing the discrimination of stacked events and their respective parental lines in food products. Perhaps statistical methods and new emerging techniques in GMO detection, like NGS, might help to tackle this problem.

8.5.6 GM animals

In recent years, genetic engineering of animals has increased significantly. In 2003, the so-called 'GloFish' was commercially available in the USA, which expresses fluorescent proteins that enable them to glow. These fishes were only available as ornamental fishes, and were not intended to be used as food or feed. GM animals are further used to produce pharmaceutical products like antithrombin (Atryn), which can be isolated from milk of GM goats (authorised in 2009 in the USA). In 2015, the US government approved transgenic chicken, which are able to produce a human drug (Kanuma) in the egg. Like the 'GloFish', these GM chicken or goats are not intended to enter the food supply.

The first GM animal to be authorised for its use as food is the GM salmon, which was deregulated in the US in 2015. The coding region of a growth hormone gene was inserted in salmon, allowing rapid growth (Ormandy *et al.*, 2011). Even though these fishes are allowed to be used as food, breeding in the US is not permitted.

Detection of genetic modification in animals follows a similar approach to that outlined above for GM crops. However, a direct transfer of suitable approaches may not always be applicable (Lievens *et al.*, 2015). Due to the absence of reference material and sequence information, limitations for the detection of GM animals are similar to those of GM crops.

8.6 Outlook

The constantly increasing number of new GM crop varieties, as well as the development of new technologies for genetic modification, lead to new challenges in the field of GM detection and quantification. To cope with these challenges, improved or new approaches need to be on hand, which ideally allow a multi-target detection.

Within the last decade, tremendous developments in the area of DNA-based food analysis have been witnessed. The rapid instrumental progress, further simplifications of the methodologies, and the obvious potential for automation, will further increase the attractiveness of these approaches. Considering the high degree of information provided by DNA analysis, applications like novel sequencing technologies or dPCR will definitely spread, and analyses of foods on the molecular biological level will be indispensable cornerstones of modern food diagnostics.

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9

Enzyme-based Sensors

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9.1 Introduction to enzymatic biosensors

The term 'biosensor' was introduced by Cammann (1977). A stricter definition of the term was set later by the International Union of Pure and Applied Chemistry (IUPAC) (Thevenot *et al.*, 2001). A biosensor is defined as 'a self-contained integrated device that is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is in direct spatial contact with a transduction element.'

Therefore, a biosensor consists of two main components: a bioreceptor and a transducer. The bioreceptor is composed of a biomolecule recognition element (an enzyme, an antibody, a protein receptor, DNA, or whole cells) that recognizes the target analyte, whereas the transducer converts the recognition event into a measurable signal (electrical, optical, thermal, and so on). A typical biosensor construct also normally incorporates signal-processing elements (amplification, filtering, data processing, and storage) and a display of the final result. A schematic diagram of a typical biosensor is illustrated in Figure 9.1.

The specificity of every biosensor is ultimately dictated to the specificity of the bioreceptor molecule used. Enzymes have been the most widely used bioreceptor molecules in biosensor applications. Enzymes are proteins that act as catalysts for biochemical reactions. Their activity is determined by their chemical composition and 3D structure. An enzyme is capable of recognizing only a specific substrate (or a class of substrates) and, therefore, is able to catalyze only a specific chemical reaction. In terms of the mechanism used in catalysis, enzymes can be categorized in six classes (Vargas-Bernal *et al.*, 2012). Among these, oxidoreductases (dehydrogenases, oxidases, peroxidases and oxygenases), which catalyze oxidation/reduction reactions of the substrate via transfer

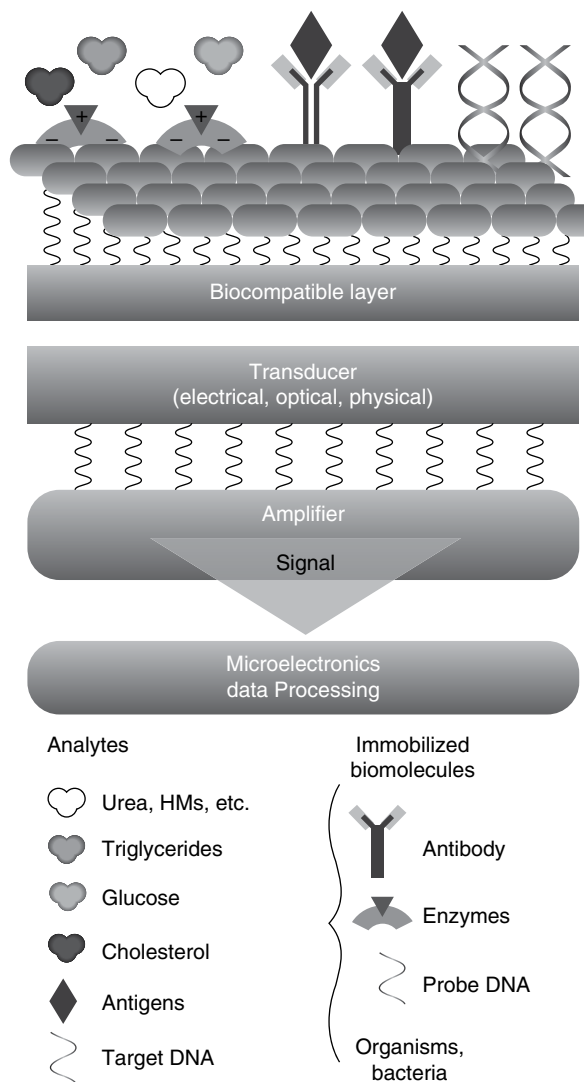


Figure 9.1 Schematic diagram describing the parts and operation of a typical biosensing device (reprinted with permission from Turdean, 2011).

of hydrogen or electrons, are the most analytically useful enzymes in biosensor applications (Vargas-Bernal *et al.*, 2012).

The popularity of enzymes as bioreceptor molecules in biosensors is due to (Borgmann *et al.*, 2011; Marazuela and Moreno-Bondi, 2002):

- a) the large number of enzymatic reactions that can be exploited for analytical purposes;
- b) the wide array of detectable species – in addition to direct detection of the substrate and product of the enzymatic reaction, inhibitors (compounds that inhibit the

- enzymatic reaction) and mediators (compounds that enhance the catalytic activity) can be monitored indirectly;
- c) the flexibility in detection – different types of transduction (electrochemical, optical, thermal) can be used to detect the analyte of interest;
 - d) the low consumption of enzymes, since they are not consumed during the analysis;
 - e) the high selectivity of enzymatic reactions;
 - f) the commercial availability of enzymes at high purity.

The disadvantages of using enzymes in biosensors devices are the following:

- a) Enzymes are bulky proteins and, often, the active site of the enzyme is not readily accessible to the substrate; therefore, the activity of the enzyme is reduced.
- b) Enzymes have an inherently limited lifetime, and can be deactivated by components in the sample, or by extreme chemical and physical conditions prevailing in the sample. Therefore, biosensors exhibit only limited long-term stability.
- c) The enzymatic activity is dependent on pH, ionic strength, chemical inhibitors and temperature.
- d) The cost of some commercial enzymes is often high.

Common enzymatic reactions forming the basis of biosensors in food diagnostics are listed in Table 9.1.

Historically, the first biosensor was described by Clark and Lyon in 1962, when the term ‘enzyme electrode’ was actually adopted (Clark and Lyon, 1962).

The development of an enzymatic biosensor involves:

- a) selection of a suitable enzyme;
- b) selection of a suitable immobilization method;

Table 9.1 Common enzymes used in food diagnostics and their reactions.

Enzyme	Enzymatic reaction
alcohol oxidase	$\text{ethanol} + \text{O}_2 \rightarrow \text{acetaldehyde} + \text{H}_2\text{O}_2$
glucose oxidase	$\text{glucose} + \text{O}_2 \rightarrow \text{gluconic acid} + \text{H}_2\text{O}_2$
lactose oxidase	$\text{lactose} + \text{O}_2 \rightarrow \text{pyruvate} + \text{H}_2\text{O}_2$
alcohol dehydrogenase	$\text{ethanol} + \text{NAD}^+ \rightarrow \text{acetaldehyde} + \text{NADH} + \text{H}^+$ $\text{acetaldehyde} + \text{NAD}^+ \rightarrow \text{acetic acid} + \text{NADH} + \text{H}^+$
lactate dehydrogenase	$\text{lactate} + \text{NAD}^+ \rightarrow \text{pyruvate} + \text{NADH}$
Acetylcholinesterase	$\text{acetylcholine} + \text{H}_2\text{O} \rightarrow \text{choline} + \text{acetic acid}$
cholineoxidase	$\text{choline} + \text{O}_2 \rightarrow \text{betaine aldehyde} + \text{H}_2\text{O}_2$
horseradish peroxidase	$\text{H}_2\text{O}_2 + \text{electron donor} \rightarrow 2\text{H}_2\text{O} + \text{oxidized donor}$ (electron donor = phenols, aromatic amines, luminol etc)
tyrosinase	$\text{phenol} + 1/2\text{O}_2 \rightarrow \text{quinone} + \text{H}_2\text{O}$
lipase	$\text{triglycerides} + 3\text{H}_2\text{O} \rightarrow \text{glycerol} + 3 \text{ fatty acids}$
nitrate reductase	$\text{NO}_3^- + \text{NADH} + \text{H}^+ \rightarrow \text{NO}_2^- + \text{NAD}^+ + \text{H}_2\text{O}$

- c) selection of a suitable transducer;
- d) optimization of the biosensor in terms of dynamic range, linearity, and minimization of interferences;
- e) packaging of the biosensor;
- f) commercialization.

The features of an ideal biosensor are (Vaddiraju *et al.*, 2010; Preda *et al.*, 2011):

- a) wide applicability to many sample matrices;
- b) high accuracy and precision;
- c) excellent sensitivity and specificity;
- d) wide dynamic range;
- e) rapid response time for real-time monitoring;
- f) high operational and physical robustness (i.e. insensitivity to variations of pH, ionic strength, temperature, pressure etc);
- g) long-term stability, lifetime and reliability;
- h) amenability to testing and calibration;
- i) low service requirements, running and capital costs;
- j) product safety (biocompatibility if the biosensor is to be used for invasive monitoring in clinical situations, and in environmental applications the host system must not be contaminated by the sensor);
- k) small size, portability and low power requirements.

As noted earlier, a main requirement for a biosensor is that the bioreceptor must be placed in close proximity to the transducer. In enzymatic biosensors, this is normally achieved by immobilization of the enzymes on the surface of the transducer. Immobilized enzymes offer some unique advantages in comparison with soluble enzymes (Ansari and Husain, 2012; Twyman, 2005):

- a) Reproducibility and low consumption of the enzyme; the same amount of immobilized enzyme can be used repeatedly for several measurements, ensuring high short-term reproducibility and low consumption.
- b) Stability: in many cases, enzymes are stabilized by the immobilization process, being active at a wider range of conditions.
- c) Simplicity: the analysis procedure is simplified and the analysis time shortened, because the use of additional solutions is not required.
- d) Sensitivity: since the transduction step is performed *in situ*, the detected species is not able to diffuse away from the transducer enhancing the sensitivity.

An ideal immobilization method should ensure:

- a) *Chemical stability*: the activity of the enzyme over time should remain constant.
- b) *Protection*: the catalytic activity of the enzyme should not be affected by matrix components.
- c) *Mechanical stability*: the immobilized enzyme should remain tightly bound to the surface of the transducer.

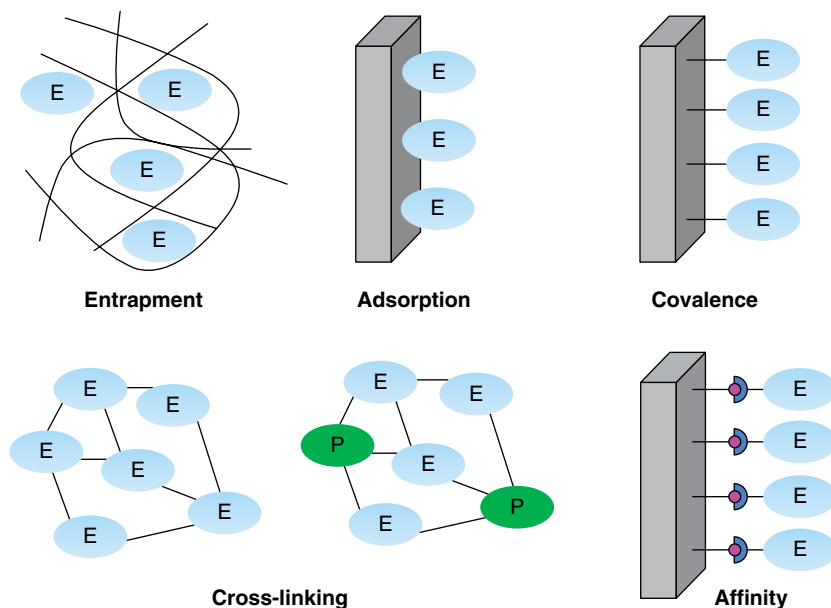


Figure 9.2 Schematic representation of the main immobilization methods. E – enzyme; P – inert protein (reprinted with permission from Sassolas *et al.*, 2012).

Since the type of immobilization method dramatically affects the analytical characteristics of enzymatic biosensors (sensitivity, accuracy, precision, lifetime, etc.), the development of suitable immobilization strategies is of critical importance. There are five main immobilization methods: adsorption, entrapment, cross-linking, covalent bonding and affinity attachment. The advantages and drawbacks of these methods have been extensively reviewed (Sassolas *et al.*, 2012; Choi, 2004; Balasubramanian and Burghard, 2006).

Figure 9.2 schematically illustrates the main enzyme immobilization methods.

9.2 Types of transducers

The majority of existing enzymatic biosensors are based on either electrochemical (amperometric and potentiometric) or optical (absorbance, fluorescence, chemiluminescence) transduction (D'Orazio, 2003), while other types of transducers (thermal, piezoelectric etc) are less frequently used. The principle of these techniques are listed in Table 9.2, whereas typical examples of the electrochemical and optical enzymatic biosensors are listed in Table 9.3.

Electrochemical biosensors can operate in turbid environments, exhibit satisfactory sensitivity, have low power requirements and are suitable for miniaturization. The two most important classes of electrochemical transducers for biosensing include the amperometric and potentiometric/ISFET devices (Zhang *et al.*, 2008; Grieshaber *et al.*, 2008).

Table 9.2 The operational principles of the main transducers used in biosensors.

Transduction system	Technique/parameter
Electrochemical	Amperometric/current at a constant voltage Potentiometric/voltage
Electrical	Conductometric/conductivity
Optical	Photometry/absorption Fluorescence/fluorescence intensity Chemiluminescence/chemiluminescence intensity
Thermal	Calorimetry/heat
Piezoelectric	Surface acoustic waves/mass absorption at the surface of a crystal Bulk acoustic waves/mass absorption at the total mass of a crystal

Table 9.3 Examples of electrochemical and optical detection in food diagnostics.

Analyte	Enzyme	Detection
aspartame	carboxylesterase, alcoholoxidase, carboxypeptidase, L-aspartase, peptidase, aspartateaminotransferase, glutamateoxidase and α -chymotrypsin	amperometric
sorbitol	sorbitol dehydrogenase, NAD ⁺	amperometric
benzoic acid	Tyrosinase	amperometric
sulphites	sulphite oxidase	amperometric
parathion	parathionhydrolase	amperometric
propoxur and carbaryl	acetylcholinesterase	optical
diazinon and dichlorvos	tyrosinase	amperometric
paraoxon	alkaline phosphatase	optical
nitrate	nitratereductase	amperometric
	nitritereductase	optical
phosphate	polyphenol oxidase and alkaline phosphatase, phosphorylase, phosphoglucomutase and glucose-6-phosphate dehydrogenase	amperometric
arsenic, cadmium, bismuth	cholinesterase	electrochemical
cadmium, copper, chrome, nickel, zinc	urease	optical
copper and mercury	glucoseoxidase	amperometric
fructose	fructosedehydrogenase	amperometric
Lactose	β -Galactosidase	amperometric
l-aminoacids	D-aminoacidoxidase	amperometric
l-glutamate	L-glutamateoxidase	amperometric
ethanol	alcohol oxidase, alcohol dehydrogenase	amperometric

Table 9.3 (Continued)

Analyte	Enzyme	Detection
cholesterol	cholesteroloxidase and peroxidase	amperometric
a-solanine	boutyrylcholinesterase	potentiometric
oxalate	oxalate oxidase, catalase	optical
polyphenols	tyrosinase, laccase	amperometric
fatty acids	lipase	amperometric
biogenic amines	amine oxidase and peroxidase	amperometric
hypoxanthine	xanthineoxidase	amperometric
inosine	xanthineoxidase	amperometric
lactic acid	xanthine oxidase, diamine oxidase, polymide oxidase	amperometric

Amperometric transduction is based on the application of a suitable potential to a working electrode, which initiates oxidation or reduction of an electroactive species. The resulting current is proportional to concentration of the target species. Enzymatic reactions often generate chemical species (e.g. H_2O_2 , NADH, quinone) that can be measured by amperometry. Depending on the electrical communication between the enzyme and the transducer, amperometric enzymatic biosensors can be classified into three generations (Borgmann *et al.*, 2011; Wang, 2008).

In biosensors of the first generation, the reactant or product of the enzymatic reaction diffuses to the transducer and causes the response. In the second generation of biosensors, intermediate compounds, called mediators, are utilized to transfer electrons between the enzyme and the transducer. Mediators can be immobilized on the surface of the transducer, together with the enzyme (Sarma *et al.*, 2008; Grieshaber *et al.*, 2008; Malhotra and Chaubey, 2003). In the case of the third generation of biosensors, direct electron transfer is possible between the active side of the enzyme and the transducer without the need of a mediator (Borgmann *et al.*, 2011; Preda *et al.*, 2011; Sarma *et al.*, 2008; Wang, 2008). Electrodes are usually made of noble metals and different forms of carbon (carbon paste, glassy carbon, carbon fibres, nanotubes or screen-printed graphite (Sarma *et al.*, 2008; Albareda-Sirvent *et al.*, 2000).

In potentiometry, the potential developed across an ion-selective membrane, forming part of an indicator electrode (hence the term 'ion-selective electrode') is measured against a reference electrode. The relationship between membrane potential and the concentration of the target species is governed by the Nernst equation (Grieshaber, *et al.*, 2008). Enzymatic potentiometric biosensors are conveniently fabricated by immobilising the enzyme on the surface of the ion-selective membrane.

Field-effect transistors (FETs) are a type of transistor that uses an electric field to control the conductivity of a channel (i.e. a region depleted of charge carriers) between two electrodes (i.e. the source and drain) in a semiconducting material. Control of the conductivity is achieved by varying the electric field potential, relative to the source and drain electrode, at a third electrode, known as the gate. Depending on the configuration and doping of the semiconducting material, the presence of a sufficient positive or

negative potential at the gate electrode would either attract charge carriers (e.g. electrons) or repel charge carriers in the conduction channel.

The current focus in biosensing applications is on ISFET (ion-selective field-effect transistor) and EnFET (enzyme field-effect transistor) devices. One of the most popular methods for the construction of FET-based biosensing devices is the immobilization of enzymes at the gate surface of ion-selective ISFET devices, creating an EnFET (Grieshaber *et al.*, 2008; Pijanowska and Torbicz, 2005; Pohanka, 2009). The majority of potentiometric and EnFET enzymatic transducers rely on the measurement of H^+ or NH_4^+ ions produced in various enzymatic reactions, by making use of pH or NH_4^+ sensitive membranes, respectively.

Optical enzymatic biosensors can be used in combination with different types of spectroscopic technique, including absorption, fluorescence and chemiluminescence (Marazuela and Moreno-Bondi, 2002; Choi, 2004; Monk and Walt, 2004). The enzymes commonly applied in optical biosensors are oxidases and oxidoreductases (which catalyze the oxidation of compounds using oxygen or NAD^+), esterases (which produce acids), decarboxylases (which produce CO_2) and deaminases (which produce NH_3). The biological receptor is immobilized directly on the surface of an optical fibre. NADH has a strong absorbance at 340–360 nm, which can be used to monitor the concentration of substrates of dehydrogenase enzymes, such as pyruvate or lactate.

Many enzymatic sensors for the determination of organophosphorous pesticides are based on the use of acetylcholinesterase, an enzyme involved in neurochemical reactions, which is inhibited by pesticides. Acetylcholinesterase can be immobilized in optical fibre biosensors, and detection is based on measuring the pH changes caused by release of acetic acid, using pH-sensitive dyes (Marazuela and Moreno-Bondi, 2002; Balasubramanian and Burghard, 2006). Fluorescence is often applied in combination with oxidase enzymes, and the decrease in the oxygen concentration is measured by the luminescence quenching of different transition metal complexes. In addition, several fibre-optic sensors have been based on detection of the fluorophore NADH at 455 nm, produced as a result of the action on different substrates of dehydrogenase enzymes (e.g. glutamate or alcohol dehydrogenases) immobilised on optical fibres (Borgmann *et al.*, 2011).

Chemiluminescence sensors, in which the analyte induces emission of light on interaction with a bioreceptor, can also be used. The popular chemiluminescence reaction between luminol and hydrogen peroxide, catalyzed by horseradish peroxidase, has served as the basis for different biosensors (Borgmann *et al.*, 2011). Electrochemiluminescence (electrogenerated chemiluminescence) biosensor systems mainly include detection of NADH using dehydrogenases and H_2O_2 using oxidases. Since many enzymes can produce H_2O_2 during their substrate-specific enzymatic reaction, electrochemiluminescence enzyme biosensors are made possible by coupling the luminol light-emitting reaction with enzyme-catalyzed reactions generating H_2O_2 . $Ru(bpy)_2^{3+}$ -based enzyme electrochemiluminescence biosensors operate on the $Ru(bpy)_2^{3+}/NADH$ co-reactant systems, in conjunction with deoxygenases.

9.3 Enzymatic biosensors and the food industry

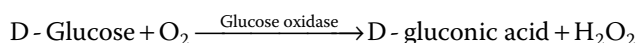
Current analytical methods in the food industry are laborious, expensive, usually slow, and require bulky instrumentation. However, the food industry requires pocket-sized

devices capable of fast, on-site measurements of undiluted samples during the production or processing of foods, or for quality control purposes. Most of these drawbacks can be conveniently addressed by enzymatic biosensors. Enzymatic biosensors are used to measure food ingredients (sugars, acids, amino acids, inorganic ions, alcohols and carbohydrates), contaminants (residues of pesticides and heavy metals), food additives (sorbitol, benzoic acid, sulfites) and indicators of food 'freshness' (such as biogenic amines). The utility of enzymatic biosensors in food analysis has been reviewed in detail (Barthemlebs *et al.*, 2010; Prodromidis and Karayannis, 2002; Cock *et al.*, 2009); Vargas-Bernal *et al.*, 2012; Jaffrezic-Renault, 2001; Upadhyaya and Verma, 2013; Sharma *et al.*, 2003).

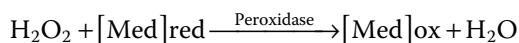
9.4 Biosensors for the analysis of main food components

9.4.1 Sugars

Electrochemical biosensors are often used for sugar detection. In these devices, either single or multiple enzymes are immobilized directly on different types of electrochemical transducers (carbon, screen-printed, etc.) using a variety of methods (Monosik *et al.*, 2012). Glucose biosensors are important in monitoring fermentation processes, and in the dairy, wine, beer, and sugar industry (Mao *et al.*, 2008). The detection of glucose is based on biosensors that utilize glucose oxidase, which catalyzes the oxidation of glucose into gluconic acid. Usually, the hydrogen peroxide produced is monitored:



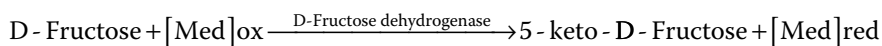
Hydrogen peroxide can then be measured by means of electrochemiluminescence reaction with luminol, or via amperometry with a suitable mediator such as Prussian blue:



A flow injection with optical fibre biosensors for glucose, based on luminol electrochemiluminescence, has been described. The sol-gel method is introduced to immobilize glucose oxidase (GOx) on the surface of a glassy carbon electrode. Glucose could be quantified in the concentration range between 50 μM and 10 mM, with a detection limit around 26 μM . The proposed method can be applied for the determination of glucose in soft drink samples. Zhu *et al.* (2002) used an amperometric probe-type glucose sensor with Pt working electrode, and an Ag/AgCl reference one, polarized at +650 mV. The results showed that cellulose acetate membranes treated with amylamine were the most convenient structures to establish single-membrane recognition layers. The linearity and response time of this electrode were found to be up to 320 mM of glucose and 500 sec, respectively, at pH 4. An electrode was used to determine the glucose content in real samples. Results were in a good agreement with the conventional measurement method (Alp *et al.*, 2000).

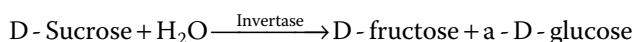
D-Fructose is a monosaccharide found in three main forms in the food: as free fructose (present in fruits and honey), in sucrose (a disaccharide with sucrose), or as

fructans – oligosaccharides (present in some vegetables and wheat). Because of its higher sweetening ability compared to sucrose and glucose, fructose is used as a diet sweetener in diabetic foods. Enzyme electrodes for fructose determination are often based on D-fructose-5-dehydrogenase, using an electron acceptor serving as an electrochemical mediator, such as ferrocene or ferricyanide:



An improved amperometric biosensor, based on a SBM composite transducer, has been used for the determination of D-fructose in some food samples. The enzyme, D-fructose dehydrogenase, was incorporated directly into a solid composite transducer containing both 2-hexadecanone as a SBM and chemically modified graphite. Ferri-cyanide was used as a redox mediator, and the amperometric signals were linearly proportional to D-fructose concentrations in the range 5×10^{-5} to 1×10^{-2} M. The use of chemically modified graphite by a mild oxidation step was shown to improve the biosensor selectivity against anionic interferences, such as L-ascorbate. The assay of D-fructose by this electrode was not influenced by the presence of sugars or other interferences. The results agreed well with those obtained with the commercial kit (Stredansky *et al.*, 1999).

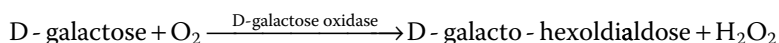
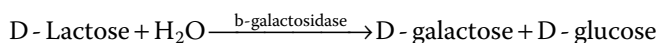
Sucrose determination requires a multienzyme system, in which sucrose is first hydrolyzed to fructose and glucose by the enzyme invertase:



α -D-glucose is converted to its β -isomer by the enzyme mutarotase, and glucose oxidase can then be used for the determination of glucose, as explained above. It must be noted that the possibility to immobilize multiple enzymes on electrodes enables multi-enzyme determinations.

One example of this is the development of a multi-enzyme electrode obtained by a two-step immobilization of the enzymes GOx, mutarotase and invertase. GOx was entrapped in a poly-1,3-diaminobenzene film on a platinum electrode by electrochemical polymerization, and a combination of mutarotase and invertase was cross-linked over the electrode via bovine serum albumin and glutaraldehyde. The sucrose concentration was determined from hydrogen peroxide oxidation. This immobilization method minimized interference from ascorbic acid. A second electrode, for glucose determination only, was constructed using an inactive invertase. The biosensor showed a good agreement with the standard LC method for sucrose and glucose analysis in soft drinks (Surareungchai *et al.*, 1999).

Lactose is the main disaccharide present in milk and its content is closely related to the quality of milk and dairy products. It is hydrolyzed by the enzyme β -galactosidase to galactose and glucose. Galactose can be further oxidized to galacto-hexodialdose:

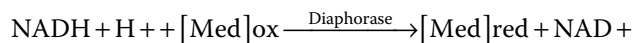
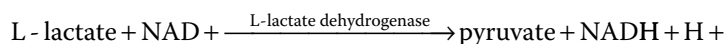
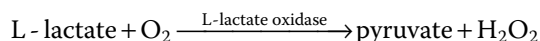


For estimation of lactose in milk and its products, an amperometric lactose biosensor was developed by immobilizing galactosidase and galactose oxidase in Langmuir-Blodgett films of poly(3-hexyl thiophene)/stearic acid. The working electrode may be used for the estimation of lactose/galactose in food and biological fluids (Sharma *et al.*, 2004).

Another research team immobilized β -galactosidase into polyelectrolyte membranes, using organic solvents and perfluorosulfonated polymer. The results of the analysis of milk whey, in a flow-injection system that included lactose biosensor based on Berlin blue (as a signal transducer) and polyelectrolyte membranes, correlated well with measurement data obtained by a standard chromatographic technique (Lukacheva *et al.*, 2007).

9.4.2 Acids

Organic acids constitute another important class of main food components. Optical, electrochemical and calorimetric biosensors can be used for their determination (Monosik *et al.*, 2012). Lactic acid is an indicator of the fermentative processes and is related to the freshness, stability, and storage quality of several products such as tomato sauces, fruits, juices, wine, and milk. Typically lactate biosensors are based on the following reactions:

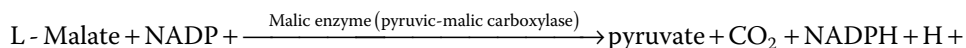
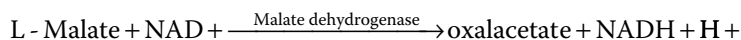


The NAD^+ can be monitored amperometrically or photometrically.

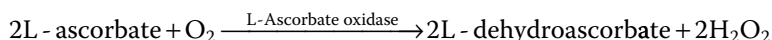
A biosensor for the selective determination of lactate in wine, based on robust solid composite transducers, was developed by Katrlík *et al.* Transducers were comprised of a solid binding matrix having a hydrophobic skeleton, with the enzymes L-lactate dehydrogenase (LDH) and diaphorase (DP) placed onto the transducer surface and covered by a dialysis membrane, which substantially reduced interferences derived from easily oxidisable compounds of wine, such as polyphenols. As a mediator, ferricyanide was used. The results obtained by the biosensors were in good agreement with those obtained by liquid chromatography (Katrlík *et al.*, 1999).

Another paper described an amperometric lactate biosensor based on a conducting polymer, poly-5,20-50,200-terthiophene-30-carboxylic acid (pTTCA), and multi-walled carbon nanotube (MWNT) composite present on a gold electrode. LDH and the oxidized form of nicotinamide adenine dinucleotide (NAD^+) were subsequently immobilized onto the pTTCA/MWNT composite film. The detection signal was amplified by the pTTCA/MWNT assembly, with immobilized enzyme. The applicability of the biosensor in commercial milk and human serum samples was demonstrated successfully (Rahman *et al.*, 2009).

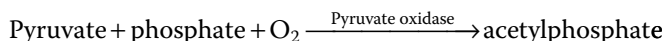
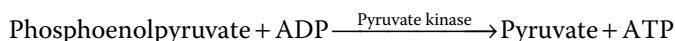
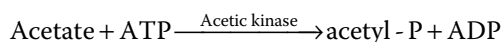
Malic acid is present mainly in fruit and vegetables, juices and other commodities. This acid is a very important parameter characterizing quality of wine, and it is often used as an acidulant. Its determination using biosensors is based on the following reactions:



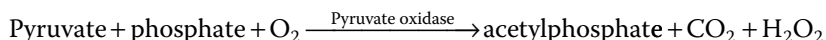
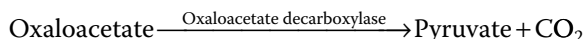
L-Ascorbic acid occurs naturally in some foods, but is also frequently added to processed foods as an antioxidant. The ascorbic acid content in food materials is an indicator of its freshness. L-Ascorbate is oxidized to dehydroascorbate, with production of hydrogen peroxide, which is ultimately detected:



Acetic acid is produced during the fermentation of alcoholic drinks, and found in soy sauce and vinegar. Among others, a widely used tri-enzymes system can be used for developing acetate biosensors (e.g. measuring the consumption of oxygen in the last reaction):



Citric acid exists in several fresh fruits such as lemons and limes and is also used as an additive in the food industry, mainly as a preservative and an acidulant. Enzymatic biosensors for citrate are based on the following scheme:

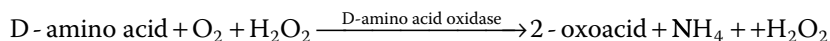


The hydrogen peroxide generated can be determined electrochemically, but the enzyme used, citrate lyase, is unstable, so this methodology is rarely used.

9.4.3 Amino acids

The presence of D-amino acids in foods is associated with a decrease in protein digestibility, thus affecting the bio-availability of essential amino acids and seriously impairing the nutritional value of the food. D-amino acids are also generally considered to be important markers of bacterial contamination of the food products. D-amino acid

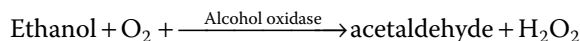
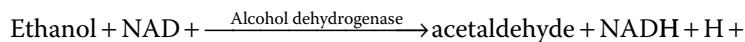
oxidase (DAAO) is a peroxisomal enzyme, containing FAD as a cofactor, that is expressed in a wide range of species, from yeasts to human, but not in bacteria nor in plants. Its function is to catalyze the oxidative deamination of D-isomer of amino acids to the corresponding 2-oxoacid and ammonia. During this step, the FAD is reduced, and the catalytic cycle is completed through its reoxidation by O₂ to yield hydrogen peroxide (Monosik *et al.*, 2012).



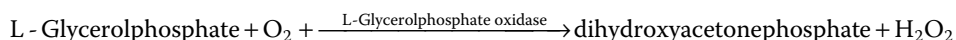
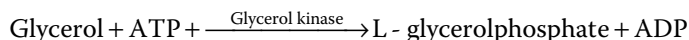
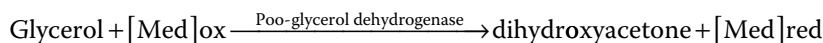
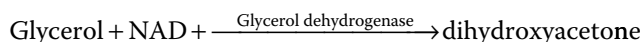
L-amino acids can be determined using L-amino acid oxidase (LAAO), while enzymatic biosensors for specific amino acids (e.g. L-glutamate) can be developed using by immobilization of L-glutamate oxidase and L-glutamate dehydrogenase on amperometric transducers.

9.4.4 Alcohols

The determination and control of ethanol is important in the brewing, wine making and distilling industries. Tax regulations also require exact determination of the ethanol content, especially in spirits. Ethanol biosensors are based mainly on immobilized alcohol oxidase or dehydrogenase, catalyzing the following reactions (Prodromidis and Karayannis, 2002; Monosik *et al.*, 2012):



In foods and beverages, glycerol serves as a humectant, solvent and sweetener, and may help to preserve foods. It is also used as a filler in commercially prepared low-fat foods (e.g. cookies), and as a thickening agent in liqueurs. Glycerol is a secondary fermentation product of alcoholic secondary fermentation, contributing to the viscosity and smoothness of a wine, with a favourable effect on the taste. Glycerol determination by an amperometric system can be based on the enzymatic reactions (Prodromidis and Karayannis, 2002; Monosik *et al.*, 2012), as follows:



The same reactions could be used for triglyceride detection, after hydrolysis and detection of the glycerol released.

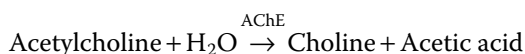
9.5 Biosensors for contaminants

9.5.1 Pesticides

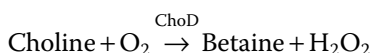
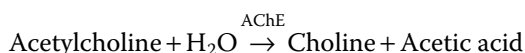
Pesticides are chemicals used in agricultural production to destroy or control weeds, insects, fungi and other pests. Pesticides are considered to be some of the most dangerous contaminants, because of their ability to accumulate, and their long-term effects on living organisms. Currently, over 800 active ingredients, which belong to more than 100 substance classes, can be classified as pesticides. Organochlorine, organophosphorus and organonitrogen pesticides (carbamates, triazines and their derivatives) are the most important of these. There are two approaches to detecting pesticides using biosensors: an indirect method based, on inhibition of enzyme activity; and a direct approach, based on measurement of the enzymatic reactants or products (Sassolas *et al.*, 2012).

The general principle of enzymatic inhibition biosensors is based on the correlation between the toxicity of pesticides and the decrease of enzyme activity. Therefore, the development of these biosensing systems relies on a quantitative measurement of the enzyme activity, before and after exposure to a target analyte. Typically, the enzyme inhibition-based biosensor includes the following steps: determination of initial enzymatic activity; incubation of a biosensor in the sample (potentially containing the target pesticides); and, finally, measurement of the residual activity. This activity can be measured using different transduction techniques, including amperometry, potentiometry, spectrometry, fluorimetry and calorimetry, depending on the enzymatic reaction used (Sassolas *et al.*, 2012; Carlo and Compagnone, 2010; Verma and Bhardwaj, 2015; McGrath *et al.*, 2012).

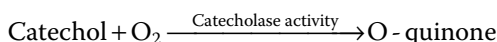
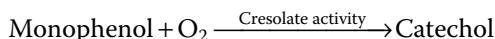
In enzyme inhibition-based pesticide biosensors, the biological receptors are usually cholinesterases, such as acetylcholinesterase (AChE), butyrylcholinesterase (BChE) or urease. For instance, AChE catalyzes the following reaction (Liu *et al.*, 2013):



Organophosphate and carbamate pesticides selectively inhibit cholinesterases by blocking the serine in the active site, through nucleophilic attack, to produce a serine phosphoester (via phosphorylation). The pH variation produced by the acid formation can be measured using electrochemical methods, such as potentiometry. This pH change can also be measured using pH-sensitive spectrophotometric indicators, or pH-sensitive fluorescence indicators. In many cases, a two-enzyme system can be used in which acetylcholine is coupled to choline oxidase (Sassolas *et al.*, 2012).

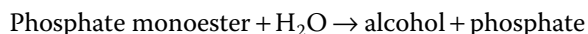


Tyrosinase oxidizes monophenol in 2-consecutive steps:



Tyrosinase is inhibited by different compounds, such as carbamate pesticides and atrazine. Numerous electro-chemical biosensors based on the inhibition of tyrosinase activity have been reported (Sassolas *et al.*, 2012).

Alkaline phosphatase (ALP) catalyzes the following reaction:



ALP is inhibited by different compounds. The substrate (phosphate monoester) can be selected so that the product (alcohol) is optically or electrochemical active. For instance, phenyl phosphate forms electrochemically active phenol

The second strategy for pesticide detection is based on organophosphorus hydrolase (OPH), which is able to hydrolyze a number of organophosphate pesticides with the formation of organophosphorus acid and alcohol (Liu *et al.*, 2013; Serna-Cock and Perenguez-Verdugo, 2011).



The alcohol formed can be electroactive or chromophoric, and can be measured by electrochemical or optical techniques, respectively.

The main weakness of the enzymatic approach for pesticide detection is the limited qualitative information acquired, since the response is related to the total amount of pesticides in the sample. Also, the interpretation of results is further complicated by the fact that each pesticide exhibits a different inhibitory effect of the enzymatic activity. Finally, the specificity of these biosensors is rather limited, as they are prone to interference by other compounds in the sample (e.g. heavy metals).

9.5.2 Heavy metals

Heavy metals and their ions are ubiquitous and, by definition, are metals having atomic weights between 63.5 and 200.6 g mol⁻¹ and a specific gravity greater than 5 g cm⁻³. Heavy metals constitute one of the most serious group of pollutants. Even in small concentrations, they are a threat to the environment and human health, because they are non-biodegradable. Metals leaching from eating utensils and cookware lead to metallic contamination of food and water. Additional sources of hazardous exposure are metallic constituents of pesticides and therapeutic agents, fossil fuels, industrial activity (metal-plating factories, mining industries, tanning, dye and chemical manufacturing industries) (Verma and Singh, 2005).

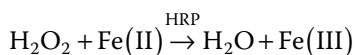
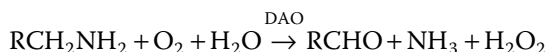
The commonest operational strategy in heavy metal biosensors is inhibition of the enzymatic activity, based on the interaction of metal ions with exposed thiol or methylthiol-groups of the enzyme's amino acids. Different enzymes, such as acetylcholinesterase, alkaline phosphatase, urease, invertase, peroxidase, L-lactate dehydrogenase, tyrosinase, and nitrate reductase, have been used. The inhibition of the immobilized enzyme can be detected via electrochemical (amperometric, potentiometric, and conductometric) or optical measurements (Upadhyaya and Vermaa, 2013; Verma and Singh, 2005; Turdean, 2011). Heavy metal enzymatic biosensors exhibit the same weaknesses as pesticide biosensors (i.e. limited selectivity), but are useful for fast screening purposes.

9.6 Food freshness indicators, antinutrients and additives

Biogenic amines (BA) are basic nitrogenous compounds, formed mainly by decarboxylation of amino acids, or by amination and transamination of aldehydes and ketones. By chemical structure, biogenic amines can either be aliphatic (putrescine, cadaverine, spermine, spermidine), aromatic (tyramine, phenylethylamine) or heterocyclic (histamine, tryptamine) compounds. The detection of biogenic amines is a valuable tool for assessing the freshness and quality of a wide variety of protein-containing products, such as fish, meat, wine, beer, vegetables, fruits, nuts, and chocolate, where BAs accumulate in the process of food spoilage.

The safety of fish and fish products has been mostly assessed by determining the histamine content, although cadaverine and putrescine have been found to potentiate histamine toxicity. The degree of histamine toxicity depends on the efficiency of the detoxification system of a body. If histamine alone is present in food intake, it is eliminated with the help of a metabolizing enzyme, diamine oxidase (DAO). However, in the presence of cadaverine and putrescine, this enzyme is inhibited. The allowed maximum residue level of histamine in food is 50 ppm (450 mM) in US and 100 ppm (900 mM) in Europe. Fish freshness has also been estimated by the level of trimethylamine (TMA).

Biosensors for biogenic amines analysis are most commonly based on diamine oxidases, although other enzymes, such as putrescine oxidase, monoamine oxidase, methylamine dehydrogenase and flavin-containing mono-oxygenase type-3 (FMO3), have been also employed (Kivirand and Rinken, 2011).



Ethanol accumulation in vegetables, and during storage of fruits, can be detected using immobilized alcohol oxidase and alcohol peroxidase, together with a chromogene. Alcohol oxidase catalyzes the oxidation of ethanol in acetaldehyde and H_2O_2 in the presence of O_2 , and the peroxidase catalyzes the oxidation of the chromogene, causing a change in colour. The maturity of fruits can also be evaluated using biosensors, by measuring the levels of glucose or sucrose. There are some compounds that give rise to disagreeable smell in food commodities that can be detected with biosensors (such as 2,4,6-trichloroanisole in wine (due to corks). Polyphenols, which are strong antioxidants, can be measured in olive oil using immobilized lacase or tyrosinase enzymes and amperometric detection.

Antinutrients are compounds that can generate health disorders to the consumer, by slowing down absorption of certain nutrients and causing deficiencies. For instance, oxalate, amygdalin and glucoalcaloids can be measured using biosensors incorporating oxalate oxidase, β -glucosidase and cholinesterase, respectively (Cock *et al.*, 2009).

The use of additives and preservatives has become an increasingly common practice in a food industry seeking increased lifetime, longer shelf-life, and better organoleptic properties. Many food additives can be detected using biosensors. Typical examples are aspartame, with catalysis by carboxyl esterase, alcohol oxidase, caboxypeptidase, L-aspartase,

peptidase, aspartate aminotransferase, glutamate oxidase and α -chymotrypsin, sorbitol with sorbitol dehydrogenase and nicotinamide adenine dinucleotide (NAD⁺), and benzoic acid with tyrosine sulphites with sulphite oxidase, all relying on amperometric transduction (Cock *et al.*, 2009).

9.7 Future perspectives

This work has demonstrated the wide applicability of enzymatic biosensors in food diagnostics. Multiplexed multidetection of several analytes (by incorporation of different enzymes on the same transducer or an array of transducers) is a promising field of research (Barthemlebs *et al.*, 2010). Although there is a vast number of research articles on biosensors, only a few commercial devices are available (Table 9.4; Barthemlebs *et al.*, 2010). These devices are mainly based on the use of oxidases, and oxygen or hydrogen peroxide-mediated detection. The development of commercial enzymatic biosensors at a large scale must tackle some important challenges, such as the limited lifetime of enzymes, standardization in calibration, long-term stability, portability, elimination of interferences by using advanced immobilization protocols, and so on.

Table 9.4 Commercial biosensors for food monitoring.

Biosensor name	Analyte	Biocomponent	Company and reference
AM2 & AM3	Ethanol	AOX	Analox instruments
AM5	Methanol	AOX	(UK & USA)
GL6	Glucose	GOX,AOX,LOX	www.analox.com
	Ethanol	GK + GPOX	
	Lactate		
	Methanol		
	Glycerol		
LM5	Lactate	LOX	
Answer 8000	Glucose	GOX + HRP	Gwent sensors(UK) www.g-s-l.co.uk
Microzyme	Lactate	n.r	Biosentec (France) www.biosentec.fr
OLGA	Glucose	n.r	Sensolytics (Germany) www.sensolytics.com
	Lactate		
	Sucrose		
	Ethanol		
	Glutamate		
Per Bacco 2000	Glucose	GOX	Biofutura s.r.l (Italy)
Per Bacco 2002	Lactate	LDH	www.biofutura.it
	Malate	MDH	
Senzytech one	Glucose	n.r	Tectronik (Italy) www.tectronik.it
	Lactate		
	Malate		

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10

Immunology-based Biosensors

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10.1 Introduction

Biosensors can be defined as devices that utilise the presence of biological or chemical receptors for the detection of analytes (molecules) typically associated with specific physiological diseases or disorders.

Immunosensors seem to be a promising and attractive technique, offering: high specificity due to the use of very specific immune molecules; a non-destructive approach to sample; simple operation; uncomplicated sample preparation; and, most importantly, high sensitivity, especially when different signal transducers have recently been combined to yield low limits of detection (Ramanaviciene *et al.*, 2012).

However, in order to achieve the characteristics mentioned above, some important issues have to be resolved, because the sensitivity, stability and longevity of immunosensors depends on: the number of the immobilised immune molecules on the surface; their conformational stability; remaining activity after the immobilisation procedure; and their orientation on the sensor surface. This is especially important considering immunoglobulins, which are asymmetrical molecules whose recognition sites take different positions in space following different immobilisation procedures, thus leading to hindered interactions with the analyte (Makaraviciute and Ramanaviciene (2013).

10.2 Antibodies and biosensors

An immunological method is one of the promising tools for the development of easy-handling biosensors. Immunoassay methods are sensitive, cost-effective, easy to perform, and require a small sample volume. However, such techniques often require long reaction times and involve multiple steps.

Antibodies are a common tool in analytical methods, and a number of polyclonal antibodies (PABs) raised to pantothenic acid (PA) have been used in enzyme-linked

immunosorbent assays (ELISA) protocols (Bertelsen *et al.*, 1988; Finglas *et al.*, 1988; Gonthier *et al.*, 1998a; Gonthier *et al.*, 1998b; Morris *et al.*, 1987; Song *et al.*, 1990) and a radio immunoassay (RIA) method (Wyse *et al.*, 1979) to determine PA levels in foods. In addition, a PAb raised to PA is the basis of a commercially available surface plasmon resonance (SPR) biosensor assay (Qflex Kit Pantothenic Acid PI), which has previously been compared favourably to liquid chromatographic mass spectrometry (LC-MS) analysis (Haughey *et al.*, 2005). This kit has achieved AOAC PTM accreditation, and has also been independently validated by Gao *et al.* (2008).

The molecular structure of the PA molecule indicates that it has two distinct terminal moieties – carboxylic acid and hydroxyl groups. The presence of these two functional groups results in a choice of chemistries, both for hapten production and for the immobilisation of the antigen onto a solid support. These synthetic chemistry approaches, used in the development of the ELISA and RIA systems, signify successful chemical pathways used during antibody production (Bertelsen *et al.*, 1988; Finglas *et al.*, 1988; Gonthier *et al.*, 1998a; Gonthier *et al.*, 1998b; Morris *et al.*, 1987; Wyse *et al.*, 1979).

In this study, a monoclonal antibody (MAb) was produced, characterised and used to develop a Biacore Q SPR-based biosensor immunoassay for the quantification of PA in foods. A comparison with a commercially available PAb based PA assay was performed, and both assays were used to analyse a wide variety of samples from different food types.

PA, vitamin B₅, is an essential B vitamin that may be fortified in food and, as such, requires robust and accurate methods of detection to meet compliance legislation. This study reports the production and characterisation of the first monoclonal antibody (MAb) specific for PA, and the subsequent development of a SPR biosensor assay for the quantification of PA. The developed assay was compared with a SPR based commercial kit, which utilised a polyclonal antibody. Foodstuffs, including cereals ($n = 43$), infant formulas and baby food ($n = 10$) and fruit juices ($n = 48$) were analyzed by both the MAb and PAb biosensor assays, and comparison plots showed good correlation ($R^2 = 0.77 - 0.99$). The results indicate that the MAb-based biosensor assay is suitable for the measurement of PA in foodstuffs, and has the added advantage of facilitating a constant, long-term supply of identical antibody. Preliminary matrix studies suggest that the MAb-based assay is an excellent candidate for further validation studies and routine quality assurance based analysis (Haughey *et al.*, 2012).

Despite the long-lasting interest and multiple efforts in immunosensor design, few examples of commercialisation have been reported (Deacon *et al.*, 1991; Ruano-Lopez *et al.*, 2009; Johnson *et al.*, 2011), the most widely known immunosensor being the pregnancy test for the detection of human chorionic gonadotropin (hCG).

10.2.1 Immunochemiluminescence biosensors

Selvakumar and Thakur (2012) described a dipstick-based immunochemiluminescence (immuno-CL) biosensor for the detection of vitamin B12 in energy drinks. The method is a direct competitive-type format, involving the immobilisation of vitamin B12 antibody on nitrocellulose membrane (NC), followed by treatment with vitamin B12 and a vitamin B12-alkaline phosphatase conjugate to facilitate the competitive binding. The dipstick was further treated with substrate disodium 2-chloro-5-(4-methoxyspiro {1,2-dioxetane-3,2A-(5A-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)-1-phenyl phosphate (CDP-Star), to generate

chemiluminescence (CL). The number of photons generated was inversely proportional to the vitamin B12 concentration.

After systematic optimisation, the limit of detection was 1 ng mL^{-1} . The coefficient of variation was below 0.2% for both intra- and inter-assay precision. Vitamin B12 was extracted from energy drinks with recovery ranging from 90–99.4%. Two different energy drinks samples were analyzed, and a good correlation was observed when the data were compared with a reference enzyme-linked immunosorbent assay (ELISA) method. The developed method is suitable for an accurate, sensitive, and high-throughput screening of vitamin B12 in energy drinks samples. The dipstick technique based on immuno-CL is suitable for the detection of several analyte in food and environmental samples.

The convenience and speed of the test have been achieved by a novel concept of immune dipstick that depends on the transportation of a reactant to its binding partner immobilised on a membrane surface (Lisa *et al.*, 2009). It combines several benefits including a user-friendly format, short assay time, long-term stability over a wide range of climates, and cost effectiveness. These characteristics make it ideally suited for on-site screening by people who are not skilled analysts (Cho *et al.*, 2005).

10.2.2 Site-directed antibody immobilisation techniques for immunosensors

Immunosensor sensitivity, regenerability and stability directly depend on the type of antibodies used for the immunosensor design, quantity of immobilised molecules, remaining activity upon immobilisation, and proper orientation on the sensing interface. Although sensor surfaces prepared with antibodies immobilised in a random manner yield satisfactory results, site-directed immobilisation of the sensing molecules significantly improves the immunosensor sensitivity, especially when planar supports are employed. The review by Makaraviciute and Ramanaviciene (2013) focuses on the three most conventional site-directed antibody immobilisation techniques used in immunosensor design.

One strategy of immobilising antibodies on the sensor surface is via affinity interactions with a pre-formed layer of the Fc binding proteins, such as protein A, protein G, Fc region-specific antibodies or various recombinant proteins. Another immobilisation strategy is based on the use of chemically or genetically engineered antibody fragments, which can be attached to the sensor surface, covered in gold or self-assembled monolayer, via the sulfhydryl groups present in the hinge region. The third most common strategy is antibody immobilisation via an oxidised oligosaccharide moiety present in the Fc region of the antibody. The principles, advantages, applications, and arising problems of these most often applied immobilisation techniques are reviewed.

A schematic representation of site-directed antibody immobilisation methods – via Fc-binding proteins, via antibody fragments and via oxidised oligosaccharide moieties – is shown in Figure 10.1.

Site-directed antibody immobilisation is a very attractive technique, especially in clinical applications, as it is simple, precise, allows direct analyte detection, and could be used in cases when the immune response is minimal.

There are two main approaches that can be used in antibody-based sensor surface preparation: random and site-directed antibody immobilisation.

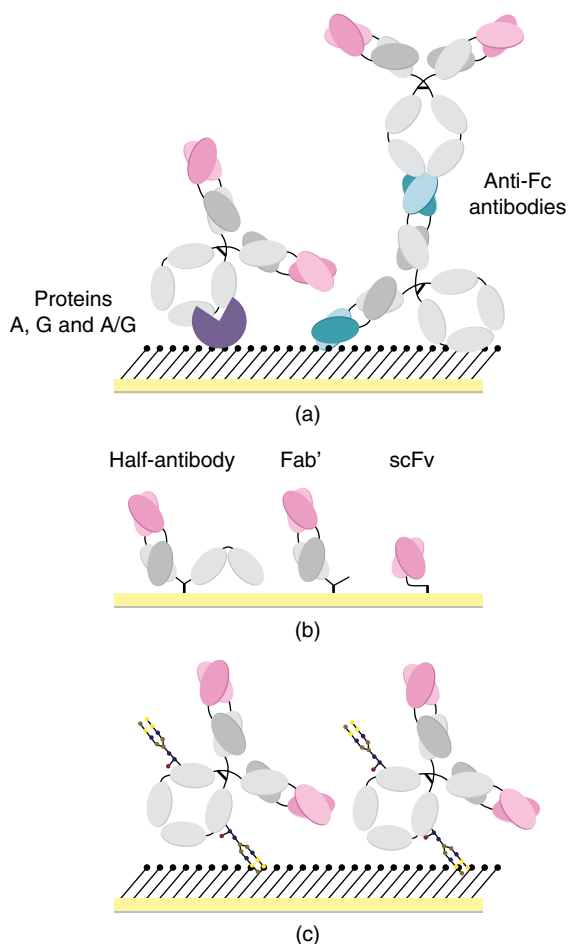


Figure 10.1 Schematic representation of site-directed antibody immobilisation methods: (a) Via Fc-binding proteins; (b) via antibody fragments; and (c) via oxidised oligosaccharide moieties. Source: Makaraviciute and Ramanaviciene, 2013.

Different supports have been reported to be used for immunosensor design – for example, noble metals, especially gold (Hafaiedh *et al.*, 2013), glass (Tedeschi *et al.*, 2003; Zhao *et al.*, 2006), silicon (Yakovleva *et al.*, 2002; Dhanekar and Jain, 2013), silicon nitride (Caballero *et al.*, 2012; Kurihara *et al.*, 2013), and indium-tin oxide (Bandodkar *et al.*, 2010). The immobilisation support is usually determined by the method used for signal transducer, and can be modified for different purposes.

The simplest sensor preparation technique is based on random adsorption of antibody molecules on the sensor surface. Although adsorption does not require the use of multiple materials and complex reactions, it does result in serious disadvantages, such as denaturation of proteins, very low stability and random protein orientation (Buijs *et al.*, 1996; Hlady and Buijs, 1996; Wiseman and Frank, 2011).

The most commonly used technique is, however, antibody amine coupling to the sensor surface previously modified with different coatings that allow biomolecule

immobilisation, such as self-assembled monolayers, dextran or various polymers (Zhou *et al.*, 2006; Kyprianou *et al.*, 2013).

Examples of site-directed antibody immobilisation methods include incubation of the antibody with its antigen prior to immobilisation, on a mixed self-assembled monolayer, so that the active sites remain protected from the active groups of the support (Yoon *et al.*, 2011). This is accomplished by cyclic voltammetry, which assists coupling of the hydroxyl groups of the oligosaccharide moieties present on antibodies to the cyano groups of the poly-(2-cyano-ethylpyrrole) (Um *et al.*, 2011). Moreover, the use of UV irradiation breaks the disulfide bridges upon adsorption by nearby tryptophan residues, freeing the sulfhydryl groups, which can then be coupled directly to the gold surface in a controlled manner (Della Ventura *et al.*, 2011).

Calixarene derivatives are able to orient an antibody in a site-directed manner (Oh *et al.*, 2005). Fusion proteins, such as pentamerised bispecific antibodies (decabodies) (Hussack *et al.*, 2009), ZZ-alkaline phosphatase-His fusion protein exhibiting Fc binding (Yang *et al.*, 2013), or cutinase-single chain antibody fusion proteins, allow the oriented immobilisation of cutinase to phosphonate ligands (Kwon *et al.*, 2004). However, the most popular and widely employed site-directed antibody immobilisation techniques are immobilisation via Fc-binding proteins, via antibody fragments and via oxidised oligosaccharide moieties.

10.2.3 Label-free arrayed imaging reflectometry (AIR) detection platform

Label-free biosensor technology has proven challenging, however, given the small molecular weight of many pollutants of interest. To address this issue, Carter *et al.* (2016) reported the development of a pollutant microarray based on the label-free arrayed imaging reflectometry (AIR) detection platform. The sensor is able to detect three common environmental contaminants (benzo[a]pyrene, bisphenol A, and acrolein) in human serum via a competitive binding scheme.

The theoretical foundations and operation of AIR have been reported elsewhere (Mace *et al.*, 2006). In brief, the technique relies on the creation of a near-perfect antireflective condition on the surface of a silicon chip. When illuminated with S-polarised laser light at the HeNe wavelength, and at an appropriate angle, an array of capture molecules spotted on the chip may be imaged with a CCD, showing minimal reflectivity in the absence of the analyte. Binding of target analytes to the appropriate capture molecule spot causes a predictable, quantitative perturbation in the antireflective condition, which may be measured as a change in reflected intensity.

Thus far, AIR has been demonstrated to be useful for detecting bacterial cell-surface proteins (Horner *et al.*, 2006), human cytokines in serum (Mace *et al.*, 2008; Carter *et al.*, 2011), and a variety of immune system markers including antibodies to human papilloma virus (Mace *et al.*, 2009) and influenza (Mace *et al.*, 2011). Quantitative analytical performance of AIR is well correlated with theory and reference techniques, such as surface plasmon resonance (SPR) and spectroscopic ellipsometry (Sriram *et al.*, 2011).

10.3 Immunoassays for detection of microorganisms

The development of sensitive assays for identification and detection of microorganisms is vital for improving diagnosis and preventing disease outbreaks. These assays also play

essential roles in control programs provided by food safety regulatory agencies and disease control and prevention agencies (Virgin and Todd, 2011). Many of the latest generation devices, such as SPR sensors (Zeng *et al.*, 2014), nuclear magnetic resonance (NMR) devices (Harel *et al.*, 2008), and surface enhanced Raman scattering (SERS) spectrometers (Han *et al.*, 2009), are regarded as promising biosensor setups, and have been commercialised to a large extent in order to meet the requirements of society. Unfortunately, the relatively high cost and complexity associated with many of today's diagnostic assay platforms have resulted in certain limitations.

The last decade has yielded breakthroughs within the development of nano-/microparticle-based biosensors, in which the affinity capture is effectively achieved by coating dispersed nano- or microparticles with capture molecules (Tekin and Gijs, 2013). Moreover, superparamagnetic particles have been increasingly used as labels in volumetric assays (Borlido *et al.*, 2013; Issadore *et al.*, 2014). Magnetoresistance sensors (Dalslet *et al.*, 2011), Hall effect devices (Østerberg *et al.*, 2014), magnetic tunnel junctions (Grancharov *et al.*, 2005), super-conducting quantum interference devices (Strömberg *et al.*, 2008) and AC susceptometers (de la Torre *et al.*, 2011) have been widely employed in biosensing platforms.

An alternative option for magnetic particle-labelled target detection is to make use of optomagnetic (magneto-optic) sensor systems, which measure modulation of an optical signal related to the Brownian relaxation dynamics of magnetic particles (Aurich *et al.*, 2007; Chung *et al.*, 2008; Donolato *et al.*, 2015a). Although the optomagnetic devices have provided significant reductions in instrumentation cost, their performance regarding magnetic particle labelled target detection is similar to the magnetic stray field detection systems discussed above. Chung *et al.* (2008) estimated that the minimum detectable number of magnetic nanoparticles (MNPs) for their optomagnetic setup was 10^7 . Aurich *et al.* (2007) employed a similar optomagnetic system and a homogeneous immunoassay strategy to detection human eotaxin and human IgG, and the LOD was found to be 1 nM and 70 pM, respectively.

Tian *et al.* (2016) presented a novel *Salmonella* detection methodology using a low-cost Blu-ray optomagnetic setup. The utilisation of a competitive turn-on immunoassay strategy associated with two kinds of magnetic particles (5 μm and 100 nm magnetic particles) gives a LOD of 8×10^4 CFU/mL, within a total assay time of three hours. Similar bioassay methods have been reported by Donolato *et al.* (2015a, 2015b) and Bejhed *et al.* (2015) for DNA detection, but this is the first time that the Blu-ray optomagnetic sensor has been reported for a turn-on immunoassay strategy. In this work, for the competitive immunoassay scheme, bacteria are affinity-captured by biotinylated-antibody coated 5 μm magnetic particles (Ab-MPs), to form immuno-magnetic aggregates. For certain bacterial concentrations, large immuno-magnetic aggregates are formed (i.e. clusters of capture MPs and bacteria). Thereafter, the aggregates are incubated with streptavidin-coated 100 nm MNPs.

The improvement of sensitivity is enabled by the formation of immuno-magnetic aggregates providing steric hindrance, protecting the interior binding sites from interaction with the magnetic nanoparticle labels. The formation of immuno-magnetic aggregates is confirmed by fluorescence microscopy. The system exhibits no visible cross-reaction with other common pathogenic bacteria, even at concentrations as high as 10^7 CFU/mL. Furthermore, they presented results when using the setup for a qualitative and homogeneous bplex immunoassay of *Escherichia coli* and *Salmonella typhimurium*.

Viruses have the ability to infect and, thereby, confer new phenotypes on host cells. *E. coli*, for example, if infected by viruses containing antibiotic resistance genes, can benefit by surviving in the presence of the corresponding antibiotics, to grow into colonies observable by the naked eye. Using this concept as a signal transduction mechanism for our immunoassay, Cheok and Jaworski (2016) have engineered ampicillin resistant virions to display a dimer of the z domain from Protein A. This zz-domain selectively binds to the conserved heavy domain of IgG across various species. As commercially available antibodies are in no short supply, this engineered virion can be used modularly with existing antibodies, for converting the presence of target antigen into a visually detectable colony forming unit.

It has been demonstrated that this scheme for zz-phage transfection and selective growth of infected *E. coli* can facilitate sub-nanomolar detection limits for target antigen. Moreover, this phage infectivity assay works over a range of concentrations competitive with existing ELISA techniques. Because this system is derived from self-regenerating components (i.e., virus and bacteria) and, furthermore, obviates the need for chromogenic substrates or spectroscopic equipment, it has been found to be particularly suitable for use in regions where cost-effective detection is a necessity.

It has been shown that engineered phage containing the zz domain can directly serve as an effective transducer of antibody binding information into visually observable colony forming units allowing detection of small amounts of protein target (Figure 10.2).

Because the z-domain (and Protein A in general) can bind a broad range of IgG classes, these have been particularly attractive components of immunosensor technologies (Jose *et al.*, 2009; Ko *et al.*, 2009; Lee *et al.*, 2003). The z-domain has even been integrated into the p3 coat of filamentous phage for a range of immunological applications, including receptor discovery (Braisted and Wells, 1996; Nord *et al.*, 1996).

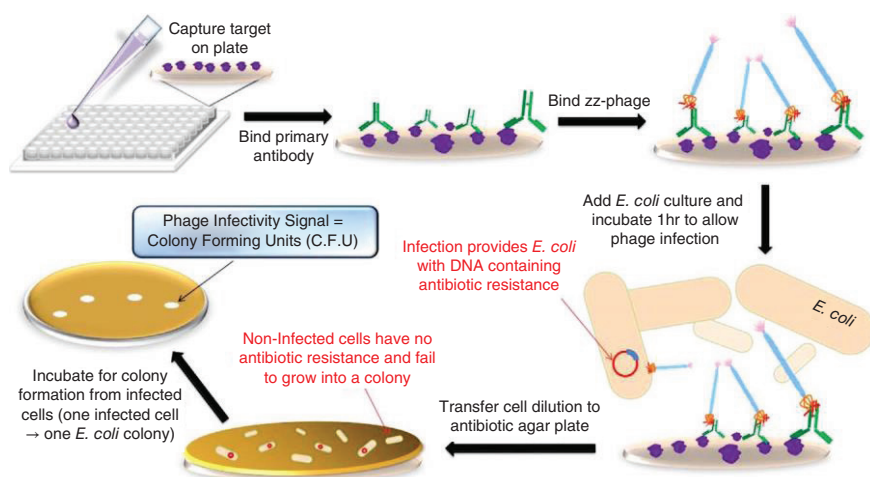


Figure 10.2 A workflow schematic of the phage infection-based method for detecting bound target antigens. Immobilized antigen is first bound by primary antibody, followed by addition of zz-phage virions able to selectively bind with the Fc region of IgG. Addition of *E. coli* to the wells results in phage infection of the *E. coli*, to confer resistance to ampicillin antibiotic. The number of phages, determined as colony-forming units, provides an effective measure of the amount of bound antigen with low detection limits. *Source:* Cheok and Jaworski, 2016.

An Electrochemical Lateral Flow Immunosensor (ELFI) has been developed, combining screen-printed gold electrodes (SPGE) and enabling quantification together with the convenience of a lateral flow test strip (Sinawang *et al.*, 2016). A cellulose glassy fibre paper conjugate pad retains the marker immunoelectroactive nanobeads, which will bind to the target analyte of interest. The specific immunorecognition event continues to occur along the lateral flow bed until reaching the SPGE-capture antibodies at the end of the cellulosic lateral flow strip. The rationale of the immunoassay consists of the analyte antigen NS1 protein being captured selectively and, specifically, by the dengue NS1 antibody conjugated onto the immunonanobeads, thus forming an immunocomplex.

Note that the *NS1 influenza protein* (NS1) is a viral nonstructural protein encoded by the NS gene segments of type A, B and C influenza viruses. With the aid of a running buffer, the immunocomplexes flow and reach the immunoconjugated electrode surface, and form specific sandwich-type detection due to specific, molecular recognition, while unbound beads move along the electrodes. The successful sandwich immunocomplex formation is then recorded electrochemically. The specific detection of NS1 is translated into an electrochemical signal contributed by a redox label present on the bead-immobilised detection dengue NS1 antibody, while a proportional increase of faradic current is observed, with increase in analyte NS1 protein concentration. The first generation ELFI prototype is simply assembled in a cassette, and successfully demonstrates wide linear range over a concentration range of 1–25 ng/mL, with an ultrasensitive detection limit of 0.5 ng/mL.

The electrochemical biosensor offers an elegant route for molecular bio-assaying, because of its possible size reduction, low cost, volume and power requirements. Hence, electrochemical biosensors are quite promising for a wide range of biomedical and environmental applications (Wang, 2005). Antigen-antibody immunosensing events can be coupled to several other detection techniques, such as piezoelectric (Garai-Ibabe *et al.*, 2011), fibre optic (Herrmann *et al.*, 2005; Konry *et al.*, 2005; Petrosova *et al.*, 2007; Sobarzo *et al.*, 2007; Atias *et al.*, 2009) and SPR (Mrksich *et al.*, 1995). These techniques are useful because of minimal sample preparation steps, but are limited to laboratory use, due to bulky equipment and stringent laboratory ambiance. On the other hand, antigen-antibody immunosensing, coupled to electrochemical techniques, are quite useful for on-site analyses, because of their simple instrumentation, affordable cost, portability and fast response time (Cosnier *et al.*, 2006; Ionescu *et al.*, 2007a, 2007b, 2006; Rai *et al.*, 2012).

In lateral flow assays, gold nanoparticles (AuNPs) provide major advantages in quantitative colorimetric immunoassays, acting as both label and carrier. This has been successfully demonstrated in detecting human IgG (HIgG) down to 5 ng/mL (Rivas *et al.*, 2014), as well as in an optical lateral flow HIgG detection, coupled with enzymatic activity of horseradish peroxidase (HRP), with detection limit of 0.5 ng/mL (Parolo *et al.*, 2013a), and in using a lab-in-a-syringe to detect HIgG down to 1 ng/mL and prostate-specific antigen (PSA) in spiked urine samples down to 1.9 ng/mL (Nunes Pauli *et al.*, 2015). Moreover, the architecture and sensitivity of AuNPs-based colorimetric immunoassays for HIgG has been successfully improved, and has achieved a detection limit of 60 ng/mL (Parolo *et al.*, 2013b).

However, colorimetric AuNPs-based immunoassays coupled with the enzymatic reaction of horseradish peroxidase (HRP) have a limited lifetime and, thus, the performance of the biosensor itself is limited, unless enhancement techniques are

incorporated (Quesada-González and Merkoçi, 2015). Integrating AuNPs lateral flow based-technology with electrochemistry results in a unique hybrid technology electrochemical lateral flow immunosensor (ELFI) for quantitative detection of analytes.

Tahir and Alocilja have integrated nitrocellulose paper strips with silver electrodes to develop an immunosensor for food-borne *Escherichia coli* O157:H7 (Muhammad-Tahir and Alocilja, 2003; Tahir and Alocilja, 2002). Yamaguchi *et al.* (2013) have developed a lateral flow immunosensor for cortisol by integrating paper pad with screen-printed flat platinum electrodes.

10.4 Immunosensors and cancer biomarkers-immunoarrays

A novel fluorescent immunosensor for determination of cancer biomarkers such as alpha-fetoprotein (AFP) was designed by Xie *et al.* (2016), by utilising both the high specificity of antigen-antibody sandwich structure and the high sensitivity of click chemistry-based fluorescence detection. Instead of an enzyme or fluorophore, the CuO nanoparticles are labelled on the detection antibody, which is not susceptible to changes in the external environments. The CuO nanoparticles which are modified on the sandwich structure can be dissolved to produce Cu^{2+} ions with the help of HCl, and then the Cu^{2+} ions are reduced by sodium ascorbate to produce Cu^+ ions. This triggers a Cu^+ -catalyzed alkyne-azide cycloaddition (CuAAC) reaction between the weak fluorescent compound (3-azido-7-hydroxycoumarin) and propargyl alcohol to form a strong fluorescent compound. A good linear relationship has been observed between the fluorescence increase factor of the system and the concentration of AFP in the range of 0.025–5.0 ng/mL, with a detection limit of 12 pg/mL (S/N = 3). The proposed fluorescent sensor has been applied to detect AFP in the human serum samples, giving satisfactory results.

Many selective biosensors have been developed on the basis of antigen-antibody (Ag-Ab) interaction, due to its high specificity. For example, Liu *et al.* (2009) introduced ELISA into a microchip to construct a sensitive and selective electrochemical sensor for AFP detection, while Zong *et al.* (2012) combined ELISA with chemiluminescence to design a novel method for multi-elemental cancer screening. These researches verified that ELISA possessed good selectivity and could be applied in biology, imaging and many other fields. However, the activity of enzyme is susceptible to the external environment.

Cu^+ -catalyzed alkyne-azide cycloaddition (CuAAC) is one of the most famous click reactions, and has been applied to produce a high fluorescent triazole complex, through ligating the non-fluorescent 3-azidocoumarins and terminal alkynes (Sivakumar *et al.*, 2004). This reaction has been applied to develop some sensitive fluorescence sensors for diverse targets. The high sensitivity and selectivity of this type of fluorescence sensor has allowed it to be used, for example, to determine copper in serum samples (Wang *et al.*, 2014), flumioxazin residues in the vegetable (Lu *et al.*, 2014), histidine in cellular homogenate (Qiu *et al.*, 2013) and mutational p53 DNA sequence (Qiu *et al.*, 2013).

An ultrasensitive immobilisation-free photoelectrochemical (PEC) biosensor was successfully developed for the first time by Ge *et al.* (2016), based on a novel enzyme-free cascaded quadratic signal amplification strategy. This rationally designed homogeneous dual amplification strategy consists of a target-analogue recycling circuit,

based on catalytic hairpin assembly (CHA), and a hybridisation chain reaction (HCR)-mediated amplification circuit. In the presence of carcinoembryonic antigen (CEA), a proof-of-concept target, target-analogue is released to trigger the upstream CHA recycling circuit.

The generated dsDNA complexes from CHA recycling could further induce the downstream HCR amplification, leading to the formation of numerous hemin/G-quadruplex DNazymes. This would accordingly stimulate the biocatalytic precipitation of 4-chloro-1-naphthol, inducing a distinct decrease in the photocurrent signal, due to the formed insoluble/insulating products on electrode surface. Under the optimal conditions, this PEC biosensor has achieved ultrasensitive detection of CEA down to the atto-gram level. The introduction of this aptamer-based cascaded quadratic amplification strategy not only remarkably improves the selectivity and sensitivity of CEA assay, but also allows the ultrasensitive detection of other proteins by designing specific aptamers, and providing a universal, isothermal and label-free PEC biosensing platform for ultra-sensitive detection of different kinds of cancer biomarkers. It holds great potential for early diagnosis of cancer.

Kadimisetti *et al.* (2016) reported a low-cost, sensitive, supercapacitor-powered electrochemiluminescent (ECL) protein immunoarray fabricated by an inexpensive three-dimensional (3D) printer. The immunosensor detects three cancer biomarker proteins in serum within 35 minutes. The 3D-printed device employs hand screen-printed carbon sensors with gravity flow for sample/reagent delivery and washing. Prostate cancer biomarker proteins, prostate specific antigen (PSA), prostate specific membrane antigen (PSMA) and platelet factor-4 (PF-4) in serum were captured on the antibody-coated carbon sensors, followed by delivery of detection-antibody-coated $\text{Ru}(\text{bpy})_3^{2+}$ (RuBPY)-doped silica nanoparticles in a sandwich immunoassay. ECL light was initiated from RuBPY in the silica nanoparticles by electrochemical oxidation with tripropylamine (TPPrA) co-reactant using supercapacitor power, and ECL was captured with a CCD camera. The supercapacitor was rapidly photo-recharged between assays, using an inexpensive solar cell. Detection limits were $300\text{--}500\text{ fg mL}^{-1}$ for the three proteins in undiluted calf serum. Assays of six prostate cancer patient serum samples gave good correlation with conventional single protein ELISAs. This technology could provide sensitive on-site cancer diagnostic tests in resource-limited settings, with the need for only moderate-level training.

Microfluidic array devices are often made using lithography, or sometimes injection moulding (Chin *et al.*, 2011; Laksanasopin *et al.*, 2015), but prototype development time and cost can be limiting factors. Both approaches require economies of scale to become cost-effective. Microfluidic arrays have been developed for multiple biomarker detection, using moulded or precision-cut microfluidic channels. Amperometric detection on gold nanostructured sensor arrays has been coupled with magnetic particles massively loaded with enzyme labels and antibodies, and the simultaneous ultrasensitive detection of up to four cancer biomarker proteins has been demonstrated (Chikkaveeraiah *et al.*, 2011; Malhotra *et al.*, 2010; Otieno *et al.*, 2014).

10.4.1 Microfluidic paper-based analytical devices (mPADs)

Remarkable efforts have been dedicated to paper-based chemosensors and biosensors over the last few years, mainly driven by the promise of reaching the best trade-off

between performance, affordability and simplicity. Because of the low-cost and rapid prototyping of these sensors, recent research has been focused on providing affordable diagnostic devices to the developing world. The recent progress in sensitivity, multifunctionality and integration of microfluidic paper-based analytical devices (mPADs) increasingly suggests that this technology is not only attractive in resource-limited environments, but also represents a serious challenger to silicon, glass and polymer-based biosensors. The review by Ahmed *et al.* (2016) discusses the design, chemistry and engineering aspects of these developments, with a focus on the past few years.

Many other research groups have investigated the fabrication of 3D electrochemical mPAD using wax-patterned paper and screen-printed electrodes for multiplexed immunoassays (Wu *et al.*, 2015). The 3D mPAD has provided good correlation between the concentration of tumour markers and the ECL peak intensity over a wide dynamic range that covered most of the levels found in human plasma and serum. Sensitive DNA detection has been developed, based on the amplification effect of graphene modified on gold-paper working electrode (Au-PWE), and further amplification upon DNA sandwich hybridisation.

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11

Graphene and Carbon Nanotube-Based Biosensors for Food Analysis

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11.1 Introduction

Recent advances in nanotechnology have driven rapid developments in electrochemical biosensors (Nikoleli *et al.*, 2013a). Nanomaterials provide many unique features and provide a route to develop biosensing devices with much higher sensitivity and selectivity at very rapid response times of less than five seconds. The nanometre dimensions of these devices, being at the scale of the target analyte, express an increased sensing surface and strong binding properties, thus allowing for substantially lower detection limits. In addition, recent developments in nanosciences have resulted in mass-fabrication of reproducible portable sensors for food toxicants and environmental pollutants.

The construction of nanosensors, and nanofabrication of the surfaces, has led to the development of a large number of disposable biosensors for the rapid detection of pesticides in the field, of bioassays in the field screening of toxic chemicals and pathogens, of bio-inspired multisensor system for improved detection of phenolic, and so on. Graphene-based nanosensing has yielded devices for the monitoring of food toxicants and analytes of clinical interest.

The present chapter describes recent developments in graphene-based technology, to the construction of highly sensitive and selective electrochemical biosensors. Although several strategies have been described in literature for graphene and carbon nanotubes applications in bioaffinity sensing (Campuzano and Wang, 2011; Lei and Ju, 2012), this chapter focuses on the various routes to immobilise a selective 'receptor' at the surface of the devices, so that its activity is retained for long lasting periods of time. It also discusses the applications of graphene-based electrodes to the rapid detection of several analytes in food analysis and clinical diagnosis. Future prospects and challenges are also included.

Graphene is a one-atom-thick planar sheet with sp^2 -bonded carbon atoms densely packed in a honeycomb crystal lattice (Shang *et al.*, 2008). Graphite, carbon nanotubes

(CNTs), and fullerenes have similar structure (Geim and Novoselov, 2007; Singh *et al.*, 2011). However, graphene possesses extraordinary properties compared with single-walled carbon nanotubes (SWCNTs), such as ultra-high mechanical strength, a tuneable electronic band gap, excellent thermal conductivity, room-temperature Hall effect, and ultra-high elasticity (Geim, 2009). Graphene is also an excellent conductor of both heat and electricity (Ambrosi *et al.*, 2010; Georgakilas *et al.*, 2012).

CNT, discovered by Iijima (1991), exhibits remarkable physicochemical, mechanical and thermal properties, due to its nano-dimensions, cylindrical structure, and high aspect ratio of length to diameter. A SWCNT is one graphene sheet wrapped around to form a cylinder (Dasgupta *et al.*, 2011); concentrically nested cylinders at an interlayer spacing of 3.4 Å form a multi-walled carbon nanotube (MWCNT), with typical diameters of 10–20 nm (Dasgupta *et al.*, 2011).

Both graphene and CNTs are excellent materials in electroanalysis. Graphene, however, has the following advantages compared with CNTs (Ambrosi *et al.*, 2010; Pumera, 2009): the purity of graphene is much higher than CNTs; and the latter contain impurities from metals that may cause uncontrolled electrocatalytic effects. Graphene is synthesised from cheaper graphite. Other advantages of graphene are its facile fabrication and modification.

Note that the edges of graphene sheets have a variety of oxygenated species that can provide support in electrical wiring of the redox centres of metalloproteins. This facilitates the adsorption of the analytes to the electrode surface (Artiles *et al.*, 2011; Bao *et al.*, 2012).

11.2 Biosensing devices based on graphene and CNTs and their applications in food analysis

Graphene and CNTs recently have gained much attention for the fabrication of biosensors with a large number of applications in food analysis. A wide range of typical examples of biosensors for the rapid detection of glucose have been provided in the literature (Kong *et al.*, 2014; Park *et al.*, 2015; Song *et al.*, 2014; Wang *et al.*, 2014; Yu *et al.*, 2014; Zhang *et al.*, 2014). Graphene and multi-walled carbon nanotubes (GR-MWNTs/AuNPs) have hosted biopolymer pectin stabilised gold nanoparticles and have been employed for the determination of glucose (Devasenathipathy *et al.*, 2015).

A variety of graphene-based electrochemical biosensors has been explored for H₂O₂ detection (Fan *et al.*, 2015; Liu *et al.*, 2014; Muthurasu and Ganesh, 2014). A novel potentiometric cholesterol biosensor has been fabricated through the immobilisation of the stabilised polymeric lipid membrane onto graphene electrode (Nikoleli *et al.*, 2013b). A miniaturised potentiometric urea lipid film-based biosensor on graphene nanosheets recently appeared in the literature (Nikoleli *et al.*, 2012).

Recently, a review article has summarised the use of electrochemical immunosensors based on graphene (Song *et al.*, 2016). A number of articles describing such immunosensors for the rapid detection of food toxicants and for applications in food analysis have been recently published (Eissa and Zourob, 2012; Eissa *et al.*, 2012, 2013).

A metallic NP/graphene nanostructure with a large specific surface area and high electrical conductivity was synthesised for electrode modification for immunoassays.

Specifically, a simple immunoassay, with one-step construction of a graphene/Au hybrid electrode, was presented by Zhu *et al.* (2015).

Shan *et al.* (2009) fabricated an electrochemical biosensor based on polyvinylpyrrolidone functionalised on graphene/polyethylenimine-glucose oxidase (GOx). The enzyme retained its activity and this glucose biosensor showed a linear range up to 14 mM of analyte.

A needle-type sensor for glucose, based on functionalised graphene, has been reported by Hasan *et al.* (2012) (Figure 11.1). Reduced graphene oxide (RGO) possesses carboxyl acid groups that can interact directly with the amines of glucose oxidase (GOx). Along with the electrostatic interactions between the positively charged polymeric ionic liquid (PIL) and GOx, the construct provides for (a) efficient enzyme immobilisation; and (b) a favourable microenvironment for bioactivity preservation. The sensor showed a sensitivity of 5.59 $\mu\text{A}/\text{decade}$ and linearity up to 100 mM of analyte concentration, and this demonstrates the feasibility of clinical applicability in the near future.

An amperometric H_2O_2 sensor has been developed through the electrostatic self-assembly of horseradish peroxidase (HRP) and sodium dodecyl benzene sulphonate (SDBS) functionalised graphene nanosheets (GSs) (Zeng *et al.*, 2010). The SDBS-modified GSs offer open and accessible two-dimensional large surfaces for enzyme tethering at a 'flexible distance', and electrostatic self-assembly make them restack to the dimensions of the biomolecules. Thus, the enzymes can be hosted at almost native conformations. The sensor exhibited excellent electrocatalytic performance, with fast response times, broad linearity, high sensitivity, low detection limit and good stability; after two months of storage at 4 $^{\circ}\text{C}$, no substantial decrease (>85%) in the sensor's response was observed. Graphene attributed excellent biocompatibility and electron transport efficiency, also allowing a high enzyme loading, to yield a synergistic catalytic effect towards peroxide. Graphene can be easily functionalised by 'designer' aromatic molecules, via non-covalent bonding, to express a variety of electrostatic properties. Thus, customised bionanocomposites can be hierarchically built through self-assembly, to yield facile platforms for biocatalytic applications.

Dey and Raj (2010) reported on an amperometric biosensor for the detection of peroxide and cholesterol with high sensitivity assigned by synthesised graphene, chemically decorated with Pt nanoparticles (GNS-nPt). Cholesterol oxidase (ChOx) and

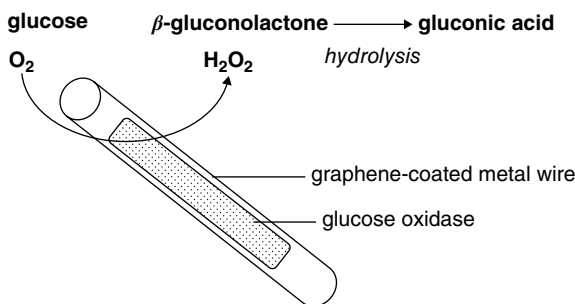


Figure 11.1 Overview of the glucose oxidase-coated graphene biosensor and the possible electrochemical reaction near the electrode.

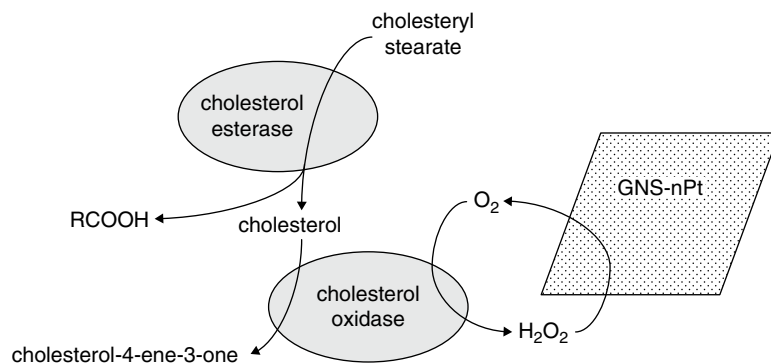


Figure 11.2 Scheme of the bioenzyme biosensor for the detection of peroxide and cholesterol, based on graphene chemically decorated with Pt nanoparticles (GNS-nPt).

esterase have been co-immobilised on the hybrid matrix (Figure 11.2), and produced a highly sensitive sensing platform, with linearity towards H_2O_2 up to 12 mM and a detection limit of 0.5 nM, without using any redox mediator or enzyme. Graphene decorated with Pt nanoparticles opens new avenues for the electroanalysis of biologically important analytes.

Alcohol dehydrogenase (ADH), immobilised on graphene-modified glassy carbon electrodes GCEs (GSs/GCE), has been developed and applied for the electrochemical sensing of NADH and ethanol (Guo *et al.*, 2011). The analytical performance for the amperometric detection of ethanol was better than conventional bio-electrodes, based on graphite functionalised and bare GCE (i.e. faster, highly selective and sensitive response, a wider linear range and lower detectability for the substrate detection).

A potentiometric and miniaturised biosensor for cholesterol, based on cholesterol oxidase (ChOx), physically adsorbed on exfoliated GSs placed on a thin copper wire, was reported by Israr *et al.* (2011). The sensor exhibited fast response times (≈ 4 sec), good operational and shelf-life stability, high sensitivity (≈ 82 mV/decade) and selectivity, reusability, and a μM to mM logarithmic range.

Nikoleli *et al.* (2012) presented a potentiometric miniaturised biosensor for urea based on graphene nanosheets (GSs) (Figure 11.3). Structural characterisation of graphene nanosheets, using atomic force microscopy (AFM), transmission electron

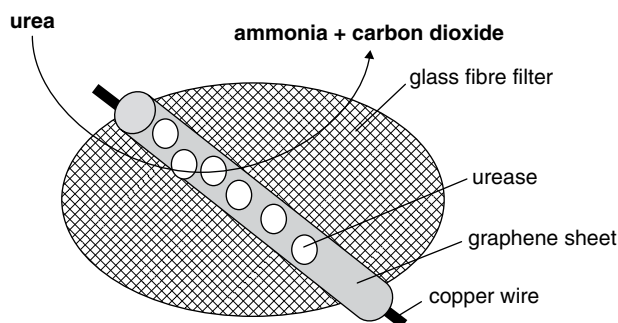


Figure 11.3 Scheme of the potentiometric urea biosensor.

microscopy (TEM), UV-Vis and Fourier transform IR (FTIR) spectroscopy, before and after conjugation to a copper wire, supported thin lipid film containing the enzyme, revealed distortions due to their attachment to the wire and enzyme adsorption. The sensor showed rapid response times (≈ 4 sec), reusability, reproducible and stable output, high sensitivity, and selectivity over a μM to mM logarithmic range.

An electrochemical immunosensor for the ultrasensitive detection of matrix metalloproteinase-2 (MMP2) was developed on gold nanoparticles (AuNPs), assembled on nitrogen-doped graphene sheets as immobilisation scaffolds for capture antibodies and polydopamine functionalised GO hybrid (PDAGO), conjugated to HRP secondary antibodies as tracers (Yang *et al.*, 2013). The proposed immunosensor displayed a detectability of 0.11 pg/mL of MMP2 and demonstrated efficiency for determination in real samples.

Eissa *et al.* (2012) reported on the use of screen-printed electrodes modified with graphene for the development of a label-free voltammetric immunosensor for β -lactoglobulin. Graphene was electrochemically reduced on the electrode surface to yield 4-nitrophenyl diazonium cations in aqueous acidic solution, and the terminal nitro groups were subsequently reduced to amines. The derivation procedure was optimised to yield one layer of nitrophenyl groups on the surface of graphene, and β -lactoglobulin antibodies were immobilised on the surface of graphene through their covalent attachment to glutaraldehyde-activated amines. The sensor was characterised with cyclic and differential pulse voltammetry in an aqueous solution of a $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox pair. Results indicated that the reduction peak current of the redox pair decreased linearly with increasing β -lactoglobulin concentration, due to the immunoreaction. The sensor, with a detection limit of 0.85 pg/mL and a wide dynamic range (1 pg/mL to 100 ng/mL), was successfully validated in packaged foods, such as cake, cheese snacks and biscuits.

A similar platform has been employed for the detection of ovalbumin, the egg white allergen (Eisa *et al.*, 2013), using ovalbumin antibodies. The immunosensor that was developed showed a wide linear range (1 pg/mL to 0.5 $\mu\text{g/mL}$), with a detection limit of 0.83 pg/mL. Validation studies in spiked cake extracts demonstrated good recovery, indicating the applicability of the sensor in the analysis of real food samples.

This voltammetric immunoplatfrom was modified to detect okadaic acid (Eissa and Zourob, 2012). Square wave voltammetry (SWV) was used to monitor the covalent immobilisation of the okadaic acid antibody on the carboxyphenyl modified graphene electrodes, via carbodiimide chemistry. The analyte was detected by a competitive assay between okadaic acid and a fixed concentration of okadaic acid-ovalbumin conjugate. Linearity of response reached ≈ 5000 ng/L, with a detectability of 19 ng/L. Validation studies were made with spiked shellfish tissue extracts, and certified reference mussel samples showed good recovery percentages.

There is an increased interest toward the development of bioelectronics devices for the detection of food toxins, especially mycotoxins. Mycotoxins are highly toxic fungi metabolites produced by *Fusarium*, *Aspergillus*, and *Penicillium* spp., and are frequently found in crops or in stored food such as cereals, nuts or fruits.

The contamination of food by mycotoxins has become an issue of increasing concern as, at high levels, they can cause acute, and chronic effects on humans and a variety of animal species. Mycotoxins tend to affect the liver, the kidney, the nervous system, the endocrine system and the immune system.

Among the 300 mycotoxins known up to today, there are a few that are considered critical for food safety, prompting the development of reliable analytical methods for their detection. Some of the most important mycotoxins include aflatoxins, ochratoxins, fumonisins, citreoviridin, patulin, citrinin, and zearalenon.

The conventional methods of mycotoxins analysis rely on sophisticated instrumentation, such as liquid chromatography with fluorescence or mass detectors, and extraction procedures for sample preparation. Hence, new tools are necessary to provide more sensitive, specific, rapid, and reliable information about the desired toxin.

Nanomaterials, such as those based on carbon (carbon nanotubes and graphene), metal nanoparticles, nanowires, nanocomposites and nanostructured metal oxide nanoparticles are starting to enjoy significant roles in the design of mycotoxin sensing and biosensing systems. The produced nanobiosystems are also bringing new food toxin detection strategies.

Singh *et al.* (2013) have recently developed a biosensor for aflatoxin detection, based on carboxylated multi-walled carbon nanotubes (MWCNT). These nanotubes were synthesised by chemical vapour deposition, generating a large number of carboxyl groups on their surface. The carboxylated MWCNT was functionalised with monoclonal anti-aflatoxin B1 (anti-AFB1). The developed electrochemical immunosensor demonstrated high sensitivity and improved detection limit (0.25 ng/mL), with a linear detection range.

A review article that describes recent results related to fabrication of nanomaterial-based biosensors for food toxin detection has been published in the literature (Malhotra *et al.*, 2014).

11.3 Future trends and prospects

Monitoring and assessing food safety require detection systems that are fast, reliable, sensitive and selective. Biosensors, built on the supreme detection capabilities of biological moieties and natural processes, show the potential to meet this demand. Unfortunately, most of the systems developed have been evaluated only in lab-made standard solutions, and only a few have been validated for real samples. Towards that end, food biosensors continue to advance with genetically modified enzymes or organisms, new immobilisation strategies and more efficient sensor interface architectures.

Although a huge variety of forms and types of food biosensors have been proposed in the literature, the field of applications has been inadequate. The diversity of research is not reflected in its applications, and has been limited mainly to glucose as a mass market. The drawbacks of current biosensors can be summarised as follows:

- a) They are not robust.
- b) They are insufficient for complex analytes.
- c) They require extensive sample preparation.
- d) Extra laboratory testing is not possible for non-trained personnel.

The monitoring of glucose is very important for maintaining blood sugar levels and, for this, a 'turn-on' fluorescence technique has been developed, where graphene oxide

(GO) interactions with ssDNA quantitate selectively glucose. Nanomaterials, such as SWCNTs, QDs and other NPs, have been used, along with GO to increase the electron mobility. The fluorescence of nanomaterials is quenched by GO, and sensing capability enhances owing to promoted charge transfer reactions. Bimetallic NPs (Pt and Pd) act as efficient glucose biosensors, as enzymes and other biomolecules can be easily immobilised on their surface.

The reproducibility and stability issues have been solved by the fabrication of enzyme-free GO biosensors. The fluorescent intensity ratio, which increases linearly with the concentration of analyte, could be used for the detection of a variety of proteins. Energy transfer is found to be a plausible mechanism for the fluorescence quenching. The limitation of the detection of ATP/GTP molecules for the visualisation of living cells has been overcome by the use of GO nanosheets, which have good cell membrane permeability and low toxicity. GO-NPs may also serve as excellent electroactive labels for the sensing of thrombin protein with greater selectivity.

GO-based biosensors have also been found useful in food safety. In the coming future, they may turn to be quite beneficial to the food industry, one of the world's major industries. The presence of pesticides is very harmful to the environment and can be detected in trace amounts with highly sensitive, robust and cost-effective GO based sensors, which lead to new insights for environmentalists. Food-borne pathogens causing diarrhoeal diseases could be rapidly detected via turn-on/off fluorescence with high specificity, without cross-reaction with other pathogens. GO biosensors, fabricated through inexpensive processes and with fast response, have been used for the detection of not only pathogenic bacteria and fungi, but also deadly viruses, which infect 70–90% of the world's human population. The GO surface mimics that of the virus cell wall and stops the infection.

Analytes such as toxins and pesticides, posing hazardous risks to the living environment, can be detected in trace amounts using GO sensors. The functional groups present on the GO surface form strong hydrogen bonds with the groups present in the analytes, thereby leading to their detection.

It is very critical to understand the mechanisms for the application of GO in the construction of biosensors, so that they could be developed in bulk. Biocompatibility studies have been reported, and still there is a need to assess their toxicity, so that they can be employed more potentially for bio-imaging and drug delivery, to curb some of the deadly diseases for which therapeutic tools have not been fully explored. GO biosensors have a lot of capability in paving a way in the medical field. Moreover, GO can act as an alternate material to solve the challenges related to the environmental issues, by exploiting its high sensitivity and selectivity properties for the detection of toxic molecules in trace amounts. The major prospect to be addressed in the future is the increasing demand of the engineering of biosensors based on GO, that will allow monitoring and detecting of analytes with high selectivity and sensitivity at low cost.

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12

Nanoparticles-Based Sensors

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12.1 Introduction

In recent years, interest has grown in the scientific community around the development of efficient sensors with the capability of carrying out food analysis. Sensor-based devices usually possess great precision and accuracy, playing a very important role in the food industry as practical tools to monitor and control food quality, as well as to guarantee consumer safety. They are non-destructive food analytical tools, allowing fast and low-cost analysis, with enhanced sensitive detection limits – in some cases down to trace levels. These advantages may be improved by the incorporation of nanomaterials or nanoparticles into sensor devices, which offer real and novel sensing opportunities (Malik *et al.*, 2013).

Particularly in agriculture and in the food sector, nanoparticles-based sensors have emerged as a technique with great potential to increase agriculture productivity, to ensure food processing control and to enhance the safety of the agro-food industry. Recent developments in the field of nanomaterials offer many technological advances for detection of food-borne pathogens and toxins for food analysis (Valdés *et al.*, 2009; López *et al.*, 2011), as methodologies for identification of microbial pathogenesis (Kaittani *et al.*, 2010) or detection of food contaminants (Sonawane *et al.*, 2014), as well as adulteration in meat and processed meat products (Ali *et al.*, 2011a, 2011b, 2012, 2014; Inbaraj and Chen, 2016).

Nanomaterials can also be used for a variety of agro-food applications, namely as food additives, flavourings, novel foods, food packaging, feed additives and pesticides. Despite the potential of nanomaterials in agro-food sector, their use, according to Kumari and Yadav (2014), may lead to toxicological risks and hazards, and nanomaterials may also pose threat to environment and human health. Therefore, standard guidelines from national regulatory agencies of food and agriculture are urgent and will be welcome, to prevent risks to the consumer.

Regarding their analytical applications, nanomaterials and nanoparticle-based sensors may be applied for monitoring the entire food chain, from agricultural or animal production through to industrial food processing, packaging and distribution. This fact is corroborated by the large number of scientific studies concerning the development of nanomaterials-based sensors, which have led to several recent reviews.

Duncan (2011) reviewed several applications of nanomaterials in food packaging and food safety, namely the use of polymer/clay nanocomposites as high barrier packaging materials, silver nanoparticles as potent antimicrobial agents, and nanosensors and nanomaterial-based assays for the detection of food-relevant analytes. Chen *et al.* (2013) provided an overview of nanoparticles-based sensors for the rapid detection of food-borne pathogens, which especially concerns the application of magnetic nanoparticle-antibody conjugates-based methods. Adley (2014) also pointed out the increase use of biosensors as monitoring technologies across the food industry, mainly for monitoring and control food moisture content, texture, pH, salt content, sugar content, microbial toxins.

Recently, Bülbül *et al.* (2015) reviewed the advancements in the design and development of portable nanoparticles-based sensors for assessing food safety. Nevertheless, regardless of their recognised advantages, the practical application of sensor-based tools in the food industry still has to compete with the standard analytical techniques in terms of cost, performance and reliability (Adley, 2014).

Nanotechnology-derived products have provided a wide range of material candidates that can be used to increase portability and enhance stability, selectivity and sensitivity of sensors and analytical measurements technologies (Bülbül *et al.*, 2015). Current applications of nanotechnology in agriculture and food sector have been revised by Sozer and Kokini (2009) and Kumari and Yadav (2014).

This chapter covers recent research advances involving the use of nanoparticles-based sensors in food diagnostics and detection of analytes. The following sections will describe, briefly, the type of nanomaterials usually incorporated in nanoparticles-based sensors, their characterisation and possible technological applications in the food field. Finally, some future trends regarding nanoparticles-based sensors applied in food science are pointed out.

12.2 Nanoparticles for sensor technology

This section aims to present an overall picture of how nanomaterials or nanoparticles are used within sensor technology for food analysis. Nanomaterials-based sensors include a wide diversity of sensors that are built using nanomaterials, regardless of their size. Macro-, micro- and nano-size sensors have been developed and, in a broader classification, these are all described as nanosensors, as they are sensors with sensitivities falling in nano-units (i.e. they allow measuring solution concentrations at nM level), even when they are without the nano-size restriction (Zhang and Wang, 2008).

Nanomaterials exhibit interesting properties, due to large surface areas, which increase significantly their reactive chemical properties, affecting the mechanical and/or electrical properties of those materials at large scales. The nanoparticles' (NPs) size and shape affect their properties, mainly electrical conductivity, wear resistance, strength, surface areas reactivity, catalytic efficiency, super-plasticity and super-paramagnetic characteristics.

The possibility of controlling the size, surface properties and assembly of NPs systems provides opportunities for the development of advanced sensing systems and portable instrumentation that incorporates nanotechnology-enabled solutions (Zhang and Wang, 2008; Khanna, 2012).

The NPs usually applied in sensor construction are materials with sizes in the range of nanometres. These NPs can be classified as:

- i) zero-dimensional nanomaterials – that is, particles formed by atom clusters with particle diameter below 100 nm (e.g. the metallic NPs of gold (Au), silver (Ag), platinum (Pt) and iron (Fe));
- ii) one-dimensional nanomaterials, which include particles with diameters in nanometre scale (less than 100 nm), but lengths in micrometer scale, such as nanowires and nanotubes (usually cylindrical in shape); and
- iii) two-dimensional nanomaterials, corresponding to nanofilms with layer thickness in the nano-range (e.g. Au thin-film prepared with Au particles (Khanna, 2012; Cao, 2004).

Nanomaterials may be in elemental form or in composite form. The nanoparticles can be classified according to their composition – for example: metallic nanoparticles (e.g., Au, Ag, Pt, among others); metal oxides nanoparticles such as antimony (SbO), cadmium (CdO) or zinc (ZnO) oxides; carbon-based nanoparticles such as fullerenes or carbon nanotubes (CNTs); semiconductor nanoparticles (e.g., quantum dots); polymer nanoparticles (e.g., dendrimers); or composite materials nanoparticles (e.g., nanoclays or nano-sized biocomposites) (Khanna, 2012).

Nanomaterials are used in sensors development to change/improve their analytical performances. In nanotechnology-based sensing approaches, the most used NPs are:

- metal nanoparticles, since they increase the surface area and conductivity of electrochemical sensors;
- metal oxides NPs, as active sensing components that allow changing redox states and surface properties, being useful in the development of new electrochemical analytical methodologies;
- CNTs and graphene, currently used in electrochemical detection systems due to their large surface area, high conductivities, and easy formation of composites with chemo/biomolecules;
- magnetic NPs that allow to develop sensors with different strategies for analyte detection, since, together with the large surface area, they may potentiate the efficiency molecular immobilisation and enhance kinetic assays;
- quantum dots (metal alloys of group III and group V elements or group IV and group VI elements), which are nano-sized fluorescent semiconductor crystals that show several advantages over conventional organic fluorescent dyes, due to their higher photoluminescence, fluorescence quantum yield and photostability (Satnami *et al.*, 2016).

12.2.1 Electrochemical techniques

In electrochemistry field, nanoparticles are used for developing new working electrodes, such as: liquid carbon paste electrodes modified with Au NPs (Afzali *et al.*, 2016); carbon paste electrodes modified with CdO NP (Arabali *et al.*, 2016); carbon

ionic liquid paste electrodes, modified with ZnO NPs (Raoof *et al.*, 2015); carbon paste electrodes with Au NPs modified with multi-walled CNTs (Afkhami *et al.*, 2014); glassy carbon electrodes immobilised with multiwall CNTs containing Au NPs (Mpanza *et al.*, 2014); glassy carbon electrodes modified with Au NPs (Thomas *et al.*, 2015); glassy carbon electrodes based on magnetism graphene oxide/ β -cyclodextrin/Au NPs composites (Wang *et al.*, 2014); glassy carbon electrodes with CNTs and NPs of antimony doped tin oxide (Ramezani *et al.*, 2016).

These examples show the vast combination of possibilities when applying different NPs with a wide range of operating properties and, therefore, the development of novel sensors and analytical methods. Usually, screen-printed transducer platforms are used for applying these nanomaterials in electrochemical sensors, due to the low-cost operation, ease of handling and reproducibility. These platforms are also commercially available with: carbon working electrodes modified with single- or multi-walled CNTs; graphene as a carbon-based nanomaterial; carbon nanofibres; carbon nanomaterials (graphene or CNTs) mixed with Au NPs and streptavidin; Ag NPs, polyaniline quantum dots core-shell ZnS/CdSe; quantum dots core CdSe; extravidin and Ag NPs (e.g., commercial screen-printed platforms from Dropsens).

As pointed out by Hayat *et al.* (2014), electrodes based on nanoparticles allow to: increase the stability and bioactivity of immobilised biomolecules (used as carriers for the biorecognition element); amplify electrochemical signals, due to increased surface area and by promoting electron transfer reactions (signal transduction and amplification); and add catalytic properties, amplifying the electrochemical signal.

These materials can be used directly in electrochemistry analysis by applying conventional techniques (Wang, 2001) such as:

- cyclic voltammetry (CV), which provides the characterisation of electrochemical reactions and chemical analysis by applying a triangular potential waveform, in single or multiple cycles, to the electrochemical cell, and being the current intensity response measured continuously with the potential for both the faradaic and the capacitive currents, which forms a cyclic voltammogram;
- differential pulse voltammetry (DPV), usually for quantitative analysis, since it allows the decay of the capacitive current through the application of a potential pulse to a voltage ramp and measuring the current at two points for each pulse, just before the application of the pulse and at the end of the pulse – being the difference between current measurements at these points for each pulse determined and plotted against the base potential;
- square wave voltammetry (SWV), a fast method for quantitative analysis presenting good sensitivity, due to the influence of the capacitive current, where the applied potential varies accordingly to a symmetrical square-wave pulse superimposed on a staircase waveform and the current difference between measured current at the forward pulse and the reverse pulse, is plotted versus the potential;
- chronoamperometry (CA), an useful technique to obtain preliminary information about nucleation process and growth mechanism in a system, by measuring its current versus time as a response to a sequence of potential pulses;
- electrochemical impedimetric spectroscopy (EIS), a technique that provides the evaluation of electrical properties of the electrode surface and data on the binding

kinetics of molecules between electrolyte and electrode surface by providing an *ac* method for non-electroactive detection of molecules, using the response of an electrochemical cell to a small amplitude sinusoidal voltage signal as a function of frequency; and

- quartz crystal microbalance (QCM), a mass sensor made of thin plate of quartz capable of measuring mass changes in the nanogram range, based upon the piezoelectric effect in quartz, where the change in the resonant frequency of the quartz is inversely related to the mass change, as it is possible to get even more information by measuring impedance characteristics.

Usually, these techniques are used in studies where the working electrode surface is modified with nanomaterials, in order to evaluate the electrode surface performance and for compound analysis. For example, the DPV technique was applied using a working glassy carbon electrode modified with AU NPs and reduced graphene oxide (Chen *et al.*, 2015); working carbon paste electrode modified with Au NPs (Afzali *et al.*, 2016), a screen-printed electrode modified with reduced graphene oxide, CNTs, and Au NPs (Wang *et al.*, 2015a). Also, a working glassy carbon electrode, modified with graphene-doped AU NPs, was used in CV analysis (Ma and Chen, 2015), and a working CdO NP ionic liquid carbon paste electrode was tested with CV, SWV and CA techniques (Arabali *et al.*, 2015).

Other approaches require further surface modifications of the working electrodes with NPs, using specific chemical or biochemical substances in order to increase the number of applications. For example:

- a strand DNA-functionalised nanoporous alumina membrane-based sensor was used for direct DNA detection with Au NP amplification via impedance spectroscopy (Ye and Yang, 2014);
- hexadecyltrimethylammonium bromide functionalised graphene-supported Pt NPs composite modified glassy carbon electrodes were also used coupled with CV and DPV (Yu *et al.*, 2015);
- a glassy carbon electrode with nanostructured Au substrate, functionalised with polyamidoaminic dendrimers and modified with the immobilisation of a conjugate atrazine-bovine serum albumin, was proposed, together with the application of CV (Giannetto and Umiltà, 2014);
- a electrode based on poly- (diallyldimethylammonium chloride) functionalised graphene and nickel (Ni) NPs composite was described and applied using CV and DPV (Yu *et al.*, 2016);
- a label-free electrochemical quartz crystal microbalance based immunosensor, using self-assembled monolayer of hexandithiol, cysteamine and Au NPs (Chauhan *et al.*, 2016);
- a 1-allyl-3- methylimidazolium chloride in functionalised reduced graphene oxide supported Au NP present in a glassy carbon electrode with CV analysis (Wang and Zhao, 2015).

12.2.2 Spectroscopic techniques

Non-electrochemical analytical techniques are also used for qualitative/quantitative analysis using sensors prepared with NPs, the optical sensors, by optically converting the sensor information into signals useful for analytical purposes (Khanna, 2012) – for

instance: UV-Vis; fluorescence; Surface Plasmon Resonance (SPR, measures adsorption of thin layers of molecules onto planar metal surfaces or onto the surface of metal nanoparticles used to characterise its thickness and monitor binding events); Surface-Enhanced Raman Scattering method (SERS, the only way to detect a single molecule and simultaneously identify its chemical structure, which blends the advantages of fluorescence spectroscopy and Raman spectroscopy, allowing characterisation, identification and detection of molecules adsorbed on rough metal surfaces or nanostructures without the interference from water molecules); and spectrophotometric analytical techniques.

Examples within the two first analytical methodologies include the development of colorimetric and fluorescence sensors, based on the use of organic ligands coupled to nanoparticles. The purpose of using colorimetric sensors is to simplify the analysis, as analytical results can be inspected with naked eye or detected with a simple portable UV-Vis spectrophotometer. Recent examples showed the application of colorimetric sensors (colour generation by the change in the absorbance) of: an aptasensor based on an assembly or disassembly of isotropic Au NP dimers aggregates using DNA probes (Xiao *et al.*, 2015); Au NPs modified with cysteamine (Kang *et al.*, 2016); and an 'artificial antibody' organophosphorous pesticide-binding aptamer connected to Au NPs (Wang *et al.*, 2016).

Kang *et al.* (2016) developed a simple visual and rapid colorimetric sensor to detect clenbuterol, based on Au NPs modified with cysteamine (CA). The solution colour change (from red to blue gray), with increasing clenbuterol concentration, resulted from the aggregation of Au NPs.

The development of fluorescence sensors with NPs may be justified by their high detection sensitivity, generally associated with a good optical stability and high fluorescence intensity. Indeed, it was proved that fluorimetric methods are powerful optical techniques for the detection of analytes at trace levels. The NPs can be utilised as an ideal acceptor of the fluorescence probe that competes with the analyte, causing the release of the fluorescent compound. As a result, there is an increase of solution fluorescence. The work of Lu *et al.* (2015), which applied Au NPs with a fluorescent probe, 7-(benzylamino)-9,9-dibutyl-9H-fluorene-2-carbaldehyde, is an example of the application of this kind of technique.

Another type of application is the use of fluorescent semiconducting material (quantum dots), as in the work of Satnami *et al.* (2016), where a fluorescence sensor, based on colloidal CdS quantum dots nanomaterial capped with L-cysteine, was applied. Si *et al.* (2016) presented a different concept – the functionalisation of polystyrene nanoparticles with fluoresceinamine, which gave fluoresceinamine-based pH-sensitive organic nanoparticles.

Examples based on SPR applications have been reported in recent literature for food safety analysis, namely, using films with metal NPs materials. The SPR sensor designs usually include thin film on a chip or glass slide, coupled with metallic NPs with ligands/receptors. The most recent works were based on Ag-Au alloy NPs immobilised on a transparent indium tin oxide glass surface, using poly vinyl alcohol as the binding material (Tao *et al.*, 2015) and a fibre optic sensor with coating layers of Ag film, Ag NPs and molecular imprinting film, prepared using tetracycline molecule as template over an unclad core of the multimode optical fibre (Shrivastav *et al.*, 2015).

The use of NPs-based sensors on the SERS method have been tested, showing possible applications in the food area due to the electronic interaction between the Raman molecule and the metal, causing a chemical effect or electronic enhancement. Some recent examples using the rough metal surfaces or nanostructures as SERS substrates with ligand molecules are: Au-Ag core-shell nanoparticles (coating Ag on the surface of Au nanoflowers was used as SERS substrate), coated with antigen Cd^{2+} -EDTA-BSA, in food safety testing (Fu *et al.*, 2015); homogeneous decoration of multi-walled CNT/Au NP hybrid nanocomposites on a commercial polyvinylidene difluoride membrane (Zhang *et al.*, 2015b) bioenabled SERS substrates, based on diatom frustules with integrated Ag plasmonic nanoparticles (Yang *et al.*, 2015); and applying Ag-coated Au NPs (used as SERS substrate) (Li *et al.*, 2015).

12.2.3 Nanoparticles characterisation

Nanoparticles can be synthesised in many ways, involving different experimental conditions, methods and reagents. The simplest method for the synthesis of metal nanoparticles involves the chemical reduction of the corresponding metal salts. For example, Ag NPs prepared using this technique (reduction of Ag nitrate by sodium borohydride) showed dimensions of 1.2 ± 2 nm, with plasmon absorbance near 400 nm (Mulfinger *et al.*, 2007; Khanna, 2012). Other methods described in the literature are electrochemical preparation, organometallic decomposition and vapour deposition methods. These procedures affect the composition, size and properties of the nanoparticles, making it necessary to characterise nanoparticles morphology and their constituents using several instrumental methods (Khanna, 2012).

Microscopic techniques are commonly used to evaluate nanomaterials surface morphologies, which can be achieved by:

- Scanning Electron Microscope (SEM), which allows characterising surface morphology, including shape, size, and positions of particles on the surface of the sample);
- Transmission Electron Microscope (TEM), which produces an image through density variations allowing direct visualisation of surface and possible defects;
- Atomic Force Microscope (AFM), which acquires a three-dimension profile of surface at nano-scale.

For chemical characterisation, the spectroscopy techniques most used to analyze the composition of nanomaterials are:

- Infrared Spectroscopy (IR), used for identifying functional groups but is not useful for nonpolar molecules;
- Ultraviolet-visible Spectroscopy (UV-Vis), commonly used to study samples that Infrared Spectroscopy cannot identify;
- Raman Spectroscopy (RS), capable of identifying nonpolar molecules);
- Energy Dispersive X-Ray Spectroscopy (EDXRS), based on the use of X-rays generated during the SEM examination of a sample, giving information about the chemical composition of the sample;
- Dynamic Light Scattering (DLS), which measures the size and size distribution of molecules and particles that have been dispersed or dissolved in a liquid;
- Surface Plasmon Resonance;

- X-Ray diffraction (XRD), used for phase identification of a crystalline material and can provide information on unit cell dimensions; and
- X-Ray Photoelectron Spectroscopy (XRPS), a surface-sensitive quantitative spectroscopic technique that measures the chemical composition.

For example:

- synthesised 4-aminobenzenethiol functionalised Ag NPs were characterised by UV-Vis, IR, DLS and TEM techniques (Patel *et al.*, 2015);
- Au NPs morphology and structure were characterised by FT-IR and TEM (Yang and Zhao, 2015);
- as-grown CdO nanostructures morphology was characterised by TEM (Arabali *et al.*, 2015);
- reduced graphene oxide decorated with Au NPs was characterised by TEM, RS, XRD and XRPS (Chen *et al.*, 2015);
- Ag film layers were characterised by SEM, AFM and TEM (Shrivastav *et al.*, 2015);
- ZnO NPs used to modify carbon ionic liquid paste electrode were evaluated by XRD (Raoof *et al.*, 2015);
- hexadecyltrimethylammonium bromide functionalised graphene supported Pt NPs composite used to modified glassy carbon electrodes were morphologically analyzed by TEM images and the composite formation characterise by UV-Vis and IR spectroscopy (Yu *et al.*, 2015);
- the morphology of Ag-coated Au NPs (used as SERS substrate) was assessed by TEM and SEM (Li *et al.*, 2015);
- poly- (diallyldimethylammonium chloride) functionalised graphene and Ni NPs composite dispersions were chemically characterised by UV-Vis Spectroscopy and morphologically by XRD and TEM (Yu *et al.*, 2016).

All this shows that, in studies in which nanoparticles are synthesised, there is a need to determine their morphological characterisation and chemical composition for a complete understanding of their properties.

12.3 Nanoparticles-based sensors: applications

As previously mentioned, nanoparticles-based sensors are an emerging research topic within food quality assessment, control and safety. Although the selectivity and the detection limits of standard reference methods are relatively satisfactory for chromatographic methods, these methods are rather time-consuming in terms of analysis, are instrumentally expensive, and require a large number of standard operating procedures (SOPs). Moreover, the majority of the classical analytical techniques are not readily adaptable to on-site detection.

The relevance of this topic was clearly pointed out in recent review papers for specific applications, like the detection of food-borne bacterial pathogens and toxins, as well as pork adulteration in meat products (Inbaraj and Chen, 2016), or for a broader range of applications concerning the potential use of portable nanoparticle-based sensors for food safety evaluation (Bülbül *et al.*, 2015). In this section, the most recent applications (after 2014) of non-enzymatic and non-immunological nanoparticles-based sensors for

food overall assessment are reviewed and discussed, apart from the use of nanoparticles in packaging, as food additives or even as nanoemulsions to encapsulate, protect and deliver additives.

12.3.1 Nanoparticles based-sensors for pesticides detection in foods

Fungicides are used for controlling fungal diseases in fruit crops being used as active ingredients in several pesticide formulations (Patel *et al.*, 2015). Unfortunately, these products, as well as organophosphorous pesticides, have known toxic effects in humans and animals, even when present in low levels, so it is necessary to develop and validate simple analytical systems for their selective, sensitive and on-site detection in foods, that could overcome the main economic and time-consuming disadvantages of traditional reference methods. Recently, different NPs based-sensors (e.g. Ag, Au and MIP) were proposed in the literature for trace level detection of fungicides, pesticides and insecticides (e.g. carbendazim, omethoate and metolcarb) in food matrices (Patel *et al.*, 2015; Wang *et al.*, 2016; Qian *et al.*, 2016).

Patel and collaborators (2015) proposed a colorimetric method for detecting carbendazim fungicide in water and food samples, using 4-aminobenzenethiol (ABT) functionalised Ag NPs as a colorimetric sensor. The ABT-Ag NPs acted as a probe for binding of carbendazim, through strong ion-pair and π - π interactions, resulting in a colour change (from yellow to orange) easily visualised by naked eye. Under optimum conditions, a linear dynamic concentration range of 10–100 μ M, with a detection limit of 1.04 μ M, was reported, based on UV-Vis spectra analysis. The colorimetric method was successfully applied to detect carbendazim in food samples (e.g. apples and carrots) which required the implementation of extraction procedures.

Wang *et al.* (2016) developed a simple and selective aptamer-based colorimetric assay for the detection of omethoate, which is a common organophosphorous pesticide. The assay is based on the fact that single-stranded DNA (ssDNA)-wrapped Au NPs are resistant to salt-induced aggregation; thus, employing an ‘artificial antibody’ organophosphorous pesticide-binding aptamer (OBA) as the recognition element, aptamer-wrapped Au NPs show high selectivity towards omethoate, resulting in the disconnection of aptamers from Au NPs and the aggregation of Au NPs. The proposed assay showed good linearity, between 0.1–10 μ mol/L, with a low detection limit of 0.1 μ mol/L.

Qian *et al.* (2016) constructed a quartz crystal microbalance (QCM) sensor-based molecularly imprinted polymer (MIP) NPs, using a MIL-101 support (a type of metal-organic framework), for detecting trace levels of metolcarb, an insecticide, in pear juice samples. Under optimal conditions, the detection limit in pear juice was 0.0689 mgL^{-1} , for a linear concentration range of 0.1–0.9 mgL^{-1} .

12.3.2 Nanoparticles-based sensors for antibiotics, growth enhancers and other veterinary drugs detection in foods

In livestock farming, the illegal use of drugs is still a dramatic reality, including synthetic steroids as growth enhancers and antibiotics to reduce bacterial and protozoal infections. Their residues in foods can cause serious side-effects on human beings, such as mutagenicity, genotoxicity, and carcinogenicity. Therefore, the European Union and

other official organisations have banned the use of several veterinary drugs in food production, and have set maximum residue limits for food safety. Different analytical methods have been established to detect veterinary drug residues in food chain production, although the great majority are time-consuming and expensive. Therefore, a great effort is being made to provide reliable and cost-effective solutions, such as the development of nanoparticles-based sensors.

Ma and Chen (2015) developed an electrochemical sensor for detection of diethylstilboestrol (DES). DES is a synthetic oestrogen that was used in veterinary medicine, and as a growth promoter in cattle, sheep, and poultry, but its use as a growth promoter has been banned since 1979. Its presence as a residue in meat and its proven carcinogenic nature for human beings has led to the regulation of its use, and the need for its detection to prevent illegal usage. These authors fabricated graphene-modified glassy carbon electrode (Gr/GCE) doped with Au NP-modified glassy electrodes (Gr/Au NP/GCE) for sensitive detection of DES. A linear relationship was established for concentrations ranging from 1.20×10^{-8} and 1.20×10^{-5} mol L⁻¹. The estimated detection limit was 9.80×10^{-9} mol L⁻¹. No interference was found in the presence of some common ions, or oestradiol, oestriol, oestrone and folic acid. The modified electrode has good selectivity and stability, and was successfully applied in the detection of DES in food samples (meat, fish and milk powder).

Wang *et al.* (2015c) developed a highly sensitive electrochemical sensor for toxic ractopamine (RAC), a growth hormone drug that has been banned in Europe and Asia, but which is still legal in the USA, using acetylene black (AB) NP-modified glassy carbon electrode (AB/GCE). The fabricated AB/GCE exhibited a remarkable signal enhancement effect toward the oxidation of ractopamine, compared to the bare GCE and the graphite modified GCE (graphite/GCE). Based on the strong signal enhancement effect of AB, a simple, sensitive and rapid electrochemical method (DPV) was established for the detection of toxic RAC. The linear range was from 1 µg L⁻¹ to 2 mg L⁻¹, and the detection limit (S/N = 3) was as low as 0.5 µg L⁻¹ (1.6 nM). The method was successfully applied in the detection of ractopamine in pork samples.

Kang *et al.* (2016) developed a simple visual and rapid colorimetric sensor to detect clenbuterol, based on Au NPs modified with cysteamine (CA). The solution colour change (from red to blue-gray) with increasing clenbuterol concentration resulted from the aggregation of Au NPs. The detection limit of clenbuterol was determined as 50 nM by the naked eye. Shrivastav *et al.* (2015) reported a novel approach for the fabrication of localised and propagating surface plasmon resonance-based fibre optic sensor for the detection of tetracycline using molecular imprinting (MIP) technique. The sensor was fabricated by coating layers of Ag film, Ag NPs and MIP film, prepared using tetracycline molecule as a template over an unclad core of the multimode optical fibre. The absorption spectra are recorded for the concentration range of tetracycline from 10⁻⁸ M to 10⁻⁵ M. The sensor works in the concentration range of tetracycline found in foods.

Yang and Zhao (2015) reported a facile method for preparing Au NPs@MIP, which was coated on mesoporous carbon (CKM-3) and porous graphene (Pr-GO) modified glassy carbon electrode, to construct an electrochemical sensor for the determination of dimetridazole. The electrochemical sensor had a good response for dimetridazole, and its linear ranges were 2.0×10^{-9} to 2.5×10^{-7} mol L⁻¹ and 2.5×10^{-7} to 3.0×10^{-6} mol L⁻¹, while its detection limit was 5.0×10^{-10} mol L⁻¹. The electrochemical sensor was applied

to determine dimetridazole in food samples (milk, honey and pork muscle samples), with satisfactory results.

Feng *et al.* (2015) proposed an electrochemiluminescence (ECL) aptasensor array, fabricated on screen-printed carbon electrodes (SPCE), for simultaneous detection of malachite green (MG) and chloramphenicol (CAP) in a single assay. MG and CAP, as a fungicide and a disinfectant, are used all over the world in the fish farming industry, MG being a potential carcinogenic and mutagenic agent, while CAP has serious side-effects on human beings, such as gray baby syndrome, leukaemia, and aplastic anaemia. For this reason, many countries have banned their use in aquaculture. The SPCE consisted of a common Ag/AgCl reference electrode, carbon counter electrode and two carbon working electrodes (WE1 and WE2). In this system, CdS quantum dots (QDs) were modified on WE1 as cathode ECL emitters, and luminol (L) with Au NPs were modified on WE2 as anode ECL emitters. The MG aptamer complementary strand (MG cDNA) and CAP aptamer complementary strand (CAP cDNA) were then attached on CdS QDs and L-Au NPs, respectively. The cDNA hybridised with a corresponding aptamer that was respectively tagged with cyanine dye (Cy5) (as quenchers of CdS QDs) and chlorogenic acid (CA) (as quenchers of L-Au NPs) using poly(ethylenimine) (PEI) as a bridging agent.

PEI can lead to a large number of quenchers on the aptamer, which increases the quenching efficiency. Upon MG and CAP adding, the targets induce strand release, due to the high affinity of analytes toward aptamers. Meanwhile, it releases Cy5 and CA, which recover the cathode ECL of CdS QDs and anode ECL of L-Au NPs simultaneously. This 'dual-potential' ECL strategy can be used to detect MG and CAP within linear ranges of 0.1–100 nM and 0.2–150 nM, with detection limits of 0.03 nM and 0.07 nM, respectively. The method was successfully applied to determine MG and CAP in fish samples.

Karaseva *et al.* (2016) reported the use of nanoparticulate MIPs as molecular recognition elements in piezoelectric chemical sensors for the detection of penicillins (antibiotics). Nanoparticles were also used as synthetic antibodies in a competitive pseudo-immunoanalysis format. The method showed a linear range at a concentration of 0.1–0.5 $\mu\text{g mL}^{-1}$ for penicillin G, and 0.1–1.0 $\mu\text{g mL}^{-1}$ for ampicillin, with limits of detection at 0.04 and 0.09 $\mu\text{g mL}^{-1}$, respectively. These analytical figures of merit are below the legal maximum residue limits (MRL) for such antibiotics in meat, therefore rendering the developed device suitable for applications in food industries, and this was verified for chicken meat samples evaluation.

12.3.3 Nanoparticles based-sensors for mycotoxins detection in foods

Mycotoxins are food contaminants, are the secondary metabolites of fungi activity, and are a serious problem in the food and agricultural industries. These toxins (e.g., aflatoxins, ochratoxins, etc.) are genotoxic and carcinogenic to humans and animals. Therefore, the exposure through food (e.g. nuts, maize, rice, vegetable oils, coffee, beans, wine, beer and meat) needs to be as low as possible. Aflatoxin B1 (AFB1) is the most common in food, and is among the most potent genotoxic and carcinogenic aflatoxins, being produced by species of *Aspergillus* that exist in areas with hot and humid climates. Ochratoxin A (OTA) is one of the most abundant food-contaminating mycotoxins, and is produced by *Aspergillus* and *Penicillium* species (Xiao *et al.*, 2105; Li *et al.*, 2016).

Maximum levels of AFB1 and OTA are regulated by the Food and Drug Administration (FDA) and the European Commission (EC) (Li *et al.*, 2016; Luan *et al.*, 2015). Thus, it is very important to develop highly sensitive detection methods for monitoring trace amounts of mycotoxins, especially AFB1 and OTA, in human food.

Luan *et al.* (2015) reported an aptasensor for AFB1 detection, using an unmodified Au NPs indicator based on the salt-induced Au NPs aggregation phenomenon. Its linear dynamic range and detection sensitivity were 0.025–100 ng/mL and 0.025 ng/mL, respectively. Li *et al.* (2016) described a SERS aptasensor, based on Au nanostar core-Ag NP satellites, for detecting AFB1. A linear detection range from 1–1000 pg mL⁻¹, with a limit of detection (LOD) of 0.48 pg mL⁻¹, were reported. The aptasensor was successfully applied for detecting AFB1 in peanut milk, demonstrating that the sensor could be applied in food analysis.

Xiao *et al.* (2015) developed an on-site colorimetric aptasensor, based on disassembly of oriented Au NP aggregates, for OTA detection. The dynamic range of the response was between 0.2–250 nM, with a detection limit of 0.05 nM. The aptasensor was applied for detecting OTA in red wine and in contaminated red wine. Soh *et al.* (2015) proposed a versatile and sensitive colorimetric assay, which integrates aptamer-target recognition and the aptamer-controlled growth of Au NPs. The method allows a visible colorimetric response and nanomolar detection limits for ochratoxin A (1 nM) in red wine samples. Moreover, the assay could be easily adapted as a chip-based platform for rapid and portable target detection.

Afzali *et al.* (2016) developed a method for the determination of OTA in different food samples, based on its oxidation responses at Au NPs modified carbon paste electrodes (GNP/CPE). The proposed electrochemical sensor showed a linear response for OTA concentration ranging from 0.5–100 nM, with detection limits of 0.2 nM. The method was applied in cereal-derived products analysis, including breakfast cereals, cereal-based baby foods and beer samples.

12.3.4 Nanoparticles based-sensors for microorganisms' detection in foods

Pathogenic microorganisms detection and growth monitoring has gained utmost importance, due to the increasing incidence rate of disease due to contamination of drinking water and food (Thakur *et al.*, 2015). There are several methods usually applied for detection of microorganisms. Those based on immunoassays using antibody-antigen interactions provide high specificity; however, monitoring viable bacteria in the presence of dead bacteria is challenging, since both participate in this type of interaction on the sensor surface, leading to erroneous results. The short self-life of such biosensors is also a limiting factor in terms of operational stability. Thus, there is a real need to develop new alternative methods and protocols that can add simplicity, improve analysis time and detection limit of bacterial monitoring procedures (Thakur *et al.*, 2015).

Thakur *et al.* (2015) report a simple and novel colorimetric sensor for detection of bacterial growth, relying on estimation of its metabolic products. Polyaniline NPs were synthesised, and the sensor films were fabricated by incorporating these nanoparticles in agarose gel. The films, when used for real-time monitoring of bacterial growth (e.g. *Escherichia coli*), exhibited a visible colour change, from blue to green. Since no

specific antibodies or receptors were incorporated, the sensor is generic in nature, and has potential for adaptation to real-life applications in the form of patch sensor on cartons, to gauge integrity and freshness of foods and beverages in real time.

Si *et al.* (2016) developed a novel kind of pH-sensitive fluorescent nanoparticles (FANPs) for fast and accurate real-time detection of *Escherichia coli* growth. The organic particles are non-toxic and highly water-soluble, and FANPs do not influenced bacterial growth, being stable over several hours in a complex medium and in the presence of bacteria. The use of FANPs enables the monitoring of bacterial growth in a real-time basis, over long time scales and in small volumes, and is suitable for screening studies.

12.3.5 Nanoparticles-based sensors for detecting food valuable constituents

The fast, low-cost and simple detection of some food compounds that play a key role in nutritional and/or physicochemical quality parameters of foods is of utmost importance. Therefore, the use of nanoparticles-based sensors contribute towards the accomplishment of this objective, namely for assessing amino acid, vitamin or flavonoid levels in food samples.

Bagci *et al.* (2015) incorporated Au NPs, synthesised at room temperature, in a colorimetric sensor device for detection of cysteine (Cys), an essential amino acid in food and biological systems. In the presence of Cys, unlike with any other amino acid, the Au NPs aggregated, yielding a red-to-purple colour change of the sample solution (in ≈ 15 minutes). The red shift of the localised surface plasmon resonance peak of the Au NPs responsible for the colour change was recorded by UV-vis spectrometer. The results showed that Au NPs can detect and quantify Cys with a linear dependency in the range of 2–100 μM , and a limit of detection of 50 nM.

Baghizadeh *et al.* (2015) proposed a fast and simple voltammetric method, based on the use of ZrO_2 NP/ionic liquids carbon paste electrode ($\text{ZrO}_2/\text{NPs}/\text{IL}/\text{CPE}$) to determine vitamin C (ascorbic acid, AA) in the presence of vitamin B6. The nanoparticles were used to prepare a carbon paste electrode, modified with 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim]BF₄), as a binder for voltammetric determination of AA and vitamin B6 in food samples. The concentration dynamic ranges were found to be 0.07–850 μM for AA, and 0.8–550 μM for vitamin B6. The detection limits for AA and vitamin B6 were 0.009 and 0.1 μM , respectively. The modified electrode was successfully used to quantify AA and vitamin B6 in food samples (e.g. tablet, fruit juice).

Raouf *et al.* (2015) showed that a new ZnO/NPs carbon ionic liquid paste electrode ($\text{ZnO}/\text{NPs}/\text{IL}/\text{CPE}$) could be used to detect vitamin B6 in food samples. A linear concentration range of 0.5–800 μM was reported, as well as a detection limit of 0.2 μM . Arabalia *et al.* (2016) reported a highly sensitive sensor based on a carbon paste electrode, modified with a CdO nanoparticle, and 1-methyl-3-butylimidazolium bromide was used for the voltammetric analysis of vitamin C in the presence of nicotin amide adenine dinucleotide (NADH).

Vitamin C is one of the safest and most effective nutrients in food science. NADH is an important coenzyme, and plays a key and virtual role in energy metabolism by accepting and donating electrons. Also, NADH oxidation in ocular tissues can be effective in the presence of ascorbic acid. The electrooxidation peak current of vitamin

C and NADH increased linearly, with their concentration in the ranges $0.07 - 480 \mu\text{mol L}^{-1}$ vitamin C and $0.5 - 700 \mu\text{mol L}^{-1}$ nicotin amide adenine dinucleotide. The LODs for vitamin C and nicotin amide adenine dinucleotide were $0.03 \mu\text{mol L}^{-1}$ and $0.1 \mu\text{mol L}^{-1}$, respectively. The proposed sensor showed good stability, sensitivity, selectivity and reproducibility, and its use could be foreseen for food sample analyses.

Akyildirim *et al.* (2015) proposed a novel electrochemical sensor based on iron nanoparticles (Fe NPs) and 2-aminoethanethiol (2-AET) functionalised graphene oxide (2-AETGO) for evaluating quercetin levels in fruits and vegetables. The nanocomposites based-sensor showed a linear response towards quercetin levels in the range of 1.0×10^{-8} to 1.0×10^{-7} M and a detection limit of 2.0 nM. The modified glassy carbon electrode (FeNPs/2-AETGO/GCE) enabled the quantification of quercetin in apple juice.

12.3.6 Nanoparticles based-sensors for detecting food contaminants and adulterations

Nanoparticles have emerged as a promising analytical tool for monitoring food adulteration and safety. The detection of food contaminants (e.g. thiocyanate, hydrazine, bisphenol A, melamine, etc.) is a major global concern.

For example, thiocyanate (SCN^-) is a small anion by-product of cyanide metabolism. It is less toxic than the lethal cyanide, is a potent inhibitor of iodide uptake by the thyroid, and can be found at μM in human bodies, due to the consumption of glucosinolate-containing brassica vegetables (broccoli, cauliflower, cabbage, etc.), cheese and milk (Song *et al.*, 2015b). Hydrazine is commonly used in chemical industry, although it is known as a carcinogenic substance (Luo *et al.*, 2015). This compound may cause water source contamination, thus affecting direct water consumption. Due to its use in food processing, the detection of hydrazine in water and/or food samples is required.

Bisphenol A (BPA) is a chemical used in the manufactory of plastics and resins, as well as food containers such as returnable beverage bottles, infant feeding (baby) bottles, tableware (plates and mugs) and storage containers (Arabali *et al.*, 2015). Thus, residues of BPA can migrate into food and beverages stored in materials containing the substance. It is therefore essential to detect trace levels of this compound in food samples.

Melamine (1,3,5-triazine-2,4,6-triamine, $\text{C}_3\text{H}_6\text{N}_6$) is a triazine heterocyclic organic compound used in the production of resins, flame retardants, fertiliser and other products (Xin *et al.*, 2015). Melamine can be hydrolyzed, leading to the formation of insoluble crystals in the kidneys and subsequent tissue injury. Therefore, its use as a food additive in human food or animal feeds is forbidden, and safety limits in milk and milk-based products were set by the Food and Drug Administration (FDA) and the European Union (Xin *et al.*, 2015). However, due to its high nitrogen content, melamine was illegally and unethically incorporated in foods. Therefore, once again, melamine detection in food is of paramount relevance for safety and health issues.

Song *et al.* (2015b) described a simple and ultrasensitive turn-on fluorescence assay of trace SCN^- , based on the fluorescence resonance energy transfer (FRET) between Au NPs and fluorescein. The detection limit was 0.09 nM and the linear concentration dynamic range was set from 1.0–40.0 nM. Furthermore, no significant interferences

were recorded from common metal ions, amino acids and sugars. The cost effective sensing system was successfully applied for the determination of SCN^- in milk products.

Luo *et al.* (2015) reported an electroanalytical method for detection of hydrazine, using graphene-cobalt hexacyanoferrate nanocomposite, functionalised into an electrode surface. The method pointed out the potential application for detecting carcinogenic compounds in food samples.

Arabali *et al.* (2015) used a CdO nanoparticle ionic liquid carbon paste electrode to detect bisphenol A electrochemically. A concentration dynamic range of 0.3–650 μM , with a detection limit of 0.1 μM , was reported by the authors. The proposed novel sensor proved to be a sensitive, high selective, simple, and precise electrochemical sensor for the determination of bisphenol A in food samples (tomato paste, tuna fish corn, stew, and water bottle).

Wang *et al.* (2015a) fabricated a highly sensitive electrochemical sensor for the determination of bisphenol A (BPA) in aqueous solution by using reduced graphene oxide (RGO), CNTs, and Au NPs-modified screen-printed electrode (SPE). GO/CNT nanocomposite was directly reduced to RGO/CNT on SPE at room temperature. Gold NPs were then electrochemically deposited *in situ* on RGO/CNT-modified SPE. Linear current responses were recorded for concentrations ranges of 1.45–20 nM and 20–1490 nM, with a detection limit of 800 pM. The sensor response was unaffected by the presence of interferents such as phenol, p-nitrophenol, pyrocatechol, 2,4-dinitrophenol, and hydroquinone.

Borase *et al.* (2015) produced biofunctionalised Ag NPs which allowed selective and rapid method for melamine detection in raw milk. The detection limit was 2 μM (252 ppb), lower than safety level recommendations of regulatory bodies demonstrating sensitivity of the method. Xin *et al.* (2015) developed a simple and rapid field-portable colorimetric method, using synthesised Au NPs mediated by methanobactin (Mb), for the detection of melamine in liquid milk. The method enabled naked-eyes melamine detection for a melamine concentration of 5.56×10^{-6} M (0.7 mg/kg). UV-vis analysis showed a dynamic concentration range of 3.90×10^{-7} to 3.97×10^{-6} M, with a detection limit of 2.38×10^{-7} M (i.e. 0.03 mg/kg). Lu *et al.* (2015) synthesised a new fluorescence probe, coupled with Au NPs, to detect melamine. Based on changes of the fluorescence intensity of the probe, the detection of melamine was possible with a linear response from 1.0×10^{-8} to 4.0×10^{-6} mol L^{-1} , and a detection limit of 3.0 nmol L^{-1} , making possible its application for detecting melamine in milk and milk-based productions.

Song *et al.* (2015a) proposed a simple, rapid and low-cost assay, with high sensitivity for melamine detection in milk samples, using sulfanilic acid (SAA)-modified Ag NPs. The interaction between exocyclic amine of melamine and SAA induced rapid aggregation of SAA-Ag NPs, accompanied by a naked-eye visible colour change, resulting in precise quantification of melamine using a simple UV-visible spectrometer. Interference from metal ions, amino acids and sugars that are common in milk was found to be negligible. The dynamic concentration range varied from 0.1 to 3.1 μM with a detection limit of 10.6 nM, which is much lower than the safety limits. The method allowed the determination of melamine in pre-treated milk products, indicating the potential practical use for the products suspected of melamine exposure.

Chen *et al.* (2015) described an electrochemical sensor for melamine, based on a glassy carbon electrode (GCE) modified with reduced graphene oxide (rGO) and covered with Au NPs. Under optimised conditions, a dynamic concentration range between 5.0–50 nM was found. The method was successfully applied to the determination of melamine in food contact materials. More recently, Rovina and Siddiquee (2016) developed a novel electrochemical method, based on ionic liquids (1-ethyl-3-methylimidazolium trifluoromethanesulfonate ([EMIM][Otf])), ZnO NPs with chitosan (CHIT), and modified Au. Under optimal conditions, a wide linear range of melamine concentrations was found (9.6×10^{-4} to 9.6×10^{-13} M), with a detection limit of 9.6×10^{-14} M. The proposed method was a fast and simple procedure technique, allowing analysis of melamine levels in milk powder products.

12.3.7 Nanoparticles-based sensors for detecting food dyes/additives

The development and use of NPs-based sensors for food additives detection has attracted a lot of attention in recent years. Indeed, several works are available in the literature regarding the detection of artificial colorants, antioxidants, sweeteners, preservatives, conditioners and bleaching agents, among others, in food samples.

Synthetic colorants have been widely used in foodstuffs. However, most synthetic colorants are pathogenic, particularly if they are excessively consumed. It is thus important to develop sensitive, rapid and simple detection platforms for toxic synthetic colorants in foods (Cheng *et al.*, 2015; Songyang *et al.*, 2015). Several synthetic colorants are used by the food industry although some of these have been legally banned. Recently, nanoparticles based-sensors have been proposed for detecting Sunset yellow (Cheng *et al.*, 2015; Songyang *et al.*, 2015; Wang and Zhao, 2015; Yu *et al.*, 2015), Tartrazine (Cheng *et al.*, 2015; Wang and Zhao, 2015), Ponceau 4R (Cheng *et al.*, 2015), Allura red (Cheng *et al.*, 2015; Yu *et al.*, 2016), Sudan I (Raoof *et al.*, 2015; Thomas *et al.*, 2015), Quinoline yellow (Fu *et al.*, 2015) and Chrysoidin (Xu *et al.*, 2015).

Cheng *et al.* (2015) used a series of porous carbon (PC), prepared using CaCO_3 NPs as the hard template, and starch as the carbon precursor, for electrochemically assessing the levels of four widely used food colourants – namely, Sunset yellow, Tartrazine, Ponceau 4R and Allura red. The highly sensitive electrochemical sensing platform developed allowed achieving detection limits of 3.1, 6.5, 3.5 and 3.4 nM for Sunset yellow, Tartrazine, Ponceau 4R and Allura red, respectively. The practical application of the new sensing platform was demonstrated using drink samples.

Yu *et al.* (2015) fabricated a new and sensitive electrochemical sensor based on a hexadecyltrimethylammonium bromide (CTAB)-functionalised graphene-supported Pt NPs composite. The CTAB-Gr-Pt composite greatly improved the oxidation activity of sunset yellow, allowing a linear concentration range of 0.08–10 μM to be set, and the detection limit was 4.2 nM. The developed method showed high sensitivity and selectivity, and was successfully applied to determine sunset yellow in soft drinks with satisfactory recoveries. Songyang *et al.* (2015) used montmorillonite calcium (MMT-Ca), functionalised with cetyltrimethylammonium bromide (CTAB) via cationic exchange effects, to enhance Sunset yellow detection. The electrochemical device was highly sensitive, rapid and simple, showed a linear range from 2.5–200 nM, and a detection limit of 0.71 nM. The potential of the proposed device was further demonstrated by analyzing soft drink samples.

Wang and Zhao (2015) proposed a new facile *in situ* reduction approach for the preparation of an ionic liquid of 1-allyl-3-methylimidazolium chloride (AMIM-Cl), functionalised reduced graphene oxide-supported Au NP (ILRGO-Au) composite for the simultaneous detection of Sunset yellow and Tartrazine. The electrochemical sensor built allowed the determination of Sunset yellow and Tartrazine in drinks within the concentration ranges of 4.0×10^{-9} to 1.0×10^{-6} and 7.0×10^{-9} to 1.5×10^{-6} M, respectively. Detection limits of 0.52 nM for Sunset yellow and 0.83 nM for Tartrazine were obtained, showing the sensor's potential for food analysis.

Yu *et al.* (2016) developed a novel and sensitive electrochemical sensor for allura red determination, based on poly (diallyl dimethylammonium chloride) (PDDA) functionalised graphene (Gr) and nickel nanoparticles (Ni) composite (PDDA-Gr-Ni). Under optimum experimental conditions, the peak current of allura red was proportional to its concentration in the range 0.05–10.0 μ M, and the limit of detection (LOD) was 8.0 nM slightly higher than that previously reported by Chen *et al.* (2015). The simple and sensitive sensor performance was checked by assessing allura red content in a strawberry juice.

Raof *et al.* (2015) constructed a new ZnO NP carbon ionic liquid paste electrode (ZnO/NPs/IL/CPE), which exhibited enhanced electrooxidation capability for detecting Sudan I. This modified electrode exhibited a potent and persistent electron mediating behaviour, followed by well-separated oxidation peaks of Sudan I and vitamin B6. The peaks current of SWV of Sudan I increased linearly with its concentration from 0.01–400 μ M, and allowed a detection limit of 8 nM. The modified electrode was successfully applied for assessing Sudan I concentrations in food samples (chilli powder, chilli sauce, tomato sauce and strawberry sauce).

Thomas *et al.* (2015) developed an electrochemical sensor based on the catalytic activity of Au NPs deposited on a glassy carbon electrode (AuNP/GCE) for the determination of Sudan I in food samples. The sensor exhibited two distinct linear response ranges (4.0×10^{-5} to 1.0×10^{-3} M, and 2.0×10^{-5} to 7.0×10^{-7} M). The reported detection limit was 10 nM – slightly greater than that described by Raof *et al.* (2015). The sensor proposed (Thomas *et al.*, 2015) allowed the accurate determination of Sudan I concentrations in food products such as ketchup and chilli sauce.

Fu *et al.* (2015) described the detection of Quinoline yellow using a novel poly (diallyldimethylammonium chloride) functionalised reduced graphene oxide (PDDA-RGO) nanocomposite modified electrode. The proposed sensor exhibited excellent electrochemical performance towards detection of quinoline yellow, with a linear range from 0.01–10 μ M and a detection limit of 2 nM. It was successfully applied in the determination of quinoline yellow in soft drinks.

Xu *et al.* (2015) used Au NPs, synthesised using hexadecyl trimethyl ammonium bromide (CTAB) as the bifunctional ligand to link the solid substrate and the Au NPs, to detect chrysoidin. A relationship between the logarithm of the concentrations and the intensity of laser was found, and the calibration curves showed good linearity in the range of 0.001–0.5 mM. The method was successfully applied to the determination of chrysoidin in yuba.

Synthetic phenolic antioxidants (e.g. propyl gallate, tertiary butyl hydroquinone, butylated hydroxyanisole; octyl gallate) have been widely used by the food industry, since they can improve food stability and may prolong food shelf-life (Cui *et al.*, 2015; Yue *et al.*, 2015; Sivasankaran *et al.*, 2016). However, monitoring synthetic phenolic

antioxidants in foods is very important, due to their being potentially harmful to human beings. Indeed, although they are powerful in protecting product quality in food distribution, excess antioxidants added to food might produce toxicities or mutagenicities, and may endanger the people's health (Cui *et al.*, 2015; Yue *et al.*, 2015; Sivasankaran *et al.*, 2016).

Cui *et al.* (2015) fabricated a novel molecularly imprinted electrochemical sensor for detecting the artificial food additive propyl gallate. The sensor determination was developed via electropolymerisation of an o-phenylenediamine membrane, in the presence of template molecules on glassy carbon electrode surface, modified by Pt-Au bimetallic NPs-capped graphene-CNTs composites (PtAu-GrCNTs). The results revealed that the sensor displayed resistance to non-specific binding, a low detection limit (25.1 nM) and a wide linear range (7×10^{-8} to 1×10^{-5} M). The MIPs sensor enabled the detection of propyl gallate in food samples (vegetable oils).

Wang *et al.* (2015b) used Au NPs and reduced graphene oxide (graphene) composites, synthesised via a simple one-pot approach, in the presence of polyvinylpyrrolidone (PVP) as a sensing device to detect butylated hydroxyanisole. The sensor, based on a Au-PVP-graphene modified glassy carbon electrode (GCE), displayed highly sensitive electrochemical responses towards butylated hydroxyanisole, in a concentration range of 0.2–100.0 μM , with a detection limit of 0.04 μM . The performance of the Au-PVP-graphene/GCE was examined by evaluating butylated hydroxyanisole contents in soybean oil and flour samples. The satisfactory results showed that the proposed approach was a sensitive and simple tool for determining butylated hydroxyanisole in complex food samples.

Yue *et al.* (2015) developed an *in situ* surface electrochemical co-reduction method to greenly and cost-effectively construct a Au NPs/electrochemical reduced graphene oxide (Au NPs/ERGO) binary nanocomposites-based electrochemical sensing platform, by using a mixture of graphene oxide (GO) and HAuCl_4 coating on the surface of electrode as a precursor for butylated hydroxyanisole and tertiary butyl hydroquinone. The sensing platform allowed the simultaneous determination of the two synthetic antioxidants in wide linear ranges (0.1–10 $\mu\text{g mL}^{-1}$ and 0.1–7 $\mu\text{g mL}^{-1}$ for butylated hydroxyanisole and tertiary butyl hydroquinone, respectively), and with low detection limits (0.0419 $\mu\text{g mL}^{-1}$ and 0.0503 $\mu\text{g mL}^{-1}$ for butylated hydroxyanisole and tertiary butyl hydroquinone, respectively). The practicability of this electrochemical sensing platform for the simultaneous detection of butylated hydroxyanisole and tertiary butyl hydroquinone was demonstrated using edible oil samples.

Recently, Sivasankaran *et al.* (2016) proposed a new sensor device for detecting octyl gallate, an ester of gallic acid and n-octanol. The approach used a glassy carbon electrode (GCE), chemically modified with Au NPs, followed by a self-assembled monolayer of dodecane thiol (DDT), which was employed for the determination of octyl gallate. Linear calibrations could be established within the concentration range of 1.2–0.2 μM , and it achieved a detection limit of 8.3 nM. Furthermore, the applicability of the method was demonstrated by analyzing octyl gallate in margarine, butter and sunflower oil.

Among food additives, artificial sweeteners are also greatly used by the food industry. Neotame (NTM) is a high-intensity artificial sweetener (near 40 and 10000 times sweeter than aspartame and sugar, respectively), but it is not yet commonly used, being considered a second-generation sweetener. An Acceptable Daily Intake (ADI) has been set at 0–2 mg/kg body weight by the Joint Expert Committee for Food Additives (JECFA)

in 2003, as well as by the European Food Safety Association (EFSA) in 2007 (Bathinapatla *et al.*, 2015).

Bathinapatla *et al.* (2015) proposed a highly sensitive and novel electrochemical sensor for detecting neotame, using a modified glassy carbon electrode with copper nanoparticles-ammonium piperidine dithiocarbamate-multiwalled CNTs, assimilated with β -cyclodextrin. The multiwalled CNTs assimilated with β -cyclodextrin/glassy carbon electrode exhibited catalytic activity towards the oxidation of neotame. Two linear concentrations ranges, from 0.03–0.15 mM and 0.2–2.0 mM, were reported, with a detection limit of 0.013 mM. The method was further validated using food samples.

Li *et al.* (2015) developed a NPs-based sensor for detecting azodicarbonamide, which is a yellow to orange crystalline powder used as a food additive (dough conditioner and bleaching agent) in flour products. Some countries (e.g. United States and China) as set a maximum level of 45 mg/kg (ppm) in flour. However, in the European Union, Australia, Singapore and Japan, it has been classified as toxic. These authors reported a simple, rapid, ultrasensitive and inexpensive approach, based on the synthesis of Ag-coated Au NPs (Au-Ag NPs) and a portable Raman spectrometer, for label-free SERS detection of azodicarbonamide in flour and flour product. The detection limits described are below the FDA's tolerance level of 45 ppm in flour (0.1 μ M (11.6 ppb), 10 μ M (1.16 ppm) and 20 μ M (2.32 ppm) for water, flour, steamed bread). Thus, the SERS-based method developed for azodicarbonamide detection offers great practical potential for the effective on-site assessments of food.

Luo *et al.* (2015) described an electroanalytical method for detection of nitrite, using graphene-cobalt hexacyanoferrate nanocomposite, functionalised in the surface of an electrode. Nitrite is widely used as food preservative, despite being known as a carcinogenic substance. The graphene-cobalt hexacyanoferrate nanocomposite-based sensor exhibited a linear concentration range from 1–100 μ M and a detection limit of 0.27 μ M. The applicability of the prepared sensor was examined by determining nitrite concentration in pickled foods.

12.3.8 Nanoparticles based-sensors for detecting metal ions in foods

Exposure to trace levels of contaminants in water and foods for long time periods may lead to undesirable health effects (Majidi *et al.*, 2015). Therefore, the detection of trace amounts of some ions in food samples is of major concern, since they are recognised as potential carcinogenic substances. For example, the bromate ion (BrO_3^-) is a powerful oxidising agent used in flour processing, to enhance maturation and improve dough. It can also be present in fish paste and fermented beverages (Majidi *et al.*, 2015).

Majidi *et al.* (2015) fabricated a novel amperometric sensor for bromate detection and determination, based on palladium nanoparticles supported by graphene oxide nanosheets (PdNPs-GO) modified glassy carbon (GC). The PdNPs-GO/GC electrode had significant electrocatalytic activity toward bromate reduction, a short response time (≈ 15 s) and a wide concentration range (1–10 μ M and 10–1000 μ M), with a good detection limit (0.105 μ M) – though this was slightly greater than the maximum acceptable bromate concentration (0.078 μ M) recommended by World Health Organization (WHO) and the United States Environmental Protection Agency (USEPA). The accuracy of the method was shown using flour and bread samples.

Tao *et al.* (2005) evaluated the analytical performance of three Ag-Au alloy nanoparticles (Ag-AuNPs) based plasmonic sensors for the detection of Hg(II) ions. Ag-AuNPs were synthesised and immobilised on a transparent indium tin oxide (ITO) glass surface, using poly dimethyl diallyl ammonium chloride (PDDA), or by embedding in a poly vinyl alcohol (PVA) film. The plasmonic sensor based on the Ag-AuNPs/PVA/ITO probe was more sensitive, compared with the Ag-Au colloid or Ag-AuNPs/PDDA/ITO probes. The changes in the peak wavelength were linearly proportional to the logarithm of the Hg(II) ion concentration in the range 0.02–100 ppb, with a detection limit of 0.01 ppb. The proposed sensor was successfully applied to the detection of Hg(II) ions in drinking water and food samples.

Zhang *et al.* (2015a) developed a sensitive and cost-effective method for the simultaneous quantification of trace amounts of Hg²⁺ and Pb²⁺ in real samples. The method uses DNA molecular beacon probes bound to *bona fide* digital video discs (DVDs), with the recognition signal enhanced by Au NP-promoted Ag deposition, which leads to quantifiable digital signals. The method exhibits a wide response range and low detection limits for both Hg²⁺ and Pb²⁺ (0.5 nM, 0.1 ppb), somewhat lower than the allowable levels recommended by the United States Environmental Protection Agency. Even so, the detection limit for Hg²⁺ is greater than that reported by Tao *et al.* (2015). In addition, the quantitative simultaneous determination of these two heavy metals in food products (e.g., rice samples) was been demonstrated.

Satnami *et al.* (2016) used surface-modified quantum dots (QDs) as a sensing receptor for Hg²⁺ and Pb²⁺ ion detection by an optical approach. Functionalised water-soluble L-cysteine-capped CdS QDs were used as a fluorescence sensor for Hg²⁺ and Pb²⁺ ions involved in the fluorescence quenching. Detection limits of 1.0 nM (Hg²⁺) and 3.0 nM (Pb²⁺) were reported, which are slightly higher than those previously found by Zhang *et al.* (2015a).

Chaiyo *et al.* (2015) proposed a novel, highly selective and sensitive paper-based colorimetric sensor for trace determination of copper (Cu²⁺) ions. Although copper ions are an essential trace element for life, excessive uptake of Cu²⁺ can cause serious health problems. Taking into account their toxicity, the concentration limit of Cu²⁺ for exposure from foods is in the range of 1.2–4.2 mg copper/day, according to the European Food Safety Authority. Thus, Chaiyo *et al.* (2015) developed a paper-based colorimetric sensor for the simple and rapid determination of Cu²⁺, using the catalytic etching of Ag nanoplates by thiosulfate (S₂O₃²⁻). Under optimised conditions, the detection limit was 1.0 ngmL⁻¹, which could be visually assessed. A semi-quantitative approach, based on image processing, was also established, allowing the detection of Cu²⁺ in the range of 0.5–200 ngmL⁻¹, with a detection limit of 0.3 ngmL⁻¹. The proposed method was applied to detect Cu²⁺ in food samples (e.g. tomato and rice).

12.4 Conclusions and future trends

As discussed, nanoparticles-based sensors are being studied and developed, with their application in the food processing field aimed at both quality and safety assessment, as increasing analytical detection sensitivities is a requirement that must be fulfilled. Indeed, the food sector represents a very significant market in Europe and worldwide, engaging food industries as well as producers, farmers and retailers.

Recently, several food scandals across the world have pointed out the need for new solutions for food quality and safety control along the entire food chain, and this requires novel fast, simple, cost-effective (but accurate and sensitive) analytical tools for in-line, on-line and at-line evaluations. Nanoparticles-based approaches may provide the answers and may contribute to achieving solutions that may cover the quality and safety control needs of the food industry. However, although their potential applicability is high, the 'intrinsic characteristics' of food industries require that costs must be kept low in order for innovations to deliver commercial uptake.

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13

New Technologies for Nanoparticles Detection in Foods

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13.1 Introduction

The past two decades have been characterised by extensive application of nanoparticles (NPs) in industry, food and medicine, as well as in fundamental research and biosensor development. The products containing NPs have been available in the market for several years, and range from clothing to mobile phones and refrigerators, with many having applications in healthcare products. For example, silver NPs (AgNPs) have been widely used as antibacterial products in toothpaste, in medical instruments, and as an antibacterial coatings in refrigerators (Maynard *et al.*, 2006; Ahamed *et al.*, 2010).

In the food industry, NPs such as titanium dioxide, silicon oxide and metallic silver have application as food additives and in packaging (Calzolari *et al.*, 2012). Currently, a wide range of organic NPs, including lipid nanospheres and micelles, are also available in food production materials, food processing and food products (Peters *et al.*, 2011). Nanoparticles have also found increased interest in biomedicine for drug delivery. For example, hydrophobic drugs can be encapsulated into the NPs, aiding their delivery into targeted issues. The surface of NPs can be modified by receptors, such as DNA/RNA aptamers, which provide a unique tool for targeted drug delivery (Maximilien *et al.*, 2016).

Nanomaterials, including nanoparticles, are defined as materials in which at least one dimension is below 100 nm. For NPs, the high surface-to-volume ratio provides unique physical properties as well as reactivity. However, once exposed to the environment, NPs can bind various organic compounds, which make them toxic for living organisms. Therefore, the development of novel methods for detecting the concentration of NPs, their size, aggregation and agglomeration is currently a rather challenging issue.

This chapter reviews the current state of the art in the application of NPs in the food industry, and NP's toxicity, as well as conventional and novel methods for determination and analysis of NPs.

13.2 Nanoparticle properties and applications in food industry

13.2.1 Preparation of nanoparticles

Nanoparticles can be obtained by two methods of preparation. The first of these is top-down, also known as breakdown technique, which consists on reducing bulk materials to smaller particles via grinding, milling, or pulverising. The second strategy is called bottom-up preparation, and produces nanoparticles due to changes in the molecular state of reactants, from atoms to packed clusters, up to hierarchical building blocks (Horikoshi and Serpone, 2013).

Both methods pursue the same objective – to increase the surface area of the material without altering its chemical composition. Top-down is more related to mechanical procedures and physical phase preparation for material separation, while the bottom-up approach deals with chemical reactions at liquid and gaseous phases, in favour of material assembling. The morphology and properties of the nanoparticles will differ, depending on the method of preparation selected. Figure 13.1 summarises the currently available techniques for obtaining nanoparticles and their corresponding categorisation.

13.2.1.1 Top-down strategy

The top-down approach usually demands mechanical and thermal cycles, but produces a broad distribution of particles in the micro and nano scale, typically ranging from

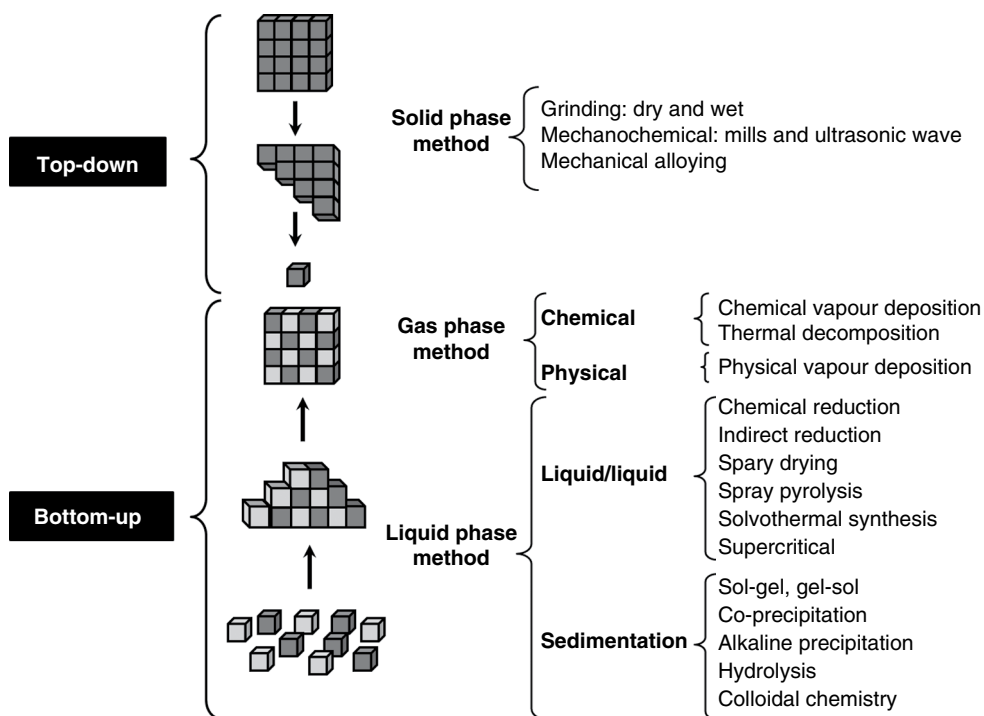


Figure 13.1 Methods of preparation nanoparticles: top-down (larger to smaller) and bottom-up (smaller to larger) approaches.

10–1000 nm (Horikoshi and Serpone, 2013). Cutting techniques, such as electron beam, photo-ion beam or X-ray lithography, as well as etching, grinding, mechanical alloying and ball milling processing, all belong to the top-down category. Certain types of nanocomposites and nano-grained bulk materials are obtained by this method. Besides production of nanoparticles, the top-down approach is used in industry for grinding and milling foodstuff. Some examples include fine-size flour with enhanced water-binding capacity (Degant and Schwechten, 2002), or micro green tea powder with improved antioxidant activity (Shibata, 2002).

13.2.1.2 Bottom-up strategy

The bottom-up method is more concerned with gathering reactants towards the production of nano-sized clusters, either in gas or liquid phases. The gas phase includes both chemical and physical vapour deposition, usually carried out by flame, plasma, laser, electric furnace, induced heating, flame hydrolysis, electron beam, or laser induction. The liquid phase comprises liquid-liquid methods based on chemical reduction, spray drying and pyrolysis, solvothermal and supercritical processes, as well as sedimentation procedures like sol-gel, gel-sol, co-precipitation, alkaline precipitation, hydrolysis, and colloidal chemistry (Horikoshi and Serpone, 2013).

Chemical reduction of metallic ions for producing colloids is a typical example of bottom-up liquid preparation. Colloidal chemistry is rather convenient for the fabrication of several structures such as nanorods, nanowires, nanoprisms, nanoplates and hollow nanoparticles, since the size and shape of nanoparticles can be controlled by varying the reducing or dispersing agent, as well as the temperature and time of reaction (Horikoshi and Serpone, 2013).

Regarding food applications, concepts derived from biology have inspired a bottom-up food nanotechnology. The organisation of casein micelles or starch and the folding of globular proteins and protein aggregates are examples of self-assembly structures that create stable entities (Dickinson and van Vliet, 2003; Sozer and Kokini, 2009). Multiphase materials such as nanocomposites, with one of the phases containing nanoscale additives, are also a result of bottom-up synthesis (Jeon and Baek, 2010).

In the last few years, following the bottom-up liquid principle, an increasing interest in the 'green' production of nanoparticles has arisen (Kharissova *et al.*, 2013). This alternative method avoids the utilisation of toxic chemicals, non-polar solvents, synthetic additives or capping agents in the synthesis of metallic nanoparticles. Stabilisation, antimicrobial and antifungal properties have been associated with phyto-synthesised nanoparticles, presumably due to the high amounts of phytochemicals and phenols contained in the reducing agents.

The chemical reduction of metal ions has been performed using various extracts of edible or non-edible parts of fruits, vegetables, flowers or plants as reducing agents (Akhtar *et al.*, 2013). Gold and silver nanoparticles produced from peels, seeds or juice of citrus fruits (Prathna *et al.*, 2011; Dauthal and Mukhopadhyay 2012; Kahrilas *et al.*, 2013; Sujitha and Kannan, 2013), grapes (Krishnaswamy *et al.*, 2014) or vegetables (Piruthiviraj *et al.*, 2015) have been reported. Extracts of flowers, including rose (Jia *et al.*, 2009; Dubey *et al.*, 2010) and gardenia (Jha and Prasad, 2013) have been also utilised for synthesis of gold, silver or palladium nanoparticles. Likewise, extracts from tea, coffee (Nadagouda and Varma, 2008), and several plants and herbs (Chandran *et al.*, 2006; Ali *et al.*, 2011) have produced nanoparticles of different morphologies and sizes.

Both methods for nanoparticles preparation have correspondent advantages and drawbacks. The bottom-up approach seems to be more efficient in producing nanostructures with fewer defects, more homogenous chemical composition, fewer impurities and better shape tuning. However, complicated chemical procedures and higher production costs seem to be associated with this technique. On the other side, top-down strategy offers the possibility of modifying the shape of material by simple solid phase methods, with an effective increase in the active surface area. Nevertheless, higher levels of impurities and reduced control of the shape in these materials could compromise the quality of nanoparticles.

Therefore, the proper method for preparation must be tactically selected, according the final use and functionality of the nanomaterials. Nanoparticles for food applications (for instance highly soluble bioactive compounds for delivery of nutrients) should be prepared considering a method that facilitates the efficient control of particle size, shape, structure, influences from the surrounding medium, as well as biocompatibility, and any possible toxicity associated with these nanoparticles (Chen *et al.*, 2006).

13.2.2 Properties of nanoparticles

Nano-scale mostly refers to dimensions lower than 100 nm. However, there is still controversy about this concept, especially related to nano-sized materials (European Commission (2006) – REACH legislation). Depending on the material, most of the nanoparticles may form aggregates. This may lead to the misinterpretation that agglomerates of nanoparticles that have external dimensions well beyond 100 nm are not considered nano-sized particles, even though they retain specific properties that are characteristic of nanoparticles. This unresolved issue might carry concerns for practical and industrial applications (SCENIHR report, 2009). In this chapter, several materials composed of two or more types of nanoparticles will be discussed; hence, the terms ‘nanoparticle’ and ‘nanomaterial’ will be used distinctly, but in a similar context.

The properties of a bulk material are usually independent of its size, but only depend on its chemical composition. However, when the material reaches nano-size dimensions, these properties start to change. Principally, there is a high increase in surface area, resulting in a larger contribution of the surface energy with regard to the overall energy of the system. This effect yields to a lower impact of the bulk atoms on the properties of the material (Halim, 2008). Furthermore, nanoparticles that are produced by self-assembly – the first step in the biomimetic approach of the bottom-up strategy – are governed by the slight balance of different non-covalent forces exhibited between molecules, such as electrostatic versus van der Waals forces.

Physical and chemical properties – such as size, shape, structure, specific surface area, aspect ratio, aggregation, size distribution, surface morphology, topography, solubility; or molecular composition, phase identity, charge, tension, reactive sites, photocatalytic properties, zeta potential, hydrophobicity and hydrophilicity of nanoparticles – may be compromised by these effects, and produce nanomaterials with different characteristics. Some of these include improved flexibility, due to enhanced mechanical strengths, better permeability, or durability (Chaudhry *et al.*, 2008; Markarian, 2005). Different melting temperatures, size distributions, optoelectronic

characteristics, and specific magnetisations, are among other particular properties that nanoparticles exhibit, in contrast to those of their bulk materials (Horikoshi and Serpone, 2013).

Nanoparticles might be classified according to diverse parameters, such as dimensionality, functionality or application, methods of preparation, structural configuration, and even source material. However, for purposes of discussing the prospective application of nanoparticles in food industry, they are grouped into organic, inorganic, or combined nanoparticles.

13.2.2.1 Organic nanoparticles

Organic nanoparticles are typically produced by spontaneous or assisted self-assembling of lipids, proteins and polysaccharides. A large number of organic nanoparticles assemble into association colloids and may adopt several configurations, such as surfactant micelles, reverse micelles, lipid vesicles, bilayers, or liquid crystals. Nano-biopolymers and nanoemulsions also belong to this group of nanoparticles. Organic nanoparticles have been extensively studied for their use in the encapsulation and delivery of nutritional and functional compounds.

- **Association colloids** are formed spontaneously, and facilitate the encapsulation and delivery of polar, nonpolar, and/or amphiphilic functional molecules simply by varying the composition and concentration of their constituents (Garti *et al.*, 2005; Weiss *et al.*, 2006). Under specific conditions of the medium, such as ionic strength, pH, radiation, or even osmotic pressure, the electrostatic interactions in the association colloids could be weakened, causing the disruption of shells. Thanks to this effect, an effective release of the encapsulated materials occurs. Colloidal systems are thermodynamically stable, exhibit particular phase transitions, are typically transparent solutions, and have diameters between 100–500 nm, but sometimes reaching up to 1000 nm (Weiss *et al.*, 2006).
- **Nanoemulsions** are a part of multiphase colloidal dispersions that do not form spontaneously, but an external mechanical force must be applied to breakdown larger droplets into smaller ones, typically ranging from 50–1000 nm (Sanguansri and Augustin, 2006). Nanoemulsions comprise two immiscible liquids, one of which is being dispersed in the other, with droplet sizes having flow properties that vary from liquid to highly solid. They also possess stability against sedimentation or creaming (Mason *et al.*, 2006). Nanoemulsions and multi-nanoemulsions composed of a combination of oil and water (O/W) phases offer great potential to encapsulate high concentrations of oil-soluble nutraceuticals or bioactive food supplements into a wide range of foodstuffs (Ezhilarasi *et al.*, 2013; Garti and Benichou, 2004).
- **Nano-biopolymers.** Nano-sized biopolymeric structures may be formed by promoting self-association or aggregation of single biopolymers, or by inducing phase separation in mixed biopolymer systems, by means of aggregative or segregative interactions (Weiss *et al.*, 2006). Natural polymers produced from proteins and polysaccharides like starch and chitosan are another group of organic particles that, at nano-scale dimensions, offer interesting properties, including improved mechanical resistance and antibacterial effects (McGlashan and Halley, 2003; Aider, 2010). The antibacterial mechanism of action seems to involve electrostatic interactions between the biopolymer and the cell membrane, increasing membrane permeability and,

eventually, causing rupture and leakage of intracellular material (Qi *et al.*, 2004). Nanotubes produced from certain proteins also belong to the group of organic biopolymers. Globular proteins of milk like lactoglobulins can self-assemble into nanotube-like structures under appropriate environmental conditions (Graveland-Bikker and de Kruif, 2006). This technique is applicable to other proteins, and has been explored to assist in the immobilisation of enzymes, or to build analogues to muscle-fibre structures (Weiss *et al.*, 2006). There also exist combinations of two or more types of organic nanoparticles, like protein-polysaccharide associates. These may occur chemically, through covalent bonds, or physically, through electrostatic interactions. Some protein-polysaccharide conjugates have excellent emulsifying and steric stabilising properties, especially under conditions where the protein alone is poorly soluble. Charged polysaccharides form soluble complexes or coacervates with proteins, depending on pH, ionic strength, and biopolymer charge distribution. Consequently, these nanoparticles also have high potential for use as delivery vehicles for nutrient encapsulation, and for the protection of adsorbed proteins and emulsified lipids against enzymatic breakdown during digestion (Dickinson, 2008).

- **Nanolaminates** are structures obtained from globular proteins and polysaccharides. They consist of two or more layers of material, with nanometre dimensions, that are physically or chemically bonded to each other (Decher, 2003). Similar to the preparation of multiple emulsions, electrostatic attraction causes polyelectrolytes and other charged substances to be deposited onto oppositely charged surfaces. This technique allows precise control over the thickness and properties of the interfacial films which, in this case, enables the creation of thin films, ranging from 1–100 nm per layer. Nanolaminates can give food scientists some advantages for the preparation of edible coatings and films over conventional technologies and may, thus, have a number of important applications within the food industry (Weiss *et al.*, 2006).

13.2.2.2 Inorganic nanoparticles

Inorganic nanoparticles are generally produced from metals or naturally occurring elements such as silicates, metals, transition metal oxides, and metallic phosphates. At the nano-sized scale, inorganic structures are mostly known as nanoclays, metallic nanoparticles and carbon nanotubes. The currently available methods are able to generate inorganic nanoparticles in a broad range of sizes, shapes and dielectric environments, with numerous functionalities and properties. Inorganic nanoparticles not only exhibit mechanical and thermal stability, but also electronic, magnetic and redox properties related to their size, structure, and chemical nature (Jeon and Baek 2010; Sanchez *et al.*, 2005).

In this type of material, as the particle size decreases, the percentage of molecules located on the surface increases enormously. Van der Waals and electrostatic forces, as well as magnetic attraction, become stronger. Subsequently, and without a proper chemical treatment to reduce the surface energy, it is very common for nanoparticles to aggregate (Jeon and Baek, 2010). To overcome this effect, various surfactants and even biomolecules have been added to nanoparticles as stabilisers or functional components. Attaching biomolecules to nanoparticles that display higher surface-to-volume ratio makes them feasible components in sensing transducers for colorimetric and optical detection of a large variety of targets (Liu *et al.*, 2011; You *et al.*, 2007).

- **Metallic nanoparticles** are capable to interact with other biomolecules, and thus alter their properties. Silver nanoparticles, for instance, exhibit a strong toxicity to various microorganisms, including bacteria and fungi, and show high temperature stability and low volatility (De Azeredo, 2009; Duncan, 2011). A common consensus about the antimicrobial mechanism of metallic nanoparticles has not yet been proposed, but the more accepted hypothesis is that some ions detach from the surface of nanoparticles, then adhere to cell membrane. Consequently, they cause changes in the membrane permeability, which provokes cellular and DNA damaging in the microorganisms (Sondi and Salopek-Sondi, 2004; Hajipour *et al.*, 2012). Antibacterial and disinfection outcomes associated to nanoparticles have been also reported for titanium dioxide and zinc oxide nanoparticles, in this case thanks to the enhanced photocatalytic activity and UV-blocking effects exhibited by the nanoparticles (Ge *et al.*, 2012; Martirosyan and Schneider 2014). Compared with the bulk material, titanium dioxide nanoparticles show signs of higher surface-to-volume ratio and provide an increased surface area at which photo-induced reactions may occur. This results in an enhanced light absorption rate, that yields higher surface photoactivity. At the same time, the higher surface-volume ratio of these nanoparticles enable better surface absorption of OH^- and H_2O , increasing the photocatalytic reaction rate (Lan *et al.*, 2013).
- **Nanoclays** are nano-structured minerals with a large aspect ratio, obtained from natural occurring silicates. This particularity makes nanoclays display specific mechanical and thermal characteristics, such as high barrier properties against the diffusion of gaseous and liquid materials. Various studies have informed about the effectiveness of nanoclays in reducing permeability to oxygen (Bharadwaj *et al.*, 2002; Adame and Beall, 2009) and water vapour (Cruz *et al.*, 2013 and references therein). Consequently, nanoclays have been employed in producing polymeric nanocomposites for food packaging, where high barrier properties, along with enhanced mechanical performance, are greatly required.
- **Nano-fillers and carbon nanotubes.** Other types of inorganic nanoparticles include nano-fillers and carbon nanotubes. Nano-fillers, commonly obtained from carbon, graphene, graphene oxide and silicates, have been added to composites in order to improve the properties of the mixed material and reduce its consumption. A uniform dispersion of nano-fillers leads to a very large matrix-interfacial area that changes the molecular mobility, relaxation behaviour, and the thermal and mechanical properties of the material. Nano-fillers with higher surface and aspect ratios are particularly interesting because of their reinforcing effects, optimal mechanical strength, flexibility, lightness, and even the possibility to be edible when made from food hydrocolloids (Dalmás *et al.*, 2007; Rojas *et al.*, 2009).

Carbon nanotubes – allotropes of carbon with a cylindrical structure – can be also incorporated into polymer structures like liquids, solutions, melts, gels, amorphous or crystalline matrices, in order to increase their mechanical properties in terms of tensile strength and elasticity (Ruoff and Lorents, 1995; Thostenson *et al.*, 2001). However, due to toxicity concerns, and owing to their excellent electrical conductivity, the use of carbon nanotubes in nanocomposites has been oriented mostly towards industrial applications in sensing devices and electronics (Smart *et al.*, 2006; Lau and Hui, 2002; Li *et al.*, 2008).

13.2.2.3 Combined nanoparticles

The mixture of two or more types of nanoparticles produces combined nanomaterials, or nanocomposites. Polymer-based inorganic nanoparticle composites generally display enhanced optical, mechanical, magnetic and optoelectronic properties (Jeon and Baek, 2010). One explicit example is polymer-clay nanocomposites. Since these are impermeable to water, water can only migrate through the polymer matrix following a torturous path. As a consequence, the nanocomposite polymer film has substantially reduced water vapour permeability.

Moreover, the introduction of dispersed clay layers into biopolymer matrices has been shown to greatly improve the overall mechanical strength of the film, making the use of these films industrially practicable (Sorrentino *et al.*, 2007; Avella *et al.*, 2005; Weiss *et al.*, 2006 and references therein). Other advantages exhibited by these nanocomposites include increased glass transition and thermal degradation temperatures, but with a possible decrease in their transparency (Cruz *et al.*, 2013; Petersson and Oksman, 2006; Yu *et al.*, 2003).

There are three types of composites derived from interaction between layered clay and silicates with polymers: phase-separated microcomposite; intercalated nanocomposite; and exfoliated nanocomposites (Cruz *et al.*, 2013). The most studied type of polymer-clay nanocomposite, known as montmorillonite, is composed of a polymer matrix and organophilic clay fillers (Kim *et al.*, 2003; Lee and Kim, 2010). Nanocomposites made by combining metallic and metallic oxide nanoparticles, like titanium dioxide, zinc oxide and silver nanoparticles, exhibit enhanced oxygen sensitivity and antimicrobial properties by effective absorption of UV light without re-emission, thus improving the stability of polymer composites (Reddy *et al.*, 2007; Cheng *et al.*, 2006; Jones *et al.*, 2008; Gordon *et al.*, 2011; Motshekga *et al.*, 2013).

13.2.3 Applications of nanoparticles in food industry

In recent years, nanotechnology has been considered as an attractive alternative for improving the quality and production of food and feed. Food structures are formed on a wide range of length scales, from millimetres down to nanometres. They can be produced naturally or artificially, by mimicking the assembly structure of biomolecules (Leser *et al.*, 2003). Nano-sized structures derived from carbohydrates, fats and proteins, the principal food constituents, have been for a long time naturally generated and metabolised by biological organisms (Chaudhry and Castle, 2011). On the other hand, milk proteins and phospholipids have been used for many decades as emulsifiers and texturising agents in food production.

These facts might persuade us to assume that the gastrointestinal tract is already acclimatised to processing nano-scale products. However, since diverse mechanisms rule the chemical and physical properties of nano-sized particles, concerns about the behaviour of synthetically produced nanomaterials in intrinsically complex biochemical environments are not yet completely elucidated. There are fundamental differences between the self-assembly occurring in living systems and that occurring in synthetic materials. Living systems follow a coded genetically assembly, ruled by consumption and dissipation of energy, which is kept thermodynamically balanced, whereas assembly of food ingredients is a purely thermodynamically driven process (Leser *et al.* 2003).

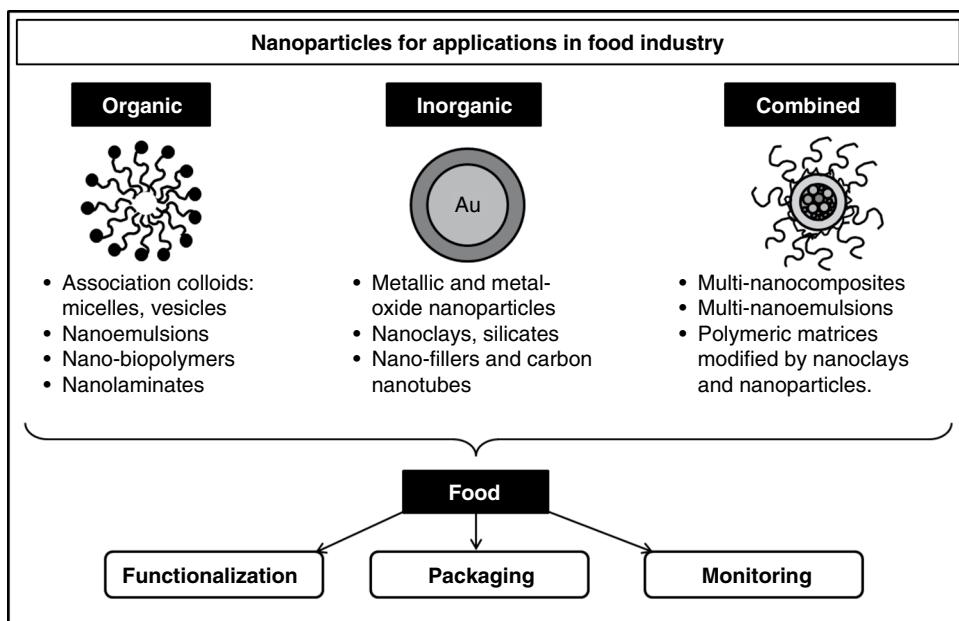


Figure 13.2 Nanomaterials that are currently utilised, and those with potential applications in food industry: organic, inorganic and combined nanoparticles for functionalisation, packaging and monitoring of food products.

Nano-sized materials are governed by molecular heterogeneity. Therefore, the challenge for integration of nanomaterials within the food industry is to understand the physical principles ruling interactions among individual components of particles of different sizes taking place in diverse scales of length and time. This could be the reason why, despite the singular properties of nanoparticles, exploring their use for practical applications in the food industry is somewhat still limited (Leser *et al.* 2003). However, and regardless of these constraints, nano-sized materials have been already applied to the functionalisation of food products. Additionally, considerable efforts have been oriented towards the improvement of the food quality and monitoring food safety issues. Applications in food industry include the use of organic, inorganic or a combination of both types of nanoparticles (Figure 13.2).

13.2.3.1 Food functionalisation

Nanomaterials have been extensively used as nanocarriers in drug delivery systems, with potential biomedical applications (Couvreur *et al.*, 1995). Translating this concept to the food industry, nutritional or functional ingredients that need to be efficiently released at nanoscale to minimise the material consumption, and maximise the cargo uptake, could be incorporated into adequate vehicles to create a modified food product with additional nutritional or practical value.

In principle, nanoencapsulation seems to be a convenient and viable method to enable the transport, protection and controlled release of various nutraceuticals, such as vitamins, enzymes, fatty acids or proteins, and functional ingredients like flavours, colorants, additives, artificial sweeteners, antioxidants, antimicrobials or preservatives. The

nanocarrier which usually exhibits particular physical and chemical properties should not only transport the ingredient but, also, protect it from biochemical degradation and sometimes serve for masking the functional material. The vehicle must be able to release the cargo with sufficient control and efficiency despite the physical, chemical and biological conditions of the medium, and it has to be compatible with the other components of the final food product (Weiss *et al.*, 2006).

Classically, organic associates, based on lipid-protein and lipid-polymer combinations, have been used for encapsulation and delivery of food and functional ingredients of different solubility. The type and resultant structure of encapsulates depends on the concentrations and molecular characteristics of the surfactants used, as well as the prevailing environmental conditions of the surroundings (Weiss *et al.*, 2006). Early efforts for encapsulation techniques did not precisely take into account important facts, such as the biocompatibility and toxicity of the carriers. However, nowadays new and better encapsulation methods, based on nanostructures, are being explored with promising applications. These methods comprise techniques like emulsification, coacervation, inclusion, complexation nanoprecipitation, emulsification-solvent evaporation, spray drying, freeze drying and supercritical fluid for food ingredients (Ezhilarasi *et al.*, 2013).

From a practical point of view, encapsulation has been already utilised to entrap enzymes in cheese production, and to fortify dairy products with vitamins (Peters *et al.*, 2011). Lipid-based capsules are currently developed for making food emulsions such as spreads, margarine and mayonnaise, with the aim of producing edible foods with lower levels of fat (Gibbs *et al.*, 1999). For similar purposes, nanotubes from hydrolyzed milk protein α -lactalbumin have been successfully generated, in order to increase the viscosity of milk while using less protein (Ipsen and Otte, 2007; Graveland-Bikker and de Kruif, 2006). Organic nanoparticles from zein have been used as edible carriers for flavour compounds, or for encapsulation of nutraceuticals (Lawton, 2002).

Additives for food products such as synthetic carotenoid lycopene, benzoic acid, citric acid, ascorbic acid, and supplements like vitamins A, D, K and E, as well as, isoflavones, luteins, phytosterols, omega-3 fatty acids, and coenzyme Q10, are some examples of nanoencapsulated nutrients and additives currently exploited by the food industry (Chaudhry and Castle, 2011). Commercially available food products containing nanoencapsulated biocompounds include canola oil designed to deliver phytosterols (Shemen Industries), selenium enriched green nano-tea powder (Qinhuangdao Taiji Ring Nano-Products), omega 3 enriched bread (Tiptop Bakeries) and chocolate diet shake (RBC Life Sciences), among others (Sekhon, 2014 and references therein).

Nanocarriers and solid lipid nanoparticles, a more general class of two-phase system colloids, have been also investigated as potential vehicles for the delivery of agrochemicals (Frederiksen *et al.*, 2003). Colloidal copper nanoparticles suspended in water have been used since 1931, in a product known as Bouisol, a fungicide in the growing of grapes and fruit trees (Hatschek, 1931). Micro- and nanoencapsulation of agrochemicals and pesticides, consisting of a combination of organic and inorganic nanomaterials such as surfactants, polymers, lipids and metal nanoparticles, are rising as a promising response to the lack of water-solubility in the development of crop-protecting agents and pesticides (Magdassi *et al.*, 2008). A nanopesticide, based on garlic essential oil loaded within polymeric nanoparticles modified by polyethylene glycol (PEG), has been proved to be an effective nanoemulsion against the beetle family *tribolium castaneum*

(Yang, *et al.*, 2009). Synthesis of silver nanoparticles by leaf extract from heartleaf moonseed plant has been reported to have larvicidal effects against malaria vectors (Jayaseelan *et al.*, 2011).

Furthermore, nanoparticles have been studied for their use as efficient fertilisers. Considerably increasing effects in the chlorophyll production of maize and spinach using titanium dioxide nanoparticles have been reported (Morteza *et al.*, 2013; Yang *et al.*, 2006). A study about silicon dioxide and titanium dioxide nanoparticles have demonstrated increased activity of nitrate reductase in soybeans, leading to improvement in plant absorption capacity (Lu *et al.*, 2002). However, most of these finding are still in the research stage, since the incorporation of nanofertilisers and nanopesticides in commercially available products might involve risky exposure of workers and consumers, and could be related to hazardous residues in the foodstuff.

13.2.3.2 Food packaging and quality preservation

The use of nanotechnology in food industry has found its mayor application in food packaging and food quality improvement (Lopez-Rubio *et al.*, 2006). Even though it is a newly emerging technology, it is estimated that up to 400 companies around the world are already developing and expanding possible applications of nanotechnology in food and food packaging (Neethirajan and Jayas, 2011). Nano-sized particles offer the possibility to create functional materials, with stronger packaging barriers, for protecting food during transportation. They might also provide prolonged shelf-life or improved quality to food products and, given that nanoparticles exhibit antimicrobial characteristics, they are also well associated with preserving foodstuffs from pathogens and contaminants (Bumbudsanpharoke and Ko, 2015).

Nowadays, inorganic nanoparticles incorporated into polymeric matrices are the principal raw material for creating nano-engineered packaging films. These materials include silica, silver and zinc nanoparticles, nanoclays, nano-zinc oxide, nano-titanium dioxide and titanium nitride nanoparticles, among others (Mohanty, 2009; Tager, 2014; Bumbudsanpharoke and Ko, 2015). Due to differences in the structure and chemical composition, each nanomaterial introduces distinct properties to the host material, which leads to diverse functional packaging applications (Sorrentino *et al.*, 2007; De Abreu *et al.*, 2007).

The conventional bulk forms of silica and titanium dioxide (SiO_2 (E551), and TiO_2 (E171)) are permitted additives used within food industry to enhance colour, brightness and, sometimes, the flavour of a variety of food products. Even nano-sized structures derived from these additives have been detected in various food products (Peters *et al.*, 2014). However, nanoparticles of zinc oxide, palladium dioxide and titanium dioxide have been widely exploited, with antibacterial, antiodorant and sterilisation purposes (Chaudhry *et al.*, 2010; Li *et al.*, 2010). For instance, titanium dioxide nanoparticles have been successfully applied to disinfect water from faecal coliform bacteria (Chawengkijwanich and Hayata, 2008). The use of nanosized zero valent irons is finding increasing applications in water decontamination (Chaudhry *et al.*, 2010).

The current use of nano-silver as an additive in antibacterial wheat flour is the subject of a patented invention (Park, 2005; Chaudhry and Castle, 2011). Silver nanoparticles and nanosilver polymers are already employed by companies around the world for manufacturing food containers with antimicrobial or antiodor characteristics, some of them even with extended shelf-life product purposes (FresherLonger, USA; Sharper

Image, USA; NewLife Co., Ltd. South Korea; Anson Nano-Biotechnology Ltd., China; Sharper Imagen, USA). Shenzhen Ibicare in China produces milk baby bottles made from nanosilver silicon polymers with antibacterial attributes. On the other hand, silica nanoparticles are suitable candidates for hydrophobic surface coating, and some reports suggest its use in clarifying beers and wines and also as a free flowing agent in powdered soups. Hydrophobic nano-coatings are also a promising tool for self-cleaning surfaces (Chaudhry and Castle, 2011).

However, the commercialisation and use of synthetically produced nanomaterials is still controversial, either due to a lack of studies, or accordance about the possible side effects on public health. In response to these concerns, several biopolymers have been exploited to develop biodegradable nanocomposites for food packaging (Silvestre *et al.*, 2011). Chitosan-based nanocomposites have been prepared in combination with different types of nanoparticles for production of films exhibiting strong antibacterial activity (Rhim *et al.*, 2006; Vimala *et al.*, 2010; Espitia *et al.*, 2012; Kolarova *et al.* 2015). Organic nanocomposites from zein have been produced for improving the strength of plastic and bioactive food packages (Shi *et al.*, 2009). Nevertheless, their use is somehow, restrained due to the poor mechanical and barrier properties of biopolymers, which may be improved by adding reinforcing compounds like fillers and nanoclays.

Nanocomposites associated to clay-polymer combinations are currently used as oxygen barrier layers in the extrusion manufacturing of bottles for fruit juices, dairy foods, beer and carbonated drinks, plastic food storages, candies and chocolates trays, or multilayer films to enhance the shelf life of a variety of foods such as processed meats, cheese, cereals, and boil-in-bag foods (Brody, 2008; De Azeredo, 2009; Bumbudsanpharoke and Ko, 2015). On the other side, synthetic polymer nanocomposites are mainly employed to improve the mechanical and barrier properties of plastic packaging (Arora and Padua, 2010). Plastic polymers composites such as nano-titanium nitride, an approved food contact material by European Food Safety Authority (EFSA), is widely used for mechanical strength and processing assistance, particularly for polyethylene terephthalate (PET), the most common thermoplastic polymer resin of the polyester family used in fibres for clothing and in containers for liquids and foods (Chaudhry and Castle, 2011).

Another practical application of combined nanocomposites related to the prolongation of food shelf life includes the use of nanolaminates to develop edible coatings and films (Park, 1999). Regularly obtained from biomolecules incorporated into polymer matrices, these films could serve as efficient moisture and carbon dioxide barriers. Edible coatings and films are currently used on a wide variety of foods, including fruits, vegetables, meats, chocolate, candies, bakery products, and French fries (Rhim, 2004 and references therein).

Other biocompatible alternatives have been recently developed for food packaging. An edible and biodegradable nanoemulsion-composite film, created with extracts of cinnamon and papaya, has been produced not only with antimicrobial packaging purposes, but also to improve the appearance and provide some texture or flavour to the food product (Otoni *et al.*, 2014a). Edible films from essential oil-loaded nanoemulsions with strong antimicrobial effects against *Escherichia coli*, prepared from thyme, lemon-grass and sage oils in alginate solution, have been also reported. Thyme-based films exhibited the strongest antimicrobial effect, and seemed to improve the water dispersion, protecting the essential oils from degradation (Acevedo-Fani *et al.*, 2015).

Edible films from methylcellulose and nanoemulsions of clove bud and oregano essential oils have been developed as shelf life extenders for sliced bread. The essential oil nanoemulsions remarkably increased the extensibility of the methylcellulose film (Otoni *et al.*, 2014b). Herbs such as ginseng, rosemary, green tea, murta, ginkgo leaf and mint have been also utilised as valuable antioxidant and antimicrobial agents in the production of edible nanoemulsions-loaded films, due to their high polyphenol content. The herbs were integrated into polymeric matrices like methylcellulose, fish skin gelatine, cassava starch with glycerol, and chitosan, among others. The effects of nanoemulsions in food packaging varied from highly antioxidant or antimicrobial, up to prevention of lipid oxidation and, in some cases, the films displayed a combination of various effects (Valdes *et al.*, 2015).

13.3 Toxicity of food-related nanoparticles

Nanoparticles (NPs) of natural origin have been present in many foods being consumed without noticeable safety risks for decades (Rogers, 2016). However, in the case of engineered NPs (ENPs), new harmful mechanisms can be introduced, which cause unexpected health problems (Bergin *et al.*, 2013; Singh, 2015). Different physicochemical characteristics of NPs, together with possible changes of these properties in various regions within the bodies of living organisms, can cause different NPs' behaviour. Knowledge about biological fate of NPs after entry into the body, translocation among organs, and intracellular behaviour, is thereby important for understanding and prediction of potential nanotoxicity effects.

13.3.1 Biological fate of ingested nanoparticles

The main physiological ways for NPs to enter the body are inhalation, dermal exposure and ingestion. For NPs present in food, water, toothpastes, health supplements and drug delivery carriers, the most predominant route of exposure is ingestion, followed by gastrointestinal track (GIT) assimilation. The GIT represents a continuous digestive tube that extends from the mouth to the anus, and is subdivided into different segments (oesophagus, stomach, small intestine, large intestine). The GIT is covered by a protective layer of epithelium and mucus (a viscous fluid of highly branched glycoproteins), of variable thickness and composition, formed by specialised GIT cells (e.g. enterocytes, goblet cells, M-cells). After the particulate components have passed the GIT epithelium, they may be transported into the blood or lymphatic systems (Bergin *et al.*, 2013). Through systemic circulation, the transported material can subsequently be distributed to different tissues, metabolised, excreted or stored.

On the cellular level, one of the most important first sites of interaction is the plasmatic membrane (PM). This is a selectively permeable barrier that controls movement of substances in and out of the cells, which is composed of specialised proteins embedded in a lipid bilayer, and is involved in variety of cell-essential processes. Particles can pass through the PM and enter the cell by various mechanisms, including non-endocytic and endocytic pathways (Yun *et al.*, 2013). Endocytosis is a cell penetration mechanism characterised by passive or receptor-mediated binding of molecules to the PM, followed by the formation of a membrane-enclosed vesicle

containing the ingested material. The vesicle is pinched off from the cell membrane and enters the cytosol.

Intracellularly, the cargo's path continues within the endosome and/or lysosome compartments. Here, it can be sorted into the recycling pathway and returned back to the cell surface, via a recycling endosome, or exposed to hydrolytic enzymes and degraded in lysosome (Kou *et al.*, 2013). Non-endocytic pathways skip the membrane-formed vesicle and deliver cargo directly into the cytosol. Depending on the intracellular localisation, NPs can potentially damage organelles or DNA.

Selection of transportation route, and the fate of NPs in general, are highly dependent on physicochemical properties of the NPs, as well as the biological system and type of cells. Importantly, the physicochemical properties of NPs may change in the GIT upon interaction with food, digestive enzymes, intestinal microbiota, and so on. As soon as NPs are suspended in biological fluids, they are affected by various pH environments, ionic strength, presence of proteins and other biomolecules that can affect their initial size and surface properties (McClements, 2013).

The gastric pH varies significantly, not just within its segment areas, but also among species and the stage of ingestion (Bergin *et al.*, 2013). For example, in the fasted state, the gastric pH of the human stomach varies from 1.2–2; in mice, the pH is approximately 4. In the fed state, the pH raise to 5 in humans while, in mice, the value drops to 3. Moving to the small intestine, which is the site of most nutrient digestion and absorption, and more specifically to duodenum, the pH is between 6–7 in humans and 4–5 in rats and mice (Kararli, 1995; McConnell *et al.*, 2008; Merchant *et al.*, 2011).

Another important fact that needs to be taken into the account is protein corona formation. Whether in the GIT or in system circulation, NPs can rapidly adsorb proteins, small molecules and ions (Pino *et al.*, 2014). Consequently, such an adsorbed corona of bioactives will modify the original characteristics of NPs and define new a biological identity and, thus, *in vivo* response.

13.3.2 Toxicity studies of engineered nanoparticles

NPs present in food can be of organic and/or inorganic nature. Organic nanoparticles are mostly lipid-based structures, such as liposomes, micelles, nanoemulsions or solid lipid nanoparticles. Polymer-based NPs are also involved, either as self-content formulations or, more often, in combination with lipid molecules. In general, the lipid-based group of NPs is well-tolerated in living systems, since they are made from physiological components. NPs can also be found in food as naturally occurring substances – for example, casein micelles in milk represent nanocapsules which are engineered by nature, with the purpose of enhanced delivery of micronutrients (Dalglish *et al.*, 2004).

The toxicity of human engineered lipid-polymer based NPs is mainly attributed to substances such as surfactants (emulsifiers) used during product synthesis (Shah, 2015). Chassaing *et al.* (2015) has reported that two commonly used emulsifiers, namely carboxymethylcellulose (Cmc) and polysorbate-80 (P80), can induce low-grade inflammation and obesity/metabolic syndrome in wild-type hosts, and promote robust colitis in mice predisposed to this disorder. Wild-type C57Bl/6 mice and Il102/2, Tlr52/2 mice strains were used. Mice were administered Cmc or P80 via drinking water (1.0% w/v or v/v, respectively) for 12 weeks.

Lu *et al.* (2014) investigated the possibility of P80 to enhance the absorption of di-(2-ethylhexyl) phthalate (DEHP), the most commonly used plasticiser in many polyvinyl chloride (PVC) consumer products, including food and beverage packaging. DEHP is also known as a causative agent for various toxicities (Caldwell, 2012; Lovekamp-Swan *et al.*, 2003). The authors reported that P80, applied in relatively high concentrations in even the most frequently consumed foods, can increase the absorption of DEHP. The mechanism to increase absorption of phthalens was studied, and it was demonstrated that P80 can induce mitochondrial dysfunction in Caco2 cells. P80 reduced mitochondrial membrane potential, diminished the production of adenosine triphosphate and decreased the activity of electron transport chain. In this context, the choice of emulsifier and condition of its use are important, and critical steps that need to be considered in the view of minimising potential adverse effects.

He *et al.* (2011) investigated the prospective of proteins, namely soybean protein isolate (SPI), whey protein isolate (WPI), β -lactoglobulin (Lg), as safer stabilisers for nanoemulsions (NEs). SPI is a plant protein obtained from soybean, and WPI is derived from milk, in which β -Lg and α -lactalbumin are the most abundant constituents. They also employed egg phosphatidylcholine (EPC), and all were compared with traditional emulsifiers, such as Cremaphor EL/RH40, Tween 80, SolutolHS15 and Poloxamer 188. Prepared NEs were characterised by means of size and zeta potential, and tested for cytotoxicity in Caco2 cells (MTT assay). The size was in the range of 150–250 nm and zeta potential below -30 mV, absolute values significantly greater than NEs stabilised by traditional emulsifiers, reflecting better prevention against self-aggregation. At low concentrations of emulsifier (0.5 mg/ml), no cytotoxicity of the protein-, EPC-, and Poloxamer-188-stabilised nanoemulsions was observed after a four-hour incubation, compared with the negative control. When the emulsifier concentration was increased to 2 mg/ml, the viability of cells treated with EPC and protein-stabilised NEs remained greater than 95% relative to the negative control, whereas the viability of cells treated with surfactant-stabilised NEs decreased to a larger extent. No concentration-dependent cytotoxicity of β -Lg-stabilised NEs was observed. Good biocompatibility of β -Lg-, SPI-, and WPI-stabilised NEs was indicated, which was likely due to the protective effect of proteins on the cells.

Yu and Huang (2013) studied interactions of WPI and Tween-20 stabilised NEs with Caco2 and HepG2 cell lines that mimic small intestinal epithelium and the liver hepatocytes, respectively. Tween-20 is a polyoxyethylene derivative of sorbitan monolaurate, which is also known as Polysorbate 20 (P20). As well as NEs (168–173 nm), micron-sized emulsions (MEs, 5–9 μ m) were also involved into the study and served as the control, since it is generally accepted that regular (not nanometre-sized) emulsions are safe. Compared with MEs, NEs did not reveal apparent toxicity on Caco2 cells, suggesting that NEs may not bear significant toxicity to the small intestine (LDH leakage test, TEER-transepithelial electrical resistance). When looking at the hepatotoxicity (MTT assay), it was revealed that NEs made with WPI caused less cell proliferation/viability than did the corresponding MEs. On the other hand, P20-NEs did not affect the viability of Caco2 cells more than their ME counterparts. The authors hypothesised that WPI-NEs, modified by biological molecules, may interact with cell-membrane specific receptors, while P20-NEs, modified by a synthetic molecule that contains multiple polyethyleneglycol (PEG) moieties, may prevent/decrease the interactions with cells.

In general, modification of NPs by PEG-like molecules is a widely used strategy when engineering NPs. PEG is a coiled polymer of repeating ethylene ether units, possessing unique physical properties such as high solubility both in water and organic solvents. In addition, it can modify mucus-penetrating properties (Xu *et al.*, 2015), particle aggregation (Shibata *et al.*, 2012) and cytotoxicity (Yu *et al.*, 2012). One noteworthy critical factor able to modulate distribution and non-specific cellular uptake of pegylated NPs is the PEG grafting density (Hak *et al.*, 2015).

A variety of material and surface functionalisation strategies can be used to formulate NPs (Singh, 2015; Ojer *et al.*, 2015). Within the group of inorganic nanomaterials, metal and metal oxide-based NPs, such as silver (AgNPs), gold (AuNPs), titanium dioxide (TiO₂NPs) and others are the most recognised and discussed in terms of potential toxicity when orally administrated in food related products (Bergin *et al.*, 2013).

Hadrup *et al.* (2014) reviewed that ingested AgNPs appear to be distributed to all organs being investigated, with the intestinal tract and liver seeming to be the main targets. Weight loss, decreased activity, altered neurotransmitter levels, impaired activity of liver enzymes, cardiac enlargement and immunological harmful effects in dose dependent manner were reported, according to various animal studies. Argyria (a bluish-grey pigmentation of skin and connective tissue) was associated with ingestion of silver in humans as in over-the-counter dietary Ag supplements (detailed characteristics of ingested Ag were, in this case, unavailable) (Bowden *et al.*, 2011). It is still not clear to what extent the contribution of intact AgNPs, or silver ions issuing from NPs, plays a role in the observed detrimental health effects (Gaillet *et al.*, 2015; van der Zande *et al.*, 2012).

Walczak *et al.* (2012) studied the behaviour of 60 nm-sized AgNPs and silver ions (AgNO₃), using an *in vitro* 'fed' gastrointestinal digestion model consisting of subsequent incubation in oral, gastric and intestinal conditions. Samples were analyzed using single particle inductively coupled plasma mass spectrometry (SP-ICPMS), dynamic light scattering (DLS), and energy-dispersive X-ray spectroscopy (SEM-EDX). The authors concluded that tested AgNPs, under physiological conditions, can reach the intestinal wall in their initial size and composition. Importantly, intestinal digestion of AgNO₃ in presence of proteins resulted in particle formation (20–30 nm) composed of silver, sulphur and chlorine. Loeschner *et al.* (2011) performed an *in vivo* study focused on the distribution of 14 nm (90% of volume, DLS) polyvinylpyrrolidone (PVP) stabilised AgNPs and silver acetate (AgAc) in rats, following 28 days of repeated oral exposure. Both tested forms of silver formed sulfur- and selenium-containing granules of similar size in the same range as the administrated AgNP, and were found in lysosomes of macrophages.

Sabella *et al.* (2014) proposed a general explanation for the toxicity of metal-containing NPs. The time-dependent ion release of a variety of metallic NPs was assessed by inductively coupled plasma atomic emission spectroscopy (ICP-AES) under two separate conditions, mimicking both lysosomal environment (37°C, pH 4.5) and cellular cytoplasmic environment (37°C, neutral pH). A significant ion release for all tested NPs was observed in the acidic conditions, with no measurable release in neutral conditions. According to obtained information, the authors predicted a model named as the 'lysosome enhanced Trojan horse effect' (LETHE), since lysosome, as a protective cellular machinery designed to degrade foreign objects, seemed to be actually responsible for

the cascading events associated with NP-induced intracellular toxicity, due to release of relatively toxic ions.

Altogether, many potential applications of nanotechnology have been identified within the food and pharmaceuticals industry but concerns are rising about the potential toxicity of ingested engineered NPs. It is the nano-scale that stands behind both technological progress and possible health risks. Other than the size, properties including chemical composition, coating, surface charge and aggregation tendency are factors responsible for NPs reactivity that in some ways can lead to harmful effects in living systems.

Generalisation of potential toxicity of engineered NPs, having a high diversity of physico-chemical properties, including environmental factors (e.g. pH, protein corona) is difficult, and requires an approach at the individual level. Nevertheless, according to *in vitro* and *in vivo* studies, organic NPs (lipid-biopolymer based) seem to be less reactive than inorganic particles. Care should be taken regarding components used during nanoparticle fabrication, such as emulsifiers, with the aim of avoiding/minimising their potential adverse effects. Moreover, in the case of metal-based nanoparticles, and in respect to the proposed LETHE model, release of metal ions from NPs should be also monitored, since the significant amount of intracellularly leaked ions may then exert ion-specific toxicity (e.g., enzyme depletion/inactivation, protein denaturation, etc.) against some cellular targets (e.g., mitochondria) and/or lysosomal damage/dysfunction. This finally results in increased reactive oxygen level, membrane damage, DNA damage, and apoptosis.

13.4 Methods of nanoparticle detection in food

The development of effective and sensitive methods of detection nanoparticles (NPs) in food is a rather urgent issue, due to increased application of nanomaterials in food industry, but also due to the possible hazard of food contamination by nanomaterials from the environment, caused by extensive industrial production. There are several problems that accompany the detection of nanomaterials in food. First, it is difficult to distinguish the engineering nanomaterials (ENM) from naturally occurring nanoparticles present in food, such as micelles of casein in milk, proteins, protein aggregates, or fatty acids and fatty acids micelles and aggregates. Second, the concentration of nanoparticles in food is as a rule rather small and below the sensitivity of analytical methods, so that pre-concentration is required. Third, the matrix effect can substantially affect the detection of nanomaterials.

Currently, there are several conventional analytical methods that have been applied for detection nanomaterials (Figure 13.3). This analysis involves in general several steps:

- 1) *Pre-treatment* is necessary, due to the complexity of food, and is performed by filtration or centrifugation.
- 2) *Separation and size fractionation* is necessary to obtain a more homogeneous sample. This is performed by various methods, such as field-flow fractionation (FFF) (von der Kammer *et al.*, 2011), size-exclusion chromatography (SEC) (Pérez *et al.*, 2009) and capillary electrophoresis (CE) (López-Lorente *et al.*, 2011).
- 3) *Particle analysis*, in respect of their size, can be performed by light scattering (LS) methods, especially using dynamic light scattering (DLS) (Brar and Verma, 2011)

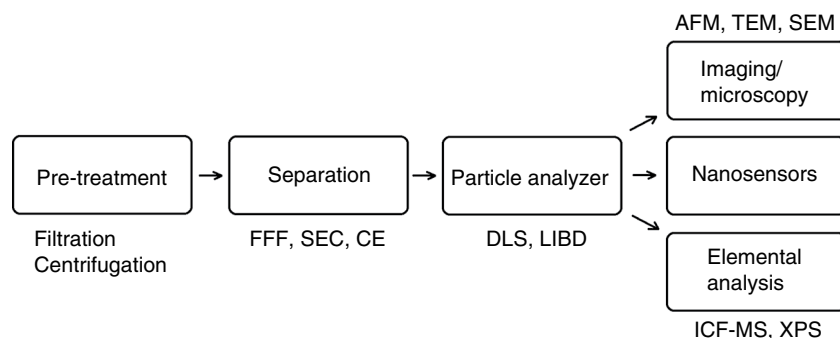


Figure 13.3 Scheme of the preparation, separation and analysis of ENMs in food samples. Partially adopted from Weinberg *et al.* (2011).

and laser-induced breakdown detection (LIBD). The latter is a high-sensitive method for the non-invasive determination of the concentration and mean diameter of NPs in liquid (Kaegi *et al.*, 2008).

- 4) *Further detailed analysis* can be performed by imaging and microscopy methods, such as atomic force microscopy (AFM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (Dudkiewicz *et al.* 2011).

These methods characterise the size and shape of nanomaterials. Elemental analysis can be performed by inductively coupled plasma mass spectrometry (ICP-MS) (Pérez *et al.*, 2009) and X-ray photoelectron spectrometry (XPS) (Tiede *et al.*, 2008). Most of the above methods are rather expensive and time-consuming. Therefore, the current trend is for the development of nanosensors that could replace these techniques. The state of the art in this area is still in its relative infancy.

In the next part, we briefly explain currently used method of nanoparticle detection and current trends toward development of nanosensors (Cheng and Compton, 2014).

13.4.1 Direct visualisations of nanomaterials

Direct visualisation of nanoparticles is one of the most relevant methods currently being used for food characterisation. Among these, transmission electron microscopy (TEM), scanning electron microscopy (SEM) and atomic force microscopy (AFM) are the most well known, as these methods have sufficient resolution to allow detection of nano objects (>0.1 nm in the case of AFM, and > 1 nm in the case of TEM and SEM).

In TEM, a high-energy electron beam (80–300 keV) is transmitted through a very thin layer of the sample. A certain fraction of the beam is scattered from the sample, while some electrons can be transmitted through the sample. The TEM image is formed on the basis of these beam-sample interactions (Dudkiewicz *et al.*, 2011). The resolution of the most sensitive TSM is about 0.05 nm. Disadvantage of TEM are artefacts that are used in preparation of the samples (for example, drying and staining by heavy metals). This can be overcome by relatively new methods that allow working with hydrated samples. For example, Liu *et al.* (2008) reported techniques for high-resolution imaging of liquid samples of 0.3 nm, with 9 nm oxide windows and 2–5 µm of water.

SEM is based on scanning the sample surface with a low energy beam of electrons (1–80 keV) and detecting the electrons scattered by the sample. This technique is used for imaging of the sample surface, and can provide images with a great depth of field, where the whole area of the micrograph is in focus. In a focused ion beam SEM (FIB-SEM), the ion beam can cut into the material, and a solid specimen can be sectioned for 3D imaging (Denk and Horstmann, 2004). Current SEM instruments can achieve resolution between 1–3 nm. For food sample analysis, cryogenic SEM (cryo-SEM) is rather effective for investigating bulk samples. The samples are kept frozen by a cryo-stage in the microscope, and are viewed under high vacuum.

Cryo-SEM can be a very useful tool for analysis of foreign nano-sized objects in food matrices, as long as their structure can be distinguished from the structure of the food product. The method has been used in characterising NP-based bio carriers (Gomes *et al.*, 2010) and polymer nanocapsules (Li *et al.*, 2009).

AFM is another direct method of sample analysis. Its resolution is 0.1–1 nm, but this depends on the tip used, as well as of the method of the sample analyzing, such as contact or tapping mode. The theory of AFM is well described in the literature (see Lamprecht *et al.*, 2014 and references therein).

The main problem in application of imaging techniques in food analysis is the heterogeneity of the food. For example, the volume of sample used in analysis by imaging methods is in the order of few μl , whereas a typical container of soft drink or yogurt is of the order of 100 ml. Therefore, for heterogeneous samples, as well as for trace concentrations of nanomaterials, it is rather difficult to obtain information on NPs contamination.

Electron microscopy methods in food analysis have been recently reviewed by Dudkiewicz *et al.* (2011), and nanoparticle imaging by AFM has been reviewed by Lamprecht *et al.* (2014). However, imaging of food samples containing ENMs is still at the development stage. None of the methods mentioned above is complete and reliable. Therefore, further research and evaluation is required.

13.4.2 Measurement of nanoparticles by light-scattering methods

An important property of NPs is their size, surface charge and size distribution. This can be measured by light-scattering (LS) methods. In particular, the particle-size distribution is of fundamental importance in controlling properties of various nanomaterials. Among LS methods, the classical static light scattering (SLS), dynamic light scattering (DLS) and laser Doppler velocimetry (LDV) are most useful for characterisation of the molecular weight, size, size-distribution and Zeta potential of NPs, respectively.

SLS, also known as Rayleigh or multi-angle light scattering, allows direct determination of particle size, and can be used for measurement of molecular mass. This is especially important for studying protein suspensions. SLS can also be used for determination of NPs aggregates, as well as for stoichiometry of complexes between different particles, such as colloidal, larger particle complexes, or particle-organic matter complexes. In a typical SLS experiment, the average intensity of the sample that is corrected for the scattering of the solvent is determined. Using the Zimm Equation, the molecular mass of NPs, including proteins, can be determined (see Brar and Verma, 2011).

DLS, also known as Photon Correlation Spectroscopy (PCS), measures Brownian motion and relates this to the size of the particles. Brownian motion is the random

movement of particles due to the collisions with the solvent molecules that surround them. The larger the particle or molecule, the slower the Brownian motion will be. Smaller particles are moved more rapidly. The temperature during measurements has to be stable, and its value should be known for determining the sample viscosity, which is required for correct analysis of the particle size. The solution containing particles is illuminated with a beam of monochromatic light, directed through the sample. The Brownian motion of the particles or molecules in suspension causes laser light to be scattered at different intensities. The time-dependent fluctuations in intensity of scattered light is then analyzed, and yields the velocity of the Brownian motion, called translational diffusion coefficient, D . This coefficient is then converted into the hydrodynamic diameter D_H , using the Stokes-Einstein equation:

$$D_H = kT / (3\pi\eta D) \quad (1)$$

where k is Boltzmann's constant, T is absolute temperature and η is the viscosity.

The diameter that is obtained by this technique corresponds to the diameter of a sphere that has the same translational diffusion coefficient as the particle. The D value will depend not only on the size of the particle, but also on its structure, concentration, and the types of ions in the solution. The ions in the medium, and their concentration, can affect the particle diffusion speed by changing the thickness of the electric double layer – the Debye length. Thus, a low conductivity medium will produce an extended double layer of ions around the particle, reducing the diffusion speed and resulting in a larger apparent hydrodynamic diameter. In contrast, a solution of higher conductivity will suppress the electrical double layer, reducing the measured hydrodynamic diameter.

A typical DLS system comprises a HeNe laser to illuminate the sample particles within the cell. Detectors measure the intensity of the scattered light, and can be arranged in the position of 173° or 90° . In order to prevent the detector from being overloaded, an attenuator is used to reduce intensity of the laser, hence measuring the intensity of scattered light within a specific range for detector. From the detector, the scattering intensity signal then passes to a digital signal processing board, called a correlator. The correlator compares the scattering intensity at successive time intervals, to derive the rate at which the intensity is varying. The correlator information then passes to the computers, where special algorithms are used to extract the decay rates for a number of size classes to produce a size distribution (Figure 13.4).

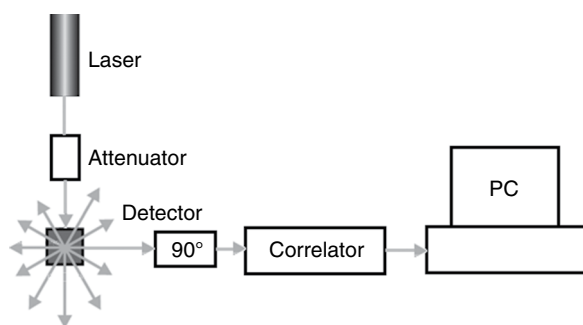


Figure 13.4 The scheme of DLS experiment in 90° optical arrangement.

The LDV method allows measurement of the Zeta potential, ζ , which is an important value characterising NPs properties. ζ is the electric potential of the solid-liquid interface between the particle surface and the surrounding fluid. This interface is also referred as hydrodynamic shear or slipping plane. Figure 13.5a shows the presence of an electrical double layer that is formed by ions around the particle. The inner region of this double layer is called the Stern layer, where the ions are strongly bound to the particles. The outer region forms a diffuse layer, with the ions less firmly attached. Within this diffuse region, a boundary, inside which the ions and particle form a stable entity, can be recognised. When the particle moves, the ions within this boundary follow the particle's movement, whereas ions beyond the boundary do not travel with particle. This boundary is defined as a slipping plane. When an electric field E is applied across an electrolyte with suspended charged particles, they start to move towards the electrode of opposite charge. The velocity of the particles, expressed in unit field strength as the electrophoretic mobility U_e , can be then used to calculate the zeta potential ζ , according to Henry's equation:

$$U_e = 2\varepsilon\zeta f(\kappa a) / 3\eta \quad (2)$$

where ε is the dielectric constant, η is the viscosity, $f(\kappa a)$ is the Henry's function that equals 1.5 for aqueous solutions with moderate electrolyte concentrations.

The movement of charged particles in an electric field can be measured by LDV that utilises the Doppler effect. For this purpose, a folded capillary cell is used (Figure 13.5b). Light scattered by moving particles experiences a frequency shift, measurable by an optical mixing or interferometric technique. In practice, this can be done by using a pair of mutually coherent laser beams, derived from a single source by means of a beam

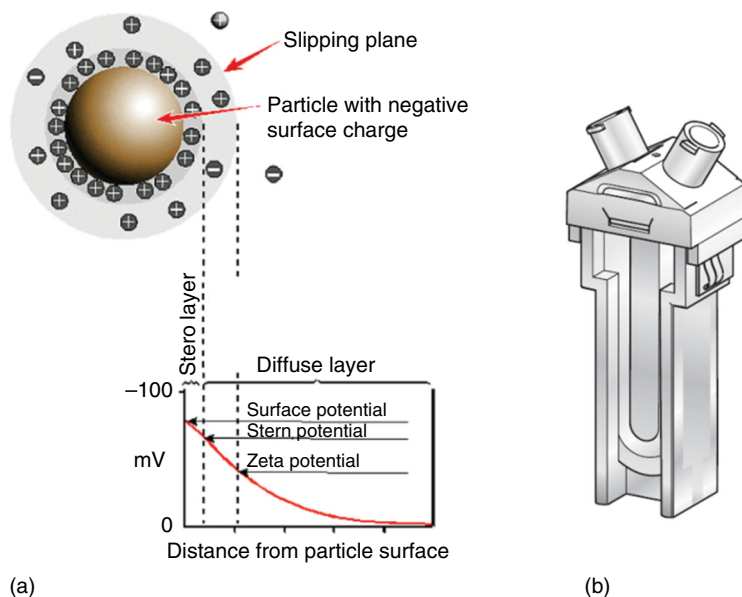


Figure 13.5 (a) Schematic representation of Zeta potential; and (b) folded capillary cell (Malvern). Partially adapted from Kaszuba and Connah (2006).

splitter. The incident beam passes through the centre of the sample cell, and the moving particles scatter the light. This scattered light is then combined with the reference beam, giving rise to a modulated beam having a 'beat' frequency. The Doppler shift is then analyzed by comparing the beat frequency with that of the reference frequency, either by Fourier transformation or the method of phase analysis light scattering (PALS). The characteristic frequencies in the scattered light are extracted by a digital signal processor, and the mobility of particles is determined.

The theory and experimental setups of DLS and LDV method can be found in Kaszuba and Connah (2006). The size, size distribution and ζ potential of NPs can be measured with high precision – for example, by the Zetasizer Nano (Malvern, UK).

The application of the SLS method is limited by transparent, rather diluted solutions. This method has been used for determination particle size in dairy components in a broad interval of 50 nm to 2 mm. For example, SLS has been used for detection of fat globules (Michalski *et al.*, 2001), lactose crystals (Mimouni *et al.*, 2005) and casein micelles (Huppertz and de Kruif, 2007). This also allows analysis of skimmed (Gaucher *et al.*, 2007) or whole milk (Ahmad *et al.*, 2008). The DLS technique can also be used for obtaining information on the structure of casein micelles (Dalglish, 1993). However, the problem remains of whether the structure of micelles change after dilution of milk by a buffer.

The DLS technique has also been used in analysis of water samples, especially waste water polluted by industrially produced NPs. In water, the NPs can change the pH, resulting in dissolution of some metals – for example, aluminium. This can cause toxicity of water and result in the contamination of agriculture products. For example, the size of titanium dioxide (TiO₂) NPs has been determined in water suspensions using the DLS method (Kato *et al.*, 2010). It has been shown that the NP sizes were comparable with values determined using other analytical methods, thus confirming the accuracy of DLS.

The resonance light-scattering (RLS) method has been demonstrated as a effective tool for analysis of NPs in environment (Weiss *et al.*, 2006). RLS is characterised by high sensitivity, ease of performance and simplicity in equipment (typically a common spectrofluorometer). The RLS technique has been widely used in determination of proteins and some metal ions, due to the strongly enhanced RLS signals of the bindings of dye proteins or dye-metal-ion complexes (Cui *et al.*, 2007).

Among new approaches in food analysis is the application of diffusing wave spectroscopy (DWS). This method can operate in a very turbid medium, and can overcome the disadvantages of the conventional SLS method. DWS allows accurate measurement of the coagulation times and gives the possibility of correlating the intensity of the scattered light to the elasticity of the structures formed in the milk gelation (Hemar *et al.*, 2004).

The main drawback of the light-scattering methods is the need to work with a liquid phase. The samples should also be treated before analysis, in order to avoid inhomogeneity and NPs aggregations. Also, it is difficult to distinguish between naturally occurring NPs and engineered NPs. Thus, *in situ* analysis of NPs using this technique is rather complicated, and may result in errors. Preferably, a multi-method approach should be used for proper food analysis (Domingos *et al.*, 2009; Brar and Verma, 2011).

13.4.3 Electrochemical methods in nanoparticle analysis

In situ detection of NPs requires the development of a novel approach, allowing determination of their size, concentration and aggregation/agglomeration in liquid phase without pre-treatment. Recently, 'nano-impact' based electrochemical methods have showed great promise for this purpose (Cheng and Compton, 2014).

So far, the NPs have been used mostly for preparation of electrochemical biosensors for detection food contaminants and toxins (see, for example, Evtugyn *et al.*, 2013). Only recently have the electrochemical methods been applied for detection of nanoparticles in liquids. The new strategy of NP detection using coulometry has been developed in the Compton laboratory. The method is based on oxidation and reduction of individual NPs at the surface of a solid ultramicroelectrode (UME). Random diffusion, due to Brownian motion, brings particles to within electron tunneling distance of an electrode (Rees *et al.*, 2012; Tschulik *et al.* 2014). The charge transfer Q between NPs and electrode surface is measured by integration of the individual current spike, and can be used for calculation of the size of metal NPs, such as silver, gold, nickel (Zhou *et al.*, 2011).

For example, to determine the size of nickel NPs (NiNPs), using a carbon-fibre microelectrode, a known concentration of these NPs is added into the degassed solution of 10 mM HClO_4 and 100 mM NaClO_4 . Chronoamperometry at the electrode potential + 1.7 V (relative to Ag/AgCl) allows measuring oxidative current peaks (Figure 13.6). But no peaks are observed at a lower potential (1.0 V). This has been attributed to Faradaic oxidation of NiNPs. This approach allows the establishment of a relationship between diameter and charge of single NiNPs, assuming the spherical shape of NPs. The relation between the NPs diameter D_{np} and charge transfer, Q , as a result of complete reduction of single NiNPs, has been established as:

$$D_{np} = 2 \left(3A_r Q / 4nF\pi\rho \right)^{1/3} \quad (3)$$

where A_r is the relative atomic mass, ρ the density of NPs and n is number of electrons participated at the oxidation reaction, F is Faraday constant.

The size distribution of NiNPs can be derived by analyzing the individual spikes. The lowest diameter of NPs that can be detected by nanoimpact method is ≈ 6 nm

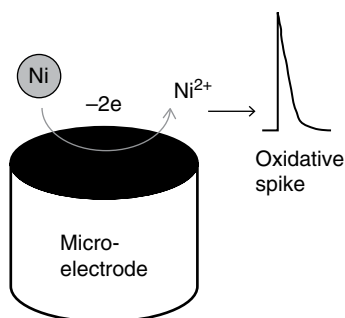


Figure 13.6 The scheme of oxidation of Ni nanoparticles at carbon microelectrode.

(resolution $\approx 1\text{--}2$ nm). However, this method is suitable for detecting even larger particles, of a diameter close to 100 nm (Bartlett *et al.*, 2015).

Recently, it has been shown that the addition of chloride facilitates the oxidation of silver nanoparticles to silver chloride in ionic liquids (1-butyl-3-methylimidazolium tetrafluoroborate), which is observed as spikes in the current that correspond to single nanoparticles (Tanner *et al.*, 2016). The nanoimpact method is able to distinguish the core and the shell of NPs, as has been demonstrated in a paper by Holt *et al.* (2016), where selectivity between gold-core and silver-shell nanoparticles was achieved on the basis of their different redox activities. An excellent agreement with electron microscopy image results has been obtained.

NPs tend to aggregate in solution as well as at the electrode surface. Chronoamperometry can be used to characterise this process. In a paper by Ngamchuea *et al.* (2015), this method has been shown as very effective for detection of NP surface aggregation and semi-quantitative sizing of the formed aggregates, based on the current measured at partially blocked electrodes (PBEs). The aggregate sizes determined were in good agreement with *ex situ* microscopic analysis.

NPs also tend to cluster, either via aggregation (irreversible) or agglomeration (reversible) processes. These processes can be hardly distinguished by means of common techniques such as DLS, TEM, SEM or nanoparticle tracking analysis (NTA), which usually only confirm the presence of large particle clusters. Sokolov *et al.* (2015) developed a novel approach for distinguishing between nanoparticle aggregation and agglomeration, by characterising a colloidal system of Ag NPs using DLS, SEM and NTA methods and the electrochemical nanoimpacts technique. In contrast to the conventional techniques, which indicate the presence of large particle clusters, electrochemical nanoimpacts provide information on individual nanoparticles in the solution phase, and reveal the presence of small nanoparticles (<30 nm), even in high ionic strength (above 0.5 M KCl). This allows more complete analysis.

The detection of small nanoparticles in high ionic strength media evidences that the clustering is a reversible process. It has been concluded that agglomeration, rather than aggregation, takes place. This observation is of general importance for all colloids, as it provides a feasible analysis technique for a wide range of systems. The nanoimpact electrochemical method has been used for determination of NPs concentration, aggregation state and detection of NPs *in situ*, in real liquids such as sea water (Stuart *et al.*, 2012, 2013).

We have already mentioned that there are difficulties in distinguishing between organic and metal nanoparticles using a conventional light scattering method. In recent work by Toh and Compton (2015), 'soft' NPs – micelles formed by cetyltrimethylammonium bromide (CTAB) – have been detected electrochemically via nanoimpacts. Short, sharp bursts of current have been used to indicate the electrical contact of a single CTAB via the oxidation of the bromide content. The variation in CTAB concentration resulted in a significant number of 'spikes' above the CMC (critical micelle concentration), attributed to the formation of micelles.

The electrochemical method is also of high importance for biomedicine. It has been shown that the size of organic NPs plays a crucial role in the drug delivery. The reduction in the size of NPs facilitates their transport through the blood vessels (Chauhan *et al.*, 2012). Recently, direct electrochemical sizing of organic NPs through direct

reduction of individual organic NPs has been demonstrated during random collision events at electrodes (Cheng *et al.*, 2013). In this work, indigo NPs were used as a model system. Under potentiostated conditions (-0.7 to -0.9 V), reductive spikes from individual indigo NPs were observed and quantified. Further application of electrochemical methods for detection NPs can be found in a recent detailed review by Cheng and Compton (2014).

13.4.4 Food monitoring and safety controls

The application of nanotechnology in the food industry might be also addressed to monitoring the processing of foods, controlling the quality or freshness, and tracking individual shipments of foodstuff. Recently, smart nanomaterials like nano-barcodes and nanosensors have emerged as integrated sensing devices for localisation, reporting and remote control of the quality of food products (Weiss *et al.*, 2006).

Regarding food monitoring, radio frequency identification tags (RFT) have been incorporated for continuous monitoring of changes in the quality of foodstuffs via wireless passive antennas on silk adhesive substrates. These sensors incorporate gold nanoparticles coatings deposited on silk for transmitting signals about fruit ripening. The silk nanofilms are easily glued to curved surfaces, and could potentially be eaten once they are applied on the fruits (Tao *et al.*, 2012).

Time temperature indicators (TTI) also appear as valuable tools for food monitoring when food is stored in less than optimal conditions, such as extreme heat or freezing. Some of these sensors are designed to follow the evolution of gases and changes in temperature along the distribution food chain, while others are designed to be used in consumer packages (Kuswandi *et al.*, 2011). TTIs based on enzymatic and colorimetric reactions of incorporated nanomaterials, or changes in the metabolic activity of bacteria, have been already reported (Rani and Abraham, 2006; Galagan and Su, 2008; Vaikousi *et al.*, 2009). Companies like 3M, LifeLines and Vitsab are already commercialising TTIs based on different working principles.

The most commonly used TTIs are thermochromic ink dots that indicate that a product is at the correct serving temperature following refrigeration or microwave heating (Kuswandi *et al.*, 2011). Nanosensors based on conducting polymers, and nanoparticles incorporated into polymer matrices linked to a colorimetric dye, have been reported for monitoring food contamination. For example, silica nano-spheres, immobilised with a fluorescent dye, were used for detecting the presence of bacterial cultures in meat via antigen-antibody interactions (Zhao *et al.*, 2007). Recently, a chemiresistive mechanism for detection of meat spoilage, based on single-walled carbon nanotubes-metalloporphyrin composites, has also been reported (Liu *et al.*, 2015). At the present, colorimetric nanoparticle-based sensors for detecting levels of O_2 , CO_2 , and diverse contaminant agents, including toxins, microbes, bacteria and other pathogens in foodstuffs, are subject of extensive investigations (Kuswandi *et al.*, 2011 and references therein).

Several of these smart nanotechnologies and nanomaterials are already causing a noticeable impact on the food industry. However, there are still several health, environmental, social and economic issues to be addressed before complete implementation and commercialisation of these functional nanomaterials into the food industry.

13.5 Conclusion

Detection of the size, aggregation and agglomeration of nanoparticles in food and in the environment is rather challenging, due to the increased application of NPs in food packing materials and food additives. In addition, extensive industrial application of NPs has resulted in pollution of the environment and groundwaters, from which they can contaminate agriculture products. Nanoparticles, due to their unique physical properties, behave differently in comparison with bulk materials. Their large surface-to-volume ratio and NPs surface charge can result in the association of NPs with organic molecules. This transformation can make NPs toxic for living organisms.

Therefore, the development of effective methods of analysis of NPs is of high importance. Conventional methods of detection, such as TEM, TSM, AFM, mass spectroscopy and light scattering techniques cannot, in most cases, be used for *in situ* analysis of samples, and require either dry samples or transparent diluted solutions. In addition, it is very difficult to distinguish between organic and engineered NPs using these methods. Also, the aggregation and agglomeration, as well as kinetics of formation NPs clusters, is impossible by these methods.

The novel approach based on electrochemical oxidation or reduction of nanoparticles at the surface of microelectrodes is rather promising, and has already demonstrated high effectivity in the detection of size, aggregation and agglomeration of NPs of various origins. The electrochemical 'nano-impact' method can be rather useful for *in situ* applications. We believe that future work in this direction will make substantial impact for food, and environmental security.

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14

Rapid Liquid Chromatographic Techniques for Detection of Key (Bio)chemical Markers

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14.1 Introduction

Modern agricultural practices and novel food processes involve the use of new additives and processing aids, as well as changes in processing, in order to improve productivity and, thus, increase competitiveness and achieve good profitability. These changes may lead to substantial changes in the nutritional value and sensory properties of foods. In addition, the presence of chemical contaminants and residues is receiving increased attention, due to environmental contamination and/or intentional addition of illegal veterinary drugs or antibiotics for animal growth promotion.

Most countries have established official regulations to control the levels of chemical additives, residues or contaminants in foods. Analytical methodologies have been developed for the qualitative and quantitative determination of these chemicals in foods. However, most of these methodologies are expensive, tedious and time-consuming. Due to the large amount of samples to be analysed, high-performance liquid chromatography (HPLC) offers the possibility of relatively rapid analysis for a good number of chemicals. In spite of the rapid development of other rapid immunological techniques, like ELISA or biosensors, HPLC is keeping a good number of applications, due to its versatility, simplicity and economy of use (Bargen *et al.*, 2014).

Furthermore, these applications are being expanded through the continue development of better column packing and equipments (automation, robustness, sensitive detectors, etc.). For instance, increased attention is nowadays given to HPLC coupled to mass spectrometry for official confirmatory purposes (Paseiro-Cerrato *et al.*, 2013). In addition, liquid chromatography can be used by the food industry as a routine technique for controlling processes, raw materials and products. In fact, day by day, large numbers of new and rapid specific applications are being published in the scientific and technical literature.

High-performance liquid chromatography (HPLC) is a technique that has become widely used for the analysis of foods. It is being used for a large number of applications, including the analysis of nutrients, chemical and biochemical contaminants, markers

for processing control, detection of adulterations and control of raw materials and products. HPLC has been traditionally used for almost all these tasks, but the actual challenge is to improve the throughput, for better competition with other techniques that have been appearing lately. The fundamentals of this technique, and a summarized description of these applications, are described in this chapter. As sample preparation is the most tedious and time-consuming step in food analysis, we mainly focus on those methodologies with less sample manipulation before HPLC analysis.

14.2 The fundamentals of liquid chromatography

HPLC constitutes a useful technique for the separation and detection at room temperatures and this is the reason why this technique is specially recommended for the analysis of thermally labile, non-volatile compounds.

Analysis by HPLC requires some sample preparation, more or less complicated, before its injection. The procedure for analyte extraction and cleanup depends on the sample, matrix complexity, column efficiency and detector selectivity. It usually includes extraction, centrifugation, isolation, derivatisation and concentration steps, prior to filtration and injection into the chromatograph.

A typical procedure for sample preparation and analysis is shown in Figure 14.1 and includes a liquid extraction by homogenisation, deproteinisation, centrifuging, clean-up by solid-phase extraction (SPE), or concentration by evaporation, filtration, chromatographic separation and detection. Depending on the mode of separation to be used, an optional derivatisation step, before or after separation, may be required. For example, amino acids need derivatisation to enhance their detection. The method of choice for separating amino acids in their native form should be cation exchange with post-column derivatisation but, if choosing partition (reversed-phase) HPLC, the derivatisation should be pre-column for both, enhancing hydrophobicity and suppressing the charge.

Based on the separation mechanisms, HPLC technique can be classified into four different types: adsorption, ion-exchange, size exclusion and partition HPLC.

14.2.1 Adsorption HPLC

Separation of solutes is based on the differential electrostatic attraction to the stationary phase molecules. Its use has been relegated to sampling and clean-up, more than for real HPLC analysis. For example, flavonoids from plant materials can be cleaned, fractionated, and enriched on alumina.

14.2.2 Ion exchange HPLC

Separation of solutes is based on their charge. Depending on the stationary phase charge, ion-exchange can be anion or cation exchange. Thus, the positively charged stationary phase will separate anions in anion exchange HPLC, while the negatively charged stationary phase will separate cations in cation exchange HPLC. It is the method of choice for charged compounds analysis, and especially for inorganic anions or cations analysis.

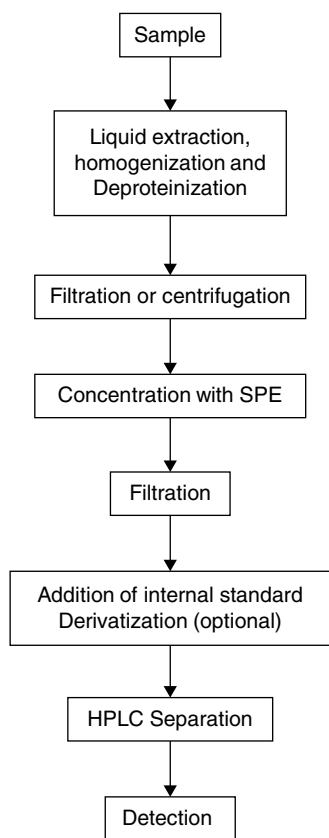


Figure 14.1 Example of typical procedure for sample preparation and HPLC separation.

14.2.3 Size exclusion HPLC

Separation of solutes is based on their size. This technique is mainly used for sample clean-up and fractionation, but it is also the method of choice in the quality control of polymers, including polysaccharides, where the molecular mass distribution is a marker of quality.

14.2.4 Partition HPLC

Separation of solutes is based on their different solubility between two solvents – the stationary and the liquid phase. Depending on the polarity of the phases, partition HPLC can be normal-phase (stationary phase more polar than mobile phase) or reverse-phase (stationary phase less polar than mobile phase). Reverse-phase HPLC is the chromatography most frequently used in food analysis, because of its efficiency and versatility. However, currently, hydrophilic interaction chromatography (HILIC) is making room in the analytical chemistry of polar and/or ionic organic molecules. This technique shares characteristics with adsorption, ionic and reversed phase chromatography, thus constituting a multimodal partition chromatography. Concretely, it is a normal-phase partition chromatography, because the stationary phase is more polar than the mobile phase.

14.3 Advances in modern HPLC

Liquid chromatography offers a wide variety of separation modes and stationary-mobile phase combinations for optimising analysis. Two solutions have been proposed in order to improve the quality control based on HPLC analysis throughput. These solutions differ on columns technology and equipment design.

Since the beginning of HPLC, the development of columns has been focused on the enhancement of sensitivity, efficiency and selectivity. A first proposal to improve sensitivity was the use of narrow-bore or micro-bore columns, which consisted of using columns of internal diameter less than 2.1 mm, instead of standard-bore (≈ 4.6 mm internal diameter) columns. The benefits are higher sensitivity and savings in the amounts of solvents needed, because the flow rate is lower (<0.5 mL/min) than in the case of the classical analytical columns (around 1 mL/min). Requirements from HPLC equipment when using these columns are higher, because the low flow rate used requires more precise and reproducible HPLC pumps, and the void volume, capillary connections and cell detector should be smaller to save the resolution of the peaks. Indeed, new designs for the detector cell, reducing its volume without losing sensitivity, have been developed.

The developments in efficiency enhancement were focused on the use of smaller-sized stationary phase support particles. The limitation in the performance of packed column is well recognized, based on the pressure limit of a solvent delivery system, leading to the current compromise, at about 3–5 μm between the column efficiency and the pressure drop. Thus, the totally porous particle in current normal use for analytical purposes has a 5 μm size, although a 3 μm particle size is also available. The benefits of reducing particle size in chromatography, known as fast-liquid chromatography, are partially neutralized, because the pressure drop necessitates a reduction in column length in order to maintain efficiency.

The appearance of chromatographic columns packed with ≈ 2 μm particle diameter, together with the Ultra Performance Liquid Chromatographic (UPLC) systems, has overcome this analytical limitation. These columns and equipment are designed to support very high pressure, and they achieve very fast analyses with very high resolution, because they are able to separate many peaks in a very short time. The resulting peaks are very narrow if they are housed in adequate HPLC systems, whose extra column volumes are low in order to avoid losses in efficiency. Another important factor is that detectors must be able to receive and process very quickly a high amount of data, to successfully maintain the resolution. An additional and very important advantage is that the consumption of organic solvents is minimal.

Monolithic columns, developed in the 1990s (Petro *et al.*, 1996), have been an interesting alternative to the use of small particle silica columns (Zacharis *et al.*, 2011; Chinnici *et al.*, 2004). This kind of column contains highly porous rods of silica with a bimodal pore structure:

- a macro porous structure that allows rapid flow (up to 9 mL/min) at low pressures with, on average, 2 μm diameter, and a dense network of pores, through which the mobile phase can rapidly flow at low pressure and dramatically reduce separation time; and
- a mesoporous structure, which forms a fine porous structure (130 Å), and creates a very large surface area, on which absorption of the target compounds can occur.

The final result is a lack of intra-particle void volume, which improves mass transfer and separation efficiency, allowing for fast, high-quality separations. The possibility of working at such high flow rates, without efficiency losses and avoiding pressure drop problems, makes run-times and column re-equilibration much shorter, resulting in increased lab throughput. These columns may also be packed with a continuous rigid polymeric rod, with a porous structure made of polystyrene divinylbenzene copolymer. This stationary phase possesses a superior pH stability (1–13), compared with that made of silica.

The combination of any of these columns, monolithic or micro-particle, together with a micro-bore capillary of nano design, in a well-designed HPLC system in which void volume has been reduced to a minimum, is ideally suited for on-line coupling to any mass-spectrometer as detector (Qi *et al.*, 2014).

14.4 Analysis of biochemical markers: applications for nutritional quality

14.4.1 Amino acids

Amino acids constitute important biochemical compounds in cells and organs, for their growth, maintenance and metabolic activity. Their requirements will vary, depending on the stage of life (Aristoy and Toldrá, 2004). The analysis of free amino acids in foods is very important, because some amino acids (known as essential amino acids) can not be synthesized in the organism, and must be supplied in the diet. Furthermore, the analysis of a specific essential amino acid that can be limited is also very important in basic foods designed for populations with specific dietary needs, such as the elderly or infants.

This is even more important in infant formulas, where an accurate compositional data for all the amino acids must be fully assured. Some foods for specific nutritional purposes can be fortified with specific amino acids, and these must be analysed to control the correct amount for each amino acid. In other cases, the control must assure the absence of a specific amino acid (i.e. phenylalanine for phenylketonuria), in diets designed for specific populations with particular metabolic defects. HPLC amino acid analysis has been thoroughly studied, and many methods have been described (Lemieux *et al.*, 1990; Calull *et al.*, 1991; Bartok *et al.*, 1994; Krause *et al.*, 1995; Liu *et al.*, 1995; Diaz *et al.*, 1996; González-Castro *et al.*, 1997; Stancher and Calabrese, 1997; Cohen and De Antonis, 1994; Cohen, 2000; Herbert *et al.*, 2000; Vicente *et al.*, 2001; Fabiani *et al.*, 2002; Aristoy and Toldrá, 2013, 2016).

The separation of amino acids for analysis can be carried out after derivatisation or in their native form. Derivatisation confers amino acids spectroscopic characteristics that will permit them to be detected by UV or fluorescence. If derivatisation is achieved after separation, amino acids are separated on the basis of polarity, using a reversed phase column, since derivatisation reagent will increase the hydrophobicity of amino acids. Alternatively, derivatisation can be done after separation. In this case, separation will be made based on the charge using a cation exchange column. The use of HILIC columns and mass spectrometry for detection allows amino acid analysis without derivatisation (Zhou *et al.*, 2013; Ge *et al.*, 2015; Joyce *et al.*, 2016), which increases the speed of analysis, as sample preparation is simplified.

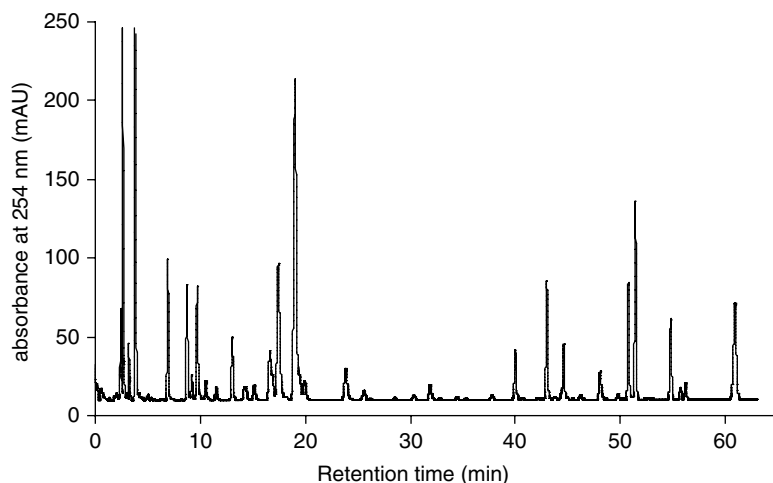


Figure 14.2 RP-HPLC chromatogram of the phenylthiocarbamyl derivatives of free amino acids from dry-cured pork loin.

The analysis of amino acids from hydrolysed proteins is relatively easy. However, the analysis of free (physiological) amino acids is fairly complicated, because many peaks, including some dipeptides, appear in the chromatogram, making it difficult to obtain a good separation for all of them. Up to now, derivatisation with phenylisothiocyanate and separation in an adequate reversed phase column seems to give the best results for the analysis of physiological amino acids. Figure 14.2 shows a chromatogram of the phenylthiocarbamyl derivatives of free amino acids from dry-cured pork loin.

A review on these methods in food analysis has been published recently by Aristoy and Toldrá (2013, 2015, 2016).

14.4.2 Carbohydrate and carboxylic acids

Knowledge of carbohydrate and carboxylic acid composition of foods is important, because these compounds are the principal constituents of many foods like fruits, cereals, honey, beverages, and so on, and are responsible for much of their flavour and quality. Furthermore, these compounds may serve as a maturity index for the optimisation of harvest time (Mikulic-Petkovsek *et al.*, 2015), authenticity (Willems and Low, 2014) and fermentative processes (Lázaro *et al.*, 1989; Giovanelli and Polo, 1994). Carbohydrates and organic acids are also routinely analysed for their use as additives. This is the case of polyols in bakery, juices and confectionary products (Corradini *et al.*, 1998; Zakharova *et al.*, 2013).

Although, traditionally, gel filtration and partition chromatography in normal phase have been used to separate carbohydrates, anion exchange is gaining ground, on the basis that sugars and polyols behave as weak acids at high pH. Even so, gel filtration is the technique of choice to separate carbohydrates by size (Gómez-Ordóñez *et al.*, 2012; Condezo-Hoyos *et al.*, 2015). Possible detection methods are refractive index (RID), evaporative light-scattering (ELSD), ultraviolet (requires the derivatisation of carbohydrates for a better sensitivity and selectivity) or pulsed amperometric detection

(PAD). Lately, mass spectrometry is also being used for the analysis of sugar alcohols, such as sorbitol, mannitol, xilitol, or inositol (Liu *et al.*, 2009; Shindo *et al.*, 2013).

RID is a universal detector, but it lacks sensitivity and does not allow the use of a mobile phase gradient if it is needed. ELSD is a semi-universal mass detector which detects the analyte after nebulisation and evaporation of the eluent and gains in performance to the RID. UV is not used for analysis of sugars or alcohol, due to their low absorbance wavelength (by 190 nm) unless they are derivatized (Nojiri *et al.*, 1999), but it is quite usual for organic acids, which absorb at 220 nm. Finally, PAD provides highly specific detection of carbohydrates, with excellent signal-to-noise ratios without requiring derivatisation (Peris-Tortajada, 2013).

Some scientific papers using HPLC for the analysis of carbohydrates and organic acids in foods are compiled in Table 14.1. In relation to the methodology used, this table shows the detection method only, because the possibility of achieving a specific HPLC analysis will depend, in many cases, on the ability of a specific detector. In fact, it is easier to purchase a chromatographic column than a detector to perform the required analysis.

Table 14.1 Applications of HPLC to the analysis of carbohydrates and organic acids in foods.

Sample	Matrix	Detection details	References
Carbohydrates	Cereals	ELSD	Young, 2002
	Beer	ELSD	Nogueira <i>et al.</i> , 2005
Monosaccharides	Fruits	UV (190 nm) or RI	Karkacier <i>et al.</i> , 2003
"	Melon	RID	Peris-Tortajada, 2013
Mono- and disaccharides	Cereal products	PAD	Menezes <i>et al.</i> , 2004
Mono-, di- and trisaccharides	Foods	ELSD	Wei and Ding, 2002
	Vegetable foods	ELSD	Shanmugavelan <i>et al.</i> , 2013
Sugars and carboxylic acids	Fruit juices	UV (214 nm)	Chinnici <i>et al.</i> , 2005
	Grapevine berry	RI-UV	Eyeghe-Bickong <i>et al.</i> , 2012
Carbohydrates, alditols and carboxylic acids	Must & wine	RI	Calull <i>et al.</i> , 1992
		UV (214 nm)	Castellari <i>et al.</i> , 2002
"	Wine	FTIR	Edelman <i>et al.</i> , 2003
Carboxylic acids	Beer	UV or RI	Mancini <i>et al.</i> , 2000
			Floridi <i>et al.</i> , 2002
	Sweet cherry	UV (210 nm)	Usenik <i>et al.</i> , 2010
	Fruits & jam	UV (214 nm)	Silva <i>et al.</i> , 2002
	Cheese	UV (214 nm)	Ergönül and Nergiz, 2010
Alditols and sugars	Fruits & Vegetables	PAD	Cataldi <i>et al.</i> , 1998
	Beverages & milk	PAD	Corradini <i>et al.</i> , 2001
	"	PAD	Bruce, 2003
	Beverages & confectionary products		

(Continued)

Table 14.1 (Continued)

Sample	Matrix	Detection details	References
Alditols	Confectionary products	PAD	Corradini <i>et al.</i> , 1998
"	Beverages & foods	Nitrobenzoylation & UV	Nojiri <i>et al.</i> , 1999
Inositol	Nuts and cereal	MS/MS	Liu <i>et al.</i> , 2009

ELSD: evaporative light scattering detector.
PAD: pulsed amperometric detector.
UV: ultraviolet.
RI: refractive index.
FTIR: Fourier transform infrared.
MS: mass spectrometry

Some reviews on the analysis of carbohydrates in foods and beverages by high performance liquid chromatography have been published (Martínez-Montero *et al.*, 2004; Peris-Tortajada, 2013).

14.4.3 Vitamins

Vitamins are biologically active compounds with relevant beneficial effects on health. The requirements for their analysis are mainly related to knowledge about the nutritive value of foods, or compliance with labelling. In many cases, their analyses require complex and time-consuming sample preparation, especially for fat-soluble vitamins, and thus the study of faster methods is progressing. Additionally, vitamins are found at very low concentrations in foods, and often are accompanied by an excess of compounds with similar characteristics or chemical properties. The use of specific detection, such as UV-visible, diode array detection that will provide spectral information, fluorescence or electrochemical detection, is thus necessary. The development of mass spectrometry, adapted as a detector for HPLC, has improved selectivity in the analysis of these compounds, making sample preparation easier and increasing throughput, due to the ability to simultaneously analyse a large number of different metabolites, including vitamins.

Fat-soluble vitamins include vitamin A (retinol, retinal and retinoic acid) and its precursors (carotenoids) currently present in vegetal foods, vitamin D, vitamin E (α -, β -, γ -, and δ -tocopherol and α -, β -, γ -, and δ -tocotrienol) and its metabolites, and vitamin K.

Some vitamins, especially fat-soluble vitamins, are labile compounds, sensitive to high temperatures, oxygen and light, which may be taken into account to analyse them. The analysis of fat-soluble vitamins requires complex sample preparation procedures that include alkaline hydrolysis (saponification), enzymatic hydrolysis, alcoholysis, direct solvent extraction, solid phase extraction, or supercritical fluid extraction of the total lipid content (Heudi *et al.*, 2004; Nollet *et al.*, 2013). Indeed, supercritical fluid extraction (SFE) methods are adequate for fat-soluble vitamins, because the extraction occurs at a lower temperature than in traditional methods, and without oxygen and light (Perretti *et al.*, 2003). The use of organic solvents like methanol, ethanol and hexane is required for the extraction or for the analysis. In these conditions, chromatographic

separation using normal-phase columns seem to be more appropriate for these hydrophobic compounds dissolved in organic solvents. Nevertheless, reversed phase is generally preferred for providing higher resolution power.

Some examples for their analysis in foods are shown in Table 14.2.

Apart from these, some reviews on vitamin E in seafood include methods for the analysis (Alfonso *et al.*, 2016), or for vitamin D analysis which includes different methods for sample preparation (Kasalova *et al.*, 2015). A complete review on HPLC methods for fat-soluble vitamins in general has been published (Nollet *et al.*, 2013).

Water-soluble vitamins, like vitamins B or vitamin C, normally require simpler sample preparation, which includes an extraction with water after homogenisation of the

Table 14.2 Applications of HPLC to the analysis of fat-soluble vitamins in foods.

Sample	Matrix	Separation/detection	References
α - and β - carotene	Carrot juices	Reverse-phase/DAD	Marx <i>et al.</i> , 2000
"	Sweet potato	Reverse-phase/PAD	Liu <i>et al.</i> , 2009
Vitamins A, D, E and β - carotene	Milk, pork, liver, infant formula	Photometric detection	Perretti <i>et al.</i> , 2003
Vitamins A and E	Infant formulas	Reversed-phase/DAD (292–235 nm)	Rodas-Mendoza <i>et al.</i> , 2003
"	Dairy products	Reversed-phase/UV	Paixao and Campos, 2004
"	Beverages	Normal-phase/FLD	Hoeller <i>et al.</i> , 2003
Vitamins A, D, and E	Infant formulas	Normal-phase/MS	Heudi <i>et al.</i> , 2004
Vitamin D	Baby food	Reversed-phase/DAD (295 nm)	Konings, 1994
"	Dairy products	Reverse-phase/DAD	
"	Milk	Reverse-phase/MS	Trenerry <i>et al.</i> , 2011
"	Meat, eggs, fish	Reverse-phase/DAD	Bilodeau <i>et al.</i> , 2011
Vitamin E	Fruit juice and vegetable extract	Post-column photochemical reaction – DAD and FLD	Terada and Tamura, 2004
"	Infant formulas	Normal-phase/FLD	Rodrigo <i>et al.</i> , 2002
"	Meat	Normal-phase/FLD	Hewavitharana <i>et al.</i> , 2004
"	Cereals	Normal-phase/FLD	Ryyanen <i>et al.</i> , 2004
"	Edible oils	Normal-phase/DAD and FLD	Gama <i>et al.</i> , 2000
"	Olive	Reverse-phase/DAD	San Andrés <i>et al.</i> , 2011
Vitamin K	Several foods	Reverse-phase/FLD	Jakob and Elmadfa, 2000
"	Olive oil	Reverse-phase/UV	Otles and Cagindi, 2007

DAD: diode-array detector

UV: ultraviolet

FLD: fluorescence detector

MS: mass-spectrometry

PAD: pulsed amperometric detector

food sample and, in some cases, a deproteinisation or solid phase extraction. Some examples dealing with multi-analysis of soluble vitamins are described in honey (León-Ruiz *et al.*, 2013), in meat products (Gratacós-Cubarsí *et al.*, 2011, 2013), in cereals (LaCroix *et al.*, 2005), in Turkish Tarhana (Ekinci, 2005), and in polyvitaminated pre-mixes used in infant nutrition (Heudi *et al.*, 2005). Although reversed-phase columns are usually employed for these analyses, the HILIC technique was used to analyse six vitamins B and vitamin C in cereals for breakfast to evaluate UV, fluorescent and coulometric detection methods (Langer and Lodge, 2014).

As mentioned, the use of mass spectrometry for detection after HPLC has made easier the analysis of several vitamins at once. Twenty-one polar vitamins in plasma were simultaneously analysed by separation in a reversed phase column (C₁₈-pentafluorophenyl), housed in an UPLC system and connected to a QTRAP-MS system in positive electrospray ionisation (ESI) operating mode for identification and quantification (Redeul, *et al.*, 2015). HILIC separation (several columns) was evaluated for polar vitamins (folic acid, 4-aminobenzoic acid, nicotinic acid, nicotinamide, riboflavin, thiamine and pyridoxine) analysis, with ESI mass spectrometric detection (Yang *et al.*, 2013).

Nevertheless, the limiting factor is the preliminary extraction from food, as the procedure is different for each vitamin and/or groups of vitamins. Whereas vitamins B₁, B₂, B₆, and PP can be extracted and analysed simultaneously, folic acid and pantothenic acid need an individual extraction procedure and, thus, must be analysed in separate LC runs (Leporati *et al.*, 2005). As an example, it has been applied to the analysis of vitamins B₁, B₂, B₆ (pyridoxine, pyridoxal, and pyridoxamine), PP (nicotinamide and nicotinic acid), pantothenic acid and folic acid in typical Italian pasta, using a reversed-phase C₁₆ amide column and triple quadrupole mass spectrometry.

A complete and detailed review on these is that from Russel (2013). In this review, each vitamin has its own section, with several methodologies for its analysis. Sample preparation is also described, giving especial relevance to recent developments in the HPLC analysis.

14.4.4 Minerals and trace elements

The determination of minerals and trace elements is usually carried out by atomic absorption or atomic emission spectroscopy. However, the analysis of mineral cations can be easily accomplished by ion-exchange chromatography, coupled to a conductivity detector. Column technology has been improved to perform the analysis in few minutes (Weiss and Jensen, 2003; Klampfl, 2004). The sample preparation is very simple, and consists of a water extraction and filtering of previously ground solid samples, or only filtering liquid samples. An example of a chromatogram with the separation of several cations in a single run is shown in Figure 14.3.

Several mono- and divalent cations have been analysed in water and beverages (Kim and Lee, 1997; Amin *et al.*, 2005) milk whey (Cataldi *et al.*, 2003) or meat products (Armenteros *et al.*, 2009).

14.4.5 Antioxidants

Control of raw materials for food processing includes the analysis of other nutritionally important compounds that support health benefits. There is a group of compounds

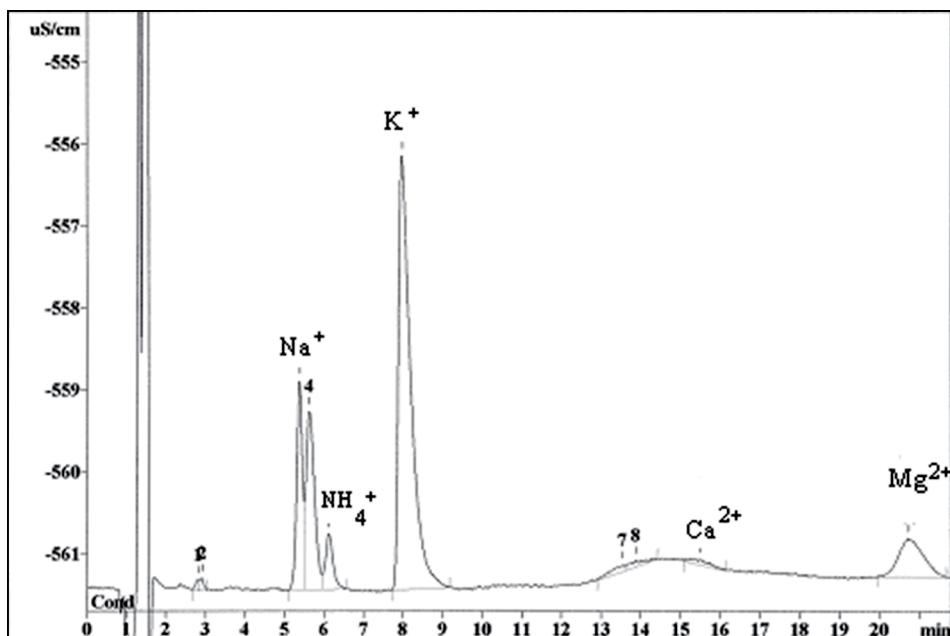


Figure 14.3 Ion exchange HPLC chromatogram of mono and divalent cations from a pork meat sample.

naturally present in foods which do not possess nutritional characteristics on their own, but which are responsible for wellness and have benefit effects in health as antioxidants, antihypertensives, antitumorals, promoters of immuno-defence, and so on.

Polyphenols are the most important bioactive compounds which are present in vegetal foods. They support health benefits as antioxidants (Burns *et al.*, 2000), preventing oxidative damage diseases (coronary heart disease, cancer, etc.). Among these, flavonoids constitute a large group of polyphenols, which are present in plant tissues as sugar conjugates or as aglycons. They are classified, according to their chemical structure, into flavonoids, flavones, flavanones, isoflavones, catechins, anthocyanins and chalcones. To analyse them, glycosides are normally hydrolysed (Nuutila *et al.*, 2002), and the resulting aglycons are identified and quantified if possible – however, there are more than 4000 flavonoids, and the main difficulty is that many of them are not available on the market.

Some methods for sample preparation and analysis of phenolic compounds in foods are available in the scientific literature (Merken and Beecher, 2000; Castellari *et al.*, 2002; Nenadis and Tsimidou, 2002; Robbins, 2003; Gyorik, 2003; Martínez-Ortega *et al.*, 2004; Li *et al.*, 2004; Chinnici *et al.*, 2004; Belajova and Suhaj, 2004; Alonso-García *et al.*, 2004; Umphress *et al.*, 2005; Hubert *et al.*, 2005; Epriliati *et al.*, 2010; Figueiredo-Gonzalez *et al.*, 2013). One interesting review is that from Villiers *et al.* (2016), in which are discussed methodologies for sample preparation, improved LC separation in terms of speed and/or resolution, including ultra high pressure LC (UHPLC), monolithic and superficially porous phases, high temperature LC (HTLC) and comprehensive two-dimensional LC (LC $\times$ LC) and methods for detection.

Saponins and ginsenosides are other important bioactive compounds, mainly present in soya and ginseng, which display hypolipidaemic activity (Rodrigues *et al.*, 2005;

Popovich, 2005) and are protective against colon cancer (MacDonald *et al.*, 2005). Some HPLC methods for their analysis have been reported (Lau *et al.*, 2003; Hubert *et al.*, 2005).

14.5 Analysis of biochemical markers: applications for food quality

14.5.1 Biochemical compounds

14.5.1.1 Amino acids

Free amino acids are important taste-active compounds that contribute to the characteristic taste of foods (Nishimura and Kato, 1988; Kato *et al.*, 1989; Kemp and Birch, 1992). Furthermore, some amino acids, such as sulphur-containing amino acids, can react during food processing, to generate aroma compounds (Flores *et al.*, 1989; Klein *et al.*, 2001). These sulphur-containing amino acids may also reduce the adverse effects of certain food ingredients (Friedman, 1994). Nevertheless, further reactions of amino acids can also cause problems. In wine, glutamine is converted into pyroglutamic acid, which causes an unfavourable flavour (Pfeiffer and Orben, 2000). Methods for the analysis of amino acids have been indicated previously.

There are some negative effects of food processing, especially if they are not correctly applied, that can be monitored through the analysis of certain amino acids or their derivatives. For instance, alkali treatment and/or heating;

- can result in the destruction of essential amino acids;
- can induce racemisation of amino acids to their D-optical isomers, making them unavailable for protein synthesis (Friedman, 1992, 1999); or
- may cause loss of digestibility by cross-linking with the lysinoalanine or histidinolalanine formation (Henle *et al.*, 1993; Wilkinson and Hewavitharana, 1997).

Thus, the HPLC analysis of these compounds has been used to monitor the correct processing of foods such as dairy products (Moret *et al.*, 1997; Ferrer *et al.*, 1999; Faist *et al.*, 2000; Calabrese *et al.*, 2009) or eggs (Chang *et al.*, 1999). Also, the irradiation used as food preservation method can cause phenylalanine oxidation to *o*-, *m*- and *p*-tyrosine, where their yield is proportional to the absorbed dose and temperature during radiation. Hence, *o*- and *m*-tyrosine are suggested as markers for irradiated protein-rich food (Krach *et al.*, 1997, 1999; Miyahara *et al.*, 2000a, 2000b, 2002).

14.5.1.2 Nucleotides and nucleosides

The analysis of ATP-related compounds offers good possibilities for quality control. Nucleotides and nucleosides are generally formed from ATP (5'-adenosine triphosphate) breakdown in foods. The nucleotides and nucleosides are extracted and filtered, and are then injected into reverse-phase HPLC, coupled to a UV or diode array detector. Di- and tri-nucleotides are retained very little in a reverse-phase column, due to their high polarity. Thus, for the analysis of ATP and ADP (5'-adenosine diphosphate), it may be worth adding a cationic ion pair to the mobile phase, to increase retention and, thereby, facilitate separation (Veciana-Nogues *et al.*, 1997). The analysis is relatively easy and fast, but the main limitation is the rapid breakdown of ATP when samples are not properly managed under careful cold conditions.

ATP disappears very quickly in *post mortem* muscle, its disappearance being correlated with the development of rigor mortis (Batlle *et al.*, 2000). The analysis of ATP-related compounds were first carried out in fish to calculate K_0 ratio (ratio between inosine + hypoxanthine to the total ATP-related compounds + inosine + hypoxanthine expressed as percentage), as a good indicator of the freshness of fish (Hattula and Kiesvaara, 1992; Veciana-Nogues *et al.*, 1997) or meat (Batlle *et al.*, 2001).

The ATP degradation process is faster in low-quality meats like those termed PSE (pale, soft and exudative meats) and, thus, the prediction of low-quality meats can be based on the analysis of ATP related compounds early *post mortem*. Some ratios, among them the 5'- inosine monophosphate (IMP)/ATP (Batlle *et al.*, 2000) or R' value, that consists of the ratio of inosine-related compounds to ATP-related compounds (Honikel and Fischer, 1977) have shown good utility for quality purposes allowing detecting exudative pork meats early *post mortem*.

Zhou *et al.* (2012) analysed the ATP related-compounds by reversed-phase UPLC as a freshness index to evaluate the change in quality of royal jelly during storage.

14.5.2 Additives

Some inorganic anions are used as food additives. The analysis of all of these has been simplified by the use of ion chromatography with anion exchange columns and electrochemical detection (Kaufmann *et al.*, 2000; Bernini *et al.*, 2001; Carinci and di Deo, 2003; Rantakokko *et al.*, 2004). A higher sensitivity can be achieved when using suppressed conductivity detection. The suppressor, which is located between the column and the detector, reduces the background conductivity of the eluent, and can also enhance the detection of the analyte (Klampfl, 2004). This method of detection, based on changes in conductivity, is a non-specific but rather sensitive technique that covers an enormous linear dynamic signal range of five orders of magnitude, which is far better than spectroscopic techniques that are generally limited to two orders of magnitude (Kissner and Koppenol, 2005). The chloride anion, which is the most common inorganic anion present in foods, has been analysed by this technique.

Nitrite and nitrate constitute two important additives used for the preservation of muscle foods in the elaboration of meat products. The harmful effects attributed to these compounds makes necessary their analysis not only in meat products, but also in some plant foods, and in water containing high amounts of them. Anion chromatography, coupled to a conductivity detector, allows a good detection of both nitrate and nitrite in 15–30 minutes. Nevertheless, the analysis of nitrite in these type of samples, as well as in some biological fluids is hampered by large amounts of chloride ions, which results in insufficient peak resolution. This difficulty could be overcome by previously removing the hydrochloride, using Ag^+ resins (Stratford *et al.*, 1997).

The type of column depends on the composition of the sample. Thus, standard anion exchange columns that use carbonate-based mobile phases are valid for low ionic strength samples, while special high-capacity columns should be used for samples of high ionic strength – for instance, those containing a high buffer concentration (Kissner and Koppenol, 2005).

Nitrate can be separated from interfering compounds with a high-capacity anion-exchange column, such as hydroxide-selective columns that are manufactured for use with electrolytically generated high-purity hydroxide. This column has a higher

capacity, and also different selectivity for commonly weakly retained solutes, than traditional IC columns that use carbonate eluents (Saccani *et al.*, 2006). They have been used in the determination of nitrate in both fresh and cured meats (Saccani *et al.*, 2006), and of nitrate and nitrite in vegetables and fruit for baby foods (McMullen *et al.*, 2005). Using this column, more than 30 anions, including chloride, nitrate, nitrite and phosphate, have been efficiently separated (McMullen *et al.*, 2005). Also, with these columns, mass spectrometry can be used for detection (Cavalli *et al.*, 2005; Saccani *et al.*, 2006).

14.5.3 Markers for process control

Process quality control is important, to detect bad practices in food processing, or in the use of preservatives, with the addition of non-permitted additives or an excess of permitted ones. Quality control also includes the detection of food contaminants, such as polycyclic aromatic hydrocarbons (PAHs), resulting from materials in contact with foods (packages), pesticides, antibiotics and other drugs. Glucose content has been reported as a potential quality marker of green coffee. This content, determined by HPLC, is linked with the increase of a woody/rubbery note in coffee cup quality (Bucheli *et al.*, 1998).

The control of Maillard reaction (MR) is a key point to ensure the quality of processed foods. Due to the presence of a primary amino group on its side chain, lysine is particularly prone to chemical modifications, with the formation of Amadori products, N ϵ -(Carboxymethyl)-l-lysine (CML), N ϵ -(Carboxyethyl)-l-lysine (CEL) in thermal processed foods. The analysis of these compounds is made after acid hydrolysis to release them from the polypeptide (Troise *et al.*, 2015). During this procedure, N ϵ -(2-Furoylmethyl)-l-lysine (furosine) is formed, and has been proposed as a marker of thermal treatment, particularly in dairy products (Goekmen *et al.*, 2009), but also in soybeans (Troise *et al.*, 2015), pasta (Giannetti *et al.*, 2013) or royal jelly (Wytrychowski *et al.*, 2014). Due to the polar and ionic nature of furosine, it can be analysed by ion pair RP-HPLC or by HILIC, and the detection is made by UV or mass spectrometry. Sample preparation includes the mandatory acid hydrolysis, and the use (or not) of solid phase extraction.

RP-HPLC has been successfully applied to the control and follow-up of proteolysis, and generation of peptides and free amino acids, by different lactic acid bacteria typical of fermented meat products (Fadda *et al.*, 1999a, 1999b; Sanz *et al.*, 1999a, 1999b). These chromatograms are useful to select the most appropriate fermentation conditions to obtain the desired proteolysis.

14.6 Analysis of biochemical markers: applications for the detection of food adulterations

The analysis of certain compounds can be used as index for the detection of food adulterations or fraudulent food additions. In this sense, HPLC constitutes a useful technique for the analysis of many of these compounds. For instance, 4-hydroxyproline is used as a marker of the addition of collagen, gelatine, or any other low-value meat extracts, to meat products. Another practice consisting in the addition of non-meat

proteins or hydrolysates to meat products can be easily detected by analysing 3-methylhistidine (Lawrie, 1988; Toldrá and Reig, 2004). In other cases, the addition of cheap amino acids to mask fraudulent fruit juice dilution (Anantharaman and Finot, 1993), or the addition of common wheat flour in making durum pasta products (Stancher *et al.*, 1995), can be detected by HPLC analysis of free amino acid profiles.

Some small peptides, like anserine, carnosine and balenine, which are naturally present in meat and fish, and not present in vegetal origin proteins, have been analysed by HPLC (Aristoy *et al.*, 2004). The content of these dipeptides differs according to the animal species, and some ratios have been proposed as markers of the presence of mammalian proteins in feeds (Aristoy and Toldrá, 2004). Figure 14.4 shows how the presence of the dipeptide carnosine can be easily detected in cat food, where proteins of animal origin are permitted, while these must be absent in ruminant (bovine) feed.

The addition of cheap commercial sweeteners to fruit juices to mask fraudulent dilutions can be detected by the analysis of the profile of carbohydrate and/or polyols, achieved by HPLC with pulsed amperometric detection (Willems and Low, 2014).

The geographical origin of wines can be identified by means of the analysis of their polyphenols or biogenic amines profile, and this can serve to detect frauds (Galgano *et al.*, 2011). The analysis of these compounds will be described in the corresponding section.

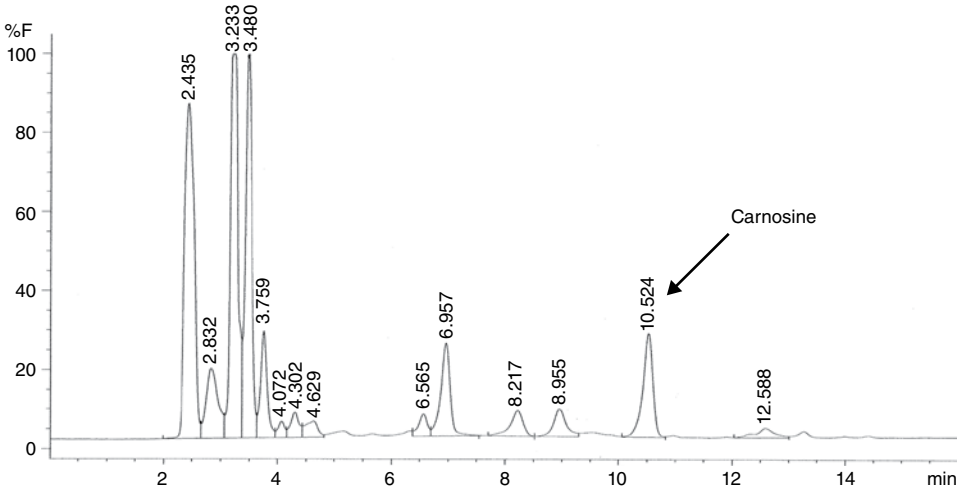
14.7 Analysis of biochemical markers: applications for food safety

14.7.1 Biochemical compounds

Some compounds have been found to exert a toxic effect on consumers and, thus, must be detected. This is the case with acrylamide, a probable carcinogen and neurotoxic agent, which has been recently found to be formed from asparagine in carbohydrate-rich foods at high temperatures and low moist conditions (Riediker, 2002). Acrylamide has been detected in potato chips, fried snacks and other cooked foods (Tareke *et al.*, 2002; Weisshaar, 2004; Shamlal and Nisha, 2014). Methods based on LC-MS are the most adequate to analyse acrylamide but, due to the low molecular mass of acrylamide, those using UHPLC and high-resolution mass spectrometry, such as Orbitrap, which operates in the full scan mode and provides accurate mass measurements, ensure the best results in terms of throughput and cost-effectiveness ratio (Omar *et al.*, 2015).

Biogenic amines are naturally occurring components in some foods. Problems arise because some specific amines can interact with some medicines, causing a health hazard. By themselves, biogenic amines can exert toxic properties when consumed at high concentrations, or can cause allergenic problems. Biogenic amines are mainly synthesized through decarboxylation of free amino acids. This pathway is frequent in fermented products such as meat, fish, cheese or wine, and is possibly strengthened by bad hygienic conditions of raw materials and manufacturing practices. Consequently, the level of biogenic amines in a food product is often considered as a marker of quality index (Vinci and Antonelli, 2002; Chytiri *et al.*, 2004). Some reviews have been published concerning biogenic amines and the factors influencing their accumulation in meat and meat

(a) FLD1 A, Ex=340, Em=455 (A:\DP\24MAY\FK-PCA-M.D)



(b) FLD1 A, Ex=340, Em=455 (A:\DP\30MAY\OVINO.D)

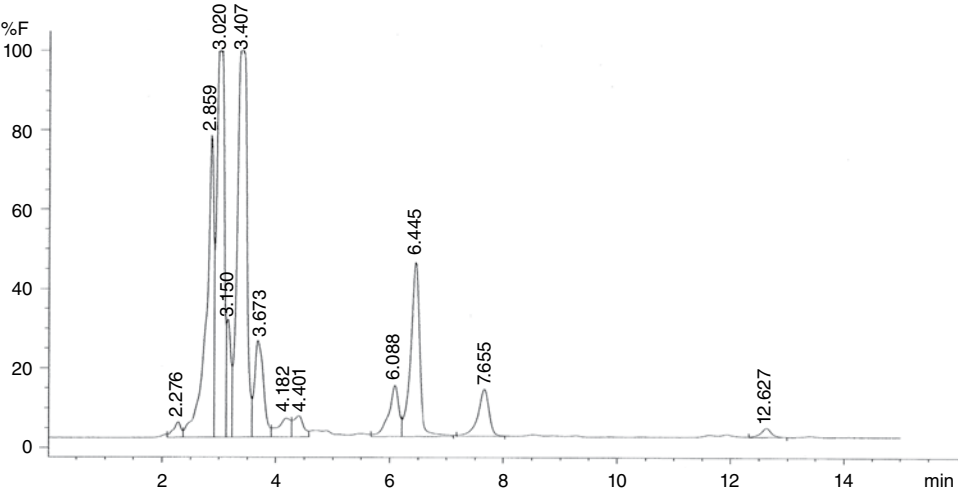


Figure 14.4 Cation exchange HPLC and OPA post-column derivatization for the detection of mammalian dipeptides in feedstuffs for cats (a) and bovines (b).

products (Suzzi and Gardini, 2003; Ruiz-Capillas and Jiménez-Colmenero, 2004) or in cheese (Stratton *et al.*, 1991).

Many methods for the analysis of amines in foods, together with some reviews (Onal *et al.*, 2013), are described in the literature. A small part of these is shown in Table 14.3 as an example. Sample preparation often includes a liquid/liquid or solid-phase extraction. As amines do not possess chromophores or fluorophores, a derivatisation, pre- or post- separation, is almost always achieved to enhance sensibility and selectivity in the UV or fluorescence detection. The derivatisation may not be necessary when using mass-spectrometry for detection (Calbiani *et al.*, 2005; Saccani *et al.*, 2005; Millán *et al.*,

Table 14.3 Applications of HPLC to the analysis of amines in foods.

Matrix	Separation technique	Detection	References
Wine/alcoholic beverages	Ion-pair-RP	OPA post-column/FLD	Vidal-Carou <i>et al.</i> , 2003
		NQS post-column/DAD	Hlabangana <i>et al.</i> , 2006
	RP	OPA pre-column/FLD	Kelly <i>et al.</i> , 2010 Arrieta and Prats-Moya, 2012
		AQC pre-column/FLD	Hernández-Orte <i>et al.</i> , 2006
		Dansyl pre-column/DAD	Dugo <i>et al.</i> , 2006 Manetta <i>et al.</i> , 2016
		Dansyl pre-column/MS	Jia <i>et al.</i> , 2012
		NQS pre-column/MS	García-Villar <i>et al.</i> , 2009
		MS	Millán <i>et al.</i> , 2007
	CE	MS	Saccani <i>et al.</i> , 2005
		OPA post-column/FLD	Triki <i>et al.</i> , 2012
Sausages	RP	OPA post-column/FLD	Straub <i>et al.</i> , 1993
		Dansyl pre-column/MS	Jia <i>et al.</i> , 2011
Fish	RP	MS	Sagratini <i>et al.</i> , 2012
Cheese	Ion-pair-RP	OPA post-column/FLD	Vale and Glória, 1997
	RP	FMOC pre-column/FLD	Aygün <i>et al.</i> , 1999
		AQC pre-column/FLD	Fiechter <i>et al.</i> , 2013

RP: reversed phase.

OPA: *o*-phthalaldehyde.

FLD: fluorescence detector.

NQS: 1,2-naphthoquinone-4-sulphonate.

DAD: diode-array detector.

AQC: 6-aminoquinolyl-*n*-hydroxysuccinimidyl carbamate.

MS: mass spectrometry.

CE: cation exchange.

FMOC: 9-fluorenylmethylchloroformate.

2007; Sagratini *et al.*, 2012). Detection of underivatized amines with electrochemical detectors has also been reported (Yashin and Yashin, 2004).

14.7.2 Veterinary drug residues in foods of animal origin

Veterinary drugs that have anabolic effect are used for therapeutic and prophylactic purposes and may also be used for improved breeding efficiency, even though they are banned in the European Union and other countries. Some of these drugs can only be administered for therapeutic purposes, and under the control of a responsible veterinarian (Van Peteghem and Daeselaire, 2004). These substances, which are illegally used, improve the feed conversion efficiency and increase the lean-to-fat ratio, due to these growth-promoting effects (Toldrá and Reig, 2006).

The detection of veterinary drugs is difficult, due to the large number of substances to be analysed. Their presence in foods is controlled by official inspection and analytical services, following EC Directive 96/23/EC on measures to monitor certain substances and residues in live animals and animal products. This directive was recently implemented by the Commission Decision, 2002/657/EC, which provides detailed guidelines for the analytical methods to be used in testing of official samples, and specific common criteria for the interpretation of analytical results of official control laboratories for such samples.

Routine inspection controls are based on rapid screening assays, and only those food samples suspected of containing a veterinary drug residue are then confirmed through methods based on the use of gas or liquid chromatography coupled to mass spectrometry, or other sophisticated methodologies and analytical instrumentation, for accurate characterisation and confirmation (Toldrá and Reig, 2014, 2015, 2016).

Liquid chromatography has been widely used in the analysis of veterinary drug residues in foods (Van Poucke *et al.*, 2013). HPLC, especially when coupled to MS, constitutes a useful technique for multi-residues screening, due to the increasing demand for analysis and the need to speed up the analysis time. The analysis is based on a solid-phase extraction clean-up, followed by filtration and injection into the system. A review on sample preparation was published by Kinsella *et al.* (2009). It has been applied for detection of veterinary drugs in several foods, including eggs, milk, fish and meat (Aerts *et al.*, 1995; Horie *et al.*, 1998), anabolic steroids in nutritional supplements (Gonzalo-Lumbreras and Izquierdo-Hornillos, 2000; De Cock *et al.*, 2001), and corticosteroids such as dexamethasone in water and meat (Shearan *et al.*, 1991; Mallinson *et al.*, 1995; Reig *et al.*, 2006). The availability of automation and computer-controlled facilities has contributed to the expansion of its use as a screening technique (Toldrá and Reig, 2006).

The use of dispersive solid phase extraction (dSPE); QuEChERS (Quick, Easy, Cheap Effective, Rugged and Safe) for sample preparation is expanding rapidly, because it simplifies the sample extraction and purification for the analysis of veterinary drugs and mycotoxins in meats and processed foods (Reig and Toldrá, 2009, 2011). These methods, linked to UPLC-MS, have improved throughput and effectiveness of residue control by allowing multi-residue analysis (Gómez-Pérez *et al.*, 2012; van Poucke *et al.*, 2013). For instance, the use of liquid chromatography, coupled to triple quadrupole mass spectrometry, has allowed the detection of 34 anabolic with a CC α within the range of 0.04–0.88 ng/g (Vanhaecke *et al.*, 2013).

14.7.3 Antibiotic residues in foods of animal origin

Reverse-phase HPLC, in combination with a wide range of different detectors, such as UV-diode array or fluorescence, constitutes a useful technique for the screening of antibiotics. Antibiotics have been successfully detected by RP-HPLC with diode array in meat, kidney and milk (Cooper *et al.*, 1995; Furusawa, 1999; Cinquina *et al.*, 2003). Fluorescence detection after HPLC separation has also been used for the detection of penicillins and avermectins in milk and liver, respectively (Bergwerff and Schloesser, 2003), for lasalocid in animal tissues and eggs (Matadubul *et al.*, 2000), and for the simultaneous determination of ten quinolone antibacterial residues in multi-species animal tissues (Verdon *et al.*, 2005). Five types of antibiotics were determined by

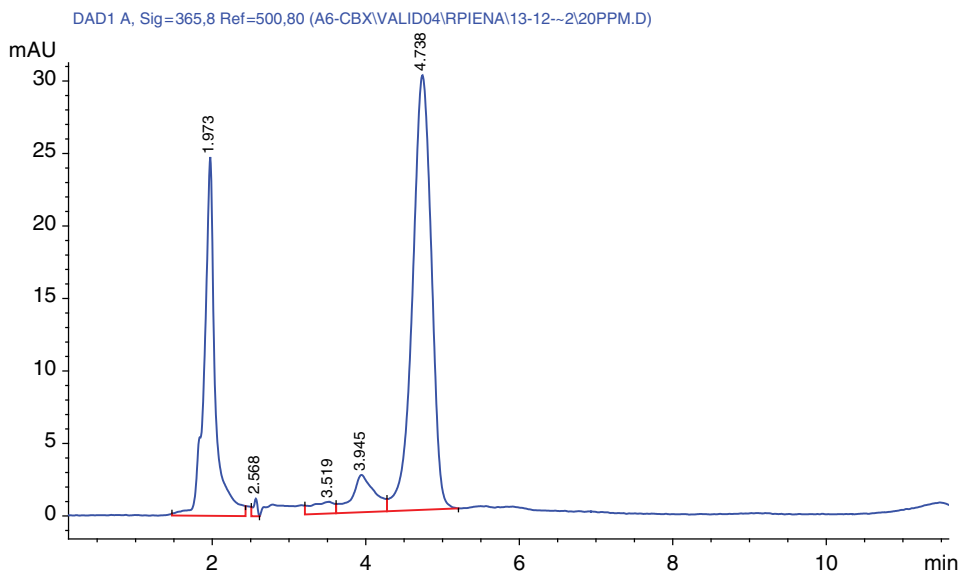


Figure 14.5 RP-HPLC chromatogram of carbadox, eluting at 4.74 min, in animal tissues.

RP-HPLC with fluorescence and UV detection in meat, liver, kidney and milk (Chonan *et al.*, 2000).

A chromatographic separation of carbadox, obtained in less than 15 minutes, is shown in Figure 14.5. The methods for the detection of antibiotics in foods were reviewed by Verdon (2008). When the analytes are not detected by light absorption or fluorescence, they may require post-column chemical modifications in order to get a detectable derivative (Bergwerff and Schloesser, 2003). The trend is towards fast analysis (Cháfer-Pericás *et al.*, 2010) and multi-residue analysis (Kaufmann *et al.*, 2011), to increase the throughput and effective detection of antibiotics.

14.7.4 Other residues

Multi-mycotoxin analysis of aflatoxins B1, B2, G1 and G2 and ochratoxin A has been performed by HPLC with fluorescence detection, after adequate clean-up procedures by immunoaffinity column (García-Villanova *et al.*, 2004; Gazioglu and Kolak, 2015). Similar reverse-phase HPLC methods for ochratoxin A have been reported (Chiaivaro *et al.*, 2002; Spotti *et al.*, 2002). Fumonisin and protein-bound fumonisins have been analysed in different foods, including cornflakes, tortilla chips and corn chips, by HPLC after extraction and partial purification by solid phase extraction (Park *et al.*, 2004). The determination of benzimidazole fungicides present in fruits and vegetables has been normalized. This method involves a clean up by liquid-liquid partition and analysis by HPLC (Prousalis, 2006). The combination of QuEChERS for sample preparation with UPLC-MS/MS was also applied to the simultaneous analysis of multi-class mycotoxins, including aflatoxins, ochratoxin A, fumonisins and zearalenone (Liu *et al.*, 2015).

Polycyclic aromatic hydrocarbons (PAH) are considered to be cancer causing agents. These compounds may be formed directly in food, as a result of heating processes like charcoal grilling, roasting or smoking (Simko, 2002). The control of PAH in smoke flavourings and smoked products is very important. Several chromatographic methodologies, including sample preparation by organic solvent extraction and cleanup through silica cartridges (Chung *et al.*, 2011; Simko, 2011), or the recently developed magnetic solid phase extraction (Shi *et al.*, 2015), have been proposed. Thus, the use of RP-HPLC with fluorescence detection has been proposed for such control in coffee (García-Falcón *et al.*, 2005; Shi *et al.*, 2015), oils (Moret and Conte, 2002), honey (Corredera *et al.*, 2011), fishery products (Moret *et al.*, 1999; Sobrado *et al.*, 2004) and meat products (Simko, 2002; Chung *et al.*, 2011; Viegas *et al.*, 2012).

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15

Olfactometry Detection of Aroma Compounds

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15.1 Introduction

The use of olfactometry in aroma analysis has allowed the identification of those volatiles occurring in a food that contribute to its aroma (Acree, 1997; Grosch, 2001). It is well known that only a small portion – less than 3–5% – of the thousands of volatile compounds identified in foods contribute to the aroma (Grosch, 2001; Dunkel *et al.*, 2014). In addition, the calculation of odour activity values (OAVs), in terms of the ratio of odourant concentration to odour threshold (Grosch, 2001), has been performed, resulting in around 230 compounds with $OAV > 1$. These compounds define the volatile stimulus of the majority of foods and beverages (Dunkel *et al.*, 2014). Nevertheless, not all the compounds identified contribute equally to food aroma, due to their differences in intensity and concentrations. Thus, the general scientific purpose has been to elucidate the contribution of individual compounds to aroma. This contribution will depend on their concentration in the matrix, limit of detection by the human nose (odour threshold) and their interaction with the food matrix (d'Acampora Zellner *et al.*, 2008).

Olfactometry is a technique used for the assessment of odour performance, coupled to a gas chromatograph that allows the separation of compounds, prior to their assessment as done by a human detector, the nose (Figure 15.1; Plutowska and Wardencki, 2008). For this purpose, the end of the chromatographic column is split and directed to two or more detectors. The results obtained from the different detectors (FID, MS and nose) will be represented in a chromatogram and an aromagram. Therefore, the characterisation of odour active compounds responsible for the aroma of food samples has been done by the use of this gas chromatography-olfactometry technique (Delahunty *et al.*, 2006; Plutowska and Wardencki, 2008; d'Acampora Zellner *et al.*, 2008). This technique was initially proposed by Fuller *et al.* (1964), and the first problems were due to discomfort from sniffing dry, hot effluent gases and the lack of sensitivity of analytical detectors.

In 1971, Dravnieks and O'Donnell proposed the use of humidified air in the sniffing port, and this design is currently used in modern sniffing ports, coupled to gas chromatographs. One should bear in mind that GC-O is a sensory evaluation method

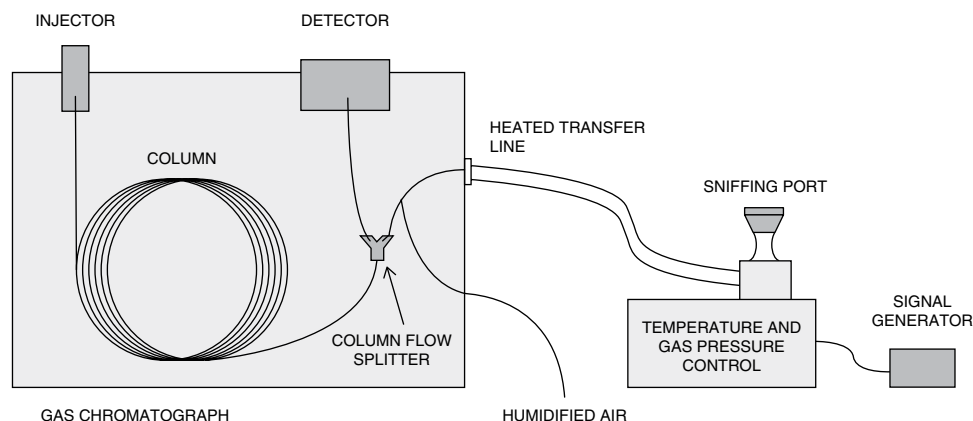


Figure 15.1 Design of a Gas chromatograph with an olfactometry detector (GC-O). Source: Plutowska and Wardencki, 2008 (Elsevier).

(Delahunty *et al.*, 2006) so, therefore, the panel's overall behaviour and the factors that affect the response of the human detector should be considered, such as the design of the sniffing port and the method to record the data. Actually, the olfactory port is integrated as a part of the GC system, including the software for word recognition, description of odours and recording. Examples of olfactory ports can be found from different suppliers; sniffer 9000 (Brechtbüeler, Germany), ODP (Gerstel, Germany), GC SNFR olfactory port (Perkin Elmer, USA), and so on.

15.2 Extraction of volatile compounds from foods for GC-olfactometry analysis (GC-O)

The extract of volatile aroma compounds used for GC-O analysis should represent the food aroma, not only when it is smelled, but also during eating (Van Ruth, 2001). Thus, the use of food extracts or headspace isolates individually only represent the composition of volatiles present in the food, or in the air above the food. The quality of the data obtained from GC-O analysis depends on this extraction procedure, since it will determine the extract composition and the availability of compounds for perception (Delahunty *et al.*, 2006). During GC-O analysis, individual compounds are assessed, so the obtained information represents the relevance of the compound for food aroma, but not its behaviour in the food matrix or in a mixture. This means that the interaction among compounds in a mixture is not considered, like the possibility of synergistic, antagonistic or additive effects (Delahunty *et al.*, 2006).

Many different isolation techniques are used, though they vary, depending on the food product. Direct chromatography is rarely used, except in simple matrices, such as alcoholic beverages. Instead, the isolation techniques used most often are: solvent extraction (SE); simultaneous distillation extraction (SDE); Soxhlet extraction, solvent-assisted flavour evaporation (SAFE); direct thermal desorption (DTD); solid phase micro extraction (SPME); and headspace analysis (HS), such as static and dynamic, among others (Plutowska and Wardencki, 2008; d'Acampora Zellner *et al.*, 2008).

Table 15.1 Advantages and disadvantages of extraction techniques.

Technique	Advantages	Disadvantages
Distillation and Solvent extraction SE	• Represents complete flavour • Fast extraction	• Decomposition of labile compounds • Loss of highly volatile • Heat induced artefact formation • Co-elution • Need of large sample amount • Presence of impurities from solvent • Solvent peak cover early eluting compounds • No suitable for fresh samples • Use of solvents. • Long time analysis.
SAFE	• Represents complete flavour • Fast extraction	• Loss of highly volatile • Need of large sample amount • Presence of impurities from solvent • Solvent peak cover early eluting compounds • Requires time to clean the SAFE apparatus • Use of solvent
Headspace static and dynamic	• Simplicity • Solvent-free procedures • Require small sample amounts • No artefact formation	• Does not represent the original sample
SPME	• Simplicity • Solvent-free procedures • Selective • Require small sample amounts • No artefact formation	• Does not represent headspace aroma • Depends on fibre type • Depends on time and temperature

The main advantages and disadvantages on the use of each technique are shown in Table 15.1. Distillation and solvent extraction, where the sample is submitted to solvent extraction, distillation and concentration steps, is the most widely used technique, as it is considered to represent the complete flavour of a food extract (Plutowska and Wardencki, 2008; d'Acampora Zellner *et al.*, 2008). The use of SAFE technique (Engel *et al.*, 1999) avoids the problems of heat induced artefact formation, as it is a solvent extraction that removes volatiles under low temperatures and high vacuum conditions. Moreover, many of the disadvantages observed for distillation and solvent extractions are counteracted by the use of headspace techniques.

These HS techniques sample the atmosphere of a food matrix previously equilibrated (static), or by removing constantly large amounts of volatiles by sweeping the food with an inert gas flow (dynamic). The flavour components obtained by these techniques rarely represent the concentration in the sample, due to the different volatilities of the aroma compounds. The use of SPME has many applications in food flavour analysis, due to its versatility, together with other microextraction techniques, such as Stir Bar Sorptive Extraction (SBSE) (Jeleń *et al.*, 2012). In SPME, a fused silica fibre with high adsorption capacity is used, with different extraction phases to extract volatile compounds. However, the volatile profile will depend on the extraction phase and time and temperature conditions used (Deibler *et al.*, 1999). Many workers recommend the use of several isolation procedures to mimic the food aroma, such as the use of headspace and solvent extractions (d'Acampora Zellner *et al.*, 2008; Grosch, 2001).

Chromatography conditions for GC-O analysis should be carefully checked to avoid the thermal breakdown of labile compounds in the heated inlet, such as the case of sulfur-containing compounds (d'Acampora Zellner *et al.*, 2008). The sniffing recommended time should be not more than 30 minutes, in order to avoid assessor's fatigue (Delahunty *et al.*, 2006), so fast GC temperature programs are used, although the separation will depend on sample composition. However, this fast program may decrease peak resolution; therefore, in order to maintain it, slow temperature programs are used and, in this case, the GC-O analysis is split into two assessors (Pollien *et al.*, 1997). Finally, the identification of the aroma compounds is confirmed by matching the retention index and odour quality of a reference standard. This process is generally performed in two different stationary phases (Acree *et al.*, 1997).

15.3 Olfactometry techniques

GC-O is a sensory evaluation method, where assessors or panel performance will affect the quality of results, although few studies deal with training protocols for assessors. Delahunty *et al.* (2006) reported that selection of appropriate assessors and training will improve the accuracy and precision of GC-O results. Generally, it has been recommended to screen assessors for their sensitivity and their ability to recognise odours. The training will depend on the method used, but it generally consists of the presentation and assessment of reference standards. Moreover, the olfactory ability among humans varies as well, as their odour thresholds. Olfactory response may be affected by many factors (Table 15.2), including the presence of bias due to different assessor conditions (fatigue, breathing cycle, etc), the specific olfactory ability of each human, previous assessor training, and several environmental conditions. All of these should be taken under consideration to control assessor performance and improve the quality of GC-O data.

15.3.1 Methodologies

Different GC-O methods have been developed to evaluate the contribution of the individual odorants by GC-O in foods (Van Ruth, 2001; Van Ruth and O'Connor, 2001; Ferreira *et al.*, 2002; d'Acampora Zellner *et al.*, 2008; Brattoli *et al.*, 2013) (Table 15.3).

Table 15.2 Different factors that affect the response of assessors (adapted from Delahunty *et al.*, 2006).

Assessor Bias	Ability of human olfactory sense	Assessor training	Recommendation
Anticipation error	Presence of specific anosmia	Screen for sensitivity	Not smoking, eating or drinking flavoured foods 1 hour before
Learning of retention times	Variation over time	Ability to concentrate	Not wear perfumes or deodorants
Lack of concentration or breathing cycle.	Health status and mood	Ability to recognise odours	
Fatigue		Training for description of odour qualities and intensities	

15.3.1.1 Dilution analysis method

Dilution analysis consists on sniffing the dilutions of an extract until no odours are detected. There are different modalities: Aroma Extract Dilution Analysis (AEDA) (Ullrich and Grosch, 1988); and Charm Analysis (Acree *et al.*, 1984). In AEDA, the last dilution where a compound is detected corresponds to its dilution factor (FD factor). In Charm Analysis, the time that the odour eluted is recorded, and the odour duration of assessors is combined to yield an aromagram with peaks and areas (Charm values). In this dilution method, the number of assessors used is generally low.

As the number of dilutions for a single extract can be around 8 to 12, only 1–2 assessors are involved in the evaluation of all dilutions (Delahunty *et al.*, 2006) while, in Charm analysis, the quantification requires three assessors (d'Acampora Zellner *et al.*, 2008). Therefore, the main limitations of results are:

- it depends on individual sensitivity.
- it does not consider odorant lost during the isolation and concentration process;
- data are not adapted for statistical manipulation;
- it is extremely laborious and time consuming; and
- it does not consider the food matrix (Curioni and Bosset, 2002).

In addition, the most potent compound is the one perceived at the highest dilution, so this technique considers the response to an odour stimulus to be linear, and all compounds have identical response with concentration (Van Ruth, 2001). This technique has been widely used in the analysis of aroma compounds in different foods (Table 15.3), such as coffee (Grosch, 1998), cheese (Curioni and Bosset, 2002) and salami (Söllner and Schieberle, 2009). In addition, a modification of this technique using SPME as extraction process, and diluting the sample by adjusting the GC injector split ratio, has been applied to Chinese dry cured ham (Xiao-Sheng Liu *et al.*, 2014).

15.3.1.2 Detection frequency method

The detection frequency method measures the intensity of an odour on the basis of the number of assessors who detect it. It was first used by Linssen *et al.* (1993), although

Table 15.3 Olfactometry studies in different foods and main identified odorant compounds.

Food	Olfactometry		Objective	Aroma compound – odour description	Reference
	Extraction technique	technique			
Coffee	Solvent extraction	AEDA	Potent odorants	● 4-Hydroxy-2,5-dimethyl-3(2H)-furanone-Caramel like ● 4-Vinylguaiaicol- Spicy ● Acetaldehyde – fruity pungent ● 2,3-Butanedione-buttery	Grosch, 1998
Cheese	Vacuum distillation	AEDA/Charm	Odorants in different cheese types	● 4-Methylphenol-musty, phenolic ● Straight chain fatty acids (C3 to C10): from sour, acetic, cheese, sweaty, sour, dirty sock, stable, wood, sweaty, fatty, rancid) ● Branched fatty acids: sweaty, rancid, goat. ● Aromatic fatty acids: flowery, honey-like.	Curioni and Bosset, 2002
Beef	Purge and trap	Detection frequency	Beef meat from different breeds	● Hydrogen sulfide-sulfury, sweaty ● Methanthiol- sulfury, sweaty ● 3-Methylbutanal-caramel, green, nutty ● Dimethyl trisulfide-sulfury, onion ● 2,3-Diethyl-5-methylpyrazine-meaty, nutty, burnt	Machiels <i>et al.</i> , 2004
Beef	SPME	Detection frequency	Beef flavour, roasted and boiled beef	● 2,4-Nonadienal-meaty, burnt ● 2-Methyl-3-furanthiol-meaty, ham, rancid ● 3-Carene-floral, smoky ● Terpinene-grassy, plastic ● 2-Ethyl-3,6-dimethylpyrazine-meat, smoky ● Nonanal-grassy, vegetable	Moon <i>et al.</i> , 2006

Dry fermented sausage	SPME	Detection frequency	Effect of different curing agents (nitrate vs nitrite) on aroma	● Methanethiol-rotten eggs ● Acetic acid-vinegar ● Hexanal-fresh cut grass ● Ethyl butanoate-strawberry ● Butanoic acid-cheese ● 1-Octen-3-ol-mushroom ● Octanal-geranium, herbal ● Acetic acid-pungent, sour ● Acetaldehyde- ● Methional-cooked potato ● Phenylacetaldehyde-honey like ● 2-Methoxyphenol-smoky ● 2-Acetyl-1-pyrroline-roast, popcorn like ● Butanoic acid-sweaty, cheese	Marco <i>et al.</i> , 2007
Hungarian salami	SAFE	AEDA	Aroma reconstitution	● 3-Methylbutanal-caramel, green ● Hexanal-green ● Nonanal-grassy, green ● 1-Octen-3-ol-mushroom ● 2-Methyl-3-methylthiofuran-meaty, onion ● 2,5-Dimethylthiophene-beef, sweet, rancid	Söllner and Schieberle, 2009
Beef-like process flavour	SPME	Detection frequency	Effect of degree of hydrolysis in aroma	● Ethanethiol-sulfurous, fruity ● Hexanal-fatty, green ● Isovaleric acid-rancid, cheese ● 1-Octen-3-one-Mushroom ● Octanal-fatty, orange ● 2-Nonanone-creamy, fatty ● 2-Decenal-oily, grassy	Song <i>et al.</i> , 2010
Cooked ham	Dynamic headspace	Modified Detection frequency, Acquisniff®	Nitrite effect on aroma		Thomas <i>et al.</i> , 2013

(Continued)

Table 15.3 (Continued)

Food	Extraction technique	Olfactometry technique	Objective	Aroma compound – odour description	Reference
Jinhua ham (dry cured ham)	Dynamic headspace and SAFE	Dynamic headspace dilution and AEDA	Generation of aroma compounds during processing	● Hexanal-cut grass ● Octanal-fatty ● 2,6-Dimethylpyrazine-toast, nutty ● Butanoic acid-cheesy ● 3-Methylbutanoic acid-sour, sweaty ● E,E-2,4-Decadienal-fatty, fried	Xiao-Sheng Liu <i>et al.</i> , 2014
Japanese soy sauce	SPME	AEDA (adjusting the GC injector split ratio)	Comparison of aroma dilution methods (diluting sample vs GC injector split ratio)	● Ethyl 2-methylpropanoate-fruity ● Ethyl 2-methylbutanoate-fruity, sweet ● Ethyl 3-methylbutanoate-fruity ● Ethyl 4-methylpentanoate-fruity ● 3-(Methylthio)propanal-cooked potato like ● 1-Octen-3-ol-mushroom ● 2-Methoxyphenol-burnt, woody	Feng <i>et al.</i> , 2015
Cooked ham	Simultaneous distillation extraction	Detection frequency (Nasal impact factor; NIF)	Effect of intramuscular fat in cooked cured pork aroma	● Hexanal-fatty, rancid ● Furfuryl mercaptan-coffee, meaty ● Benzaldehyde-almond, sweet ● E,E-2,4-Decadienal-fatty, broth	Benet <i>et al.</i> , 2016

Pollien *et al.* (1997) developed it in order to have a method without a panel training requirement. Pollien *et al.* (1997) not only recorded the description of the odour, but also the odour duration, in order to combine and normalise the individual data. This resulted in what is called nasal impact frequency (NIF) – that is, the number of assessors who detect the odour. Additionally, peak area ($\text{NIF} \times \text{odour duration}$) was defined as the surface of NIF (SNIF). Simplicity, and the absence of assessors' training, are the main advantages. However, the main limitation is the difficulty in appreciating differences with increasing concentrations when all assessors detect the same odour, because results are not based on real intensities.

Detection frequency has been applied to different foods (Table 14.3), beef (Machels *et al.*, 2004; Moon *et al.*, 2006), dry sausages (Marco *et al.*, 2007), beef-like flavour (Song *et al.*, 2010) and cooked ham (Benet *et al.*, 2016). The aromagram of a reduced salt dry fermented sausage obtained by GC-FID-O, and using the detection frequency method, is shown in Figure 15.2 (Corral *et al.*, 2013). The left part of the figure represents the FID signal while, on the right hand, is shown the aromagram

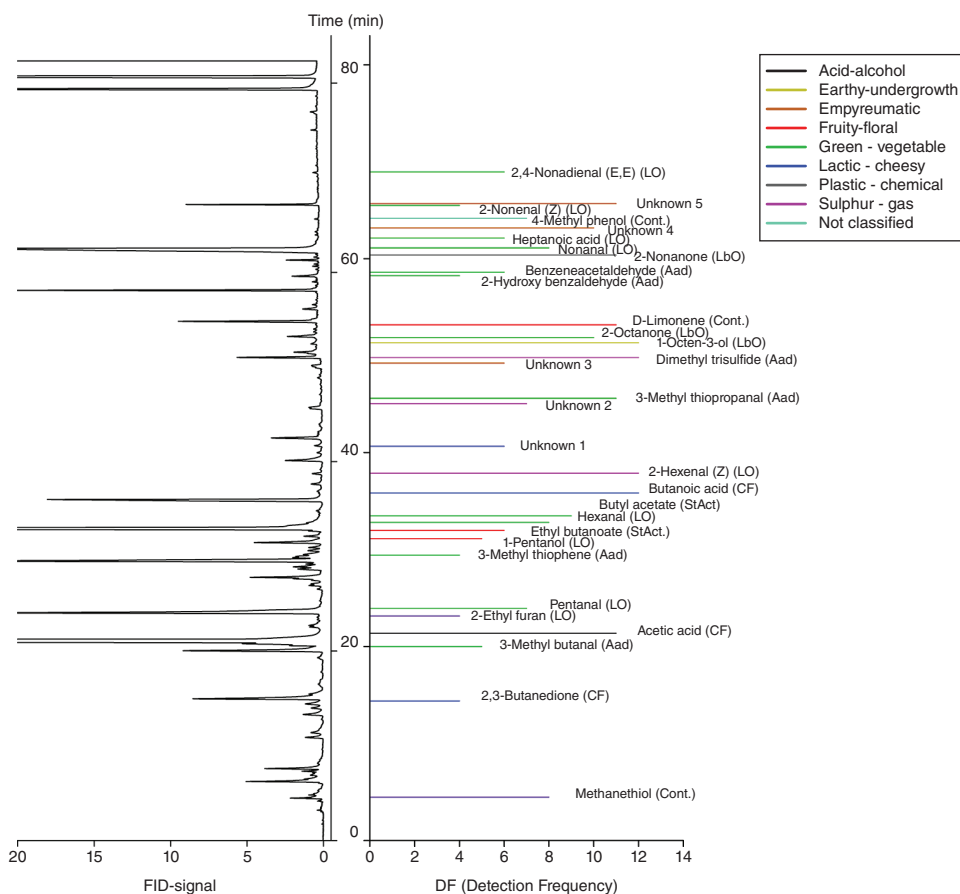


Figure 15.2 Aromagram of a reduced salt dry fermented sausage obtained by GC-FID-O and using the detection frequency method.

with the detection frequency values and chemical identity of compounds for each recognised aroma. Aromas were classified by colours, depending on the evolved aroma class.

15.3.1.3 Direct intensity method

The method that measures the intensity of the odour is called the direct intensity method (Delahunty *et al.*, 2006). Two different methods have been defined: posterior intensity and time intensity methods. Posterior intensity methods measure the perceived intensity of an odour eluting from the GC based on a scored scale. Their main limitation is the high variability among assessors using the scored scale, due to a lack of training. However, it has been proposed to use scale anchors (although this is not feasible during a GC run) (Van Ruth, 2001).

On the other hand, in time intensity methods (OSME), assessors directly record the intensity and the duration for each aroma compound (McDaniel *et al.*, 1990; Van Ruth and O'Connor, 2001) so that a peak area for each odour can be calculated. This technique uses several assessors (4–6), who record the intensity using a slide bar, and the signal is recorded. The results are averaged among assessors, and produce peaks with odour intensities as a function of time. Several modifications of the recording intensity method have been used by Delahunty *et al.* (2006) and Etiévant *et al.* (1999) to improve the use of the scale. However, this method requires a degree of assessor training in order to have reproducible results.

15.3.2 Use of GC-O methodologies

The most widely used methods in aroma analysis are dilution and detection frequency methods (Table 15.3). However, as results also depend on the isolation technique used, they may be different. This is so in the case of the aromagrams of a dry fermented sausage obtained using SAFE and SPME, and using AEDA and detection frequency methods, respectively (Corral *et al.*, 2015). Odours with high linear retention indices were obtained using SAFE/AEDA (Figure 15.3a), while a high number of aroma compounds with low linear retention indices were obtained using SPME and detection frequency methods (Figure 15.3b).

Furthermore, several authors have used a combination of methodologies, where detection frequency and direct time intensity methods have been used. This modified detection frequency method takes into account the detection frequency and the average intensity indicated by the panellists (Resconi *et al.*, 2010), as also proposed by the acquisition and processing software AcquiSniff® for data analysis (Berdagué *et al.*, 2002).

The comparison of different GC-O methods by Van Ruth and O'Connor (2001) showed that the data obtained using posterior intensity and frequency methods resulted in similar odour intensities to those evaluated by headspace analysis. Nevertheless, these authors emphasised the necessity of using a large number of assessors to obtain reliable data, due to differences in odour threshold among individuals. In addition, d'Acampora Zellner *et al.* (2008) indicated that detection frequency and posterior intensity methods provide similar odour intensities/concentration relationships, while the dilution method provides the attribute odour potency.

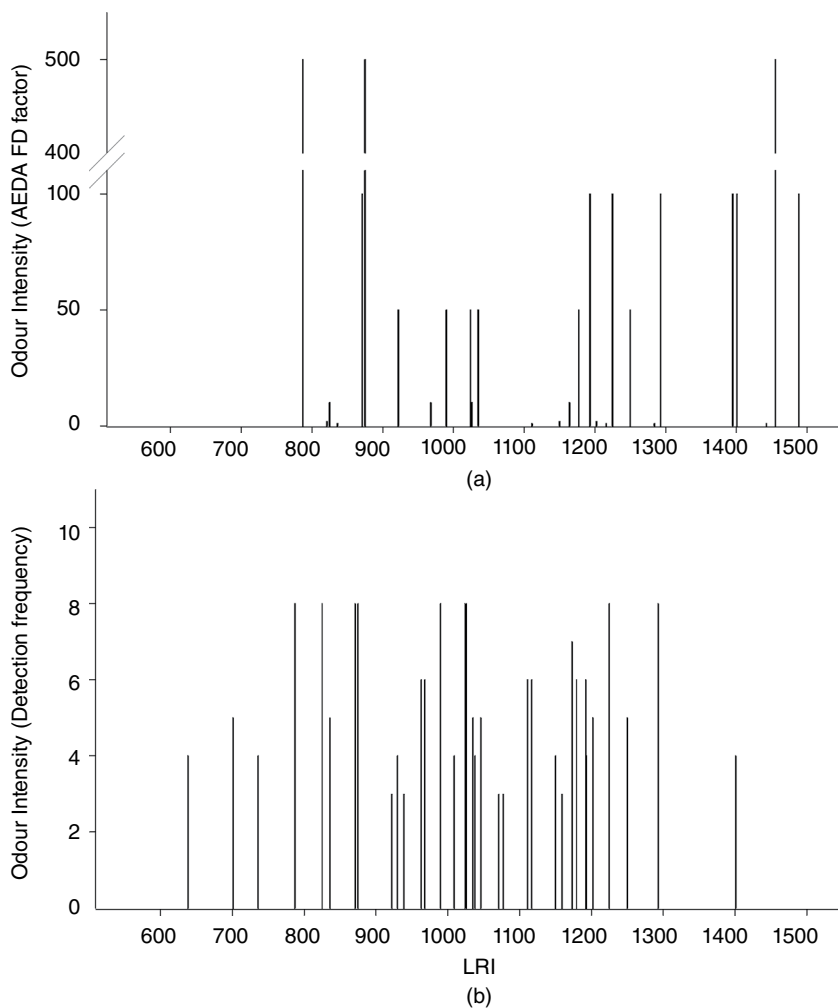


Figure 15.3 Comparison of the aromagram of a dry fermented sausage obtained using SAFE/GCO-AEDA (A) and SPME/GCO-detection frequency (B) methods.

15.4 Applications of GC-O in food industry

The goal of using GC-O in food industry may have different approaches, but it is the most frequently used to reveal the composition of the volatile compounds with aroma impact in the food, and to reformulate food aromas. Other applications may be the identification of off-flavours, control production processes, and control of raw materials.

15.4.1 Identification of key aroma compounds in different foods

The application of GC-O in aroma analysis allows the preparation of synthetic blends whose aromas are close to the original food (Grosch, 2001). The screening for potent

odorants in foods starts with the separation of the volatile in an extract by chromatography analysis, detection by GC-O using AEDA or other techniques, quantification and calculation of OAVs, reformulation of the synthetic blend and comparison of the aroma with the original and, finally, evaluation of the synthetic blend with omitting one or more compound by omission experiments (Grosch, 2001).

Generally, the odorants with high OAVs are essential for the aroma. When odorants are mixed, there is a large loss of sensory properties of the individual constituents; hence, the reason for finding odorants with lower OAVs as important contributors (Laing, 1994). GC-O is also used to look for potent odorants present at trace levels (Delahunty *et al.*, 2006). This is the case of nitrogen and sulfur compounds, which have been detected as potent odorants in meat (Rochat *et al.*, 2007) and meat products (Corral *et al.*, 2016), due to their low odour thresholds and low concentrations. These compounds contribute to different aroma notes obtained by olfactometry, as shown in Table 15.4. The aroma notes that have been described in meat products varies from onion and garlic notes to earthy and roasted notes coming from sulfur and nitrogen compounds, respectively. However, the odour notes depend on the chemical structure, and these compounds have been found in many different type of foods, including cheese, meat, coffee, wine and snacks (Vermeulen *et al.*, 2005; Curioni and Bosset, 2002).

Table 15.4 Nitrogen and sulfur aroma compounds detected in meat products by GO-O and odour description.

Chemical group	Descriptor by GC-O	Authors
Sulfur compounds		
Dimethyl disulfide	Garlic, caramel, bouillon, toasted	4,10
Dimethyl trisulfide	Cabbage, sulfur, unpleasant	6
Diallyl sulphide	Garlic	4,5
Diallyl disulphide	Garlic	4,5
Methyl ethyl sulfide	Rotten onion, unpleasant	11
Methyl ethyl disulfide	Onion, pungent	1
Methyl allyl sulfide	Garlic	1,3,4,5,9
Methyl allyl disulfide	Garlic	4
Dipropyltrisulfide	Meaty, bread, toasted	3
bis(2-methyl-3-furyl)disulfide	Cooked meat-like	9
Thiols		
Methanethiol	Rotten eggs, cauliflower	8,11
2-Furylmethanethiol	Roasty, sulfurous	9
2-Methyl-3-furanthiol	Roasted meat	6,9
Others		
Methional	Onion, cooked-potato, meat, broth, savoury snacks, rancid	7,8,9,10,11
2-Methyl-3-methyldithiofuran	Roasty, meaty	6

Table 15.4 (Continued)

Chemical group	Descriptor by GC-O	Authors
Thiazolines		
2-Acetyl-2-thiazoline	Roasty, popcorn-like	9
Pyrrole derived		
1-Methylpyrrole	toasted, boullion	10
2-Acetylpyrrole	Roasted nuts, fried snacks	10
2-Acetyl-1-pyrroline	Roasty, popcorn	7,9
Pyrazines		
Dimethylpyrazine	Coffee, toasted	2
2-Ethyl-3,5(6)-dimethylpyrazine	Earthy	9
Tetramethylpyrazine	Toasted sugar	10
2,3-Diethyl-5-methylpyrazine	Earthy	9
3-Isopropyl-2-methoxypyrazine	Earthy, pea-like	9
3-Isobutyl-2-methoxypyrazine	Earthy, pea-like	9
Indoles		
3-Methylindole	Naphthalene	5

- 1) Stahnke (1994)
- 2) Stahnke (1995)
- 3) Meynier *et al.* (1999)
- 4) Schmidt and Berger (1998a)
- 5) Schmidt and Berger (1998b)
- 6) Chevance *et al.* (2000)
- 7) Blank *et al.* (2001)
- 8) Marco *et al.* (2007)
- 9) Söllner and Schieberle (2009)
- 10) Gianelli *et al.* (2011)
- 11) Olivares *et al.* (2011).

In recent years, comprehensive two-dimensional gas chromatography with olfactometry has been used to assess the quality of food products. Although its complexity in instrumental design and data analysis reduce its application in routine flavour research (Cordero *et al.*, 2015), this methodology has been used to characterise key odorants, to correlate key odorants with sensory data and to identify marker compounds to predict aroma profiles. This technique improves the capacity of conventional CG-O techniques to resolve complex odour zones where odours co-elute or are masked by an odourless peak (Delahunty *et al.*, 2006). Giri *et al.* (2015) showed an example of an aroma zone in cooked meat, with several aroma compounds co-eluting (Figure 15.4). The upper part of the chromatogram represents the MS signal, while the bottom part represents the olfactory signal. The use of heart-cut GC-GC-MS/olfactometry allows the identification of the individual compounds, as shown in the second dimension, where the compounds are identified and their odours individually shown.

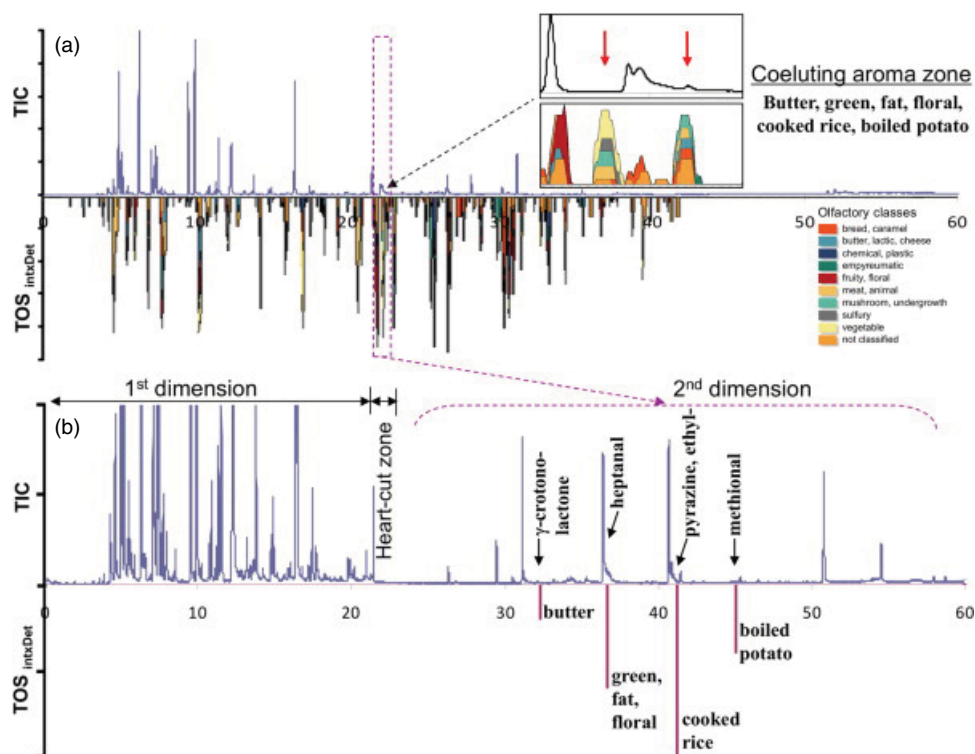


Figure 15.4 Cooked meat sample showing the aroma zone where different odours co-eluted analysed by GC-MS/O (a) and heart-cut GC-MS/O (b). Source: Giri *et al.*, 2015. Reproduced with permission of Elsevier.

15.4.2 Identification of off-flavours for quality control

Gas chromatography with olfactometry is often used for quality control of off-flavours in foods like fish samples, heated milk, and so on (Wilkes *et al.*, 2000), and also for the identification of musty odours in water and wine (Callejón *et al.*, 2016).

A recent study on fresh beef meat characterised by the presence of strange odours was carried out by GC-O and detection frequency method. The study resulted in the identification of unusual cabbage and rancid odours (Figure 15.5) that are not usually detected in raw beef meat (Corral and Flores, unpublished data). The use of GC-O is essential to determine the compounds responsible for the off-flavour; however, fast analytical techniques have been proposed to be applied in quality control; such as the use of electronic noses or direct mass spectrometry techniques (PTR-MS or SIFT-MS) (Flores *et al.*, 2013), due to their advantages, especially in fast measurements.

Another example of the application of GC-O is the detection, in the manufacture of dry-cured ham, of an off-flavour called 'bone taint', or deep spoilage that produces expensive losses in production. This problem is produced by an undesirable microbial population. The odorants responsible for this off-flavour in spoiled hams were identified by the GC-O and detection frequency method. These taint odorants were also

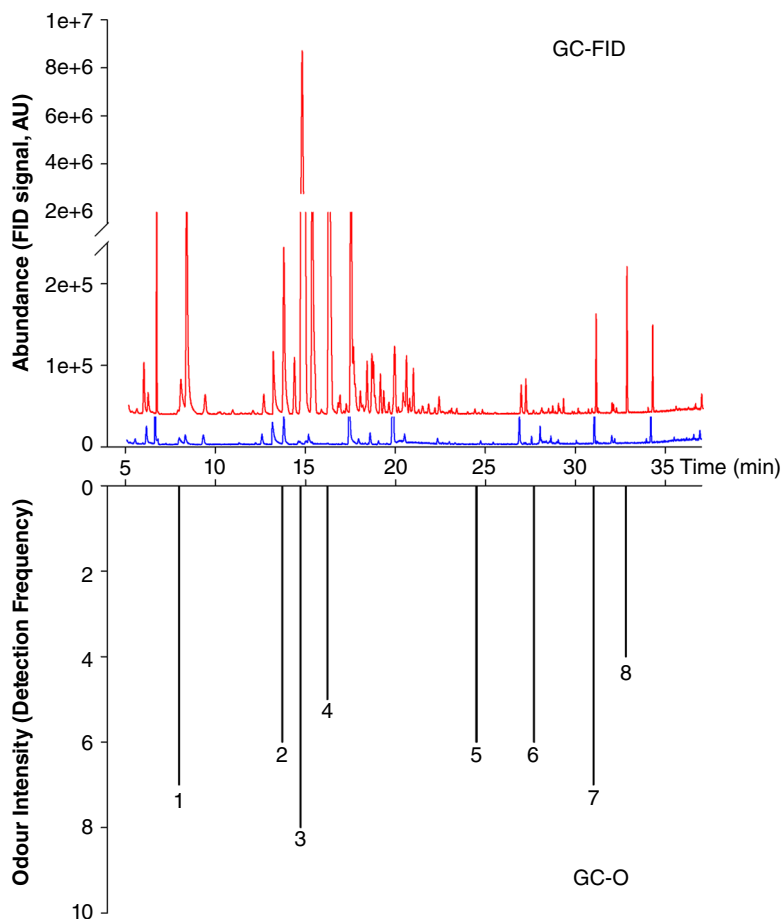


Figure 15.5 Comparison of raw beef meat flavour from normal (lower signal) and off-flavour (upper signal) and aromagram (detection frequency) of the off-flavour raw beef. Odours: 1: butter, oxidized, 2: rotten, oxidized iron, 3: garlic, cabbage, rancid, 4: garlic, cabbage, rancid, 5: cabbage, rancid, 6: stable, dung, 7: spicy, herbal, 8: toasted, cured.

detected in unspoiled hams, although, in this case, the detection frequency values were different than from spoiled hams (Carrapiso *et al.*, 2010).

15.4.3 Application of GC-O to production processes

Knowing the key aroma impact compounds in foods allows the identification of their metabolic origin and, consequently, this knowledge is used to improve food processing.

The generation of aroma compounds in meat products has been studied, as well as the many processing factors that affect it (Toldrá and Flores, 2007). Generally, in meat products, odour-active compounds are derived from protein and lipid degradation processes (Flores, 2009). GC-O, combined with AEDA, has been used to study the aroma generation in different meat products. The processing of dry cured ham has been evaluated to determine the key odorants and the ripening days necessary to obtain this

odorants (Flores *et al.*, 1997; Carrapiso *et al.*, 2002). Not only long ripening times are involved in the development of aroma compounds, but also several processing factors, such as salt concentration, ripening temperature, intramuscular fat content and so on, affect the generation of key aroma compounds, as analysed by GCO and detection frequency method (DF) (Carrapiso and García, 2004).

In other types of meat products, such as dry fermented sausages, the study of the aroma compounds generated through processing has been reviewed, indicating the most potent odorants (Flores and Olivares, 2015). However, the effect of processing factors, such as the use of different curing salts, nitrate versus nitrite, has shown an effect on the generation of aroma compounds when studied by GC-O (Marco *et al.*, 2007). The use of nitrite or nitrate has not been related to a specific odour compound, but differences in detection frequency values have been detected in several odorants.

A similar approach in cooked ham was studied by Thomas *et al.* (2013), who reported the effect of the use of nitrite on the generation of key odour compounds, by GC-O and a modified detection frequency method (Acquisniff®). These authors showed that the aroma of cooked ham is a balance between sulfur compounds and oxidation compounds, but this balance is disturbed in the absence of nitrite. In addition, the effect of intramuscular fat content on cooked cured pork ham aroma was evaluated by GC-O and detection frequency method (Benet *et al.*, 2016). This study showed that not only the intramuscular fat content, but also its composition in polyunsaturated fatty acids, affects the generation of odour compounds derived from Maillard reaction, affecting the sensory characteristics of the hams.

The identification and quantification of potent odorants in cheeses with different fat content was studied by Milo and Reineccius (1997) by GC-O and AEDA method, revealing the role of specific aroma compounds in cheese flavour. Another approach in GC-O analysis is the study of the aroma changes produced during the thermal treatment of different foods. A study of coffee flavour by GC-O and AEDA, together with GC-O and headspace analysis, was performed on roasted coffee to identify the key odour compounds (Grosch, 1998). The study identified several sulfur odour compounds during roasting, which increased in concentration, and the contribution of various phenols to smoky notes. In addition, the GC-O and AEDA analysis of a brew prepared from ground coffee revealed that the changes in aroma profile are due to changes in odorant concentration, and not in the formation of new odorants.

Other studies applied GC-O to study the generation of carbonyl compounds during grilling of beef meat from different feeds (Resconi *et al.*, 2012), using GC-O and a modified detection frequency method that takes into account the detection frequency and the average intensity indicated. The results showed that the diet system used in beef production – pasture or concentrates – affects the generation of aldehydes aroma compounds. The effect of diet has also been studied in lamb meat by GC-O and modified detection frequency method (Resconi *et al.*, 2010), revealing that feeds finished on pasture had less lipid-derived unsaturated aldehydes and ketones, probably due to the antioxidants present naturally in the pasture diet. However, there are many other processing factors that affect meat aroma.

GC-O was also applied to study the key aroma compounds in fresh and frozen lamb meat by GC-O and a modified detection frequency method (Bueno *et al.*, 2011). The study revealed that several pyrazine aroma compounds were richer in fresh lamb grilled samples than in frozen ones.

Nowadays, GC-O may be also applied to control the materials used for food processing. In wine production, the use of rotten or mouldy fruits is controlled by GC-O, as a way to prevent the appearance of undesirable off-flavours such as geosmin, 2-methylisoborneol and chloroanisoles (Plutowska and Wardencki, 2008). GC-O is also applied to evaluate the character-impact aroma compounds produced in reaction flavours or also named as process flavours. The production of process flavours is a Maillard reaction process, where a reducing sugar and a food-grade nitrogen source react under controlled conditions to produce an aroma similar to the one produced in thermally processed foods (Kerler *et al.*, 2010). This is an important process for the flavour industry that requires the use of GC-O, together with the organoleptic evaluation, as shown in the evaluation by GC-O and detection frequency method of a beef-like process flavour (Song *et al.*, 2010), or by GC-O and AEDA applied to a meat-like process flavour (Yang *et al.*, 2015). All of these examples have summarised the applicability of GCO in food processing.

15.4.4 Application of GC-O to reformulation of food aromas

The aroma analysis concept indicates that GC-O is not only applied for the identification of key aroma compounds, but also for the reformulation of food aromas (Grosch, 1998, 2001). The use of GC-O and dilution techniques allows for the identification of key aroma impact compounds. These compounds are then quantified using isotopomers, by the procedure called isotope dilution assay (IDA), where the aroma extract is spiked with a known quantity of the isotopomers. This process allows the calculation of the odour activity values (OAV) of the compounds. Those compounds with the highest OAVs are used to prepare a model mixture that is sensory-analysed to resemble the food aroma. Moreover, the contribution of each component to the food aroma is evaluated through omission experiments, where a single component is omitted. This process has been performed to reformulate the aroma of a high number of foods, such as boiled beef, tomato paste, wine, olive oil, French fries and roasted coffee (Grosch, 2001).

Moreover, in the last few years, several studies have used this sensomic approach to study the aroma active compounds by using GC-O and AEDA analysis in hazelnuts (Burdack-Freitag and Schieberle, 2012), wheat beers (Langos *et al.*, 2013), Sherry wine (Marcq and Schieberle, 2015), rape honey (Ruisinger and Schieberle, 2012), Andes berry (Sinuco *et al.*, 2013), roasted duck liver (Straßer and Schieberle, 2014) and dry sausage (Corral *et al.*, 2015), among other foods. In summary, GC-O is an essential technology of the aroma analysis concept, as it is the first step for the selection of key aroma compounds.

15.5 Conclusions

Gas chromatography-olfactometry (GC-O) for food aroma analysis has been used for many years, and has demonstrated its applicability in the food sector from the identification of key aroma compounds, applicability to production processes and quality control, and aroma reformulation. The advancement in GC-O has been the design of an olfactometry port integrated into the GC software, and the design of olfactometry

methodologies capable of overcoming the disadvantages of assessors. Nowadays, new instrumental designs, such as comprehensive two-dimensional gas chromatography coupled to olfactometry, improve the capacity of conventional CG-O techniques to resolve complex odour zones. However, the complexity and data analysis reduce its application in food aroma research.

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16

Data Handling

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16.1 Introduction

In this chapter, the fundamentals of chemometrics will be presented by means of a quick overview of the most relevant techniques for data display, classification, modelling and calibration. The goal of the chapter is to make people aware of the great superiority of multivariate analysis over the commonly used univariate approach. Mathematical and algorithmical details will not be presented, since the chapter is mainly focused on the general problems to which chemometrics can be successfully applied in the field of food chemistry.

Many of the readers of this book may not be familiar with chemometrics, and a significant percentage of them may have never even heard of this 'new' science (quite strange that it is still considered a 'new' science, when the Chemometrics Society was founded more than 60 years ago, and the most basic algorithms date back to the beginning of the 20th century). Furthermore, some of them could be quite put off by anything involving mathematical computations higher than a square root, or statistical tests more complex than a *t*-test.

Therefore, the goal of this chapter is simply that of being read and understood by the majority of the readers of this book. This goal will be completely achieved if some readers, after having read it, could say: 'Chemometrics is easy and powerful indeed, and from now on I will always think in a multivariate way'.

Of course, to accomplish this goal in the limited space of a chapter, the attractive sides of chemometrics must be highlighted. Therefore, the intuitive aspects of each technique will be shown, without giving too much relevance to the algorithms.

First of all, what is chemometrics? According to the definition of the Chemometrics Society, it is '*the chemical discipline that uses mathematical and statistical methods to design or select optimal procedures and experiments, and to provide maximum chemical information by analyzing chemical data*'.

One of the major mistakes that people make about chemometrics is thinking that, to use it, one has to be a very good mathematician, and to know the mathematical details of the algorithms being used. From the definition itself, it is clear instead that a chemometrician is a *chemist* who can *use* mathematical and statistical methods.

If we want to draw a parallel with everyday life, how many of us really know in detail how a TV set, a mobile phone, a car or a washing machine works? But everybody watches TV programs, makes phone calls, drives a car and starts a washing machine. Of course, what is important is that people know what each instrument is made for, and that they can use it properly, no matter how complex the algorithms regulating them can be.

Although chemometrics makes available a very wide range of techniques, some of them being very difficult to fully understand and use correctly, the great majority of the real problems can be solved by applying one of the basic techniques. Understanding these, at least from an intuitive point of view, is relatively easy and does not require high-level mathematical skills.

16.2 Data collection

Chemometrics works on data matrices. This means that, on each sample, a certain number of variables have been measured (in the ‘chemometrical jargon’ we say that each object is described by v variables). Although some techniques can work with a limited number of missing values, a chemometrical data set must be thought of as a spreadsheet in which all the cells are full.

Sometimes, instead, if data are gathered without having any specific project, it happens that the result is a ‘sparse’ matrix, containing some blank cells. In such cases, if the percentage of missing data is quite high, the whole data set is not suitable for a multi-variate analysis. As a consequence, the variables and/or the objects with the lowest number of data must be removed, and therefore a huge amount of experimental effort can be lost.

All the chemometrical software allows the import of data from ASCII files or from spreadsheets. It is, therefore, suggested to organise the data in matrix form from the start, as shown in Figure 16.1, in such a way that the import can be performed in a single step.

If, by contrast, the data are spread in several files or sheets (e.g. one file for each sample or for each variable), then the import procedure would be much longer and more cumbersome.

Together with the numerical data that will be elaborated by the different chemometrical techniques, all the possible sort of metadata should also be collected. The definition

	date	batch number	operator	var. 1	var. 2	var. 3	var. 4	...	var. v
obj. 1									
obj. 2									
obj. 3									
obj. 4									
obj. 5									
obj. 6									
.....									
obj. n									

Figure 16.1 The structure of a chemometrical data set.

of metadata is ‘*data that provide information about the other data*’, and some examples are:

- date of sample collection
- date of sample analysis
- instrument on which the analysis has been performed
- operator who has performed the analysis
- type and size of the packaging of the sample
- ...

All this information can be extremely useful in successfully interpreting anomalies or trends detected by the data analysis.

It is also very important that the order of the samples in the file corresponds to the chronological order (or at least that a metadata allowing to go back to it is present). If not, all the time-related information will be lost.

16.3 Data display

The human mind can digest much more information when looking at plots rather than numbers. This is easily demonstrated by looking first at the sequence of numbers reported in Table 16.1, and then the plot in Figure 16.2.

It is very clear that, even in a very simple data set like this one (just ten samples, and only one variable) the information obtained by looking at the plot is superior, and much more easily available, than the information one can get by analysing the raw numbers. From the plot, it becomes evident that the samples are clustered into two groups of the same size, with the one at higher values being much tighter than the one at low values. Much more time and effort is required when we want to get the same information from the table.

Let us now take into account a more complex data set – the one reported in Table 16.2, where each object is described by two variables. The same data are plotted in Figure 16.3.

This bivariate data set, beyond showing once more that a plot is much more easily handled by the human brain than a data table, demonstrates that, when dealing with more than one variable, the analysis of just one variable at a time can lead to the wrong

Table 16.1 Ten samples described by one variable.

Sample	1	2	3	4	5	6	7	8	9	10
Value	25.3	22.1	25.5	25.6	19.4	25.7	20.2	21.3	25.9	21.8

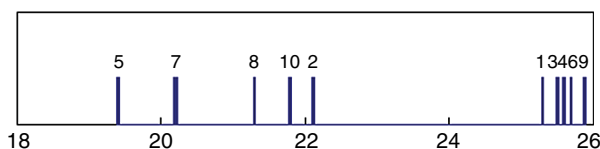


Figure 16.2 Scatter plot of the data in Table 16.1.

Table 16.2 Twenty samples described by two variables.

Sample	Variable 1	Variable 2
1	21.2	32.5
2	16.2	21.0
3	13.1	21.7
4	11.6	21.3
5	20.8	29.9
6	10.4	20.6
7	19.5	26.8
8	9.8	25.2
9	15.2	31.2
10	12.0	26.0
11	17.6	28.5
12	24.0	30.0
13	17.8	33.1
14	15.0	24.0
15	11.0	24.2
16	24.8	25.3
17	12.8	23.3
18	26.5	30.6
19	22.9	27.5
20	9.7	22.8

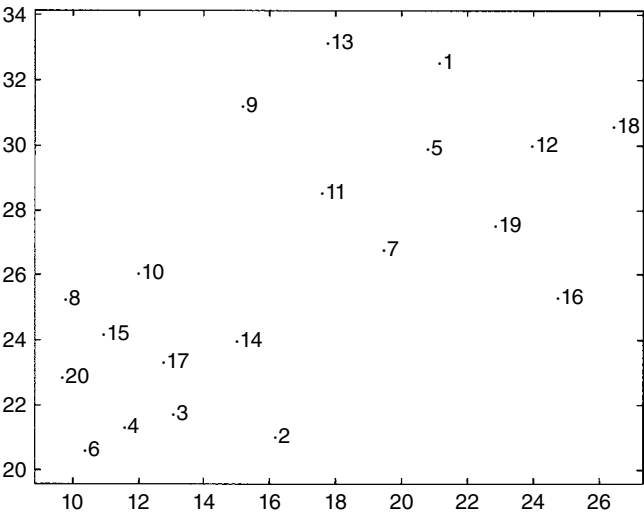


Figure 16.3 Scatter plot of the data in Table 16.2.

results. In this data set, we have 20 samples, supposedly belonging to the same population. When looking at the plot, we realise that we are in a situation very similar to what we found with the univariate data set. The samples are split into two clusters of the same size, with the objects of the first one more tightly grouped than the objects of the second one. This conclusion cannot be reached when looking at one variable at a time, since neither of the two variables is able to discriminate between the two groups.

If we had a data set with three variables, it would still be possible to visualise the whole information by a three-dimensional scatter plot, in which the coordinates of each object are the values of the variables. But what to do if there are more than three variables? What we need, therefore, is a technique permitting the visualization by simple bi- or tridimensional scatter plots of the majority of the information contained in a highly dimensional data set. This technique is Principal Component Analysis (PCA), one of the simplest and most used methods of multivariate analysis. PCA is very important, especially in the preliminary steps of an elaboration, when one wants to perform an exploratory analysis in order to have an overview of the data.

It is quite common to have to deal with large data tables, in which a set of o objects (samples) is described by ν chemico-physical parameters. Examples of such data sets can be samples of olive oils from different origins described by their content in fatty acids and sterols, or samples of wines described by Fourier-Transformed Infra-Red (FT-IR) spectra. It is easy to realise how, especially in spectral data sets, ν can be very high (>1000). In such cases, it would be impossible to obtain valuable information without the help of multivariate techniques.

From a geometrical point of view, we can consider a ν -dimensional space, in which each dimension is associated to one of the variables. In this space, each sample (object) has coordinates corresponding to the values of the variables describing it.

Since it is impossible to visualize all the information at once, one should be content with the analysis of several bi- or tridimensional plots, each of them showing a different part of the global information.

It is also evident that not all possible combinations of two or three variables will give the same quality of information. For instance, if some variables are very highly correlated, then the information brought by each of them would be almost the same. If two variables are perfectly correlated, then one of them can be discarded, losing no information at all. In this way, the dimensionality of our space will be reduced from ν to $\nu - 1$. If two variables are very highly correlated, then taking into account a new variable which is a combination of the two original ones would produce only a slight loss of information, while the dimensionality of the space would be reduced to $\nu - 1$.

It is quite apparent now that not all the dimensions have the same importance and that, owing to the correlations among the variables, the 'real' dimensionality of our data matrix is somehow lower than ν . Therefore, it would be very valuable to have a technique capable of concentrating, in a few variables – and therefore in a few dimensions – the bulk of our information. This is exactly what is performed by PCA: it reduces the dimensionality of the data and extracts the most relevant part of the information, placing into the last dimensions the non-structured information – that is, the noise. According to these two characteristics, the information contained in very complex data matrices can be visualised in just one or a few plots.

From a mathematical point of view, the goal of PCA is to obtain, from ν variables ($X_1, X_2, \dots, X_\nu$) and o samples, a certain number of linear combinations having two

important features: to be uncorrelated and to be ordered according to the explained variance (i.e. to the information they contain). The lack of correlation among the linear combinations is very important, since it means that each of them describes different ‘aspects’ of the original data. As a consequence, the examination of a limited number of linear combinations (generally the first two or three) allows us to obtain a good representation of the studied data set.

From a geometrical point of view, what is performed by PCA corresponds with looking for the direction which, in the ν -dimensional space of the original variables, brings the greatest possible amount of information (i.e. explains the greatest variance). Once the first direction is identified, the second one is looked for – it will be the direction explaining the greatest part of the residual variance, under the constraint of being orthogonal to the first one. This process goes on until a stop criterion (number of directions or total variance explained) has been satisfied, and c directions have been computed.

These new directions can be considered to be the axes of a new orthogonal system, obtained after a simple rotation of the original axes. In the original system, each direction (i.e. each variable) brings with it, at least in theory, $1/\nu$ of total information. However, in the new system, the information is concentrated in the first directions and decreases progressively so that, in the next ones, no information can be found except noise.

The global dimensionality of the new system (c) is smaller than that of the original data (ν), since only the first dimensions (the ‘significant components’) bring information and are taken into account. The projection of the objects in this space of reduced dimensionality retains almost all the information that can now also be visualized by bi- or tri-dimensional plots. These new directions, linear combinations of the original ones, are the Principal Components (PC) or Eigenvectors.

With a mathematical notation, we can write:

$$\text{var}(Z_1) > \text{var}(Z_2) > \dots > \text{var}(Z_c)$$

where $\text{var}(Z_i)$ is the variance explained by component i .

The first PC is formed by the linear combination:

$$Z_1 = a_{11}X_1 + a_{12}X_2 + \dots + a_{1\nu}X_\nu$$

explaining the greatest variance, under the condition:

$$\sum a_{1i}^2 = 1$$

This last condition notwithstanding, the variance of Z_1 could be made greater simply by increasing one of the values of a .

The second PC:

$$Z_2 = a_{21}X_1 + a_{22}X_2 + \dots + a_{2\nu}X_\nu$$

is the one having variable (Z_2) as large as possible, under the conditions that:

$$\sum a_{2i}^2 = 1$$

and that:

$$\sum a_{1i}a_{2i} = 0$$

(this last condition ensures the orthogonality of components one and two).

The lower order components are computed in the same way, and always under the two conditions previously reported.

It is now interesting to locate each object in this new reference space. The coordinate on the first PC is computed simply by substituting into equation $Z_1 = a_{11}X_1 + a_{12}X_2 + \dots + a_{1c}X_c$ the terms X_i with the values of the corresponding original variables. The coordinates on the other principal components are then computed in the same way.

These coordinates are named scores, while the constants a_{ij} are named loadings.

By taking into account the loadings of the variables on the different principal components, it is very easy to understand the importance of each single variable in constituting each PC. A high absolute value means that the variable under examination plays an important role for the component, while a low absolute value means that it has a very limited importance.

If a loading has a positive sign, it means that the objects with a high value of the corresponding variable have high positive scores on that component. If the sign is negative, then the objects with high values of that variable will have high negative scores. As already mentioned, after a PCA, the information is mainly concentrated on the first components. As a consequence, a plot of the scores of the objects on the first components allows the direct visualisation of the global information in a very efficient way. It is now very easy to detect similarity between objects (similar objects have a very similar position in the space), or the presence of outliers (which are very far from all other objects), or the existence of clusters. Taking into account at the same time scores and loadings, it is also possible to interpret very easily the differences among objects or groups of objects, since it is immediately understandable which are the variables making the greatest contribution to the phenomenon under study.

Mathematically speaking, we can say that the original data matrix $X_{o,v}$ (having as many rows as objects and as many columns as variables) has been decomposed into a matrix of scores $S_{o,c}$ (having as many rows as objects and as many columns as retained components) and a matrix of loadings $L_{c,v}$ (having as many rows as retained components and as many columns as variables). If, as usual, $c < v$, a matrix of the residuals $E_{o,v}$, having the same size as the original data set, contains the differences between the original data and the data reconstructed by the PCA model (the smaller the values of this matrix, the higher the variance explained by the model).

We can, therefore, write the following relationship:

$$X_{o,v} = S_{o,c} * L_{c,v} + E_{o,v}$$

Now, let us see the application of PCA to a real data set (MacNamara, 2005). Twelve variables have been measured by gas chromatography on 43 samples of Irish whiskeys, of two different types. Nineteen samples were from type A, while 24 samples were from type B, with the samples ordered according to the production time. The data are reported in Table 16.3.

Table 16.3 Chemical composition of 43 whiskey samples.

Sample	Type	1) Acetaldehyde	2) Ethyl acetate	3) Acetal	4) Propanol	5) Isobutanol	6) Isoamyl acetal	7) Butanol-1	8) 2-Me-1butanol	9) 3-Me-1butanol	10) Et caproate	11) Ethyl caprylate	12) Et caprate
1	A	80	408	37	583	466	24	15	388	988	3	13	45
2	A	76	327	40	507	483	25	18	396	1033	3	12	46
3	A	79	296	43	467	397	20	17	323	859	4	13	44
4	A	74	415	28	569	407	24	15	352	921	4	13	46
5	A	69	381	29	510	367	21	14	329	870	4	13	46
6	A	66	340	35	428	387	26	13	339	910	4	14	50
7	A	82	373	17	401	337	23	11	297	813	4	13	42
8	A	78	385	34	459	371	19	12	313	843	3	12	41
9	A	67	374	34	458	385	22	12	326	868	3	13	47
10	A	50	331	32	422	345	17	12	307	835	3	12	42
11	A	66	342	30	423	341	17	13	305	846	3	13	43
12	A	54	321	28	408	354	20	13	310	874	4	13	41
13	A	68	344	33	429	333	16	12	300	824	3	11	38
14	A	69	358	37	446	347	17	13	311	855	3	11	37
15	A	78	346	40	411	320	16	12	287	796	3	11	36
16	A	77	387	51	427	345	22	12	290	805	3	10	32
17	A	104	322	72	432	353	18	13	303	823	3	10	35
18	A	84	333	55	421	340	17	13	292	787	3	10	31
19	A	82	382	47	457	328	18	10	278	765	3	10	31
20	B	65	403	18	496	529	19	19	365	1014	3	11	35
21	B	58	352	18	434	457	17	17	312	907	3	8	26
22	B	71	394	25	555	560	18	20	391	1083	3	11	33
23	B	69	369	25	497	500	16	18	349	1005	3	10	29
24	B	83	344	28	489	479	15	17	352	957	3	10	29
25	B	93	344	31	500	481	15	18	352	990	3	10	29
26	B	65	453	18	503	529	21	17	390	1017	3	10	31
27	B	62	405	17	500	488	18	17	357	965	3	9	27
28	B	58	435	16	501	548	21	17	415	1056	3	10	31
29	B	63	459	17	544	575	21	19	426	1100	3	10	28
30	B	99	462	26	490	500	22	16	403	1057	3	10	30
31	B	81	357	21	402	396	16	14	310	814	2	7	17
32	B	80	380	23	497	483	18	17	395	1041	3	10	28
33	B	76	425	22	486	475	22	17	379	1007	4	10	25

Table 16.3 (Continued)

Sample	Type	1) Acetaldehyde	2) Ethyl acetate	3) Acetal	4) Propanol	5) Isobutanol	6) Isoamyl acetal	7) Butanol-1	8) 2-Me-1butanol	9) 3-Me-1butanol	10) Et caproate	11) Ethyl caprylate	12) Et caprate
34	B	79	446	24	446	418	18	14	319	803	3	9	25
35	B	78	461	24	478	458	19	16	352	908	3	9	23
36	B	108	477	29	493	430	16	14	329	811	3	11	28
37	B	111	481	28	494	429	16	15	330	833	3	9	22
38	B	82	408	22	473	431	18	12	317	774	3	10	27
39	B	73	428	20	493	445	18	13	327	804	3	8	20
40	B	102	469	25	490	457	20	11	327	776	3	10	27
41	B	90	463	22	491	452	20	12	324	774	3	9	21
42	B	50	410	14	440	419	19	12	300	704	3	11	28
43	B	61	425	17	445	432	20	12	318	758	3	10	23

Since a trained assessor can easily discriminate a whiskey of type A from a whiskey of type B, it is interesting to know whether this discrimination is possible also on a chemical basis, just taking into account the variables obtained by a routine analysis. When looking separately at each of the 12 variables, it can be seen that none of them completely separates the two types. Therefore, when thinking on a univariate basis, one could say that it is not possible to discriminate between the two types of whiskey. As a consequence, one could look for different (and possible more expensive to determine) variables.

After a PCA (Figure 16.4), it is, instead, evident that the information present in the 12 variables is sufficient to clearly discriminate the two whiskeys.

Once more, it has to be pointed out that taking into account all the variables at the same time gives much more information than just looking at one variable at a time.

Now let us go one step back and try to understand how this result has been obtained. First, since the variables have different magnitudes and variances, a normalization has to be performed, in such a way that each variable will have the same importance. Autoscaling is the most frequently used normalization, which is done by subtracting from each variable its mean value, and then dividing the result by its standard deviation. After that, each normalized variable will have mean = 0 and variance = 1. Table 16.4 shows the data after autoscaling.

The results of the PCA are such that PC1 explains 38.4% of the total variance, and PC2 26.4%. This means that the PC1-PC2 plots shown in Figure 16.4 explain 64.8% of total variance.

Table 16.5 shows the loadings of the variables on PC1 and PC2. From it, the loading plot in Figure 16.4 is obtained.

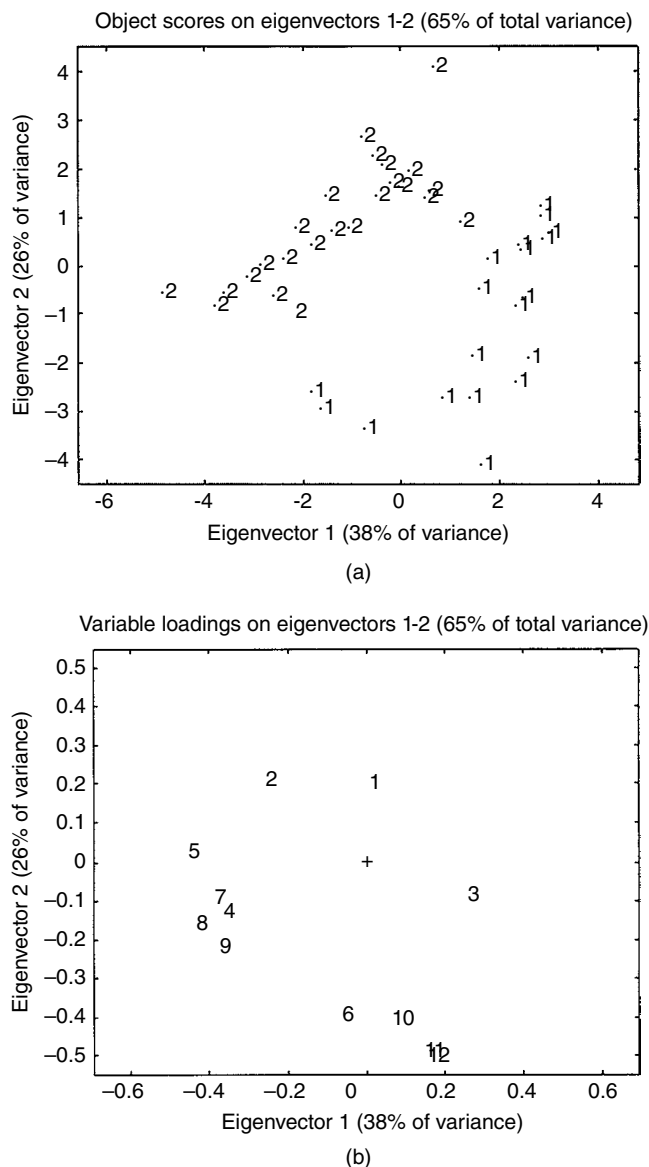


Figure 16.4 PCA of the data of Table 16.3. On top, the score plot of the objects (coded according to the whiskey type), at the bottom the loading plot of the variables (coded according to the order in Table 16.3).

From the score plot in Figure 16.4, it can be seen that the plane PC1-PC2 perfectly separates the two categories. By looking at the loading plot and at Table 16.5, it is possible to know which are the variables mainly contributing to each of the PCs. Variables 4, 5, 7, 8 and 9 (propanol, isobutanol, butanol-1, 2-Me-1-butanol and 3-Me-1-butanol – the alcohols) have the loadings with the highest absolute value on PC1, all of them being negative. This means that the alcohols are higher in those samples having the highest negative scores on PC1. Variables 6, 10, 11 and 12 (isoamyl acetal, ethyl caproate, ethyl

Table 16.4 Autoscaled data.

Sample	Type	1) Acetaldehyde	2) Ethyl acetate	3) Acetal	4) Propanol	5) Isobutanol	6) Isoamyl acetal	7) Butanol-1	8) 2-Me-1butanol	9) 3-Me-1butanol	10) Et caproate	11) Ethyl caprylate	12) Et caprate
1	A	0.288	0.340	0.672	2.507	0.553	1.743	0.187	1.348	0.936	-0.338	1.441	1.418
2	A	0.013	-1.285	0.927	0.791	0.796	2.105	1.335	1.559	1.366	-0.338	0.821	1.536
3	A	0.219	-1.907	1.183	-0.112	-0.435	0.295	0.952	-0.365	-0.297	2.084	1.441	1.301
4	A	-0.125	0.481	-0.095	2.190	-0.291	1.743	0.187	0.399	0.296	2.084	1.441	1.536
5	A	-0.469	-0.202	-0.010	0.858	-0.864	0.657	-0.196	-0.207	-0.192	2.084	1.441	1.536
6	A	-0.675	-1.024	0.501	-0.993	-0.578	2.467	-0.579	0.056	0.190	2.084	2.060	2.005
7	A	0.426	-0.362	-1.032	-1.602	-1.293	1.381	-1.344	-1.051	-0.737	2.084	1.441	1.066
8	A	0.150	-0.121	0.416	-0.293	-0.806	-0.067	-0.961	-0.629	-0.450	-0.338	0.821	0.949
9	A	-0.606	-0.342	0.416	-0.316	-0.606	1.019	-0.961	-0.286	-0.211	-0.338	1.441	1.653
10	A	-1.776	-1.205	0.246	-1.128	-1.178	-0.791	-0.961	-0.787	-0.526	-0.338	0.821	1.066
11	A	-0.675	-0.984	0.075	-1.106	-1.236	-0.791	-0.579	-0.840	-0.421	-0.338	1.441	1.184
12	A	-1.501	-1.406	-0.095	-1.444	-1.050	0.295	-0.579	-0.708	-0.154	2.084	1.441	0.949
13	A	-0.537	-0.944	0.331	-0.970	-1.350	-1.153	-0.961	-0.972	-0.631	-0.338	0.202	0.597
14	A	-0.469	-0.663	0.672	-0.586	-1.150	-0.791	-0.579	-0.682	-0.335	-0.338	0.202	0.480
15	A	0.150	-0.904	0.927	-1.377	-1.536	-1.153	-0.961	-1.314	-0.899	-0.338	0.202	0.363
16	A	0.082	-0.081	1.864	-1.015	-1.178	1.019	-0.961	-1.235	-0.813	-0.338	-0.418	-0.106
17	A	1.939	-1.386	3.654	-0.903	-1.064	-0.429	-0.579	-0.893	-0.641	-0.338	-0.418	0.245

(Continued)

Table 16.4 (Continued)

Sample	Type	1) Acetaldehyde	2) Ethyl acetate	3) Acetal	4) Propanol	5) Isobutanol	6) Isoamyl acetal	7) Butanol-1	8) 2-Me-1butanol	9) 3-Me-1butanol	10) Et caproate	11) Ethyl caprylate	12 Et caprate
18	A	0.563	-1.165	2.205	-1.151	-1.250	-0.791	-0.579	-1.183	-0.985	-0.338	-0.418	-0.224
19	A	0.426	-0.182	1.524	-0.338	-1.422	-0.429	-1.727	-1.552	-1.195	-0.338	-0.418	-0.224
20	B	-0.744	0.240	-0.947	0.542	1.454	-0.067	1.718	0.742	1.184	-0.338	0.202	0.245
21	B	-1.225	-0.784	-0.947	-0.857	0.424	-0.791	0.952	-0.655	0.162	-0.338	-1.657	-0.810
22	B	-0.331	0.059	-0.351	1.874	1.897	-0.429	2.100	1.427	1.844	-0.338	0.202	0.011
23	B	-0.469	-0.442	-0.351	0.565	1.039	-1.153	1.335	0.320	1.098	-0.338	-0.418	-0.458
24	B	0.494	-0.944	-0.095	0.384	0.739	-1.515	0.952	0.399	0.640	-0.338	-0.418	-0.458
25	B	1.182	-0.944	0.160	0.633	0.767	-1.515	1.335	0.399	0.955	-0.338	-0.418	-0.458
26	B	-0.744	1.243	-0.947	0.700	1.454	0.657	0.952	1.401	1.213	-0.338	-0.418	-0.224
27	B	-0.950	0.280	-1.032	0.633	0.867	-0.429	0.952	0.531	0.716	-0.338	-1.037	-0.693
28	B	-1.225	0.882	-1.117	0.655	1.726	0.657	0.952	2.060	1.586	-0.338	-0.418	-0.224
29	B	-0.881	1.364	-1.032	1.626	2.112	0.657	1.718	2.350	2.006	-0.338	-0.418	-0.575
30	B	1.595	1.424	-0.265	0.407	1.039	1.019	0.570	1.743	1.595	-0.338	-0.418	-0.341
31	B	0.357	-0.683	-0.691	-1.580	-0.449	-1.153	-0.196	-0.708	-0.727	-2.759	-2.276	-1.866
32	B	0.288	-0.222	-0.521	0.565	0.796	-0.429	0.952	1.533	1.442	-0.338	-0.418	-0.575
33	B	0.013	0.681	-0.606	0.317	0.681	1.019	0.952	1.111	1.117	2.084	-0.418	-0.927
34	B	0.219	1.103	-0.436	-0.586	-0.134	-0.429	-0.196	-0.471	-0.832	-0.338	-1.037	-0.927

35	B	0.150	1.404	-0.436	0.136	0.438	-0.067	0.570	0.399	0.171	-0.338	-1.037	-1.162
36	B	2.214	1.725	-0.010	0.475	0.038	-1.153	-0.196	-0.207	-0.756	-0.338	0.202	-0.575
37	B	2.420	1.805	-0.095	0.497	0.023	-1.153	0.187	-0.181	-0.545	-0.338	-1.037	-1.279
38	B	0.426	0.340	-0.606	0.023	0.052	-0.429	-0.961	-0.524	-1.109	-0.338	-0.418	-0.693
39	B	-0.194	0.742	-0.777	0.475	0.252	-0.429	-0.579	-0.260	-0.823	-0.338	-1.657	-1.514
40	B	1.801	1.564	-0.351	0.407	0.424	0.295	-1.344	-0.260	-1.090	-0.338	-0.418	-0.693
41	B	0.976	1.444	-0.606	0.429	0.352	0.295	-0.961	-0.339	-1.109	-0.338	-1.037	-1.396
42	B	-1.776	0.380	-1.288	-0.722	-0.120	-0.067	-0.961	-0.972	-1.778	-0.338	0.202	-0.575
43	B	-1.019	0.681	-1.032	-0.609	0.066	0.295	-0.961	-0.497	-1.262	-0.338	-0.418	-1.162

Table 16.5 Loadings of the variables on PC1 and PC2.

	1) Acetaldehyde	2) Ethyl acetate	3) Acetal	4) Propanol	5) Isobutanol	6) Isoamyl acetal	7) Butanol-1	8) 2-ME-1-butanol	9) 3-ME-1-butanol	10) ET caproate	11) Ethyl caprylate	12) ET Caprate
PC1	0.006	−0.253	0.261	−0.363	−0.452	−0.067	−0.385	−0.429	−0.378	0.071	0.146	0.159
PC2	0.196	0.206	−0.086	−0.129	0.023	−0.395	−0.096	−0.162	−0.221	−0.404	−0.493	−0.498

caprylate and ethyl caprate – the esters) have the loadings with the highest absolute value on PC2, all of them being negative. This means that the esters are higher in those samples having the highest negative scores on PC2.

Therefore, it can be said that the esters are mainly responsible for the separation between the two types, while the alcohols are mainly responsible for the variability inside each type. The fact that all the alcohols have very similar loadings means that they are very much correlated, as is the case for the esters. This is a further demonstration of the superiority of multivariate analysis on univariate analysis. Indeed, it will be possible to adulterate a product in such a way that all the variables, singularly taken, fall inside their individual range of acceptance, although it will be much more difficult (not to say impossible) to have an adulterated product in which also the correlations among the variables will be preserved. Therefore, adulterated products that will be unnoticed by the ‘classical’ univariate analysis will be easily detected by a multivariate analysis (see Section 16.7, ‘Modelling’).

Table 16.6 reports the scores of the objects on PC1 and PC2.

As previously shown, the scores of an object are computed by multiplying the loadings of each variable by the value of the variable. As an example, let us compute the score of sample 1 on PC1 (since the autoscaled data have been used, these are the values that must be taken into account):

$$\begin{aligned} &0.288 * 0.006 + 0.340 * (-0.253) + 0.672 * 0.261 + 2.507 * (-0.363) + 0.553 * (-0.452) \\ &+ 1.743 * (-0.067) + 0.187 * (-0.385) + 1.348 * (-0.429) + 0.936 * (-0.378) \\ &+ (-0.338) * 0.071 + 1.441 * 0.146 + 1.418 * 0.159 = -1.778 \end{aligned}$$

So, we have demonstrated that the two types of whiskeys are also really different from the chemical point of view.

Now, let us look at Figure 16.5.

In it, the samples are coded according to Table 16.3 – that is, following the production order. It can be seen that, for both types, there is a trend from the left hand side of the plot (negative values of PC1) to the right hand side of the plot (positive values of PC1), with this effect being much clearer for type A. As has been previously said, PC1 is mainly related to the alcohols. Therefore, it can be concluded that, throughout the production period taken into account, there has been a progressive decrease of the alcohol content

Table 16.6 Scores of the objects on PC1 and PC2.

Object	Category	Score on PC1	Score on PC2
1	A	-1.778	-2.654
2	A	-1.581	-2.974
3	A	1.477	-2.730
4	A	-0.679	-3.359
5	A	0.921	-2.744
6	A	1.737	-4.096
7	A	2.675	-1.889
8	A	1.673	-0.432
9	A	1.534	-1.811
10	A	2.526	-0.650
11	A	2.395	-0.793
12	A	2.395	-2.350
13	A	2.488	0.350
14	A	1.850	0.113
15	A	3.080	0.722
16	A	2.446	0.409
17	A	2.955	0.603
18	A	2.891	1.031
19	A	2.903	1.231
20	B	-2.546	-0.658
21	B	-0.426	1.448
22	B	-3.730	-0.862
23	B	-1.805	0.400
24	B	-1.093	0.748
25	B	-1.392	0.723
26	B	-3.069	-0.258
27	B	-2.090	0.724
28	B	-3.556	-0.589
29	B	-4.814	-0.585
30	B	-2.815	0.020
31	B	0.677	4.098
32	B	-2.361	0.140
33	B	-2.148	-0.925
34	B	0.180	1.939
35	B	-1.527	1.444
36	B	-0.173	1.729

(Continued)

Table 16.6 (Continued)

Object	Category	Score on PC1	Score on PC2
37	B	−0.747	2.663
38	B	0.575	1.484
39	B	−0.510	2.283
40	B	0.012	1.648
41	B	−0.314	2.114
42	B	1.251	0.927
43	B	0.514	1.368

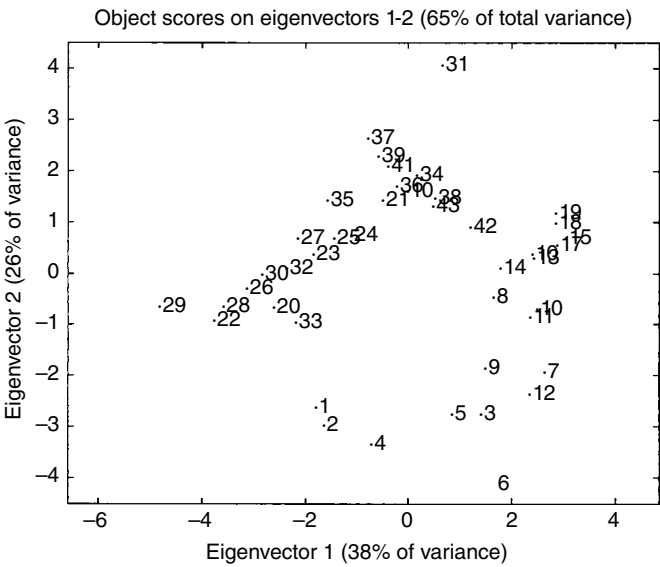


Figure 16.5 Score plot of the data of Table 16.3. The samples are coded according to the order in Table 16.3.

(or that something happened in the analytical system, decreasing the response factor for alcohols). While the previous finding was the answer to a question that was explicitly formulated by the producer (‘are the two types of whiskey different?’), this result came out totally unexpected. This shows very well what is mentioned in a paper by Bro *et al.* (2002):

‘Usually, data analysis is performed as a confirmatory exercise, where a postulated hypothesis is claimed, data generated accordingly and the data analysed in order either to verify or reject this hypothesis.

‘No new knowledge is obtained in confirmatory analysis except the possible verification of a prior postulated hypothesis. Using exploratory analysis the data are gathered in order to represent as broadly and as well as possible the problem under investigation.

'The data are analysed and through the, often visual, inspection of the results, hypotheses are suggested on the basis of the empirical data. Consequently, exploratory data analysis is an extraordinary tool in displaying thus far unknown information from established and potential monitoring methods.'

16.4 Process monitoring and quality control

When running a process, it is very important to know whether it is under control (i.e. inside its natural variability) or out of control (i.e. in a condition that is not typical and can, therefore, lead to an accident).

Analogously, when producing a product, it is very important to know whether each single piece is inside specification (i.e. close to the 'ideal' product, inside its natural variability) or out of specification (i.e. significantly different from the 'standard' product and, therefore, in a condition possibly leading to a complaint by the final client).

PCA is the basis for a multivariate process monitoring and a multivariate quality control, much more effective than the usually applied univariate approaches (Kourti and MacGregor, 1995).

After having collected a relevant number of observations describing the 'normally operating' process (or the 'inside specification' products), encompassing all the sources of normal variability, it will be possible to build a PCA model, defining the limits inside which the process (or the product) should stay.

Any new set of measurements (a vector $\mathbf{x}_{1,v}$) describing the process in a given moment (or a new product) will be projected onto the previously defined model by using the following equation: $\mathbf{s}_{1,c} = \mathbf{x}_{1,v} * \mathbf{L}_{c,v}'$. From the computed scores, it can be estimated how far from the barycenter of the model (i.e. from the 'ideal' process [or product]) it is.

Its residuals can also be easily computed: $\mathbf{e}_{1,v} = \mathbf{x}_{1,v} - \mathbf{s}_{1,c} * \mathbf{L}_{c,v}$ ($\mathbf{e}_{1,v}$ is the vector of the residuals, and each of its v elements corresponds to the difference between the measured and reconstructed value of each variable). From these, it can be understood how well the sample is reconstructed by the PCA model – that is, how far from the model space (a plane, in case $c = 2$) it lies.

Statistical tests make possible the automatic detection of an outlier in both cases (they are defined as T^2 outliers in the first case, and Q outliers in the second case). With these simple tests, it is possible to detect a fault in a process, or to reject a bad product by checking just two plots, instead of as many plots as variables (as in the case of the Shewhart charts commonly used when the univariate approach is applied). Furthermore, the multivariate approach is much more robust, since it will lead to a lower number of false-negatives and false-positives, and it is also much more sensitive, since it allows the detection of faults at an earlier stage.

Finally, the contribution plots will easily outline which variables are responsible for the sample being an outlier.

16.5 Three-way PCA

It can happen that the structure of a data set is such that a standard two-way table (objects versus variables) is not enough to describe it. Let us suppose that the same samples have

been tasted by different assessors, each giving scores on different descriptors. A third way needs to be added to represent the data set adequately, and this can be imagined as a parallelepiped of size $I \times J \times K$, where I is the number of samples (objects), J is the number of descriptors (variables) and K is the number of assessors (conditions) (Geladi, 1989; Smilde, 1992).

To apply standard PCA, these three-way data arrays $\underline{X}$ have to be matricized to obtain a two-way data table. This can be done in different ways, according to what one is interested in focusing on.

If we are interested in studying each 'sampling' (i.e. each combination sample-assessor), a matrix $\mathbf{X}'_b$ is obtained, having $I \times K$ rows and J columns. This approach is very straightforward in terms of computation but, since $I \times K$ is usually a rather large number, the interpretation of the resulting score plot can give some problems.

To focus on the samples, the data array $\underline{X}$ can be matricized to $\mathbf{X}'_a$ (I rows, $J \times K$ columns). The score plot is quite easily interpretable but, since also in this case $J \times K$ is usually a rather large number, the interpretation of the loading plot is very difficult.

The same considerations can be made when focusing on the assessors; in this case, $\mathbf{X}'_c$ is obtained (K rows, $I \times J$ columns).

Three-way PCA allows much easier interpretation of the information contained in the data set, since it directly takes into account its three-way structure. If the Tucker3 model is applied, the final result is given by three sets of loadings, together with a core array describing the relationships among them. If the number of components is the same for each way, the core array is a cube. Each of the three sets of loadings can be displayed and interpreted in the same way as a score plot of standard PCA.

In the case of a cubic core array, a series of orthogonal rotations can be performed on the three spaces of the objects, variables and conditions, looking for the common orientation for which the core array is as much body-diagonal as possible.

If this condition is sufficiently achieved, then the rotated sets of loadings can also be interpreted jointly by overlapping them.

An example of application of three-way PCA is a data set from the field of sensory evaluation (Cordella, 2011). In it, eight types of noodles, each corresponding to a different formulation, were produced in four independent replicates, with each replicate tested by the panel in two independent sessions. Each of the 12 panellists gave a score to eight descriptors (1: yellow colour, 2: translucency, 3: shininess, 4: surface smoothness, 5: firmness, 6: chewiness, 7: surface stickiness, 8: elasticity). The data set can, therefore, be seen as a $64 \times 8 \times 12$ data set.

By taking into account the loading plots of the objects (Figure 16.6), it can be seen that the regions occupied by the eight samples of each noodle (4 replicates $\times$ 2 sessions) never overlap. This means that the global variability (production + sensory evaluation) of each noodle is always smaller than the differences among the noodles. The fact that the region spanned by each noodle is approximately the same (with the exception of noodle 2) indicates that the global variability can be considered as independent of the type of noodle.

It can also be seen that the variability between sessions is smaller than the variability among replicates, this meaning that the 'instrumental error' of the judges is smaller than the variability of the production.

On the first axis, noodles 7 and 8 have the lowest loading, followed by noodle 6, and then by the remaining five types, all with very similar loading. This ranking ($7 = 8 > 6$) corresponds to the content of glyceryl monostearate (GMS, 2.8%, 2.8% and 1.4%,

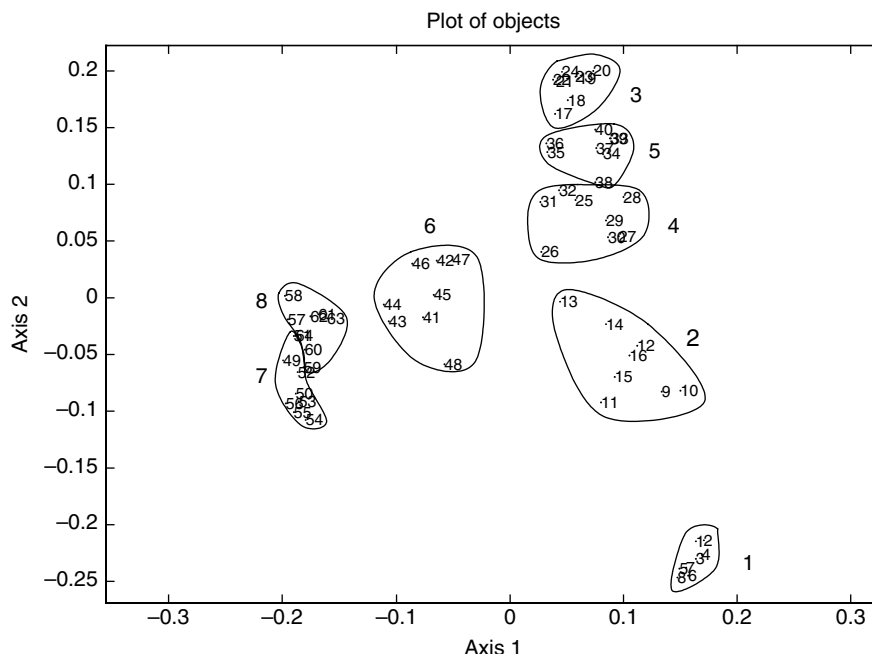


Figure 16.6 Scatter plot of the loadings of the objects. Objects 1–8: noodle 1; objects 9–16: noodle 2; ...; objects 57–64: noodle 8.

respectively, with the other noodles having no GMS). It can be concluded that the loadings of each noodle group on the first axis is directly related to the GMS content.

On the second axis, the five formulations having no GMS are discriminated, with noodle 3 having the highest loading and noodle 1 having by far the lowest loading. Noodle 3 is made only by durum wheat four (DWF), while noodle 1 is the only one containing wheat starch (WS). On the same axis, noodles 5, 4 and 2 have decreasing loadings, and this corresponds to their amounts of wheat gluten (WG, 6%, 3% and 0%, respectively).

Figure 16.7 shows the scatter plot of the loadings of the variables. Variables 5–8 (the texture-related descriptors) have the highest values on the first axis. This means that the first axis is mainly related to the texture of the product.

Variables 1 and 4 (colour and smoothness, both positive attributes) have positive loadings on axis 2, in contrast with variables 2 and 3 (translucency and shininess, both negative attributes). Therefore, the second axis is mainly related to the appearance attributes of the noodles.

It should also be noticed that variables 5–8 (the texture-related descriptors) have very similar loadings on both axes and, therefore, are very highly correlated.

As a result, it can be concluded that axis 1 is related to the amount of GMS and to the texture of the product; it can be seen that the addition of GMS gives a worse product.

Axis 2 is related to this aspect; it can be seen that noodle 3, made with DWF, is the product with the best appearance (the most yellow and the smoothest), while noodle 1, made with a large amount of WS, has the worst appearance (the most translucent and the most shiny). The addition of WG also improves the appearance, since it results in an increase of the yellow colour and the smoothness.

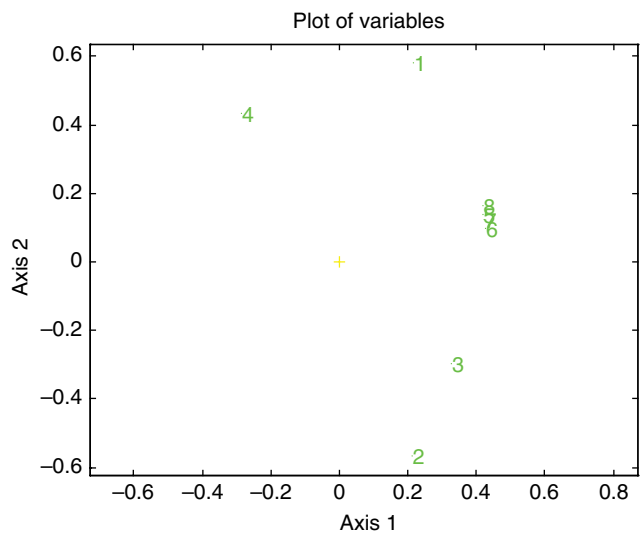


Figure 16.7 Scatter plot of the loadings of the variables.

Table 16.7 Data sets on which three-way PCA can be applied.

Field of application	Objects	Variables	Conditions
Environmental analysis	Air or water samples	Chemico-physical analyses	Time
Environmental analysis	Water samples (different locations)	Chemico-physical analyses	Depth
Panel tests	Food products (oils, wines)	Attributes	Assessors
Food chemistry	Foods (cheeses, spirits, ...)	Chemical composition	Ageing
Food chemistry	Foods (oils, wines, ...)	Chemical composition	Crops
Sport medicine	Athletes	Blood analyses	Time after effort
Process monitoring	Batches	Chemical analyses	Time

By taking into account both axes, it is easy to detect noodle 3 as the best one. Table 16.7 shows some types of data sets on which three-way PCA can be successfully applied.

16.6 Classification

In Section 16.3 of this chapter, ‘Data display’, we verified that the two types of whiskey are, indeed, well separated in the multivariate space of the variables. Therefore, we can say that we have two really different classes. Let us suppose, however, that we now get

some unknown samples and we want to know what their class is. After having performed the chemical analyses, we can add these data to the previous data set, run a PCA and see where the new samples are placed. This will be fine if the new samples fall inside one of the clouds of points corresponding to a category, but what if they fall in a somehow intermediate position? How can we say with 'reasonable certainty' that the new samples are from type A or type B? We know that PCA is a very powerful technique for data display, but we realize that we need something different if we want to classify new samples. What we want is a technique that produces some 'decision rules' discriminating among the possible categories.

While PCA is an 'unsupervised' technique, the classification methods are 'supervised' techniques. In these techniques, the category of each of the objects on which the model is built must be specified in advance.

Some examples of problems of classification in the field of food are the detection of the geographical origin of a wine, a milk or a cheese; the detection of the vintage of a wine or of the cultivar used to produce it; the detection of the floral origin of a honey; and so on.

The most commonly used classification techniques are Linear Discriminant Analysis (LDA) and Quadratic Discriminant Analysis (QDA). These define a set of delimiters (according to the number of categories under study) in such a way that the multivariate space of the objects is divided in as many subspaces as the number of categories, and that each point of the space belongs to one, and only one, subspace. Rather than describing in detail the algorithms behind these techniques, special attention will be given to the critical points of a classification.

As previously stated, the classification techniques use objects belonging to the different categories to define boundaries delimiting regions of the space. The final goal is to apply these classification rules to new objects for their classification into one of the existing categories. The performance of the technique can be expressed as classification ability and prediction ability. The difference between 'classification' and 'prediction', though quite subtle at first glance, is actually very important, and its underestimation can lead to very bitter deceptions.

Classification ability is the capability to assign to the correct category the same objects used to build the classification rules, while prediction ability is the capability to assign to the correct category objects that have not been used to build the classification rules. Since the final goal is the classification of new samples, it has to be clear that the predictive ability is by far the most important figure of merit to be looked at.

The results of a classification method can be expressed in several ways. The most synthetic one is the percentage of correct classifications (or predictions). Note that, in the following, only the term 'classification' will be used, but it has to be understood as 'classification or prediction'. This can be obtained as the number of correct classifications (independently of the category) divided by the total number of objects, or as the average of the performance of the model over all the categories. The two results are very similar when the size of all the categories is very similar, but they can be very different if the size is quite different. Let us consider the case shown in Table 16.8.

The very poor performance of category 3, by far the smallest one, almost does not affect the classification rate computed on the global number of classifications, while it produces a much lower result if the classification rate is computed as the average of the three categories.

Table 16.8 Example of the performance of a classification technique.

Category	Objects	Correct class	Percentage correct class
1	112	105	93.8
2	87	86	98.9
3	21	10	47.6
Total	220	201	91.4/80.1

A more complete and detailed overview of the performance of the model can be obtained by using the classification matrix, which also allows us to know the categories to which the wrongly classified objects are assigned (in many cases, the cost of an error can be quite different, according to the category the sample is assigned to). In it, each row corresponds to the true category, and each column to the category to which the sample has been assigned. Continuing with the previous example, a possible classification matrix is the one shown in Table 16.9.

From it, it can be seen that the 112 objects of category 1 were classified in the following way: 105 correctly to category 1, none to category 2, and 7 to category 3. In the same way, it can be deduced that all the objects of category 3 which were not correctly classified were assigned to category 1. Therefore, it is easy to conclude that category 2 is well defined, and that the classification of its objects gives no problems at all, while categories 1 and 3 are quite overlapping. As a consequence, to have a perfect classification, more effort must be put into better separating categories 1 and 3. All this information cannot be obtained from just the percentage of correct classifications.

If over-fitting occurs, then the prediction ability will be much worse than the classification ability. To avoid it, it is very important that the sample size is adequate to the problem and to the technique. A general rule is that the number of objects should be more than five times (at least, no less than three times) the number of parameters to be estimated. LDA works on a pooled variance-covariance matrix; this means that the total number of objects should be at least five times the number of variables. QDA computes a variance-covariance matrix for each category, which makes it a more powerful method than LDA, but this also means that each category should have a number of objects at least five times higher than the number of variables. This is a good example of how the more complex, and therefore 'better', methods, sometimes cannot be used in a safe way, because their requirements do not correspond to the characteristics of the data set.

Table 16.9 Example of a classification matrix.

Category	1	2	3
1	105	0	7
2	1	86	0
3	11	0	10

16.7 Modelling

In classification, the space is divided into as many subspaces as categories, and each point belongs to one and only one category. This means that the samples that will be predicted by such methods must belong to one of the categories used to build the models; if not, they will be assigned to one of them anyway. To make this concept clearer, let us suppose the use of a classification technique to discriminate between water and wine. Of course, this discrimination is very easy. Each sample of water will be correctly assigned to the category 'water', and each sample of wine will be correctly assigned to the category 'wine'. But what happens with a sample of orange squash? It will be assigned either to the category 'water' (if variables such as alcohol are taken into account) or to the category 'wine' (if variables such as colour are considered). The classification techniques are, therefore, not able to define a new sample as being 'something different' from all the categories of the training set. This is, instead, the main feature of the modelling techniques.

Though several techniques are used for modelling purpose, UNEQ (one of the modelling versions of QDA) and SIMCA (soft independent model of class analogy) are the most used. While in classification every point of the space belongs to one and only one category, with these techniques the models (one for each category) can overlap and leave some regions of the space unassigned. This means that every point of the space can belong to one category (the sample has been recognised as a sample of that class), to more than one category (the sample has such characteristics that it could be a sample of more than one class), or to none of the categories (the sample has been considered as being different from all the classes).

Of course, the 'ideal' performance of such a method would not only be to classify correctly all the samples in their category (as in the case of a classification technique), but also be such that the models of each category could be able to accept all the samples of that category, and to reject all the samples of the other categories. The results of a modelling technique are expressed the same way as in classification, plus two very important parameters: specificity and sensitivity. For category c , its specificity (how much the model rejects the objects of different categories) is the percentage of the objects of categories different from c rejected by the model, while its sensitivity (how much the model accepts the objects of the same category) is the percentage of the objects of category c accepted by the model.

While classification techniques need at least two categories, modelling techniques can also be applied when only one category is present. In this case, the technique detects if the new sample can be considered as a typical sample of that category or not. This can be very useful in the case of Protected Denomination of Origin products, to verify whether a sample, declared as having been produced in a well-defined region has, indeed, the characteristics typical of the samples produced in that region.

The application of modelling will greatly reduce the possibility of frauds. While an 'expert' can adulterate a product (e.g. by adding olive oil to an extra virgin olive oil, or by adding less aged whisky to aged whisky) in such a way that all the variables, independently considered, still stay in the accepted range, it is almost impossible to adulterate a product in such a way that its multivariate 'pattern' is still accepted by the model of the original product, unless the amount of the adulterant is so small that it becomes unprofitable from an economic point of view.

16.8 Calibration

Let us imagine we have a set of wine samples and that, on each of them, the FT-IR spectrum is measured, together with some variables such as alcohol content, pH or total acidity. Of course, chemical analyses will require much more time than a simple spectral measurement. It would, therefore, be very useful to find a relationship between each of the chemical variables and the spectrum. This relationship, after having been established and validated, would be used to predict the content of the chemical variables. It is easy to understand how much time (and money) this would save since, in a few minutes, it would be possible to have the same results as previously obtained by a whole set of chemical analyses.

Generally speaking, we can say that multivariate calibration finds relationships between one or more response variables y , and a vector of predictor variables x . As the previous example should have shown, the final goal of multivariate calibration is not just to 'describe' the relationship between the x and the y variables in the set of samples on which the relationship has been computed, but to find a real practical application for samples that, in a following time, will have only the x variables measured.

The model is a linear polynomial ($y = b_0 + b_1x_1 + b_2x_2 + \dots + b_Kx_K + f$), where b_0 is an offset, the b_k ($k = 1, \dots, K$) are regression coefficients and f is a residual. The 'traditional' method of calculating $\mathbf{b}$, the vector of regression coefficients, is Multiple Linear Regression (MLR). However, this method has two major limitations that make it inapplicable to many data sets:

- 1) It cannot handle more variables than objects.
- 2) It is sensitive to collinear variables.

It can be easily seen that both these limitations do not allow the application of MLR to spectral data sets, where the samples are described by a very high number of highly collinear variables. If one wants to use MLR to such data anyway, the only way to do it is to reduce the number of variables and their collinearity through a suitable variable selection (see Section 16.9, 'Variable selection').

When describing the PCA, it has been noticed that the components are orthogonal (i.e. uncorrelated), and that the dimensionality of the resulting space (i.e. the number of significant components) is much lower than the dimensionality of the original space. Therefore, it can be seen that both the aforementioned limitations have been overcome. As a consequence, it is possible to apply MLR to the scores originated by PCA. This technique is Principal Component Regression (PCR).

It has to be considered that PCs are computed by taking into account only the x variables, without considering at all the y variable(s), and are ranked according to the explained variance of the ' x space'. This means that it can happen that the first PC has little or no relevance in explaining the response we are interested in. This can be easily understood by considering that, even when we have several responses, the PCs to which the responses have to be regressed will be the same.

Nowadays, the most favoured regression technique is partial least squares regression (PLS, or PLSR). As happens with PCR, PLS is based on components (or 'latent variables'). The PLS components are computed by taking into account both the x and the y variables and, therefore, they are slightly rotated versions of the principal components. As a consequence, their ranking order corresponds to the importance in the modelling

of the response. A further difference with MLR and PCR is that, while the former must work on each response variable separately, PLS can be applied to multiple responses at the same time.

Because both PCR and PLS are based on latent variables, a very critical point is the number of components to be retained. Though we know that the information is ‘concentrated’ in the first components, and that the last components explain just noise, it is not always an easy task to detect the correct number of components (i.e. when information finishes and noise begins). Selecting a lower number of components would mean removing some useful information (under-fitting), while selecting a higher number of components would mean to incorporate some noise (over-fitting).

Before applying the results of a calibration, it is very important to look for the presence of outliers. Three major types of outliers can be detected:

- outliers in the x -space (samples for which the x -variables are very different from those of the rest of the samples – they can be found by looking at a PCA of the x -variables);
- outliers in the y -space (samples with the y -variable very different from that of the rest of the samples – they can be found by looking at a histogram of the y -variable); and
- samples for which the calibration model is not valid (they can be found by looking at a histogram of the residuals).

The goodness of a calibration can be summarised by two values – the percentage of variance explained by the model, and the Root Mean Square Error in Calibration (RMSEC). The former, being a ‘normalized’ value, gives an initial idea about how much of the variance of the data set is ‘captured’ by the model; the latter, being an absolute value to be interpreted in the same way as a standard deviation, gives information about the magnitude of the error.

As already described in the classification section, and as pointed out at the beginning of this section, the goal of a calibration is essentially not to describe the relationship between the response and the x -variables of the samples on which the calibration is computed (training, or calibration, set), but to apply it to future samples, where only the cheaper x -variables will be measured. In this case, too, the model must be validated by using a set of samples different from those used to compute the model (validation, or test, set). The responses of the objects of the test set will be computed by applying the model obtained by the training set, and then compared with their ‘experimental’ response (i.e. the response measured by the reference analytical method). From these values, the percentage of variance explained in prediction and the Root Mean Square Error in Prediction (RMSEP) can be computed. Provided that the objects forming the two sets have been selected flawlessly, these values give the real performance of the model on new samples.

It has, anyway, to be kept well in mind that the error of the reference method is the limiting factor, and that the RMSEP of any method will never be smaller than the standard deviation of the reference method. Therefore, in many cases, a much substantial improvement of the multivariate calibration model could be obtained by trying to improve the reference method, rather than trying to ameliorate the model itself (with serious risks of over-fitting).

Spectroscopic methods, such as NIR or FT-IR, are extremely fast and cheap (they do not require solvents, and samples can be analysed as such, or with a very simple pre-treatment). The fact that they are also non-destructive makes them ideal for food

samples, since they allow us to get very detailed information about a sample in a very short time (less than one minute), and at no cost.

In a recent paper (Caredda *et al.*, 2016), Fourier Transformed Mid-Infrared spectra were employed to predict the acidic composition of sheep milk. The models so obtained allowed replacing the much more time-consuming and expensive GC-FID methodology, making it possible to analyse a much greater number of samples and to evaluate the nutritional profile of each of them, at only a fraction of the original cost.

16.9 Variable selection

Usually, not all the variables of a data set bring useful and non-redundant information. Therefore, a variable (or feature) selection can be highly beneficial, since from it the following results can be obtained:

- 1) Removal of noise and improvement of the performance.
- 2) Reduction of the number of variables to be measured and simplification of the model.

The removal of noisy variables should always be looked for. Though some methods can give good results, even with a moderate amount of noise disturbing the information, it is clear that their performance will increase when this noise is removed. Therefore, feature selection is also now widely applied for those techniques (PLS and PCR) that, in the beginning, were considered to be almost insensitive to noise.

While noise reduction is a common goal for any data set, the relevance of the reduction of the number of variables in the final model depends very much on the kind of data constituting the data set, and a very wide range of situations is possible. Let us consider the extreme conditions:

- 1) Each variable requires a separate analysis.
- 2) All the variables are obtained by the same analysis (e.g. chromatographic and spectroscopic data).

In the first case, each variable not selected means a reduction in terms of costs and/or analysis time. The variable selection should, therefore, always be made on a cost/benefit basis, looking for the subset of variables leading to the best compromise between performance of the model and the cost of the analyses. This means that, in the presence of groups of useful but highly correlated (and therefore redundant) variables, only one variable per group should be retained. With such data sets, it is also possible that a subset of variables giving a slightly worse result is preferred, if the reduction in performance is widely compensated by a reduction in costs or time.

In the second case, the number of retained variables has no effect on the analysis cost, while the presence of useful and correlated variables improves the stability of the model. Therefore, the goal of variable selection will be to improve the predictive ability of the model by removing the variables giving no information, without being worried by the number of retained variables. It has, anyway, to be taken into account that a simpler model is more robust and less prone to over-fitting.

Intermediate cases can happen, in which 'blocks' of variables are present. As an example, take the case of olive oil samples on each of which the following analyses have been

run: a titration for acidity; the analysis of peroxides; a UV spectroscopy for ΔK ; a GC for sterols; and another GC for fatty acids. In such a situation, what counts is not the final number of variables, but the number of analyses one can save.

The only possible way to be sure that the 'best' set of variables has been picked up is 'all-models' technique, testing all the possible combinations. Since, with k variables, the number of possible combinations is $2^k - 1$, it is easy to understand that this approach cannot be used unless the number of variables is really very low (e.g. with 30 variables, more than 10^9 combinations should be tested). In any case, one must be aware that 'the best' combination would be such only for that specific data set (and once more, one must be careful of the risk of over-fitting).

The simplest (but least effective) way of performing a feature selection is to operate on a 'univariate' basis, by retaining those variables having the greatest discriminating power (in the case of a classification) or the greatest correlation with the response (in the case of a calibration). By doing that, each variable is taken into account by itself, without considering how its information 'integrates' with the information brought by the other (selected or unselected) variables. As a result, if several highly correlated variables are 'good', they are all selected, without taking into account that, owing to their correlation, the information is highly redundant and, therefore, at least some of them can be removed without any decrease in performance. On the other hand, those variables are not taken into account that, though not giving by themselves significant information, become very important when their information is integrated with that of other variables.

An improvement is brought by 'sequential' approaches. These select the best variable first, then the best pair formed by the first and second, and so on, in a forward or backward progression. A more sophisticated approach applies a look back from the progression to reassess previous selections. The problem with these approaches is that only a very small part of the experimental domain is explored, and that the number of models to be tested becomes very high in the case of highly dimensional data sets, such as spectral data sets. For instance, with 1000 wavelengths, 1000 models are needed for the first cycle (selection or removal of the first variable), 999 for the second cycle, 998 for the third cycle, and so on.

More 'multivariate' methods of variable selection, especially suited for PLS applied to spectral data, are currently available. Among them, we can cite interactive variable selection (Lindgren *et al.*, 1994), uninformative variable elimination (Centner *et al.*, 1996), iterative predictor weighting PLS (Forina *et al.*, 1999), and interval PLS (Nørgaard *et al.*, 2000).

Very good results are obtained by Genetic Algorithms (Leardi, 2000). GAs are a general optimisation technique with good applicability in many fields, especially when the problem is so complex that it cannot be tackled with 'standard' techniques. GAs try to simulate the evolution of a species, according to Darwinian theory. Each experimental condition (in this case, each model) is treated as an individual, whose 'performance' (in the case of a feature selection for a calibration problem, it can be the explained variance) is treated as its 'fitness'. Through operators simulating fights among individuals (the best ones have the greatest probability of mating and, thus, spreading their genome), mating among individuals (with the consequent 'birth' of 'offspring' having a genome that is derived from both the parents), and the occurrence of mutations, the GAs result in a pattern of search that, by mixing 'logical' and 'random' features, allows a much more complete search of complex experimental domains.

When applied to spectral data sets, a selection of spectral regions is produced, rather than individual wavelengths, as per the majority of the other methods. This is very important, since the final solution is much more logical from the chemical point of view, and much more interpretable from the spectroscopic point of view.

16.10 Conclusion: future trends and the advantages and disadvantages of chemometrics

In future, multivariate analysis should be used more and more in everyday (scientific) life. Until a few decades ago, experimental work resulted in a very limited amount of data, the analysis of which was quite easy and straightforward. Nowadays, it is common to have instrumentation producing an almost continuous flow of data. One example is process monitoring performed by measuring the values of several process variables, at a rate of one measurement every few seconds. Another example is quality control of a final product of a continuous process, on which an FT-IR spectrum is taken every few minutes (or seconds).

In Section 16.8 ('Calibration'), the case of wine FT-IR spectra was cited, from which the main characteristics of the product can be directly predicted. It is, therefore, clear that the main problem has shifted from obtaining a few data to the treatment of a huge amount of data. It is also clear that standard statistical treatment is not enough to extract the entirety of the information buried in these data.

Many instruments have some chemometric routines built into their software, in such a way that their use is totally transparent to the final user (and sometimes the word *chemometrics* is not even mentioned, to avoid possible aversion). Of course, they are 'closed' routines, and therefore the user cannot modify them. It is quite obvious that it would be much better if chemometric knowledge were much more widespread, in order that users could better understand what kind of treatment their data have undergone, and eventually modify the routines in order to make them more suitable to their requirements.

In one of his papers, Workman (2002) very efficiently depicts the advantages and disadvantages of multivariate thinking for scientist in industry. From the eight advantages of chemometrics he clearly outlines, special relevance should be given to the following ones:

- 1) Chemometrics provides speed in obtaining real-time information from data.
- 2) It allows high quality information to be extracted from less resolved data.
- 3) It promises to improve measurements.
- 4) It improves knowledge of existing processes.
- 5) It has very low capital requirements – it is cheap.

The last point, especially, should convince people to give chemometrics a try. No extra equipment is required – just an ordinary computer and some chemometrical knowledge (or a chemometrical consultancy). It is certain that, in the very worst cases, the same information as found from a classical analysis will be obtained in a much shorter time, and with much more evidence. In the great majority of cases, instead, even a simple PCA can provide much more information than was previously collected. So

why are people so shy of applying chemometrics? In the same paper previously cited, Workman gives some very common reasons:

- 1) There is widespread ignorance about what it is and what it can realistically accomplish.
- 2) This science is considered too complex for the average technician and analyst.
- 3) Chemometrics requires a change in one's approach to problem solving, from univariate to multivariate thinking.

So, while chemometrics leads to several real advantages, its 'disadvantages' lie only in a general reluctance to use it, and accepting the idea that the approach that has been followed over many years can turn out not to be the best one.

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17

Automated Sampling Procedures

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17.1 Introduction

Nowadays, food analysis is more important than ever, because of the changing perspectives on customer behaviours, and enhancement in market expectations. The demand for higher quality, safety and traceability within the food supply chain, the need for food products which are fully compatible with government regulations, and conscious consumers who request to be informed about nutritional composition of foods via labelling, are the main driving forces of market competition in the global markets, and are major pressures on the food industry.

These new tendencies bring with them an obligation for more sensitive, accurate, reliable, robust and effective analytical approaches. An analytical procedure generally consists of six steps: sampling; sample preparation; separation of desired compounds from matrix; identification; quantification; and data collection. Recent technological advancements and design of modern and sensitive analytical instruments provide detection limits sensitive enough to detect trace amount of analytes in samples. Unfortunately, all these improvements still have an important deficiency. Most of them need multistage sample preparation steps, because of an inability to handle directly samples from matrix, such as foods, which consist of non-homogenous, complex and numerous chemical substances. Due to this requirement, sample preparation procedure becomes the one of the most critical factors that affects the analysis quality, time and cost of labour.

Characteristic features of sample preparation step generally depend on food matrix, properties of analyte, level of analyte, and the accuracy level which is needed for the instrumentation step (Ridgway *et al.*, 2007). All procedures may have their own techniques, but nearly all of them have the same common aims: the removal of potential interferants; increasing the concentration of the analyte; obtaining suitable forms of analytes for a better detection; and forming valid methods which are usable independently from the sample structure (Smith, 2003). Sampling, mechanical processing/homogenisation, decomposing of matrix, isolation of analyte, clean-up, enrichment and, in some cases, derivatisation are typical parts of sample preparation procedures (Ridgway *et al.*, 2007; Ramos, 2012).

Despite all the advances in design of instruments and other equipment, the techniques used for sample preparation still involving manual and *off-line* applications. This neglected point represents the main reason why sample preparation is the most time-consuming part of an analytical procedure. Manual manipulation of samples is a potential source of error because of several reasons, such as the human factor, transportation of samples to the laboratory, and possible alteration of sample components and/or contamination of samples during multi-step procedures. All these factors also have an undeniable and considerable influence on the total cost of the analysis.

Despite the traditional and prevailing sample preparation methods, new goals and expectations – for instance, miniaturisation, hyphenation of various steps, integration with identification and quantification, and high-level automation – dominate recent trends in this field (Chen *et al.*, 2008). It is recognised that higher speed, accuracy, selectivity and reproducibility, smaller sample sizes for trace analyses, and an environmentalist approach with a lower amount of solvent, can be achieved with automated processes (Smith, 2003; Chen *et al.*, 2008). An increasing number of samples and on-line treatments are other important advantages of automated sampling (Buldini *et al.*, 2002).

17.2 Extraction techniques for sample preparation

Sample preparation aims to isolate desirable components from sample matrix, due to the inability of most of analytical instruments to handle the sample matrix directly. For that reason, sample handling typically consists of extraction procedures which differ in degrees of selectivity, convenience and speed. Extraction can be generally described as the isolation of analytes from a sample while removing interfering sample components, and obtaining analytes in a suitable form for the analysis (Raynie, 2006).

The physical and chemical properties of a sample and sample compounds of interest have a great influence on the selection of extraction methods. However, nowadays, not only is selection of a proper sample preparation procedure dependent on sample properties, but some other requirements are also demanded. These requirements include; improving the sensitivity while reducing the sample loss; less labour, together with a shorter analysis time; reducing sample contamination and sample degradation during sample preparation; higher reproducibility; precision; accuracy; miniaturised procedures, to reduce the amount of organic solvents under a concept of green approach; and reduction of sample amounts (Pan *et al.*, 2014). All of these expectations require an improvement in sample preparation methods towards automation, which is the direct coupling of sample preparation and analytical detection systems.

17.2.1 Extraction from liquid samples

In contrast to solid samples, sample preparation for liquid samples can be generally performed without pre-treatments, and based on partitioning into an immiscible solvent or trapping of analytes onto a solid surface. Conventional liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are two of the dominant methods which are used in a wide range of food analysis procedures. Liquid-phase microextraction (LPME) and solid-phase microextraction (SPME) emerged from LLE and SPE, respectively, to

overcome the limits and disadvantages of these traditional methods, and to obtain a diverse range of automation and miniaturisation. All these novel techniques also have their own advantages and disadvantages.

17.2.1.1 Liquid-liquid extraction

Liquid-liquid extraction (LLE) is one of the oldest sample preparation methods used in analytical chemistry. LLE is based on two immiscible liquid phases and, usually, one of them is an aqueous phase and the other is an organic phase. These two phases are mixed traditionally in a separatory funnel, and the analyte can be extracted from aqueous phase into an organic solvent, according to its distribution behaviour in the phases (Hii and Lee, 2010). Miscibility, density and solubility are the main considerations while selecting the extraction solvents (Wells, 2003). Despite the fact that the LLE technique is utilised on a large scale, it has obvious disadvantages, such as: a requirement for large volumes of organic solvent; repeating the extraction several times to complete; limited selectivity; and a need for a clean-up or concentration step after sample extraction (Ridgway *et al*, 2007). LLE can be automated, but usually in a limited and impractical ways. Today, solvent microextraction (SME) is a main tendency for minimising the disadvantages of LLE, via miniaturisation and automating of LLE.

17.2.1.2 Solvent microextraction (SME)

The SME technique, which is also referred to as liquid-phase microextraction (LPME) by some authors in the literature, has emerged as a consequence of the solvent minimising approach over the last two decades. This sample treatment method uses solvent volumes of 100 μL or less during extraction, or concentration of the samples (Kokosa, 2013). SME eliminates the problem of large volumes of solvent, while providing analytes in a concentrated form. The phase which is immiscible with water is usually termed the acceptor phase, while the aqueous phase, which contains the sample, is usually called the donor phase (Sarafraz-Yazdi and Amiri, 2010). The categories of SME involved in many applications include: single-drop microextraction (SDME); dispersive liquid-liquid microextraction (DLLME); liquid-liquid microextraction (LLME); and hollow-fibre microextraction (HF-LPME), as a membrane based extraction method (See Figure 17.1)

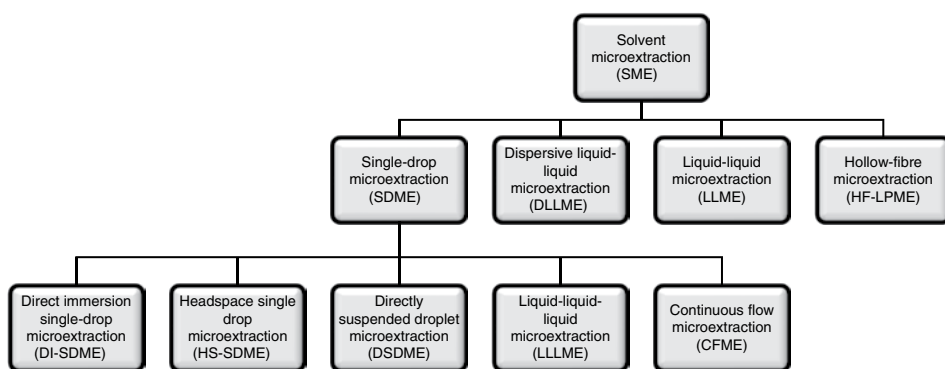


Figure 17.1 Classification of solvent microextraction (SME).

17.2.1.2.1 Single-drop microextraction (SDME)

In the late 1990s, the idea of using drops with the purpose of extraction began appearing in the sample treatment procedures. SDME is based on the immersion of a microlitre drop of water-immiscible organic solvent into the sample using a microsyringe. The aqueous medium is stirred and, when the distribution of analytes from aqueous phase to microdrop is done, the microdrop is retracted of back into the syringe and transferred for chromatography analysis (Andruch *et al.*, 2012a). The volume of current extractant is generally 1–8 μL , and volume of aqueous phase is generally 1–10 ml in the application of SDME (Ramos, 2012).

The principal advantages of SDME are simplicity, analysis speed, low cost, low amount of organic solvent required, and being capable of using with gas chromatography (GC) and high-performance liquid chromatography (HPLC) systems. The disadvantage is the drop volume variations under extreme conditions of extraction, such as high speed, temperature or extraction time (Liu and Dasgupta, 1996; Asensio-Ramos *et al.*, 2011). The choice of solvent, drop volume, temperature, extraction time, stirring rate and ionic strength of the solution are the main factors affecting SDME efficiency (Wardencki *et al.*, 2007).

SDME is a useful approach for sample preparation in food analysis, and has drawn attention in most of the studies in recent years. The analysis of organophosphorus pesticides (OPPs) in water, fruit juice and wine samples (Xiao *et al.*, 2006; Zhao *et al.* 2006; Garbi *et al.*, 2010), analysis of organic acids in fruits and fruit juices involving in-syringe derivatisation and GC-MS application (Saraji and Mousavinia, 2006), and determination of lead (Manzoori *et al.*, 2009) and cadmium (Chamsaz *et al.* 2013) in food samples are a few examples of SDME procedures for food matrix.

The classification of SDME can rely on a number of phases (two phases or three phases) or modes of operations, according to immersing in:

- 1) the aqueous media;
- 2) flowing liquid media;
- 3) immiscible organic solvent placed above the aqueous media; or
- 4) exposing the headspace above the sample (Pena-Pereira *et al.*, 2010a; Asensio-Ramos *et al.*, 2011).

Direct immersion single drop microextraction (DI-SDME), drop-to-drop microextraction (DDME), headspace single drop microextraction (HS-SDME), liquid-liquid-liquid microextraction (LLLME), directly suspended droplet microextraction (DSDME) and continuous flow microextraction (CFME) are the main types of SDME, which has attracted considerable attention with different benefits. On the other hand, a dynamic approach in SDME methods began to appear with the survey of He and Lee (1997), in contrast with static modes based on diffusion of the analytes.

The two types of dynamic SDME are unexposed-drop (in-syringe) and exposed-drop (in-needle). In the unexposed-drop method, the aqueous sample solution or headspace is withdrawn into a microsyringe. The organic solvent, which was already pre-loaded into the syringe, forms a thin film along the wall of the syringe barrel. The aqueous phase is then pushed out, and the procedure is repeated several times. In the exposed-drop method, the organic solvent drop is pushed out from the microsyringe, instead of withdrawing the sample inside. The solvent is exposed to the sample solution at the needle. After a pre-determined time, the solvent is withdrawn into the microsyringe again, and the procedure is repeated for more sample exposure.

In comparison with the static LPME methods, the dynamic approach provides a higher enrichment with a shorter extraction time. However, lower reproducibility, because of repeated manual application, needs to improve (He and Lee, 1997). For this purpose, semi-automated and fully automated systems have driven the following progress in this field. Hou and Lee (2002) used a semi-automated dynamic LPME for the HPLC analysis of polycyclic aromatic hydrocarbons in water samples. A syringe pump was programmed for a controlled extraction. Saraji (2005) developed a semi-automated dynamic HS-LPME system for GC analysis of nine alcohols from water samples. The system contained a variable speed stirring motor, attached to a circular plate, and rotation of this plate was used to make the movement of the syringe plunger uniform.

Mohammadi and Alizadeh (2006) introduced an automated dynamic headspace-organic solvent film microextraction (DHS-OSFME) system, which had computer software for controlling the plunger speed, the number of extraction cycles and the dwell time. Ouyang *et al.* (2007) compared fully automated dynamic/static HS-LPME and DI-LPME systems. An autosampler and software were used for controlling the plunger, solvent volume, agitation speed, temperature, position of the needle tip, and dwell time during extraction. Lee and Lee (2011) described a fully automated dynamic in-syringe LPME procedure for the detection of carbamate pesticides with gas chromatography/mass spectrometric analysis (GC-MS) in water samples. This procedure consisted of automatic control of the extraction, injection and analyte derivatisation steps, with considerable enrichment factors (between 57 and 13) and low limits of detection (ranging from 0.05 to 0.1 µg/L) for carbamates.

Direct immersion single-drop microextraction (DI-SDME) DI-SDME contains a direct immersion of a drop of water-immiscible organic solvent into a stirring sample solution, waiting during a pre-determined time to reach system equilibrium, and retraction of the drop, which is suspended from a needle tip into a microsyringe. The same microsyringe is utilised for both extraction and injection of analytes, which removes the disadvantage caused by using different apparatus. The polarity of samples is a limiting factor for DI-SDME applications; non-polar, or moderately polar, liquid samples are suitable for the extraction (Sarafraz-Yazdi and Amiri, 2010). The amount of solvent is usually 1–3 µL, in order to avoid manipulation and falling problems and, also, requiring a longer extraction time to reach system equilibrium. Stirring may enhance the extraction yield, but the high speed may cause droplet deformation and dislodgement (Jain and Verma, 2011).

Pena *et al.* (2008) described a system which consisted of immersed single-drop microextraction, coupled with sequential injection analysis (SIA) and electrothermal-atomic absorption spectrometry (ETAAS), for the automated extraction and determination of Cr(VI) in water samples. The pre-concentration factors were found to be 20 and 70 for microextraction times of 5 and 20 minutes, respectively. The detection limit of procedure was 0.02 µg/L of Cr(VI).

Headspace single drop microextraction (HS-SDME) HS-SDME is aided by direct exposure of the microdrop to the headspace of the sample solution. The drop suspended from the syringe is placed in the headspace of the solution, to extract volatile or semi-volatile compounds. HS-SDME is a three-phase technique which requires equilibrium between the organic solvent and the aqueous phase with the head space (Luque de Castro and Molina, 2012). Compared with DI-SDME, droplet stability is a less critical factor for a successful

HS-SDME extraction, which allows faster stirring of the aqueous solution (Xu *et al.*, 2007). Moreover, the drop is not immersed in the aqueous sample phase, and this advantage allows extraction of a complex sample matrix without interferences arising from non-volatiles; also, the solubility of the drop in the aqueous solution need not be considered (Pena-Pereira *et al.*, 2010a; Sarafraz-Yazdi and Amiri, 2010).

Since it can be utilised from water as a solvent in HS-SDME, the range of extractable analytes can be extended. However, the vapour pressure of the solvent, which must be chosen properly to avoid evaporation during sampling, is a limitation of HS-SDME. Increasing the droplet size in the case of a water-soluble solvent is another demanding point that may cause falling of the droplet from the needle (Xu *et al.*, 2007; Sarafraz-Yazdi and Amiri 2010).

HS-SDME has been proposed for the determination of analytes in food samples in several studies. A few example in this field include:

- iodine determination in non-fat milk powder and wheat flour, using UV-vis spectrophotometry (Pena-Pereira *et al.*, 2009);
- the determination of benzoic acid and sorbic acid in soy sauce and soft drink samples, with a procedure involving coupling of HS-LPME and capillary zone electrophoresis (CZE) (Ling *et al.*, 2010);
- analysis of trimethylamine-nitrogen (TMA-N) in fish samples via microvolume UV-vis spectrophotometry (Pena-Pereira *et al.*, 2010b);
- determination of cyclamate in artificial sweetener and soft drink samples, with instrumental GC analysis (Hashemi *et al.*, 2011);
- determination of fluoride in milk samples through ion chromatography (Yiping and Caiyun, 2010);
- the extraction of flavour compounds from fruit juices by ultrasonic nebulisation headspace ionic liquid-based single drop microextraction (UN-HS-IL-SDME) (Jiang *et al.*, 2013);
- the determination of volatile oxidation compounds in mayonnaise with HS-SDME coupled with GC-MS (Enteshari *et al.*, 2014).

Xie *et al.* (2010) used an in-line coupling system involving a headspace liquid-phase microextraction (HS-LPME) and capillary electrophoresis (CE) with the purpose of determination of phenols in water sample. They investigated influencing factors on the extraction efficiency, and obtained the optimal enrichment factors with a sample volume of about half of sample vials.

Šrámková *et al.* (2014) studied an automated in-syringe HS-SDME procedure for determination of ethanol in wine samples. In this method, the syringe itself was used as an extraction chamber, and slow stirring was applied inside the syringe to homogenise solutions. Applying the negative pressure to increase the transfer of analyte is another considering point in the system. A sequential injection analysis (SIA) was also coupled with in-syringe HS-SDME for an automated solvent-free system. The time needed for one analysis was five minutes, and the detection limit of ethanol in samples was determined as 0.025% (v/v).

Directly suspended droplet microextraction (DSDME) Yangcheng and co-workers (2006) described DSDME as a new sampling method, and practised this technique for extraction of 1,8-dioxanthraquinone, using 1-octanol as the solvent phase. While an aqueous sample phase was stirring, and a vortex being generated via a stirring bar inside the

sample cell, a drop of an organic solvent which had a lower density than water was dripped on the centre of the liquid surface. After a certain time, a HPLC sample injector was used to uptake the microextract from solvent droplet and transfer it for HPLC analysis. In DSDME, the microdroplet itself starts to rotate around a symmetrical axis, and this rotation may cause a better mass transfer and improve the extraction efficiency. A high amount of solvent, and high stirring rates, are the main advantages of DSDME in comparison with other static LPME methods.

Liquid-liquid-liquid microextraction (LLLME) LLLME application is a three-phase type of SDME, which consists of extraction of analytes from aqueous solution to organic phase and, simultaneously, back-extraction into an aqueous drop that serves a final acceptor phase at an appropriate pH. Generally, an organic immiscible solvent with a lower density than water is confined inside a Teflon ring (Ma and Cantwell, 1999), or forms directly a thin layer above an aqueous sample solution. An aqueous droplet is also immersed into this organic solvent. Firstly, analytes are extracted from the aqueous sample solution to this organic solvent and, at the same time, back-extracted into an aqueous droplet, which usually suspended at the end of a syringe needle. LLLME can be applicable without using a microsyringe as a supporting device (Sarafraz-Yazdi *et al.*, 2009). LLLME is a suitable choice for the extraction of hydrophilic organic compounds, and has a good compatibility with reverse-phase HPLC and capillary electrophoresis (Jeannot *et al.*, 2010).

Choi *et al.* (2009) performed 3-phase SDME and 2-phase SDME using an automated SDME-CE technique. This in-line coupling of SDME and two different types of commercial CE provided an enrichment factor of 2000 (10 minutes extraction) in 3-phase SDME with stirring, while 2-phase SDME yielded 110-fold enrichments using a Teflon sleeve and without stirring (30 minutes of extraction for analytes). Zhu *et al.* (2010) proposed a novel coupling approach, with single-drop liquid-liquid-liquid microextraction (SD-LLLME), to improve the efficiency of the CE instrument. This on-line technique gained a 550-fold enrichment in adenine analysis under the optimised conditions. Also, it was mentioned that this on-line sample preparation method can be utilised for the determination of adenine in green tea samples.

Continuous flow microextraction (CFME) In CFME, the continuous flow of the sample solution replaces the stirring of the aqueous sample solution, and sustained interaction between sample and organic solvent is the main factor for high enrichment. Liu and Lee (2000) introduced the CFME method as a novel approach in a liquid-liquid microextraction field. CFME contains a glass extraction chamber with a connected PEEK (polyetheretherketone) tube for obtaining a continuous flow of aqueous sample solution. Sample solution is pumped continuously via a HPLC solvent delivery system into this chamber. After filling the chamber with sample solution, organic solvent is introduced via a microsyringe. The solvent moves, together with the sample solution, to the outlet of the PEEK tubing, virtually immobilises at this place and forms a drop. This solvent drop is exposed to continuous flow of sample solution, interacts with it, and extraction of the analytes is performed. Some of the advantages of CFME, in comparison with other LLME methods, are: a high enrichment factor in a short extraction time (10 minutes); smaller volume of sample solution; and the use of an HPLC injection valve, which allows the droplet size to be controlled.

Anthemidis and Adam (2009) developed an automatic sequential injection single-drop micro-extraction (SI-SDME) system for analysis of trace metals in water samples.

For this purpose, a flow-through microextraction cell was designed and coupled with a sequential injection system. Final determination of the analyte was performed through the use of electrothermal atomic absorption spectrometry (ETAAS). This system involved on-line complex formation of metals with chelating agents. Ammonium diethyldithiophosphate (DDPA) was chosen as a chelating agent for cadmium determination in samples. The detection limit of system was 0.01 µg/L, and enhancement factor was 10 for 10 minutes extraction time.

17.2.1.2.2 *Dispersive liquid-liquid microextraction (DLLME)*

DLLME represents a major type of SME technique that has attracted considerable attention in recent years. A conventional DLLME system consists of the interaction of three components:

- 1) water-immiscible extraction solvent;
- 2) water-miscible disperser solvent;
- 3) sample aqueous solution.

The mixture of µL of solvent and mL of dispersive solvent is rapidly injected into an aqueous sample solution with a syringe. The solution adopts a cloudy appearance because of fine droplets of solvent, which disperse in the aqueous phase and form an emulsion. Centrifugation of the system separates the two phases and the solvent, with the analytes accumulating at the bottom of the sample tube in the final stage of the procedure.

Increasing of the extraction surface area by means of fine droplets of solvent is one of the important advantages of DLLME, and gives rise to fast and effective extraction. High enrichment factor, the use of a few microlitres of solvent, simplicity of procedure, and low cost are the other considerable advantages of DLLME (Sarafray-Yazdi and Amiri, 2010; Kocúrová *et al.*, 2012). On the other hand, the system needs certain requirements that limit the choice of solvent and disperser. The extraction efficiency of the solvent has to be high enough for all analytes at the same time, it has to be immiscible with water, it has a higher density from water and it has to be applicable for instrumental analysis.

Furthermore, a disperser which is miscible with both of sample phase and solvent phase has to be chosen for obtaining a fine emulsion (Andruch *et al.*, 2012a; Kocúrová *et al.*, 2012). For this purpose, chlorinated solvents, such as chlorobenzene, dichloromethane and carbon tetrachloride are preferred as extraction solvents, while acetone, acetonitrile, MeOH, and ethanol are preferred as disperser phase in the application of DLLME (Asensio-Ramos *et al.*, 2011). The use of solvents with a density lower than water, and removing the need for centrifugation or disperser solvent, are some of the different strategies to overcome the limitations of these techniques (Kocúrová *et al.*, 2012). Although non-polar analytes are the primary target compounds for DLLME applications, an ionic liquids (ILs) approach and *in situ* derivatisation are also used to expand DLLME for polar analytes (Ramos, 2012).

As well as other liquid phase extraction methods (SDME, hollow-fibre microextraction (HF-LPME), etc.), ILs approach finds a wide range of application in the field of DLLME literature. ILs are important alternatives to organic solvents, due to their limited miscibility with water, high affinity for numerous compounds of interest, and they commonly have a high density than water (Ruiz-Aceituno *et al.*, 2013).

The extraction of pesticides is an example of DLLME applications in food analysis. Food matrices, such as cucumber and watermelon (Zhao *et al.*, 2007), apple (Zang *et al.*, 2008), water and fruit juices (Fu *et al.*, 2009), tea (Moinfar and Milani Hosseini, 2009) and banana (Ravelo-Pérez *et al.*, 2009) are just a few examples in the literature. The other field that performs DLLME is the determination of metals, especially in water samples (Gharehbaghi *et al.*, 2008, 2009; Wen *et al.*, 2011).

Automation of conventional DLLME is an important challenge for DLLME procedures, because of the nature of the application, which is composed of multiple steps. Incorporation of DLLME into sequential injection analysis (SIA) is one of the main trends in the field of automation of the procedure (Andruch *et al.*, 2012b; Anthemidis and Ioannou, 2009, 2010, 2011). Martinis *et al.* (2011) utilised an on-line ionic liquid (IL) dispersive microextraction system for determination of selenium species in water and garlic extract samples. On-line separation and pre-concentration was performed by IL-DLLME and detection was performed with coupled electrothermal atomic absorption spectrometry (ETAAS).

Maya *et al.* (2012) introduced a fully automated system with a syringe-based approach. This 'Lab on syringe' system carried out microextraction and detection steps inside a syringe, using an automatic syringe pump, two optical fibres and a solvent with a density lower than water. Optical fibres were placed on the head of the syringe, facing each other, in order to quantify the analytes through a UV-vis spectrophotometer. This integrated system enabled detection of solvent droplets, which accumulated on the head of the syringe with extracted analytes. The extraction of rhodamine B was performed for the characterisation of DLLME, using different operation parameters (selection of extractant, selection of disperser solvent, volume and percentage of extractant/disperser mixture, pH, extraction time, etc.). In the next step, water and soft drink samples were used for extraction and quantification of the dye rhodamine B. The limit of detection was 0.007 mg/L, and the enrichment factor was 23 (for 1 mg/L rhodamine B standard).

17.2.1.2.3 Hollow-fibre microextraction (HF-LPME)

In HF-LPME systems, a thin supported liquid membrane (SLM), sustained into the pores of a porous hollow fibre (HF), is used as the extraction phase. The analytes are firstly extracted into SLM, and then into an acceptor phase filled into the lumen of the HF. A piece of HF (u-shaped or rod-like) is first dipped into extractant phase for a few seconds, then the acceptor phase is filled inside the lumen of the HF and, finally, the whole system is placed in a sample solution (Pedersen-Bjergaard and Rasmussen, 2008). The porous structure of HF protects and stabilises the extraction phase, while increased extraction yield is obtained through its high surface area. A high amount of solvent that flows and distributes within the pores of HF by capillary forces can be immobilised into this structure (Ghambarian *et al.*, 2012).

When the acceptor phase and the extraction solvent are the same organic solvent, this procedure is called two-phase HF-LPME (HF(2)-LPME), according to the number of phases. In the same way, an aqueous solution (acidic or alkaline) that is immiscible with SLM may be used for extraction of polar and ionisable analytes, and this procedure is called three-phase HF-LPME (HF(3)-LPME). Furthermore, ionisable compounds can be extracted by the electromembrane extraction (EME), which is a type of HF(3)-LPME technique involving electrode wires placed inside and outside the fibre (Kokosa, 2013).

Several examples involving the applications of HF-LPME are found in the literature for the sample preparation step of food analysis. Zhu *et al.* (2002) described a HF-LLLME procedure, with the aim of pre-concentrating phenoxy herbicides in bovine milk, and the final separation was performed with a HPLC system. Also, HF-LPME coupled with HPLC for determination of melamine in milk products was used by Wang *et al.* (2010) and Gao and Jönsson (2012). Sanagi *et al.* (2013) analysed the polycyclic aromatic hydrocarbons (PAHs) in fresh milk, through the HF(2)LPME method, combined with GC-MS.

In 2014, Sun and co-workers introduced a graphene-reinforced hollow fibre liquid phase microextraction (G-HF-LPME) technique for pre-concentration of phenylurea herbicides in milk samples, prior to HPLC-UV detection. Enhancing adsorption via a nanomaterial such as graphene, while obtaining certain clean-up efficiency, was the main purpose of the research. Romero-González *et al.* (2006) applied a hollow fibre-supported liquid membrane (HFSLM) procedure, in combination with LC/MS, for solid food matrices like cucumber, pepper and tomato for the determination of pesticide residues. Saaïd *et al.* (2009) accomplished the determination of biogenic amines in shrimp sauce and tomato ketchup samples via HF-LPME and HPLC-UV determination. The procedure also included both the *in situ* derivatisation and extraction of samples in a single step. Barahona *et al.* (2010) utilised the HF-LPME technique for the analysis of fungicides from orange juices. The optimisation procedure was performed with capillary electrophoresis (CE), and the LC/MS system was used for evaluating the analytical performance.

Although sampling procedures of food analyses contain the abovementioned conventional HF-LPME applications, a limited number of studies involving automatization of HF-LPME are found for food matrices comparing environmental (Hou *et al.* 2003; Jiang *et al.* 2005; Wu *et al.* 2005) and biological samples (Cui *et al.*, 2009). Ouyang and Pawliszyn (2006) developed an automated HF-LPME procedure to investigate the kinetic calibration method for quantification of the procedure. All of the extraction steps were accomplished automatically with an autosampler and software. The calibration method and automated system were used to quantify carbaryl in red wine samples, and final instrumental analysis was performed by GC/MS. Pezo *et al.* (2007) employed an automatic HF-LPME procedure to carry out the determination of the migrants from certain prototypes of active packaging. The automatic extraction system contained a multichannel syringe pump which supplied the processing of several samples concurrently, a three-way valve with online programme, and a dynamic approach by pumping fresh solvent through the lumen of fibre in a continuous way.

17.2.1.3 Solid-phase extraction (SPE)

The trapping of analytes onto an immobilised phase is one of the traditional approaches for sample preparation from liquid or gaseous samples (Figure 17.2). The SPE technique is a sorbent-based extraction technique, which traps the analytes by using different kinds of sorbents. These include polar sorbents, apolar polymeric resins, silica, graphitised carbon sorbents and, for specific purposes, ion-exchange sorbents, immunoaffinity sorbents, controlled-access sorbents, functionalised polymeric resins and molecularly imprinted polymers (Wells, 2003).

In SPE, the partitioning of analytes between two phases is the fundamental principle, similar to LLE, but the analytes are separated and concentrated on a solid surface, and then desorption is performed by an organic solvent. Important advantages of SPE, in

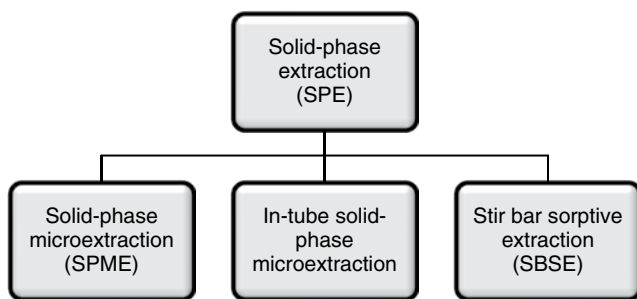


Figure 17.2 Methodologies based on trapping the analytes.

comparison with LLE applications, are interferences removal, fast and sensitive separation, the use of reduced amounts of organic solvent, reducing emulsion formation, and convenience for automatization. Conditioning of the sorbent to interact with analytes in a proper way, retention of the analytes via bounding to the solid surface, washing the system to remove the undesired compounds, and desorption and collection of analytes are the main steps of SPE procedures (Buldini *et al.* 2002).

Disposable and pre-packed cartridges, and syringe-barrel formats, are widely used devices in SPE. The cartridges can be designed with different adsorption characteristics for different systems and different automatization levels. Weight of the packaging material may vary between 35 mg and 2 g, and the volume above the packing material is within the range 0.5–10 ml (Majors, 2010). A syringe barrel is composed of 20 μm polypropylene frits at the top and bottom of a 40 μm sorbent bed, while the syringe barrel terminates with a male luer tip. The sample reservoir is generally polypropylene (Thurman and Mills, 1998).

The other widely used format of SPE is SPE discs with different diameters (a standard disc size is 47 mm). An SPE disc is a flat, thick membrane (usually $\leq 1\text{mm}$ thickness), but these discs differ from membrane filters due to their structure. PTFE (polytetrafluoroethylene) discs involve embedded packing material in PTFE fibrils. Glass fibre discs that are more rigid and thicker are also found for higher flow rates (Żwir-Ferenc and Biziuk, 2006). The disc cartridges are also a modified format, included the sorbent-containing discs, which are placed in a syringe-barrel format. A Teflon barrel with 20 μm frit, the disc, and a pre-filter, are the main components of the disc cartridges (Thurman and Mills, 1998). The main advantages of SPE discs, compared with the restricted flow rate of the cartridges, are a high flow rate and decreased extraction time. Also, the smaller sorbent particles of the discs ensure a more efficient separation of the analytes (Żwir-Ferenc and Biziuk, 2006).

The SPE method is widely used for analysis of different compounds in numerous food matrices, due to the variety of sorbents types needed for specific sample preparation requirements. For pesticide analysis, SPE was performed in wine (Soleas *et al.*, 2000; Jiménez *et al.* 2001); in fruits and vegetables (Schenck *et al.*, 2002; Štajnbaher and Zupančič-Kralj, 2003); in essential oils of citrus fruit (Barrek *et al.*, 2003); and in egg samples (Schenck and Donoghue, 2000), for the purpose of extraction and clean-up. In acrylamide analysis, the SPE method was used for clean-up purposes for coffee samples (Andrzejewski *et al.*, 2004; Granby and Fagt, 2004) and cereal-based foods (Zhang *et al.*, 2005).

A few examples involving the SPE applications in the sample preparation step include: purification and fractionation of berry extracts, using SPE for determination of anthocyanin (Huopalahti *et al.*, 2000; Denev *et al.*, 2010); analysis of folic acid in fortified beverages (Breithaupt, 2001; Pérez Prieto *et al.*, 2006); determination of Ochratoxin A in roasted coffee (Sibandaa *et al.*, 2002); and determination of patulin in apple products (Boonzaaijer *et al.*, 2005).

Gallart-Ayala *et al.* (2011) developed an automated on-line system for the extraction and LC-MS/MS determination of bisphenols in canned soft drinks. The extraction of bisphenols was accomplished by an on-line SPE system, which involved a C18 column with 12 μm particle diameter, with pre-concentration being performed without any pre-treatment to samples. The system analysed 11 samples simultaneously, and the results could be obtained even at a very low concentration level (ng/L). Jurado-Sánchez *et al.* (2007, 2011) presented a continuous SPE approach with the purpose of clean-up and pre-concentration of analytes in two different studies. The analysis of N-nitrosamines in beverages and the detection of N-nitrosamines, aromatic amines, and melamine in milk, cheese, yogurt, and cream samples were performed with a continuous SPE device, followed by GC equipped with nitrogen-phosphorus detection (NPD) and GC-MS, respectively.

Tao *et al.* (2012) utilised a semi-automated system for the extraction of aminoglycosides in animal derived food samples. An automated SPE system was applied for the purification of analytes before LC-MS/MS instrumental analysis. The SPE system allowed five different solvents and extraction of four samples simultaneously. Niu *et al.* (2012) detected bisphenol A, nonylphenol, and octylphenol in food samples, including rice, maize, and wheat, with a automated approach. They set up a procedure including clean-up and instrumental analysis of samples with an on-line SPE equipped with LC-ESI-MS/MS after ultrasonic extraction and centrifugation of samples.

17.2.1.3.1 Solid-phase microextraction (SPME)

The SPME technique is based on the miniaturisation approach in the field of SPE. Simple, cheap, solvent free and efficient one-step nature and easy automation are the main advantages of SPME procedures. SPE applications need an elution step for separation of analytes from SPE columns before the detection and quantification steps. An organic solvent is usually used for this purpose. However, in SPME, a fibre coated with an appropriate stationary phase carries out the extraction of desirable analytes, and this fibre can be placed directly into GC systems, in which thermal desorption of analytes can occur (Smith, 2003). In LC systems, desorption of analytes can be performed by exposing the fibre to a moving stream of mobile phase (dynamic mode), or by dipping into the mobile phase/organic solvent for a predetermined time (static mode) (Balasubramanian and Panigrahi, 2011).

SPME application consists of an equilibrium between the adsorbent layer and the sample by partition. The analyte is adsorbed onto the sorbent layer by exposing the fibre to the headspace of the sample, or by direct immersion into it (Ramos, 2012).

There are many factors that have a considerable effect on the performance of SPME. These include: type of fibre; thickness of the stationary phase; sampling mode (headspace or direct immersion); volume of sample; temperature; duration of extraction; desorption conditions; derivatisation; and salting out. Different operation modes of SPME may have a certain influence on the effectiveness of SPME (Wardencki *et al.*, 2007).

Analysis of volatile compounds is the primary field of SPME application in food matrix. as Examples in recent years include: analysis of furan in commercial canned and jarred food products by SPME-GC/MS system (Kim *et al.*, 2010); analysis of volatile compounds via headspace solid-phase microextraction (HS-SPME) in wine samples (Noguerol-Pato *et al.*, 2009; Pino and Queris, 2010; Sagratini *et al.* 2012; Burin *et al.*, 2013; Xiao *et al.*, 2015); in citrus fruits (Nardini *et al.*, 2013); in cucumber juice (Cho and Buescher, 2011); in tomato puree (Viljanen *et al.* 2011); in olive oil (Cecchi and Alfei, 2013; Romero *et al.* 2015); in meat samples (Ma *et al.*, 2013; Lorenzo, 2014). Pesticides analysis in fruits and vegetables (Menezes Filho *et al.*, 2010; Abdulra'uf and Tan, 2013, 2015), and amino acid analysis in food samples (Mudiam *et al.*, 2012; Mousavi and Pawliszyn, 2013) are among other efforts to utilise SPME in food analysis.

Ravid *et al.* (2010) used an autosampler of a GC-Pal system in order to obtain automation in HS-SPME, and combined it with GC-MS for identification of the origin of fruit flavours in food and beverage samples. A stereoselective column provided the separation of chiral flavour compounds and detection of synthetic flavourings in food samples. Cagliero *et al.* (2012) developed a total analysis system (TAS), which included an online coupling of automated HS-SPME and an enantioselective (Es)-GC-MS system. The research investigated optimisation and speeding up of the system, and then its application for the determination of the authenticity of fruit-flavoured food samples. This automatic system gave rise to an important decrease in the analysis time (ranging between 67–87% according to sample matrix), but needed development in the analysis condition for the chiral markers which were specific for each fruit sample.

Barros *et al.* (2012) performed the analysis of volatile compounds in wine samples, using an autosampler and software to achieve a certain level of automation in the HS-SPME procedure coupled with the GC-IT/MS system. A central composite experimental design (CCD) was used for the optimisation of automated HS-SPME. The extraction parameters (salt addition, extraction time, incubation time and temperature) were determined as the important parameters for the optimisation. Also, five different fibres were tested for the procedure. The optimised procedure was applied to wine samples for detection of 20 important aromatic compounds.

Mirnaghi *et al.* (2013) studied, using 96 thin-film SPME and an autosampler (together called a Concept-96-autosampler) for the analysis of phenolic compounds in food samples. The automated system, which enabled analysis of up to 96 samples simultaneously, was coupled with LC-MS/MS. The phenolic compounds in grape, berry, and wine samples were analysed in less than 2.2 minutes per sample, and a significant decrease in matrix effect was observed, compared with other solvent extraction techniques.

17.2.1.3.2 In-tube solid-phase microextraction

SPME applications may be limited by the amount of stationary phase, which restricts the extraction capacity. Also, transferring analytes to HPLC and LC-MS systems may be more complex, compared with GC-MS systems. The in-tube approach uses an open and tubular fused-silica capillary column, in which the extraction of analytes is performed by the stationary phase of the column, instead of a fibre. The sample solution is introduced into the internally coated capillary column continuously, the analytes are trapped by the column, and desorption of the analytes was accomplished by the mobile phase via direct connecting with LC (Kataoka *et al.*, 2000). Continuous feeding of sample solution to the system can be provided by an autosampler device, and automation of

the system can be achieved easily with a standard HPLC instrument (Ouyang and Pawliszyn, 2006). The capillary SPME column is placed between the injection loop and injection needle of the HPLC autosampler, and this automated in-tube SPME-LC-MS system is found as commercial equipment.

SPME and in-tube SPME approaches have their own advantages and disadvantages. In-tube SPME needs a centrifugation or filtration step for the sample solution, in order to remove particles and prevent capillary column, while the particles can be removed from the fibre of SPME by washing before the injection to instrument, so that filtration is then not necessary. On the other hand, the fibres of SPME can be damaged because of their fragile nature and, also, the coating may be damaged because of agitation of the sample solution. The extraction time of in-tube SPME systems is less than SPME, and a great variety of stationary phase is available as commercial capillary columns, compared with limited commercial SPME fibres (Kataoka, 2002).

A desorption chamber is generally utilised for the coupling of SPME with HPLC, with the aim of solvent desorption of analytes from fibre. This chamber is assigned as an interface and, after desorption of analytes in the chamber via a mobile phase or a solvent, the analytes are introduced to the HPLC column. Eisert and Pawliszyn (1997) demonstrated the first attempt to automate the SPME technique through an in-tube SPME approach, using a piece of GC capillary column. The non-coated capillary column of LC autosampler was replaced with the GC capillary column, and the autosampler was controlled with a software programme. Desorption of analytes from the capillary column was achieved by flowing methanol from a second vial into the injection loop of HPLC.

Wu *et al.* (2002) used a similar system for the determination of polar pesticides in water and wine samples. A polypyrrol (PPY)-coated capillary column was used for an in-tube SPME device, and electrospray ionisation mass spectrometry (HPLC-ESI-MS) was chosen as the detection method. Nonaka *et al.* (2009) utilised an automated in-tube SPME method for the analysis of aflatoxins in food samples such as peanuts, tree nuts, cereals, dried fruits, and spices. The SPME capillary column was placed between the injection needle and the injection loop of the autosampler. Drawing and ejecting of the sample was performed repeatedly, until equilibrium was reached, and the mobile phase was used for the desorption and transferring of the analytes to LC-MS system.

Another microextraction method which was developed involving in-tube application is headspace in-tube extraction (ITEX), for the analysis of volatile and semi-volatile compounds. In the ITEX method, a packed sorbent material in a tube attached to a needle is used, instead of an internally coated capillary tube. A wide variety of commercial sorbent material, and a high amount of stationary phase, giving a higher extraction yield, are the main advantages of the system. An ITEX system consists of two parts. The upper part contains: the sorbent material in a tube which has a bigger diameter; a heater surrounding this tube for the thermal desorption of the analytes; and a headspace syringe, which is connected to tube. The lower part of the system contains an ordinary needle. For the desorption step, a certain amount of desorption gas is aspirated into the syringe to occur a desorption volume, then the extraction tube is heated rapidly to the desorption temperature. After desorption is completed, the desorption volume is ejected into the GC system (Jochmann *et al.*, 2008).

Thanks to its fully automated and solventless nature, ITEX systems have seen application in analysis of water contaminants and aroma compounds in food samples in recent years. Laaks *et al.* (2010) developed an ITEX method with a Combi-PAL autosampler

and GC-MS instrument for the determination of water contaminants. The aspirating/dispensing cycle was performed by the syringe several times, for the extraction of analytes at a certain flow rate, and desorption was carried out with the flow of nitrogen. Five different sorbent materials were evaluated during the study, and the detection limits were obtained as 10 ng/L for volatile organic compounds (VOCs) in samples. In another study, Laaks *et al.* (2014) investigated the nine different sorbent materials for the extraction of volatiles of beer samples with an automated ITEX system coupled with GC-MS.

Zapata *et al.* (2012a) carried out the determination of volatile compounds (mainly acetaldehyde, ethyl acetate and diacetyl) in wine and beer samples, with a commercial automated ITEX installed in an autosampler and coupled with GC-MS. The critical factors effecting the extraction procedure were determined as the vial size, sample size and the sorbent material, because of the saturation effect of ethanol in the study. Zapata and co-workers (2012b) also developed a combined system which contained ITEX and multiple headspace extraction (MHE) together, and the analysis of aroma compounds in wine samples was achieved by an automated MHS-ITEX-GC-MS system.

17.2.1.3.3 *Stir-bar-sorptive extraction*

One of the factors which limits SPME applications is the amount of the stationary phase derived from the coated fibre. A small amount of stationary phase may cause an uncompleted extraction of analytes, because of the current phase ratio between the sample solution and fibre (Smith *et al.*, 2003). Stir-bar-sorptive extraction (SBSE) was developed to overcome this limitation at the end of the 1990s. A coated stir bar is used for the stirring and extraction purpose, and the amount of adsorbent obtained is much more than with SPME fibres. Although it is mentioned that the extraction time for the SBSE is generally longer than SPME, the main advantages of SBSE method are higher phase ratio, higher efficiency, and lower LODs (Jeleń *et al.*, 2012; Ramos, 2012).

In the SBSE procedure, a magnetic stir bar is coated with an adsorbent layer, which is usually polymethyl dimethyl siloxane (PDMS), or the magnetic stirrer may be inserted into a short tube contained PDMS. When the sample solution is stirred, the stir-bar rotates into it and the extraction is carried out (direct SBSE). Alternatively, the stir-bar can be exposed to the headspace of a sealed vial involving the gas, liquid or solid sample (HS-SBSE). The stir-bar extraction systems are also commercially available, and known as 'twister'. The desorption of analytes from the stir-bar can be performed by a thermal GC desorption system, or by using a solvent with the aim of elution.

The commercial limited coatings and difficulties in the effort of automation are the fundamental drawbacks of SBSE technique. The apolar character of PDMS makes this technique more suitable for non-polar or slightly polar analytes, or for needs derivatisation in some applications. Dual-phase twisters are one of the recent efforts to expand the field of application via combination of SDME with another sorbent, to increase the selectivity of the system (Sánchez-Rojas *et al.*, 2009).

A recent review prepared by Kawaguchi and co-workers (2013), describes the applications of SBSE in food analysis in detail. Aroma compounds and volatiles, VOCs, OPPs, pesticides, fungicides, PAHs, and phenols are some of the analysed compounds in several food matrices using SBSE in the literature. The general approach for the automation of SBSE is the combination with the desorption units, especially in thermal desorption systems. This system is also commercially available.

Sandra *et al.* (2003) performed SBSE for the enrichment step of pesticides analysis in food samples after methanol extraction. A thermal desorption unit (TDU), which was installed on a xyz robot, was placed on the top of GC system equipped with a programmed temperature vaporisation (PTV) injector. This coupled equipment provided unattended desorption of 98 twisters automatically. The detection of pesticides in fruit, vegetables and baby food samples was analysed by capillary-GC-MS, using the retention time locking (RTL) software. The detection limits from ppm to ppb amounts was evaluated by the multi-residue method (MRM) described in the study.

Kende *et al.* (2006) studied the pesticides levels of fruit and vegetables samples using a SBSE-thermal desorption-GC-MS. A twister desorber unit was mounted on the injector of the GC-MS instrument. The migration of alkylphenols (APs) and phthalate esters (Pes) for fresh ready-to-eat (RTE) vegetables samples was investigated by Cacho *et al.* (2012). After extraction of samples, a PDMS coating stir-bar was used for SBSE pre-concentration. A commercial and automated TDU system, coupled with a programmed temperature vaporisation (PTV) injector, was used for thermal desorption and injection of samples to the GC-MS instrument. The recoveries obtained from the proposed method ranged between 83–118%.

17.2.2 Extraction from solid samples

In the case of solid sample handling, drying, grinding, homogenisation, and so on, sample treatments steps can be needed, due to the varying physical forms (powder or animal/plant tissue) of food samples. Also, after most of the liquid extraction procedures, additional clean-up and concentration may be required to supply appropriate samples, in order to facilitate analytical detection by removing interferences or undesirable substances and, also, to meet the detection limit of the instrument. The extraction of volatile compounds from solid matrix can be performed by several techniques, including static headspace, purge and trap (dynamic headspace), membrane extraction, and headspace-SPME.

For less volatile compounds, solid-liquids extraction methods and soxhlet extraction find a wide range of application in routine food analysis. Although these techniques have been accepted in several reference methods, they have important disadvantages, such as large amount of solvent and sample utilisation, high analysis time, containing high degree of sample manipulation, and the need of solvent evaporation. To overcome these disadvantages, the automation approach, including automated soxhlet extraction, has appeared in recent years as a promising improvement in this field, but there has been an important decrease in the use of these techniques over the last two decades. Contrary to this decrease, enhanced (or acceleration) techniques, involving solvents under high temperature and pressure, or shaking the sample to obtain a high diffusion rate during extraction, have started to appear in food analysis (see Figure 17.3). The easy on-line coupling aspects of these techniques with chromatography applications are the main driving force in this accelerating interest.

17.2.2.1 Matrix solid phase dispersion (MSPD)

The extraction, elution and fractionation of desired compounds from solid, semi-solid and viscous samples can be achieved in a single step with MSPD procedures. The MSPD technique relies on the mixing of sample with a solid support material to disperse the components on the sorbent surface, and a washing/elution step with a smaller amount

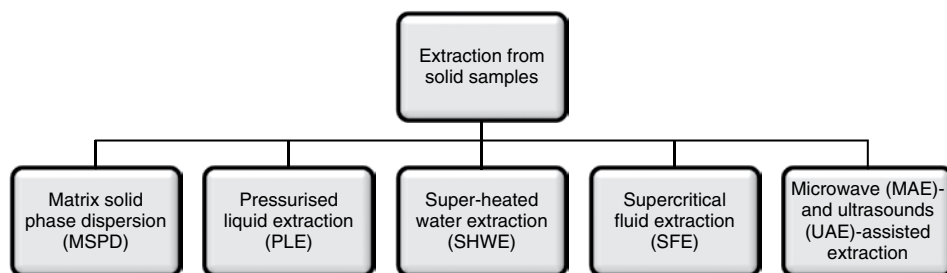


Figure 17.3 Methodologies for the extraction from solid samples.

of solvent than is used in liquid-liquid extraction methods. C18 bonded silica, silica gel, florisil, and alumina are examples of the sorbent materials in the MSPE applications.

The solid support allows the disruption step which is often essential for solid and semi-solid samples prior to extraction to be removed. The blending mixture of solid support and sample, which is packed in a column after mixing, provides a structure that is porous to solvent, in order to penetrate and extract the analytes. This structure may also allow the retention of undesired and interfering components of sample matrix, and visa versa (Ridgway *et al.*, 2007).

The selectivity and efficiency of the MSPE depends on the type of sorbent material, the type of elution solvent, the sample structure and composition and the ratio between the sample and sorbent material (Kristenson *et al.*, 2006). Most of the MSPD procedures particularly use apolar sorbents, such as reverse-phase materials (C18 and C8) for samples with high lipid content. The solid or liquid nature of the sample is an important factor influencing the strength of the analyte-matrix interactions. Although the ratio between sorbent and the sample generally ranges from 1 : 1 to 1 : 4 in the literature, the most common application is 1 : 4, which can be also modified, along with the sample and sorbent nature (Capriotti *et al.*, 2010). Also, pressurised liquid extraction (PLE) and solid-phase extraction (SPE) may be employed in conjunction with MSPD procedures, to gain a higher recovery and clean-up in the extraction process, respectively.

Automation is a difficult approach for the MSPD processes, due to the requirements for the blending step, which has to be carried out manually. The establishment of on-line systems have found a wide range of interest as a primary step through the automation of MSPD until recently. Lu *et al.* (2011) investigated sulfonamides (SAs) residues in grass carp tissues by an on-line analytical protocol, consisting of MSPD coupled with HPLC-MS/MS. After blending of a homogenised sample with C18 sorbent material, the mixture was packed into an extraction vessel, and the vessel was placed into the system, which was established via on-line coupling of MSPD and LC/MS/MS with a 10-port switching valve. The separation of analytes was achieved within seven minutes, and the system had a recovery range between 69.0–96.3%.

Gutiérrez Valencia *et al.* (2011) developed a coupled system that allowed the extraction of organophosphorus pesticides (OPPs) in bovine liver tissue samples through MSPD, pre-concentration with SPE, and instrumental analysis with HPLC-DAD systems. For this purpose, the coupled analytical system contained:

- 1) two reciprocal pumps, of which one was used for pumping the cleaning and elution solvents to the MSPD stainless steel cartridge, and the other for pumping the diluted extract to SPE stainless steel column for pre-concentration;

- 2) two six-port switching valves to connect MSPD cartridge and SPE column; and
- 3) a chromatographic injection port switching valve for the subsequent analysis that was done by the HPLC/DAD system.

The method had a recovery between 91–101% and the limits of detection of the OPPs were in the range of 0.04–0.25 µg/g.

17.2.2.2 Pressurised liquid extraction (PLE)

PLE is a technique which involves organic solvents at elevated temperatures. The system also needs high pressure, owing to the necessity to keep the solvent in its liquid form. Temperature is one of the important factors influencing the extraction yield. Temperatures above atmospheric boiling point of the solvent, obtained via high pressure, give rise to a decrease in the viscosity of the solvent. This decrease contributes the wetting properties of solvent in terms of the solid sample matrix. It also enhances the diffusion rates of analytes from sample to solvent, by breaking the interactions between analytes and solid matrix.

Static mode, dynamic mode and a combination of both modes are the three types of PLE applications. In static mode, solvent and sample maintain in contact with each other under specific conditions (temperature and pressure) and during a pre-determined time. After the system reaches equilibrium, which occurs between compounds found in the sample and the compounds transferred to the solvent, the efficiency of extraction is limited by this equilibrium. As a consequence of this, several consecutive extraction cycles are applied to the same sample in the static mode (Herrero *et al.*, 2013). Cleanup and enrichment steps may be needed after static mode PLE, because of undesired interferences from the sample matrix, such as fats. Low selectivity of organic solvents brings about the performing of column chromatography, gel-permeation chromatography or SPE applications, with the purpose of cleanup after extraction. The addition of adsorbents in PLE cells to retain fats or other undesired components is another way with respect to the single-step approach (Chen *et al.*, 2008).

In dynamic mode, the sample is placed into a stainless-steel cell and is exposed to a continuous flow of fresh solvent in a closed flow-through system. The increase in the efficiency of extraction via flowing solvent is an important advantage of dynamic mode, while dilution of the extract and use of more organic solvent, compared with static mode, are the main disadvantages of this approach. PLE application mostly includes an inert sorbent material that is employed for the dispersing of sample, to prevent the tendency of aggregation, and then the sample, with sorbent material, is packed into PLE cell. Diatomaceous earth, sand, glass beads and Teflon particles are some of the examples in the literature used as sorbent materials. Furthermore, drying agents (sodium sulphate, cellulose, etc.) can be added as a desiccant into the PLE cell, to avoid pre-treatment of wet samples (Carabias-Martínez *et al.*, 2005). High extraction efficiency, high speed, low extraction time and reduced organic solvent consumption are the benefits supplied by the wide range of utilisation of PLE in food analysis.

Determination of contaminants, such as polycyclic aromatic hydrocarbons (PAHs), pesticides, metals and mycotoxins, is the primarily field of PLE in the analysis of food, and also in biological samples. The extraction of several food components, such as polyphenols, lipids, essential oils and bioactive compounds (flavonoids, tocopherols, etc.) represents a new trend of PLE utilisation in food analysis (Carabias-Martínez *et al.*, 2005; Herrero *et al.*, 2013). Due to this wide range of opportunities for the extraction of diverse components

and the appropriate nature of PLE procedures, automation (semi-or full-) has been the main focus of research for more selective and fast analytical methods in recent years.

Most of these studies described in the literature of PLE have included automatic commercial PLE equipment for the analysis of different compounds, such as: polyphenols in apple peel and pulp (Alonso-Salces *et al.*, 2001); pesticides in fruits (Blasco *et al.*, 2005) and vegetables (Frenich *et al.*, 2005); tocopherols and tocotrienols in cereals (Bustamante-Rangel *et al.*, 2007); ochratoxin A in bread (Osnaya *et al.*, 2006); and in breakfast and infants cereals (Zinedine *et al.*, 2010) and OCPs and PCBs in marine samples (Helaleh *et al.* 2012).

Papagiannopoulos and Mellenthin (2002) and Papagiannopoulos *et al.* (2002) evaluated an on-line coupling system involving in PLE, SPE and HPLC steps for extraction of polyphenols in hop pellets and proanthocyanidins in malt samples, respectively. These studies particularly indicated the decreased analysis time, with respect to using an automation approach.

17.2.2.3 Super-heated water extraction (SHWE)

Water, as a solvent that is used in PLE procedures, can be efficient for the extraction of many analytes. Thanks to its unique properties, organic solvent consumption can be eliminated or reduced in SHWE applications. This method is also known as subcritical water extraction (SWE), high temperature water extraction (HTWE), hot water extraction (HWE) and pressurised hot water extraction (PHWE) in the literature. When the temperature of water is increased under pressure, its dielectric constant starts to decrease, which directly causes a decrease in the polarity of water. Thus, water can act as a medium/non-polar solvent.

The temperature and pressure of water is kept under critical point (generally, 100–250°C and moderate pressure). In a static system, water is filled into the extraction cell and heated. However, in dynamic systems, a sample that is kept under pressure is heated to extraction temperature, and water is passed through from the extraction cell. In static mode, the water has to be cooled in the extraction cell, and this application can result in inefficiency, because of re-adsorption of analytes. In dynamic systems, a cooling unit acts to decrease the temperature of water below 100°C (Smith, 2010).

17.2.2.4 Supercritical fluid extraction (SFE)

Supercritical fluid extraction (SFE) is one of the extraction methods that involves elevated temperature and pressure. Supercritical fluids are substances that are subjected to pressures and temperatures above their critical points. At this region, the substances possess a combination of both liquid and gas properties. They have a lower viscosity than the liquid form, but have good solvating ability like liquids. At the same time, they have good penetration ability into solid matrix, like gases with a high diffusivity. Supercritical fluids can lead to faster extraction, through their solvating power and their transport property coming from liquid and gas behaviours, respectively.

Solvents such as carbon dioxide, nitrous oxide, ethane, propane, n-pentane, ammonia, fluoroform, sulfur hexafluoride can be used in SFE applications (Tobiszewski *et al.*, 2012). However, CO₂ is the most preferred solvent, due to its low supercritical temperature and pressure values. Moreover, its low viscosity and high diffusion rate, as well as its non-toxic, non-flammable and inexpensive nature, are the other important advantages (Kou and Mitra, 2003; Smith, 2003)

SFE is an important alternative to solid-liquid extraction methods such as soxhlet extraction, with respect to non-hazardous and inexpensive solvent utilisation. The solvent strength can also be controlled by the density, which is a function of temperature and pressure, and a variety of solvent strength can be obtained with a single supercritical fluid (Buldini *et al.*, 2002). The addition of modifiers is another way to manipulate the solvating power of the fluid. CO₂ is an available supercritical fluid for the extraction of non-polar substances as PAHs, lipids and so on. A small amount of polar organic solvents are added to CO₂ to improve its extraction efficiency for the polar substances, and methanol is mostly used as a modifier for this purpose.

SFE allows several advantages during its applications. When selecting a supercritical liquid with a low critical temperature, extraction can take place for analytes of thermally unstable substances. A faster extraction rate can be obtained, due to the high penetration ability of supercritical fluids. In addition, after extraction, these fluids are easily removed from solid samples, by reducing the pressure and thus allowing the evaporation of fluid.

In the application of the SFE method, an extraction cell containing the sample and pressurised fluid above its critical point is heated for a pre-determined of time. At the end of the extraction, the extract is collected by a vial containing a solvent, or a solid sorbent trap. SFE can be operated in static and dynamic modes. In static mode, an extraction cell is loaded with sample and supercritical fluid at a constant pressure and temperature, and in a specific time. In dynamic mode, the sample is subjected to continuous flow of the supercritical fluid.

Collections of the analytes occurs through depressurisation of the supercritical fluid in different ways. Off-line trapping is performed by a sorbent with the analytes absorbed onto it, or by a solvent placed into a vial. However, on-line trapping provides direct transfer of the analytes to the chromatographic systems. For SFE-GC systems, the fluid is depressurised directly into a conventional injection port or an on-column injector, which includes a restrictor for depressurising the fluid. In SFE-LC systems, solid-phase trapping is established (Chen *et al.*, 2008; Kroon and Raynie, 2010).

The increasing attention to extracting food components (such as antioxidants, carotenoids, lycopene, etc.) and pesticides through SFE methods has been summarised recently by Pourmortazavi *et al.* (2014). Also, a comprehensive review of supercritical extraction of lipids and fatty acids has been published by Martinez and Aguiar (2014). Meanwhile, the on-line applications of SFE have received considerable attention during the last few decades, with the aim of developing automated and integrated analytical systems. On-line coupling of SFE with infrared (IR) spectroscopy techniques, or chromatographic techniques such as GC, LC and supercritical fluid chromatography (SFC), have been the main trends in this field, due to their simple aspects.

In on-line SFE-GC systems, depressurisation of CO₂ to obtain a gas phase, and to easily separate and eliminate it from the analytes, can be achieved by different interfaces. The trapping of analytes by a SPE or membrane, using a GC injector or programmable temperature vaporiser, are some of the examples for GC interfaces (Hyötyläinen, 2008; Martinez and Aguiar, 2014).

However, interfaces for SFE-LC applications are commonly based on solid phase trapping. Also, other interfaces, such as the impactor interface, open-tubular trapping, and the sample loop interface, are present in the literature (Hyötyläinen, 2007).

Aro *et al.* (2002) utilised an on-line coupled system containing SFE-SFC-GC to analyse the volatile and semi-volatile compounds in fish samples. After the volatiles were

extracted in the extraction cell, pressure restriction was achieved by fused silica capillary tubing placed after the extraction cell, and the extract was collected at the front of the ODS C18 column. SFC was used for the lipid extraction and, when the volatiles and lipids were separated, the volatile fraction was introduced directly into a gas chromatograph. Pól *et al.* (2004) used an on-line coupled system composed of SFE and HPLC-DAD instruments with an interface, which was a monolithic column placed in a thermostated chamber and six-port valve. Shorter analysis time and a good selectivity were obtained, together with any degradation in lycopene, due to the closed and automated nature of the analytical system.

Zougagh *et al.* (2008) evaluated an automatic system involved in sample treatment and instrumental analysis with on-line coupling approach. Due to the polar characters of paraquat and diquat (bipyridyl herbicides), a clean-up step, followed by extraction, was required for olive oil samples. The SFE system firstly provided a clean-up procedure for non-polar substances in the sample, and the polar pesticides were trapped into extraction vessel. Secondly, these polar pesticides were passed to the mobile phase that flushed through the extraction vessel and filled the loop of the electrochemical valve. After electrochemical screening, the positive samples were subjected to HPLC detection for separation and quantification. The detection limit of the confirmation method was 0.003 and 0.002 µg/g for PQ and DQ, respectively, and total analysis time was much shorter in comparison with LLE method (35 minutes for SFE and 160 minutes for LLE).

Zougagh and Rios (2008) also published a report for the extraction of riboflavin vitamins from food samples using the SFE method in a similar manner. SFE was on-line, coupled to continuous flow CE. This automated SFE-CF-CE-FD system contained SFE, with the aim of clean-up and extraction. The system used an interface composed of a capillary placed in the CF-CE coupling device. Yamaguchi *et al.* (2011) analysed piperine in peppers samples by coupling SFE to UHPLC, and the column-switching techniques were employed for this purpose. The study also compared three different approaches (solvent extraction, soxhlet extraction and SFE). According to results, SFE was the simplest and the most high-speed approach, with 0.25 hours of analysis duration within these three methods.

17.2.2.5 Microwave- and ultrasound-assisted extraction

Microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE) are general approaches which are employed to facilitate and enhance the efficiency of solvent extraction techniques. MAE methods use microwave energy to heat the extraction solvent or samples, which contain dipole molecules or microwave absorbents. The fast and uniform heating that occurs from the inside to the outside of the sample, and the agitation obtained during procedure, are the main advantages of the MAE. An open or closed vessel can be configured for focus (FMAE) and pressurised (PMAE) applications respectively (Ramos, 2012). UAE utilises acoustic cavitation to improve the extraction of analytes from the sample to the solvent. The formation of gas bubbles results in an increase in temperature and pressure in the local microenvironment.

Both MAE and UAE methods can be on-line, coupled with LC systems. Static and dynamic modes of MAE allow an online coupling with LC, while only the dynamic mode of UAE is available for online integration. One of the dynamic modes of MAE is performed by continuously passing sample and extraction solvent through the

microwave-assisted system. After extraction, filtration and LC analysis are carried out by the following steps. In dynamic UAE applications, the extraction solvent flows through the solid samples placed into the extraction vessel, or both the extraction solvent and the sample continuously flow through an ultrasonic bath, as per the dynamic MAE procedures (Pan *et al.*, 2014).

Chen *et al.* (2007a) reported a dynamic microwave extraction that was on-line and coupled to SPE-HPLC for the analysis of OCPs in grain samples. While the extraction of samples was performed by recirculating of solvent through the vessel, microwave heating was applied to the extraction vessel. The extract was carried toward to SPE column via a peristaltic pump, and on-line dilution with water was achieved in the sample loop. The sample was introduced to the SPE column for clean-up and concentration purposes. Then, the analytes trapped in the column were eluted with the mobile phase for analysis in HPLC. The automated on-line system involved in a six-port valve, an automatic 10-port switching valve and an injection valve, together with three pumps in general.

Chen *et al.* (2007b) also performed another study for the extraction of flavonoids in herbal plant samples. This time, dynamic microwave extraction was coupled to on-line derivatisation, which carried out into a sample loop, and the final step was UV detection. The detection and quantification limits of the automated system obtained were 0.28 and 0.92 mg/g, respectively, with a mean recovery of 98.5%. In 2009, the determination of formaldehyde in aquatic products was performed by the same research group, by a technique based on on-line integration of MAE, derivatisation, clean-up using restricted access material (RAM) and HPLC detection (Chen *et al.*, 2009).

The application of UAE in food analysis, such as lipids, polyphenols, antocyanins, polysaccharides and so on from plant sources, and bioactives compounds from animal sources, has been reviewed in a recent study by Vilku *et al.* (2008). In addition, several researches involved in on-line coupling approach with UAE can be found in the literature. Caballo-López and Luque de Castro (2003) used a dynamic UAE approach for the extraction of N-methylcarbamates from soil and food samples. The on-line coupled system contained a leaching step via UAE, a clean-up step involved in filtration and pre-concentration, a separation step with analytical column and derivatisation, and fluorescence detection as the last step. Yebra-Biurrun *et al.* (2005) coupled a continuous UAE system with a flame atomic absorption spectrometer (FAAS), using three switching valves and one injection valve in the whole system. The determination of zinc in meat samples could be carried out with a detection limit as 0.6 µg/g (for a 5 mg sample amount).

Chao *et al.* (2013) included an ultrasound-assisted push/pull perfusion (UPP) approach into HF-LLLME-HPLC instrumental system to accelerate the extraction rate of neutral analytes and to minimise fluid loss and/or fluid gain during the process. This on-line system provided the analysis of target phthalate esters in bottled drinks samples. Also, Falkova *et al.* (2014) developed on-line ultrasound-assisted surfactant-mediated extraction, coupled with spectrophotometric determination, by setting up two different injection methods for the analysis of anthraquinones in plant samples. One of these was sequential injection (SIA), while the other was stepwise injection (SWIA). According to the results obtained, both of the applications gave comparable results, with the reference methods and the sampling frequencies for SIA and SWIA were 12 per hour and 6 per hour respectively.

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18

The Market for Diagnostic Devices in the Food Industry

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18.1 Introduction

The food industry is, in general, driven by consumer demands and by governmental legislation. The impact of (social) media and NGOs is, nowadays, of high importance towards public perception. Therefore, consumers are increasingly aware of food safety issues and the effect of food on well-being. They are demanding higher-quality, fresher products, with fewer additives. Aspects like vegan, ethics and animal welfare are the focus of many consumer discussions. Moreover, it is known now that a large part of the population is allergic to some foods. In rare cases, allergens may have severe – even life-threatening – consequences. Consumers, therefore, need to know what is in the food they buy – hence, regulations in many countries require that manufacturers declare the presence of potential allergens on the label. This puts severe pressure on the industry, because it needs to comply with accurate labelling and stringent tracking and tracing systems to be able to respond instantly to any (real, potential, or perceived) incident.

On top of these requirements, the food industry is faced with the complexity of today's supply chains. Ingredients are sourced from all over the world (globalisation), spreading food-related hazards as fast as the ingredients and products move. Consequently, surveillance must be stepped up to be able to keep hazards due to micro-organisms, allergens (labelling), and chemical and physical contamination under control. From an economic point of view, food production has to face market increasing price volatilisation for primary agro products, while prices in the supermarkets remain stable. Furthermore, growing competitiveness has to face global quality standards, as well as environmental concerns, and more stringent regulations will boost the demand for analytical instrumentation (Frost and Sullivan, 2014). Some of the requirements both parties (consumers and legislation) put onto food manufacturers are listed in Table 18.1.

Table 18.1 Quality and safety requirements put onto the food industry.

Consumer demands
Absence of chemical residues (e.g. pesticides, veterinary drugs, cleaning agents)
Absence of chemical contaminants (e.g. heavy metals, persistent organic pollutants like dioxins, polyaromatic hydrocarbons) Absence of non-declared allergens (e.g. traces of peanuts)
Absence of foreign bodies (metal, glass, plastic, insects or parts thereof)
Correct labelling, including nutritional data
Microbial stability as well as absence of pathogens
High quality, culinary experience
Long shelf life, without noticeable quality loss or sensorial impact
Acceptable cost
Aspects like animal welfare, regional production, GMO-free products

18.2 Food diagnostics

The consequence of all this is that the food industry needs to know more about the product than ever before. Traditionally, food processors would like to be able to control the process. However, to measure process parameters at the place of production and to obtain maximum certainty with respect to regulatory requirements and consumer demands, many more diagnostic devices are needed or highly desirable. At the place of production, it means that these devices – in contrast to external commercial analysis – work at least at-line or by-line, and better on-line or in-line during food production. There is a market for devices that:

- measure in a given range (e.g. EU regulations – based on Reg (EG) No 178/2002) everything that helps to comply with the preceding requirements;
- can be implemented in an existing quality assurance system and also with respect to data management;
- work accurately, reliably, and are long-lived;
- have a sufficiently short response time;
- are hygienic (do not adversely affect the runtime of the process line or the quality of the product);
- are easy to operate, maintain, dismantle, and reassemble; and
- are affordable for the food industry.

In the following paragraphs, examples of what would be used by the industry if available, and complying with these requirements, are provided.

18.3 Product composition

It is important to be able to detect any undesirable substances in the raw materials and intermediate and final products (Table 18.2).

Table 18.2 Undesired substances in raw materials and intermediate and final products.

Threats	Primary production	Processing	Logistics	Retail
Biological:				
• Bacteria	• Identification and quantification by plate count agar, PCR	• Identification by plate count agar, PCR	• Intelligent packing	• Radio-frequency identification (RFID)
• Virus		• Treatment by:	• Transport temperature control	• Time temperature indicator (TTI)
• Yeast		• Heat		• Cooling storage
• Fungi		• Blanching	• Antimicrobial packing	• Temperature control
		• Spray nozzles (sanitation)	• Self- cooling/ self- heating packages	• Intelligent shelves
		• Ozone generation for sanitation systems	• Atmosphere control	• Information via smartphone
				• Combined with customer service
Chemical:				
• Pesticide	• Identification and quantification by reference analysis like chromatographic and spectroscopic methods – conducted by external commercial labs or in-house QA in a chemical lab	• Identification and quantification by reference analysis like chromatographic and spectroscopic methods – conducted by external commercial labs or in-house QA in a chemical lab	• Control not mandatory and therefore, in principle, not conducted	• Information via smartphone combined with customer service
• Veterinary drug				• In the case of 'own brands': QA by external labs – controlling the standard parameters (see left)
• Contaminant (e.g. heavy metals)				
• Cleaning agents				
Physical:				
• Bones	• Human inspection	• Human inspection	• Appropriate handling of packaging material, proper shipping	• Information via smartphone
• Metal	• Raw material inspection and specification	• Raw material inspection and specification		• Combined with customer service
• Wood	• Metal detectors, X-ray technology	• Metal detectors, X-ray technology		
• Plastics	• Magnetic traps	• Magnetic traps		
• Glass	• Electronic bottle inspection (for empty glass)	• Electronic bottle inspection (for empty glass)		
	• Bone separator	• Bone separator		

18.3.1 Physical hazards

Any extraneous object or foreign matter in a food item that may cause illness or injury to a person consuming the product. Sources for such contaminants include raw materials, badly maintained facilities and equipment, improper production procedures and poor employee practices.

Foreign bodies have been the cause of incidents many times. Metal detection – in contrast to plastics and bones – is relatively easy, although calibration and sensitivity are still causing problems. For other foreign bodies, there are no generally applicable and reliable detection devices. Some progress has been made with optical methods to detect foreign bodies present in empty glass jars and bottles. However, most manufacturers still rely on prevention and the use of sieves/strainers to capture and remove foreign bodies. Detection devices are preferably placed close to the filling machine, to minimise the chances of contamination. For aseptic ultra-high temperature (UHT) treatment lines, this often means that the device has to be resistant to sterilisation by steam or water of over 120°C and 3–5 bars of pressure. This can be a problem for in-line metal detectors, as these often have fibre-reinforced plastic housing to allow transmission of the sensor waves.

18.3.2 Biological hazards

Food products should not contain undesirable microorganisms in concentrations that may cause harm to the product and consumer. Being able to know the concentration of microorganisms at any time will provide the information needed to decide when a process needs to be stopped for cleaning and sanitation of the processing equipment. Therefore, devices that are able to detect microbes by smelling (electronic noses) are of great interest for the food industry, but are not yet commercially available. It is also important to be able to distinguish between pathogenic/toxigenic and spoilage microorganisms. Combining immunological principles and electronic devices with a high sensitivity for changes in their direct environment has led to some very specific sensors for particular pathogenic microorganisms. It may be expected that combination with nanotechnology will provide very advanced sensors that will enable food processors to detect the presence of harmful microbes, even before a product is packed. For such sensors, the market will be huge, as it will greatly help to prevent food-borne safety incidents. Self-evidently, they need to be developed into robust industrial devices.

18.3.3 Chemical hazards

These occur when chemicals are present in foods at levels that can be hazardous to humans. Some potential chemical hazards could occur prior to a processor receiving product, such as the improper use of pesticides, antimicrobial and veterinary drug residues, or toxins (mycotoxins, natural toxins, and marine toxins). Others could be chemicals used on processing equipment, such as oils used on equipment or sanitisers.

18.3.3.1 Metals

Raw materials may be transported in metal containers, and may be stored in stainless steel tanks, and processing, in most cases, takes place in stainless steel machinery. Stainless steel consists of a variety of metals, including iron, chromium, nickel, and molybdenum. Processing equipment with moving parts is likely to have components

made from other materials, such as bronze (often used for bearings). Depending on product and processing conditions, there may be chemical and electrochemical reactions, as well as mechanical wear.

Most food products contain sodium chloride and water, and that results in chemistry, even with most, if not all, qualities of stainless steel. If the food is acidic and processing is at elevated temperatures, the reaction rates may be quite high. If materials in the same equipment in a process line differ, there will be electrochemistry. This all may result in an increase in the metal concentration of the product. In principle, this need not be alarming, as the human body actually needs the metals. The concentration, however, should never be so high as to have a significant influence on the healthy daily uptake. Another reason for watching metal concentrations is that they provide information on wear of the equipment, and the need for maintenance (e.g. lubrication) or replacement.

18.3.3.2 Pesticides

Although much effort is placed on avoiding the use of pesticides, in the vast majority of agricultural areas, their use is unavoidable for the production of the amount of food needed. Where cultivars are selected that are more resistant to pests and, hence, may need no or less pesticides ('organic farming'), there is a chance that the variety contains high 'natural' concentrations of pesticides, which may be counterproductive from a food safety point of view. Concentrations of pesticides, if properly used, should be present only in very low, harmless concentrations. They may be high, however, as a result of carelessness. Nevertheless, nothing is known of metabolites generated during food production. Simple and affordable methods for detecting pesticides and their metabolites will no doubt find a market in the food industry.

18.3.3.3 Organic contaminants

Besides persistent organic pollutants (e.g. dioxins, polyaromatic hydrocarbons), microbial toxins are undesirable and should not exceed acceptable concentrations. Because of the nature of the world, there will always be microorganisms that grow where food is harvested; without them, there would not be any soil to grow our food. A consequence is that toxins – and, particularly, mycotoxins – are abundant, and measures should be in place to source food and ingredients with harmless concentrations. Because it is not realistic to transport and store raw materials aseptically, moulds will always be present. Giving them the chance to grow may result in an increase in the concentration of such toxins.

Microbial growth may be restricted by control of relative humidity (RH) and temperature. At $RH < 60\%$, most moulds are unable to grow. Cooling to reduce the growth rate of microorganisms may be useful, provided that care is taken to prevent significant differences in temperature between parts in the bulk of raw materials. Moisture will migrate to cold spots, and increase the water activity locally to 1 (equivalent to RH of 100 %). Measurement of RH and temperature is, thus, important but, in addition, direct measurement of the concentration of toxins, providing proof that the susceptible product is safe or unsafe, is highly desirable.

18.3.3.4 Allergens

The number of people suffering from food allergenicities is increasing. Even the apple that should 'keep the doctor away' may be unhealthy for many. Most allergens are

proteins, and their selective detection in an environment of a mixture of other proteins is based on antibody technology, and is quite complex. In some cases, detection of indicator molecules may be more rewarding, such as lactose being an indicator of the (likely) presence of milk and, thus, milk's proteins. It would, of course, not help if lactose-free milk products have been used on a process line that is subsequently used for 'milk-free' products. There is obviously scope for devices that instantly can detect proteins selectively, and devices that are able to measure other metabolites, indicative for allergens, instantly. Up to now, it seems – due to the fact that there is no specific threshold for allergens – it is quite challenging to find an analytical solution.

18.3.4 Metabolites

- A) Resulting from enzymatic or microbial activity, these can be used to determine the freshness of a product. This could be used at the level of the consumer, or upstream in the supply, during selection of ingredients. At the consumer level, it could, for example, allow the consumer to follow the ripening of fruits and signal the optimal moment of consumption. In other products, it might be as simple as indicating a pH change caused by the presence of lactobacilli. During manufacturing, it could be used for the selection of ingredients – for example, to measure the ripeness of batches of fruits and vegetables in a non-intrusive way. Only batches at optimum ripeness would be used in processing. In many processes, enzymatic and microbial activities are essential. Examples are the fermentation of tea, yoghurt, cheese, and sauerkraut. Many times, very basic measurements are done to control the process. The fermentation of tea is controlled by watching the development of the colour during fermentation. The final test takes place after the tea has been dried, by a taste expert. Obviously, this is relatively late. Ideally, measurement should be done in-line, be directly related to the desired property, and be suitable for direct process control. Some gas sensor arrays, also called electronic noses, are already capable of detecting volatile compounds – major impact compounds, as well as off-flavours. The major challenge will be to implement them into a production surrounding.
- B) **Process metabolites:** since 2002, when acrylamide was identified in many heating processes, this kind of contamination has been receiving more and more attention. The change of technologies fostered by resource efficiency approaches will generate new threats. Beside this, the impact of primary and secondary metabolites of plant protection chemicals is still unclear.

18.3.5 Desired product constituents

As consumers increasingly realise the importance and the effect of food on well-being, more and more products will target their concerns. Ingredients include the well-known vitamins and minerals, but currently unknown ingredients will probably be discovered. It is, however, also useful to know the actual concentration of desired, valuable substances, such as those listed in Table 18.3.

18.3.6 Source of constituents

In the near future, an array of new ingredients contributing to the health benefits of foods is expected to emerge. The quest for new health-stimulating ingredients has only

Table 18.3 Desired product constituents and product structure.

Product constituents
Proteins
Individual amino acids
Sugars
Healthy fatty acids as well as the total concentration of fatty acids
Compounds with an additional health impact, e.g. antioxidants
Vitamins, especially those that address a population's deficiencies
Flavour and taste compounds

just begun. Instead of synthesising these ingredients in a chemical way, much is expected from extraction of plant material. When the plant material has a history of consumption, this will facilitate clearance of the ingredients for use in foods significantly. Plant material is known for its variation (depending on the season, soil, fertilisers, etc.). When ingredients are prepared from fluctuating sources, measurement (and control) is key to ensuring the right level of active ingredients.

18.4 Product structure

To be acceptable, product structure needs to comply with consumer expectations. Therefore, it is important to ensure that the structure-determining parameters in Table 18.5 are within limits.

18.4.1 Viscosity

There are many measurements to quantify the texture of food. Some are based on physical principles like viscosity, while other methods are developed to mimic a consumer-desired attribute – for example, the ability of ketchup to flow from a bottle onto food. As pourability is difficult to relate to a simple viscosity measurement, companies have developed their own practical ways to measure this property. In the case of ketchup, this can be done by placing a small reservoir of product on a defined slope. After removing the container, the ketchup will flow downward from the slope. The length of the trail can be used to indicate the pourability of the product. Sensors that could measure viscosity in-line would be ideal.

The most straightforward solution to measure its properties in-line would be measuring the pressure loss of the product when it flows through a pipe. This is difficult to accomplish, and further complicated by the fact that many products do change texture during storage.

18.4.2 Air/gas

For some products, like ice cream, the amount of air per volume of product is an important parameter, as it has a major influence on the product texture (and also on the cost of

the product per volume). In-line measurement of aeration is desirable, but is complicated by pressure fluctuations in the process line, leading to fluctuating gas/liquid volume ratios. Also, when the product is in the supply chain, monitoring the air content (or crystal size) is useful to check for any temperature abuse.

In other products, the presence of air is undesirable, and a lot of effort is paid to de-aeration and gentle filling. Sensors that could check for the presence of air/gas in a non-intrusive way could find application.

18.4.3 Crystal size

Ice cream is probably the product influenced most by crystal size, although fat crystals play a role in other foods. Better measurement during production would make it possible to deliver a more constant quality to the consumer. Probably more important are the changes during distribution that could severely degrade the quality. Predicting the crystal size only by temperature history is difficult or impossible. A simple, non-intrusive crystal size sensor could be used when the product is placed in the freezer, or when bought by consumers.

18.5 Influence of processing on product composition

Processing may result in the formation of desirable and undesirable substances, or these may be the result of contamination during processing (Table 18.4).

18.5.1 Reactions between naturally present substances in food

Much food requires heating to become palatable, and to make the nutrients that the human body needs available. Heating, however, may have undesirable side-effects, such as some of the Maillard reaction products (e.g. acrylamide). Often, the production of such substances can be controlled by careful selection of cooking (or, in this case, frying) conditions. In many vegetables, nitrates are converted to nitrites and, in the presence of proteins, into nitrosamines. Both may be carcinogenic in high concentrations and, therefore, their formation should be prevented.

In products with both ascorbic acid and benzoate then, with time, benzene will be formed, even at ambient temperature. Food products are, by nature, very complex, and a vast number of chemical reactions, some known and many unknown, will take place during processing. With time, more of these reactions will become known, and there will be an ever-increasing demand for methods of analysis and, hence, diagnostic devices that can be used industrially.

Table 18.4 Substances resulting from food processing.

Maillard products
Nitrite
Nitrosamines
Polycyclic aromatic hydrocarbons (PAHs)
Cleaning and disinfection agents

18.5.2 Contamination with cleaning and disinfection agents

Although contamination with chemicals during processing ought to be avoided by the correct design of the process line and skilful operation by trained operators (Lelieveld *et al.*, 2016), incidents may occur (e.g. failure of seals) that can only be noticed through measurement, and not by observation. Therefore, measurement of such contamination is highly desirable. Contamination of a product by cleaning-in-place (CIP) fluids could be measured by an in-line pH (or equivalent) measurement just before the filling machine.

18.6 Processing parameters

Processing is done to convert raw materials into desired products that are safe. Obviously, accurate control of the processing parameters and, hence, the capability of measuring these parameters, are essential. To inactivate microorganisms and to arrest or reduce enzymatic activity (e.g. blanching), heat treatments are traditionally applied. Novel processing methods, also with respect to resource efficiency, have been introduced that are based on other physiological effects than that of heat, such as (very) high pressures or (pulsed) electric fields. Also, methods have been developed that still use heat, but reduce the total heat input by (for example) faster heating (ohmic and micro-wave). Such methods require control of other parameters, such as pressure, field strength and flow distribution. Process parameters that may need control and, hence, diagnostic devices, are listed in Table 18.5.

Table 18.5 Process parameters to be controlled.

General

Temperature and temperature distribution

Flow rate and velocity profile/distribution

pH, pO₂

Humidity

Pressure

Colour

Turbidity

Viscosity

Structure

Droplet, bubble, crystal size and distribution

Fouling of product contact surfaces

Additional parameters for high-pressure processing

Pressure (up to 1000 MPa)

Temperature distribution in the high-pressure vessel

Pressurizing rate

18.6.1 General

Most of the parameters listed under 'general' in Table 18.5 need no further explanation. Their importance was established long ago, and measurement of most of them is possible, although there is scope for improvement, in particular with respect to hygienic design. Many temperature and pH probes used in the food industry are of a design that results in products accumulating in dead zones that are difficult to clean and where, during processing, microorganisms multiply and contaminate the passing product (Lelieveld *et al.*, 2014). The availability of hygienic devices is limited, and there is scope for affordable designs that do not affect the flow pattern of the product.

18.6.2 Flow rate and velocity distribution/temperature and temperature distribution

All thermal preservation processes are determined by exposure of the product to a heating process for a certain time. Long heating times will reduce the quality of the product. The trend is to reduce the heat treatment as much as possible, by making it fast and homogeneous. The heating process can be conventional, based on conduction and convection, or advanced and based on induction of heat by an electric field (ohmic, radio-frequency, microwave heating). The intensity of the heat input, coupled with the residence time, determines the final temperature of the product.

For conventional heating of a product flowing through a straight pipe and heated from the outside, the coldest product will be found in the centre, which would be the appropriate place for mounting a thermocouple, to monitor and control the temperature. To increase the homogeneity of the temperature, mixing perpendicular to the flow is used (e.g. by bending the pipe and inducing secondary flows or by application of static mixers). Now, however, the position of the coldest particle is unknown and, ideally, the cross-section should be scanned to identify the coldest particle. After heating, the product is held at an elevated temperature to obtain the desired inactivation. The minimum heat treatment is given to the particle with the shortest residence time.

The preceding process is complicated by the presence of particles in the fluid. This is because heat penetrates only slowly into the particles, and particles have a velocity different from that of the fluid and are not homogeneously distributed over the cross-section. To determine the effectiveness of a heating process, experiments are done by sending small, floating temperature loggers through the process – or, indirectly, by sending particles filled with a known chemical species through the line, and deriving the heat treatment from the chemical conversion. Although this provides much insight, it should be realised that the aim of the preservation step is a reduction of microorganisms by a factor of 10^6 – 10^{12} , implying that the tiniest particle that does not receive the right heat treatment may cause problems. To determine an accurate description of the process, high numbers of sensors should be sent through the process.

To minimise the thermal damage, temperatures are raised and holding times are shortened. In the extreme case heating is done by steam injection, and the holding time reduced to less than a second. Determining velocity and temperature distributions in such a case is still a challenge.

18.6.3 Droplet, bubble, crystal size and distribution

Apart from the influence on viscosity and mouth-feel, for water-in-oil emulsions, water distribution often plays an important role in preservation. If the droplet size is small, bacteria are trapped in a small amount of aqueous solution containing limited nutrients. Consequently, growth is nutrient-limited, and spreading of the bacteria is prevented – unless, of course, it has sufficient lipase activity. In combination with other hurdles for microbial growth, like a sufficiently low pH, margarine is often preserved this way, reducing or eliminating the use of preservatives.

18.6.4 Additional parameters for high-pressure processing

High-pressure processes that are currently applied are done at room temperature, with the aim of inactivation of vegetative microorganisms. The inactivation is mainly based on pressure, and hardly on temperature. For control purposes only, the pressure needs to be measured which, in the case of liquid product, is homogeneously distributed throughout the vessel, therefore allowing a single measurement position. Although pressures are high (typically 500–700 MPa), reliable sensors exist. When the product is not a liquid but a solid, such as meat/bone, it would be interesting to measure the pressure inside a piece of meat/bone.

More recently, the use of high pressure for sterilisation has been shown to be reliable, in combination with heat (Bermúdez-Aguirre *et al.*, 2016). Here, inactivation of microbial spores is the aim, and temperature plays a key role. The process is based on the use of adiabatic heating of the product. Under pressurisation, the product will rise in temperature, which is essential for the inactivation of microorganisms. Most products will rise 3–5°C per 100 MPa. Starting temperature is typically around 90°C, and the target end temperature is 110–120°C. The wall of the vessel will not rise in temperature during compression, and will act as a heat sink. Immediately after compression, products close to the vessel wall will drop in temperature and, therefore, fail to sterilise. In the case of sterilisation, the measurement of the temperature in high-pressure vessels is essential. Drilling holes in the cover of the vessel for mounting temperature probes is not feasible from a mechanical point of view. There is a clear need for a temperature-logging device that would resist 120°C and 700 MPa.

18.6.5 Pulsed electric field (PEF) processing

After 60 years of research and development, this novel technology has found industrial applications. Contrary to thermal preservation methods, PEF has no effect on flavour and nutrients and, hence, PEF treated products cannot be distinguished organoleptically from fresh products. This is of particular interest for fruit juices; PEF treated juices cannot be distinguished from freshly squeezed juices, so the technology is used for the extension of the shelf life of fruit juices. The first company making PEF-treated fruit juices was Genesis Juice Cooperative in Oregon, USA, who put a range of PEF-treated fruit juices on the market in 2005. The company was taken over a few years later, and the PEF process was replaced by a high-pressure process.

In the Netherlands, however, research and development continued, which resulted, in 2011, in a Dutch company starting to apply PEF on a large scale to enhance the shelf life of

fruit juices (Smit, 2012). Today, the products are on the market in the Netherlands and many other European countries. In 2010, a Dutch company started to develop equipment for cooking using PEF. It appeared to be possible to cook meat, fish, potatoes and vegetables in an extremely short time (seconds rather than minutes), and to obtain a quality that is appreciated by top cooks and others, to the extent that the company has received several prizes/awards. The technology is nicely described in 'e-Cooking' (IXL Netherlands, 2016).

The effect of PEF is based on the detrimental influence of electric fields on the membrane surrounding the cells of both microbes and higher organisms. Microbial membranes consist of a double layer of phospholipids that are polarised and neatly arranged, to separate the contents of the microbial cell from the environment. To allow the access of selected nutrients and excretion of metabolites, dedicated 'pumps', consisting of special proteins, are needed. Electrical fields, in the order of 1 volt per micrometre, affect the integrity of the phospholipid membrane, causing it to leak and 'bleed to death'. The effect is achieved by using pulses with a duration of a few microseconds. If the electrical field strength is insufficient, or the time is too short, the cell is able to repair the damage. On the other hand, if the field strength is too high and the duration too long, too much energy will be put into the product, raising the temperature to values that cause thermal damage the product, as with traditional thermal pasteurisation.

Another effect of PEF is that it destroys the connections between cells of both plant and animal products, making the product softer (vegetables) or tender (meat). Heat has the same effect and, for many foods until now, heating has been the only way to make them palatable, be it by conventional methods (boiling, steaming) or by using newer techniques (microwave, radio-frequency, ohmic). Meat is difficult to digest without cooking, and some meat (e.g. stewing meat) needs hours of thermal cooking to become tender enough for consumption. With PEF, this process takes less than a second. There are other – meanwhile also industrial – applications of PEF that require sensors to allow process control.

For a PEF treatment to be effective, it is essential that all cells, whether microbes or cells of higher organisms (vegetables, meat) receive the correct treatment – that is, the required number of pulses, of the correct shape and duration, and at the required field strength – so that, for microbes, the damage to the cell is irreversible, and for the foods, all parts of it are palatable. Consequently, for treatment of liquids, the residence time distribution and, thus, the flow pattern, are important.

For both liquid and solid foods, the degree of homogeneity of the field strength is important. Because the field strength is affected by differences in conductivity which, in turn, is influenced by differences in temperature, it would be of great interest to be able to measure the temperature in the product without disturbing the electric field, and without heating the probe more than the product surrounding it. Ideally, there should be sensors that could be suspended in a product to facilitate the adjustment of the correct (i.e. energetically most economical) process conditions.

18.7 Packaging parameters

Quantities of product sold must be measured, which is why we have weighing scales and volume measurements. In-line weight measuring and control are widely used, and are flow meters that dispense measured quantities of liquid or fluid solids into containers. For aseptic packing, there are additional parameters to control:

- air flow, to certify that air is going out of the sterile zone and not into it;
- air quality, to ensure that the air entering the sterile zone is sterile;
- concentration and temperature of the sterilising agent, often hydrogen peroxide; and
- container seal integrity.

18.7.1 Sterility testing

To have at least some assurance that an aseptically packed product is safe and free from relevant microorganisms, packs are sampled for analysis. Sample containers opened for analysis, however, are wasted, and present a significant loss of product, as well as an environmental burden. Moreover, unless 100% is sampled and, thus, nothing is left for sale, sampling provides only a *chance* that all packs are sterile and, thus, are indeed safe. Non-destructive methods would, therefore, offer great savings.

In the dairy industry, non-destructive testing has been applied for several decades: a device called Elektester measures energy absorbed by mechanically vibrated packs. Deviations in the energy absorbed means that the viscosity of the product in the pack is different and, thus, something must be wrong with its contents. The method has limited application, as many products do not change in viscosity as a result of microbial activity. There are, however, other parameters that do change as a result of microbial activity, such as electrical impedance, which can also be measured in a non-invasive way (Nihtianov and Meijer, 1997). Regrettably, at this time, this method is not yet commercially available. A third method, using similar electronics, measures volume changes (Nihtianov *et al.*, 2001). The growth of microorganisms may result in the production of gases or change the density of the product, resulting in changes in volume that, even if very minor, can easily be measured accurately. Finally, the temperature rise caused by microbial activity can be measured, using so-called 'smart sensors' that allow reliable detection of mK differences in temperature.

The combination of a series of non-destructive methods would cover a large range of microorganisms, and make sterility testing of products efficient. It would allow a larger sampling rate at affordable costs, due to the savings resulting from elimination of labour-intensive, destructive testing, avoiding product loss and reduction in waste.

18.8 Conclusion

It is clear from this chapter that the *potential* market for food diagnostics is huge. Analysing complex food samples will be a challenge, as well impact Industry 4.0. Countries such as China, India, and Brazil are acting as production hubs for food manufacturing and as sources for raw materials (Frost and Sullivan, 2015). The majority of parameters that the industry would like to measure cannot be measured in an industrial way. This is a task for research, development, and particularly industrialisation of diagnostic possibilities that have been developed, but of which application remains restricted to laboratories. The possibilities that micro- and nanotechnology offer will, hopefully, stimulate the availability of the diagnostic tools that the industry needs.

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Index

Note: *f* following a page number indicates a figure and *t* a table.

a

- Absolute limits 217, 218
- AC susceptometers 256
- Acetaldehyde 43
- Acetic acid 80, 242
- Acetone 438
- Acetonitrile 438
- Acetylcholinesterase 233*t*, 238, 244
- 2-Acetylfuran 42, 43
- Acidity 33, 121, 424, 427
- Acrylamide 357, 441, 470
- Additives 246–247, 294–297, 305, 314, 355–356
- Adenine 437
- Adenosine triphosphate (ATP) 162, 354–355
- Adulterants 63, 64, 71*t*, 74, 75, 83
- Adulterations 13, 100, 292, 356–357
- Affinity recognition 117–120
- Affymetrix 189
- Aflatoxin 274
- Aflatoxin B1 289, 361
- Aflatoxin B2 361
- Aflatoxin G1 361
- Aflatoxin G2 361
- Aflatoxin M1 83
- AFLP 195–196
- Agarose gel electrophoresis 189, 194, 195, 215
- Agilent Technologies 189–190
- Aging 42, 84
- Agrobacterium tumefaciens* 207
- Air/gas 471–472
- Albumen 110
- Alcohol dehydrogenase (ADH) 233*t*, 272
- Alcohol oxidase 233*t*, 246
- Alcohols 243
- Alcoholysis 350
- Alditols 349*t*, 350*t*
- Alkaline hydrolysis 350
- Alkaline phosphatase 245
- Allergens 469–470
- Allura red 294, 295
- 1-Allyl-3-methylimidazolium chloride 295
- Amines 357–358
 - biogenic 246, 357
- D-Amino acid oxidase 242–243
- L-Amino acid oxidase 243
- Amino acids 242–243, 347–348, 354, 355
- 4-Aminobenzenethiol 287
- 4-Aminobenzoic acid 352
- Ammonia 76, 77*t*, 81, 83, 243, 449
- Amperometric H₂O₂ sensor 271
- Amplified fragment length polymorphism (AFLP) 195–196
- Amylopectin 14, 15*f*
- Amylose 14, 15*f*
- Anion exchange 348
- Anserine 357
- Antennas 329
- Antibiotic residues 360–361
- Antibodies 119, 163, 251–255
- Antinutrients 246
- Antioxidants 352–354
- Antithrombin (Atryn) 221
- Anthrax lethal factor 122

- Apples 64, 74, 82, 83, 439, 469
 - carbendazim in 287
 - contamination 84
 - patulin in 442
 - polyphenols in 449
 - quercetin in juice 292
- Aptamers 172
- Argyria 320
- Aroma 76–77, 82–83
 - see also* flavour
- Aroma compounds
 - nitrogen 390–391*t*
 - olfactometry detections 379–400
 - sulphur 390*t*
- Aroma extract dilution analysis (AEDA) 383, 388
- Arrayed imaging reflectometry (AIR) 255
- Artificial neural networks (ANN) 79, 84
- ASCII files 402
- Ascorbic acid 472
- L-Ascorbic acid 242
- Aspartame 246
- Aspergillus* 289
- Atomic absorption spectroscopy 352, 435, 438, 439
- Atomic emission spectroscopy 320, 352
- Atomic force microscopy (AFM) 272, 285, 286, 322, 323, 330
- ATP 162, 354–355
- Au nanoparticles (NPs) *see* gold nanoparticles (NPs)
- Automated sampling procedures 431–464
- Avermicilins 360
- Azodicarbonamide 297
- b**
- Bacillus* 103, 170
- Bacillus anthracis* toxin 122
- Bacillus cereus* 102, 103, 104, 111, 113, 121, 123
- Bacillus licheniformis* 103
- Bacillus subtilis* 103, 123, 124
- BacLight 159
- BacT/Alert system 155
- Bacteria
 - as food-borne pathogens 98–101
 - food contamination 97–137
 - Gram-negative 106–109, 119*t*, 121
 - Gram-positive 101–106, 118–119*t*
 - lactic acid bacteria (LAB) 98, 154, 356
 - omics methods for detection of 116
 - probiotic 98
 - spore-forming 103
 - viable but non-culturable (VBNC) 101
- Bacterial toxins 110–114
 - analysis of 121–126
 - endotoxins 110–111
 - exotoxins 111–114
- Bakery items 99, 218, 316, 348
- Balenine 357
- Banana 78
- BapL 104
- Batch sequence 3
- Batches 3
- Beef 64, 85, 384*t*, 387, 394
- Beer 41–44, 75, 445
- Benzene 472
- Benzimidazole 361
- Benzoate 472
- Benzoic acid 436
- Beverages 67–70*t*, 73–74*t*
 - alcoholic 84–85, 359*t*
 - non-alcoholic 84
- Bile salts 110
- Bioactive compounds 141, 308, 353, 448
- Biochemical markers
 - for process control 356
- Biochemical markers analysis 357–359
 - antibiotic residues 360–361
 - biochemical compounds 357–359
 - food adulteration detection 356–357
 - food quality 354–356
 - food safety 357–362
 - nutritional quality 347–354
 - veterinary drug residues 359–360
- Biochip 164
- Biofilms 101, 106
- Biological techniques 75
- Bioluminescence/ATP detection 162
- Biomass 108, 171
- BioRad 169
- Bioreceptors 231
- Biosensors 170, 231, 239–243, 247*t*, 251–268

- advantages of 232–233
- for amino acids detection 242–243
- for analysis of organic acids 241–242
- aptamer-based 172
- based on graphene 270–274
- and cancer biomarkers-
immunoarrays 259–261
- carbon nanotubes (CNTs) *see* carbon
nanotubes (CNTs)
- for contaminants 244–245
- to detect alcohols 243
- disposable 269
- electrochemical 239
- electronic nose sensors *see* electronic
noses (eNose)
- for ethanol determination 243
- features of ideal 234
- fibre optic 172
- Fourier transform infrared spectroscopy
(FTIR) 171–172
- graphene oxide-based 275
- immobilized 234–235
- immunochemiluminescence 252–253
- immunology-based 251–268
- lateral flow immunosensors 259
- mass-sensitive 170–171
- nanotechnology for pathogen
detection 172
- Raman spectroscopy 171
- site-directed antibody immobilisation
techniques for 253–255
- for sugar analysis 239–241
- surface plasmon resonance
(SPR) 171
- transducers 235–238
- using enzymes in 233
- Biotyping 195
- Bisphenol A (BPA) 292, 293
- Bisphenols 442
- Bone taint 392
- Book and claim approach 10
- Botulinum neurotoxin (BoNT) 114, 121
- Botulinum toxin 111
- Botulism 63, 103
- Bouisol 314
- BRC standard 7
- British Retail Consortium 7
- Bromate ion 297
- Brownian motion 323–324
- Bubbles 475
- Bulk acoustic wave sensors (BAW) 80
- Burkholderia caryophylli* 108
- 2,3-Butanediol 80
- Butter 64
- Butyrylcholinesterase (BChE) 244
- c**
- Cabbage 83
- Cadaverine 246
- Cadmium 434
- Calcium 475
- Calibration 424–426
- Calixarene 255
- Campylobacter* 103, 106, 108, 126
- Campylobacter coli* 109
- Campylobacter fetus* 109
- Campylobacter jejuni* 63, 106, 109, 126
- Campylobacteriosis 109
- Canola 217
- Canola oil 314
- Capillary electrophoresis 215, 321, 436,
437, 440
- Capillary gel electrophoresis via laser-
induced fluorescence 215
- Carbadox 361
- Carbohydrates 14, 348–350
- Carbon electrodes 48, 272, 282, 283,
286, 289
- Carbon nanotubes (CNTs) 269–270, 281,
310, 311
 - food analysis biosensors 270–274
 - multi-walled 270
 - single-walled 270
 - SnO₂ sensors 85
- Carbon tetrachloride 438
- Carboxylic acids 348–350
- Carboxymethylcellulose 318
- Cardiovascular diseases 141, 145
- Carnosine 357
- Carrageenan 14, 16f, 17, 18
- Carrots 287
- Casein 307, 318, 321, 326
- Catalytic hairpin assembly (CHA) 260
- cDNA 164, 172, 189, 190, 193, 289

- Centers for Disease Control and Prevention 64
- Cereals 252, 273, 316, 348, 352, 444, 449
- Cereulide 104, 113, 123
- Certified reference material 219–220
- Cetyltrimethylammonium bromide (CTAB) 206, 207, 294, 295, 328
- Chain cooperation 2
- Chain-encompassing quality assurance schemes 2
- Charm analysis 383
- Cheese 64, 105, 359*t*, 384*t*, 394
- Chemical agents 64
- Chemical hazards 468–470
 - allergens 469–470
 - metals 468–469
 - organic contaminants 469
 - pesticides 469
 - see also* Hazards
- Chemiluminescence 172, 238, 253, 259
- Chemometrics 13, 16, 52, 84, 401–402, 428–429
- Cherries 64
- Chickens
 - ammonia in chicken meat 77*t*
 - transgenic 221
- Chilli 83
- Chitosan 294, 316
- Chloramphenicol 289
- Chloride anion 355, 356
- Chlorobenzene 438
- Chloroform 206
- Cholesterol 271
- Cholesterol oxidase 272
- Cholineoxidase 233*t*
- Cholinesterases 244
- Chromatography
 - anion chromatography 355
 - fast-liquid chromatography 346
 - gas chromatography *see* gas chromatography
 - hydrophilic interaction 345, 347, 352
 - ion-exchange chromatography 352
 - ion extraction chromatography 43
 - partition chromatography, normal phase 348
 - size-exclusion chromatography 321
 - see also* High-performance liquid chromatography (HPLC); Liquid chromatography
- Chromium 468
- Chromium(VI) 435
- Chronoamperometry 282, 328
- Cinnamon 316
- Citric acid 242
- Classification ability 421
- Classification matrix 422
- Cleaning and disinfection agents 473
- Cleaning-in-place (CIP) fluids 473
- Clenbuterol 288
- Cloned plasmid fragments 210
- Closed system concept 6
- Clostridium* 103
- Clostridium botulinum* 63, 103, 121
- Clostridium difficile* 124
- Clostridium epsilon* toxin (ETX) 121
- Clostridium perfringens* 101, 102, 103, 113, 124
 - analysis of 121
 - filtration detection of 156
 - nested PCR detection of 167
- Clove bud essential oil 317
- Cluster analysis procedure 50
- CNTs *see* carbon nanotubes (CNTs)
- Codex Alimentarius Commission 4
- Coefficient of variation 253
- Coffee 33–38, 75, 84, 384*t*, 394
- Collagen 190, 356
- Colloidal chemistry 307
- Colloidal copper nanoparticles 314
- Colloidal systems 309
- Colorimetric sensors 284
- Comparative genomic hybridisation (CGH) 164
- Complementary DNA (cDNA) 164, 172, 189, 190, 193, 289
- Conducting polymers 77, 80, 83, 84, 241, 329
- Conductivity 75, 237, 324, 355
 - electrical 78, 270, 280, 311
 - thermal 270
- Confocal scanning microscopy 74
- Contaminants
 - cleaning and disinfection agents 473

- determination of 448
- heavy metals 245
- organic 469
- pesticides 244–245
- Continuous flow microextraction (CFME) 437–438
- Controls 2, 105
 - food safety 1
 - PCR amplification 201
 - process 5, 187
 - routine inspection 360
- Copper
 - catalysed alkyne-azide cycloaddition 259
 - ions 298
- Cork
 - Mancha Amarela/Yellow spot (MA) 45
 - wine interactions 44–52
- Corn 218
- Corn oil 83
- Corticosteroids 360
- Cotton 220
- Cows 80
- Cryogenic scanning electron microscopy (Cryo-SEM) 323
- Cryptosporidium* 169
- Crystal size 472
- CTAB 206, 207, 294, 295, 328
- Cucumber 83, 439, 440, 443
- Cyanide 292
- Cyclamate 436
- Cysteamine 283, 284, 288
- Cytokine K 113
- Cytokines 255
- Cytosine 188
- Cytotoxins 108, 109, 114
- d**
- Dairy products 74, 75, 240, 354, 356
 - vitamin fortified 314
 - volatile compounds 80
- β-Damascenone 42
- Data
 - analysis 416–417
 - autoscaled 411–413
 - markets 10
 - ownership 10
 - processing systems 79
 - sets 405, 420t
- Data handling 401–430
 - calibration 424–426
 - classification 420–422
 - data collection 402–403
 - data display 403–417
 - modelling 423
 - process monitoring and quality control 417
 - three-way PCA 417–420
 - variable selection 426–428
- Deaminases 238
- Deamination 243
- Dehydration 83, 101
- Density 433
- Deoxynucleoside triphosphates 190
- Deoxyribonucleic acid *see* DNA
- Deoxyribose 188
- Detection capabilities 274
- Detection frequency method 383–388
- Developing countries 97
- Dexamethasone 360
- Di-(2-ethylhexyl) phthalate 319
- Diagnostic devices 465–478
- Diamine oxidase 246
- Diarrhoea 106, 108, 275
- Dichloromethane 438
- Dictionary of Natural Products 140
- Dielectric constant 325, 449
- Diet 139, 144
- Diethylstilboestrol (DES) 288
- Diffusing wave spectroscopy (DWS) 326
- Dilution analysis method 383
- Diode array detection 350, 360
- Diquat 451
- Direct epifluorescent filter technique (DEFT) 157–158
- Direct immersion single-drop microextraction (DI-SDME) 435
- Direct intensity method 388
- Direct solvent extraction 350
- Direct thermal desorption 380
- Directly suspended droplet microextraction (DSDME) 436–437
- Disaccharides 14, 16, 240

- Dispersive liquid-liquid microextraction (DLLME) 433, 438–439
- Dispersive solid phase extraction 360
- Distillation 77, 78, 381
- DNA 165, 188, 205
 - extraction robots 207
 - hybridisation methods 188–189
 - microarrays 164–165, 189–190
 - quality determination of meat products 75
- Doppler effect 325
- Dot-blot technique 189
- Dried fruits 444
- Dry sausages 387, 388, 389*f*, 394, 395
 - Escherichia coli* in 196
 - olfactometry studies 387
- Duck liver 395
- Durum pasta products 357
- Durum wheat flour (DWF) 419
- Dyes 191, 208, 211, 212, 215
 - detection of 294–297
 - DNA intercalating 190, 215
 - fluorescent 161, 168, 189, 212, 281, 329
 - live-dead assay 158–159
 - pH sensitive 238
 - RT-PCR 193–194
 - SYTO9 dye 159
 - viability 193–194
- Dynamic headspace organic solvent film microextraction (DHS-OSFME) 435
- e**
- Edible coatings and films 316–317
- Egg phosphatidylcholine 319
- Eggs 63, 74, 100, 108, 221, 441
 - antibiotic residues in 360
 - bacterial food poisoning from 99
 - HPLC analysis of 354
 - veterinary drug residues in 360
- Eigenvectors 406
- Electric fields 195, 237, 325, 473, 474, 476
- Electrical properties 280, 282
- Electrochemical detection 236*t*
- Electrochemical impedimetric spectroscopy (EIS) 282–283
- Electrochemical lateral flow immunosensor (ELFI) 258
- Electrochemiluminescence (ECL) 238, 260, 289
- Electrodes 281–283
 - see also* Carbon electrodes; Glassy carbon electrodes
- Electronic noses (eNose) 75–77, 170–171
 - analytical distillation methods 78
 - applications for food quality 79–85
 - Artificial Olfactory System (AOS) ISE Nose 2000 80
 - chemical sensors for gaseous environment 78–79
 - data processing systems 79
 - detection system 78–79
 - direct extraction methods 78
 - headspace analysis methods (HS) 78
 - instrumentation of 77–79
 - PERES for beef, pork, poultry and fish 85
 - for quality and safety control 63–96
 - sampling system 77–78
- Electrophoresis
 - 2D 120, 124, 125, 126
 - capillary electrophoresis 215, 321, 436, 437, 440
 - gel *see* gel electrophoresis
- Electrospray ionisation mass spectrometry 444
- Electrothermal-atomic absorption spectrometry (ETAAS) 435, 438, 439
- Elektester 315
- Elemental analysis 322
- ELISA 75, 120, 122, 159, 251–252
- Endocytosis 317
- Endonucleases 195, 196
- Endotoxins 121
- Energy dispersive x-ray spectroscopy (EDXRS) 285
- EnFET (enzyme field-effect transistor) 238
- Enteritidis* 64
- Enterobacteriaceae 154
- Enzymatic hydrolysis 350

- Enzyme-based sensors 231–250
 development of 233
 and the food industry 238–239
 introduction to 231–235
- Enzyme-linked immunosorbent assay (ELISA) 75, 120, 122, 159, 251–252
- Enzymes 231, 233*t*
 detection 75
 immobilized 234
 soluble 234
- Enzymes-based sensors
 for analysis of main food components 239–243
 for contaminants 244–245
 food freshness indicators, antinutrients and additives 246–247
 future of 247
- Epsilon toxin (ETX) 121, 123
- Escherichia coli* 63, 83, 97, 106, 108, 110
 biofilm production 106–108
 control of 99
 detection of 120, 125, 257
 edible film with antimicrobial effect against 316
 enterotoxin peptides 111
 Shiga-like toxins (Stx) 114
- Esterases 238
- Esters 414
- Ethanol 29, 83, 272, 436, 438, 445
 biosensors 243
 in fruit and vegetables 246
 sensors to detect the levels in whiskeys 85
 using in analysis of vitamins 350
- Ethidium bromide 190
- Ethidium monoazide 193
- Ethyl acetate 81
- Ethyl butanoate 82
- Ethyl caprate 414
- Ethyl caproate 410
- European Centre for Disease Prevention and Control (ECDC) 99, 101
- European Commission (EC) 290
 FI-PPP program 9
 Reference Laboratory 206
- European Community 4
- European Food Safety Authority (EFSA) 99, 101, 298, 316
- European Network of GMO Laboratories (ENGL) 206–207
- European Union (EU) 287, 292
 Commission Decision, 2002/657/EC 360
 EC Directive 96/23/EC 360
 GM sequence information 220
 Novel Foods Regulation 205–206
 outbreaks of food-borne diseases 97
- Exotoxins 111–114, 121
- Exponentially modified protein abundance index (emPAI) spectral counting method 124
- Extraction
 from liquid samples *see* Liquids, extraction from
 from solid samples *see* solid samples, extraction from
- f**
- Farming 287, 469
- Fat 84, 314, 326, 472
- Fatty acids 75, 76, 77, 79, 81, 427
 carnitine 144
 MALDI-TOF MS profiling of 160
 nanoencapsulation 313
 nanoparticle detection of 321
 non-esterified 143
 olive oil 405
 omega-3 314
 polyunsaturated 80, 394
 short-chain 141
 supercritical extraction of 450
- Ferricyanide 240, 241
- Fertilisers 292, 315
- Field-effect transistors (FETs) 237
- Films with metal NPs materials 284
- Filtration 156
- Fish 85
 analysis of amines 359*t*
 farming 289
 freshness of 246
 ‘GloFish’ 221
 histamine content 77, 246
 quality of 81–82
 smoked 105

- FISH (fluorescent *in situ* hybridisation) 158, 189
- Flavonoids 140, 452
- Flavour 76, 392–393, 436
- Flow cytometry 160–161
- Flow rate 346, 347, 441, 445, 474
- Fluorescence 238, 284, 360
 - of nanomaterials 275
 - RP-HPLC with 362
- Fluorescence imaging (FI) 74
- Fluorescence resonance energy transfer (FRET) 191, 292
- Fluorescence sensors with NPs 284
- Fluorescence spectroscopy 70, 74
- Fluorescent immunosensors 259
- Fluorescent *in situ* hybridisation (FISH) 158, 189
- Fluorescent intensity ratio 275
- Fluorescent semiconducting material (quantum dots) 284
- Fluoride 436
- Fluorimetric detection 284
- Focus microwave-assisted extraction (FMAE) 451
- Folic acid 288, 352, 442
- Food and Drug Administration (FDA) 64, 290, 292
- Food authentication 20
- Food-borne illnesses 63, 100, 187
- Food chain, tracking and tracing of food products 2–3
- Food Database FooDB 140
- Food diagnostics, market for devices 465–466
- Food dyes *see* dyes
- Food emulsions 314
- Food industry, quality and safety requirements 466*t*
- Food monitoring
 - radio frequency identification tags (RFT) 329
 - and safety controls 329
 - time temperature indicators (TTI) 329
- Food packaging and quality preservation 315–317
- Food poisoning 97
- Food preservatives *see* preservatives
- Food processing
 - contamination with cleaning and disinfection agents 473
 - substances resulting from 472*t*
- Food toxins *see* toxins
- Foodomic methods 97, 126
- Foreign bodies 468
- Fourier transform infrared spectroscopy (FTIR) 14–21, 23–33, 171–172, 425
- Fourier transform ion cyclotron MS technology 126
- Fourier Transformed Mid-Infrared spectra 426
- Freshness indicators 246
- Fructans 240
- Fructose 16, 17, 29, 239, 240
- Fruit 64
 - ethanol accumulation in 246
 - quality assessment of 66*t*, 82–83
 - quantifying adulterants/pesticides in 72–73*t*
 - quercetin levels in 292
- Fruit juices 252, 316, 434, 436, 439
 - apple 292
 - bacterial contamination of 84
 - citrus fruit 307
 - freshness determination 75
 - orange 84, 440
 - pear 287
 - pulsed electric field (PEF) processing of 475–476
 - SEA and SEB in 122
 - strawberry 295
- FT-IR spectroscopy *see* Fourier transform infrared spectroscopy (FTIR)
- Fullerenes 270
- Fumonisin 274, 361
- Functional foods 141
- Fung, Daniel 153
- Fungicides 287, 289, 314, 440, 445
 - benzimidazole 361
 - carbendazim 287
- Furan 443
- Furfural 42, 43

- Furosine 356
Fuzzy logic 79
- g**
- Galactans 17
Galactose 16, 17, 24, 240, 241
Galacturonic acid 16, 17, 23, 24, 25
Gas 471–472
Gas chromatography 13, 70, 140, 380*f*, 434
Gas chromatography-olfactometry
 detection frequency
 method 383–388
 dilution analysis method 383
 direct intensity method 388
 food industry applications 389–395
 identification of key aroma
 compounds 389–391
 identification of off-flavours 392–393
 production processes
 applications 393–395
 reformulation of food aromas
 applications 395
 use of 388
GC-O *see* gas
 chromatography-olfactometry
Gel electrophoresis 124, 168, 190, 208,
 212, 217
 agarose 189, 194, 195, 215
 capillary 215
 coupled with MS 120
 detection of DNA 167
 separation of amplicons 166
Gel filtration 348
Gelatine 356
Gene chip 164
Gene Expression Profiler (GeXP) genetic
 analysis system 166
Gene Trak kit (Neogen) 165
GeneQuence (Neogen) 189
Genetic algorithms 427
Genetically modified animals 221
Genetically modified (GM)
 ingredients 205–230
Genetically modified organisms
 (GMO) 205
 analysis of 205–206
 certified reference material 219–220
 choice of target sequences 207–208
 copy numbers 219
 detection challenges 218–221
 digital PCR 210–212
 end-point PCR 208
 food composition and
 processing 218–219
 multiplex analysis 212–215
 outlook 221–222
 quantification of 215–217
 real-time PCR 208–210, 218
 sampling and DNA
 extraction 206–207
 sequence information 220
 stacked events 220–221
 validation 217–218
Genomics 139
Genomotyping 164
Ginseng 317, 353
Ginsenosides 353
Glassy carbon electrodes 288, 294, 296
Global Food Safety Initiative 7
Global G.A.P (international standard) 7
Globalization 97, 100, 115
Glucose 17, 239, 271, 274–275
Glucose oxidase 233*t*
Gluten 419
Glycerol 243
Glyceryl monostearate 418
Goats 80, 221
Gold nanoparticles (NPs) 284, 288, 292,
 293, 294, 295, 296, 320
 Ag-coated 297
 carbon paste electrodes modified
 with 281, 282, 283
 cysteine detection 291
 isotropic 284
 matrix metalloproteinase-2
 detection 273
 ochratoxin A detection 290
 pesticides detection 287
 quantitative colorimetric
 immunoassays 258
Gold standard method 115
Good Manufacturing Practices
 (GMP) 114
Grape berry 29, 443

Grapes 29, 64, 83, 84, 307, 314
 cell wall changes during processing 15
 must 84
 phenolic compounds 443
 terpenoids 82
 tracing origin of 41
 Graphene 269–270, 281, 288
 Graphene oxide 274–275, 296
 Graphite 269
 Guanine 188
 Gut, the 141

h

HACCP 3, 4, 5, 114, 115, 187
 Haemolysin BL 123
 Haemolysin listeriolysin O 126
 Haemolytic enterotoxin 113
 Hall effect devices 256
 Ham 385*t*, 386*t*, 392, 393, 394
 Hazard Analysis and Critical Control Points (HACCP) 3, 4, 5, 114, 115, 187
 Hazards
 biological 468
 Hazards, physical 466–468
 Hazelnuts 395
 Headspace 33–38, 435
 Headspace analysis 78, 162, 380, 381*t*
 Headspace in-tube extraction (ITEX) 444, 445
 Headspace liquid-phase microextraction (HS-LPME) 436
 Headspace single drop microextraction (HS-SDME) 435–436
 Headspace-solid phase microextraction
 beer volatile profiling 41–44
 gas chromatography PCA 13, 33–38
 Heavy metals 63, 245, 298
 Herbal plants 452
 Herbicides 440
 Hexadecyltrimethylammonium
 bromide 294, 295
 Hexylacetate 82
 High-performance liquid chromatography (HPLC) 70, 343–344, 434
 adsorption 345
 advances in 346–347
 amines analysis 359*t*

amino acids analysis 354
 carbohydrates and organic acids
 analysis 349–350*t*
 ESI MS/MS 122
 fat-soluble vitamins analysis 351*t*
 fundamentals of 344–345
 ion exchange 345
 monolithic columns 346
 partition 345
 reverse-phase 345, 354, 356, 360, 361, 362, 437
 size exclusion 345
 use of mass spectrometry after 352
 veterinary drug residues analysis 360
 High-pressure processing 475
 High temperature water extraction (HTWE) 449
 Histamine 77, 246
 Histidine 77
 Histidinoalanine 354
 Hollow-fibre microextraction (HF-LPME) 433, 439–440
 Honey 75, 207, 348, 352, 362, 395, 421
 Horseradish peroxidase 165, 233*t*, 271
 Hot water extraction (HWE) 449
 Human campylobacteriosis 108, 109
 Human Metabolome Database 140–141
 Hybrid molecules 219
 Hybridisation 188–190
 DNA methods 188–189
 RNA methods 189–190
 Hybridisation chain reaction (HCR) 260
 Hybridisation probes 191, 209
 Hydrazine 292, 293
 Hydrogen peroxide 239
 Hydrogen sulfide 81
 Hydrophilic interaction chromatography (HILIC) 356
 3-hydroxy-2-butanone 81
 4-hydroxyproline 356
 Hyperspectral imaging (HSI) 74

i

Ice cream 472
 IFS standard 7
 IKB concept 6
 Imaging techniques 74–75

- Immersed single-drop
 - microextraction 435
 - Immunoassays 172, 251, 270–271, 290
 - for detection of
 - microorganisms 255–259
 - gold nanoparticles (AuNPs) *see* gold nanoparticles (NPs)
 - multiplexed 261
 - PCR-based 155
 - radio immunoassay (RIA) 252
 - SPR-based 252
 - Immunoglobulins 251
 - Immunomagnetic separation (IMS) 163–164
 - Immunoprecipitation 163
 - Immunosensors *see* biosensors
 - Impedance 283, 477
 - In-tube solid-phase
 - microextraction 443–445
 - Inductively coupled plasma mass spectrometry (ICP-MS) 322
 - Infant formulas 347
 - Information clusters 7–9
 - Information infrastructures 9, 10
 - Infrared spectroscopy (IR) 13, 70, 74, 285, 425
 - Ingestion, of nanoparticles 317
 - 5'-Inosine monophosphate (IMP) 355
 - International Standards Organisation (ISO) 191
 - International Union of Pure and Applied Chemistry (IUPAC) 231
 - Iodine 436
 - Iron 145, 281, 292, 468
 - Irradiation 255, 354
 - ISFET (ion-selective field-effect transistor) 238
 - Isothermal amplification 194
 - loop-mediated isothermal amplification (LAMP) 194
 - nucleic acid sequence-based amplification (NASBA) 194
 - Isotope dilution 122, 395
- j**
- Jams 23
 - Jellies 23
 - Juices 103, 241, 242, 348, 357
 - cucumber 443
 - fruit *see* fruit juices
 - multivitamin 84
 - PEF-treated 475
- k**
- Kitchen surfaces 100
 - Kiwi 78
- l**
- Label-free arrayed imaging reflectometry (AIR) 255
 - Lactate dehydrogenase 233*t*
 - Lactic acid 29, 81, 99, 241
 - Lactic acid bacteria 98, 154, 356
 - Lactococcus lactis* 98
 - β -Lactoglobulin 273
 - β -lactoglobulin 319
 - Lactose 240, 241, 469–470
 - Lactose oxidase 233*t*
 - Lamb meat 394
 - Lasalocid 360
 - Laser-induced fluorescence
 - imaging 74
 - Lateral flow assays 119, 155, 172, 258
 - Lateral flow kits 156, 163
 - Lateral flow test strips 258
 - Latex agglutination assays 163
 - Lead 434
 - Lemon 83
 - Lemon-grass oil 316
 - Ligation-dependent probe amplification (LPA) 214
 - Light-scattering methods
 - dynamic light scattering (DLS) 285, 320, 321, 323–324, 326
 - evaporative light-scattering (ELSD) 348, 349
 - laser Doppler velocimetry (LDV) 323, 325, 326
 - to measure nanoparticles 323–326
 - Photon Correlation Spectroscopy (PCS) 323–324
 - Rayleigh/multi-angle (SLS) 323
 - resonance light-scattering (RLS) 326
 - static light scattering (SLS) 323, 326

- Limits of detection 39, 123, 126, 251, 289, 435, 448
 - Linear discriminant analysis (LDA) 421, 422
 - Linearity 234, 240, 271, 272, 273, 287, 295
 - Lipase 75, 233*t*, 475
 - Lipids 140, 206, 309, 310, 314, 451
 - degradation of 79
 - extraction of 448, 450
 - UAE in analysis of 452
 - Lipopolysaccharides (LPS) 110–111, 121
 - Liquid chromatography 140
 - electron spray ionisation tandem mass spectrometry (LC ESI MS/MS) 122
 - rapid 343–377
 - tandem mass spectrometry (LC-MS/MS) 123
 - ultra performance 346
 - Liquid-phase microextraction (LPME) 432, 433
 - Liquids, extraction from 432–446
 - continuous flow microextraction (CFME) 437–438
 - direct immersion single-drop microextraction (DI-SDME) 435
 - directly suspended droplet microextraction (DSDME) 436–437
 - dispersive liquid-liquid microextraction (DLLME) 438–439
 - headspace single drop microextraction (HS-SDME) 435–436
 - hollow-fibre microextraction (HF-LPME) 439–440
 - in-tube solid-phase microextraction 443–445
 - liquid-liquid extraction (LLE) 432, 433
 - liquid-liquid-liquid microextraction (LLLME) 437
 - single-drop microextraction (SDME) 434–435
 - solid-phase extraction (SPE) 440–442
 - solid-phase microextraction (SPME) 442–443
 - solvent microextraction (SME) 433
 - stir-bar-sorptive extraction 445–446
 - Listeria* 64, 158
 - Listeria monocytogenes* 63, 104, 105, 126
 - Listeriosis 64, 104–105
 - Live dead assay 158–159
 - Liver 144, 320, 360, 361, 395, 447
 - Logistics elements 3
 - Loop-mediated isothermal amplification (LAMP) 167, 194, 217
 - Luciferase 162
 - Luciferin 162
 - Luffa 83
 - Lysine 356
 - Lysosome enhanced Trojan horse effect (LETHE) 320, 321
- m**
- Magnetic resonance imaging (MRI) 74
 - Magnetic tunnel junctions 256
 - Magnetoresistance sensors 256
 - Maillard reaction 356
 - Maize, Bt176 217, 218
 - Maize oil 83
 - Malachite green 289
 - MALDI-TOF MS 117, 120, 121, 122, 125, 126, 159–160
 - MALDI-TOF TOF 124
 - Malic acid 242
 - Malnutrition 143–145
 - deficiencies in particular nutrients 145–148
 - nutritional assessment studies 146–147*t*
 - Malt 449
 - Mangoes 64, 82, 83
 - Mannoproteins 29
 - Mannose 29
 - Marmalades 14
 - Mass-sensitive biosensors 170–171
 - Mass spectrometry 120–121, 140, 349
 - hybrid quadrupole/linear ion trap (LTQ) 122
 - MS/MS MRM 123
 - ultra-high resolution mass spectrometry (UHRMS) 126
 - Matrix-assisted laser desorption ionisation-time of flight mass spectroscopy *see* MALDI-TOF MS
 - Matrix metalloproteinase-2 (MMP2) 273

- Matrix solid phase dispersion (MSPD) 446–448
- Maturity 83, 246, 348
- Mayonnaise 314, 436
- Meat 65–66*t*, 76, 79–80, 85, 108, 359*t*, 394
 - see also* Pork; Sausages
- Meat products 357, 362, 390, 393, 394
 - fermented 356
 - FISH detection of pathogens in 158
 - fungal development of dry cured 80
 - minerals and trace elements in 352
 - nanoparticles-based sensors for analysis of 279, 286
 - nitrites and nitrates in 355
 - quality determination of 74, 75
 - RTE 105
 - shelf life, factors influencing 79
 - vitamins in 352
- Medium-infrared spectroscopy (MIR) 74
- Melamine 292, 293–294
- Messenger RNA (mRNA) 168, 189, 193
- Metabolic phenotype 141, 142
- Metabolites 114, 142, 143, 148, 470, 476
 - fungi 273, 289
 - metabolite complement in human studies 140–141
 - pesticides 469
 - tryptophan 148
 - vitamins 350
- Metabolomics 126
 - in assessment of nutritional status 139–152
 - relationship between diet and health 141–142
 - usability in nutrition sciences 139–140
- Metadata 402, 403
- Metal ions, chemical reduction of 307
- Metal oxides 78–79, 83
- Metallic nanoparticles 310, 311
- Metals 439
 - chemical hazards 468–469
 - contaminants 448
 - detection of 468
- Methanol 85, 350, 438
- Methicillin-resistant *S. aureus* (MRSA) 102, 123
- 3-Methyl-1-butanol 81
- 3-Methylbutanal 42
- 3-Methylbutyl Acetate 81
- Methylcellulose 317
- Methylesterification, degree of 23–24, 25, 26, 27*t*, 28*t*
- 3-Methylhistidine 357
- Metlin 140
- Micro-bore columns 346
- Microarrays 189–190
- Microbial food contamination 100
- Microbial growth 82, 98, 469, 475
- Microbial toxins 469
- Microchips 259
- Microfluidic array devices 260
- Microfluidic paper-based analytical devices (mPADs) 260–261
- Microorganisms
 - immunoassays for detection of 255–259
 - nanoparticles-based sensors for detection of 290
- Microwave-assisted extraction (MAE) 451–452
- Milk 64, 241, 360
 - adulterants/pesticides quantification in 71*t*
 - quality of 65*t*, 80–81
 - SEA and SEB detection in 122
 - sheep 426
 - sheep milk 426
- Milk powder 83, 294, 436
- Minerals 139, 311, 352, 470
- Miscibility 433
- Modelling 423
- Moisture 280, 316, 469
- Molecular beacons 209, 298
- Molecular imprinting polymer (MIP) 288
- Molecular technology 187–203
 - amplified fragment length polymorphism (AFLP) 195–196
 - characterisation methods 195–198
 - hybridisation-based methods 188–190
 - molecular characterisation methods 195–198
 - multi-locus sequence typing (MLST) 196–197

- Molecular technology (*contd.*)
- multi-locus variable-number tandem repeat analysis (MLVA) 196
 - nucleic acid amplification
 - methods 190–194
 - pulse field gel electrophoresis (PFGE) 195
 - restriction fragment length polymorphism (RFLP) 196
 - whole genome sequencing (WGS) 197–198
- Molecularly imprinted polymer (MIP) 287
- Molybdenum 468
- Monoclonal antibody (MAb) 252
- Monolithic columns 346, 347, 451
- Monoterpenes 82
- Montmorillonite 312
- Montmorillonite calcium 294
- Moulds 98, 154, 469
- mRNA 168, 189, 193
- Multi-locus sequence typing (MLST) 196–197
- Multi-locus variable-number tandem repeat analysis (MLVA) 196
- Multi-walled carbon nanotubes (MWCNT) 241, 274
- Multidrug-resistant (MDR)
 - Campylobacter* 106
- Multiple headspace extraction (MHE) 445
- Multiple linear regression (MLR) 424
- Multiple-locus VNTR analysis (MLVA) 196
- Multiple reaction monitoring (MRM) 122
 - MS/MS MRM 123
- Multiplex FISH (mFISH) 158
- Multiplex ligation-dependent probe amplification (MLPA) 216f
- Multivariate analysis 428
- Mushrooms 74
- Mycobacterium avium* 120
- Mycobacterium avium* subspecies *paratuberculosis* (MAP) 120
- Mycotoxins 273–274, 289–290, 448, 469
- n**
- NADH 238, 272, 291–292
- Nano-biopolymers 309–310
- Nano-fillers 311
- Nanoclays 310, 311
- Nanocomposites 312, 316
- Nanoemulsions 309, 317
- Nanoencapsulation 313–314
- Nanolaminates 310, 316
- Nanomaterials 279, 280, 305
 - analysis of the composition of 285–286
 - direct visualisations of 322–323
 - evaluation of surface morphologies 285
 - pre-treatment of food, prior to
 - detecting 321
 - separation and size fractionation 321
 - use in drug delivery systems 313
- Nanoparticles
 - Ag-Au alloy 298
 - agglomeration 328
 - aggregation 328
 - applications in food industry 312–317
 - association colloids 309
 - biological fate of ingested 317–318
 - carbon-based 281
 - carbon nanotubes (CNTs) *see* carbon nanotubes (CNTs)
 - characterisation of 285–286
 - classification of 281, 309
 - clusters 328
 - combined 312
 - composite materials 281
 - definition of 305
 - detection in food 321–329
 - electrochemical analysis 281–283, 327–329
 - engineered 317
 - exfoliated nanocomposites 312
 - gastrointestinal track (GIT) assimilation of 317
 - inorganic 310–311
 - intercalated nanocomposite 312
 - magnetic 281
 - measurement by light-scattering
 - methods 323–326
 - metal oxides 281
 - metallic 281, 310, 311, 320
 - nanoclays 311
 - naturally occurring 321
 - nickel 295, 327

- organic 309–310
- pH-sensitive fluorescent nanoparticles (FANPs) 291
- phase-separated microcomposite 312
- polymer 281, 312
- preparation of 306–308
- properties of 308–312
- semiconductor 281
- for sensor technology 280–286
- silver *see* silver nanoparticles
- spectroscopic techniques 283–285
- top-down preparation of 308
- toxicity of food-related 317–321
- Nanoparticles-based sensors *see* Nanosensors
- Nanosensors 269, 279–304, 329
 - applications 286–298
 - food contaminants and adulterations detection 292–294
 - food dyes/additives detection 294–297
 - food valuable constituents detection 291–292
 - metal ions detection 297–298
 - microorganism detection 290–291
 - mycotoxin detection 289–290
 - pesticide detection 287
 - veterinary drugs detection 287–289
- Narrow-bore columns 346
- Nasal impact frequency 387
- National Control Programmes 101
- Near-infrared (NIR) spectroscopy 74, 425
- Neotame (NTM) 296–297
- Neurotoxins 64
- Newborns 144
- Next generation sequencing (NGS) 197, 220
- Next-generation technologies 169–170
- Nickel 283, 295, 468
- Nicotinamide 352
- Nicotinamide adenine dinucleotide (NADH) 238, 272, 291–292
- Nicotinic acid 352
- Nitrate reductase 233*t*
- Nitrates 108, 355, 356, 472
- Nitrites 297, 355, 356, 394, 472
- Nitrogen-phosphorus detection (NPD) 442
- Nitrosamines 442, 472
- NMR spectroscopy *see* nuclear magnetic resonance (NMR) spectroscopy
- Noise reduction 426
- Non-typhoidal *Salmonella enterica* 106, 108
- Non-typhoidal serovars (NTS) 108
- Noodles 418–420
- Norovirus 63, 64
- Northern hybridisation analysis 189–190
- NS1 influenza protein (NS1) 258
- Nuclear magnetic resonance (NMR) spectroscopy 65*t*, 70, 74, 78, 140, 256
 - ¹³C-NMR 24
 - ¹H-NMR 24
 - metabolites measurable with 148
 - metabolomics study 140, 144, 145
- Nucleic acid amplification
 - methods 190–194
 - isothermal amplification 194
 - polymerase chain reaction *see* polymerase chain reaction (PCR)
 - RNA-based amplification assays 193–194
- Nucleic acid sequence-based amplification (NASBA) 194
- Nucleosides 354–355
- Nucleotides 188, 196, 354–355
- Nutrition, personalised 142, 143
- Nutritional status 143–148
 - deficiencies in particular nutrients 145–148
 - malnutrition 143–145
- Nuts 246, 273
- o**
- Obesity 101, 141, 143, 318
- Ochratoxin A 289, 290, 361
- Octyl gallate 296
- Odour 75, 379, 395
- Oil *see* fat
- Okadaic acid 273
- Olfactometry
 - aroma compound detection 379–400
 - detection frequency method 383–388
 - dilution analysis method 383

- Olfactometry (*contd.*)
 direct intensity method 388
 extraction of volatile
 compounds 380–382
 factors that affect the response of
 assessors 383*t*
 methodologies 382–388
 techniques 382–388
- Olfactory ports 380
- Oligonucleotides 164, 172, 191, 208, 212, 215
- Oligosaccharides 110, 170, 172, 240, 253, 254*f*, 255
- Olive oil 83, 426
- Olives 23–29
- Omic technologies 116, 188
- Onions 83
- Open sector system concept 6–7
- Optical detection 236*t*
- Optical enzymatic biosensors 238
- Optomagnetic (magneto-optic) sensor systems 256
- Orbitrap 126
- Oregano essential oil 317
- Organic acids 241–242, 434
- Organisational alternatives 9
- Organophosphorus hydrolase 245
- Orthogonal signal correction (OSC)-PLS1 regression 29–33
- Orthosiphon stamineus* 84
- Outer product 13, 23–29
- Outliers 425
- Oxidases 238
- Oxidoreductases 231–232, 238
- P**
- Packaging 74, 305, 476–477
- Palladium dioxide 315
- Pancreatic lipase 75
- Pantothenic acid 251, 252
- Papaya 83, 316
- Paraquat 451
- Parasites 64
- Paratuberculosis 120
- Partial least squares regression 13, 424
- Particle analysis 321–322
- Pasta 123, 352, 356, 357
- Pathogens, bacteria as food-borne 98–101
- Pattern recognition techniques 70
- PCA *see* principal component analysis (PCA)
- Peach 74
- Peanut milk 290
- Peanuts 444
- Pears 16, 23–29, 64, 74
- Pectic polysaccharides, methylesterification of 23–29
- Pectin 14, 18
- PEEK (polyetheretherketone) 437–438
- Penicillins 360
- Penicillium* 289
- Peptide nucleic acids probes (PNAs) 189, 212
- Peptidomics 97, 116–117, 126
- Peroxides 271
- Pesticides 244–245, 275, 439, 443, 448, 469
 detection of 287
 organophosphate 238, 244, 287, 434, 447
- Phage typing 195
- Phenol-Explorer 140–141
- Phenolic acids 140
- Phenylalanine 347
- Phenylethyl alcohol 81
- Phenylketonuria 347
- Phosphate 356
- Phospholipids 312, 476
- Photoelectrochemical (PEC) biosensor 259
- Phtalens 319
- Plasmatic membrane 317
- Plate count 154, 157
- Platelet factor-4 (PF-4) 260
- Point mutations 196
- Poly-5,20-50,200-terthiophene-30-carboxylic acid (pTTCA) 241
- Poly dimethyl diallyl ammonium chloride (PDDA) 298
- Poly-*N*-acetyl glucosamine (PNAG) 104
- Poly vinyl alcohol (PVA) film 298
- Polyclonal antibodies (PABs) 251
- Polycyclic aromatic hydrocarbons 362, 448
- Polydopamine functionalised GO hybrid (PDAGO) 273

- Polyethylene terephthalate (PET) 316
- Polyethyleneglycol (PEG) 319–320
- Poly(ethylenimine) (PEI) 289
- Polymerase chain reaction (PCR) 116, 166–167, 190, 205
 - digital 168–169, 192, 210–212
 - droplet digital 169, 217
 - end-point 208, 210, 211, 212
 - multiplex 166, 168, 192–193, 212, 214, 215
 - nested 167
 - qualitative 218
 - quantitative 116, 168, 192
 - real-time 167–168, 190–192, 208–210, 218, 219
- Polymeric mannose 31*f*, 32*f*
- Polymethyl dimethyl siloxane (PDMS) 445–446
- Polynucleotides 188
- Polyols 348, 357
- Polyphenols 353
- Polysaccharides 13, 16–20
 - FT-IR spectroscopy analysis of 14–22
 - in olive and pear pulps 23–29
- Polysorbate-20 319
- Polysorbate-80 318–319
- Polyunsaturated fatty acids 80, 394
- Polyvinyl chloride (PVC) 319
- Polyvinylpyrrolidone (PVP) 296
- Ponceau 4R 294
- Pork 64, 74, 80, 85, 348, 355
 - aroma 394
 - enterotoxins in 167
 - nanoparticles-based sensors 286
 - ractopamine detection in 288
 - Salmonella* on 192
 - see also* Meat
- Potatoes 64, 74, 83, 357, 476
- Poultry 64, 85
 - meat 108
- Practical limits 218
- Prediction ability 421
- Preservatives 105, 246, 294, 313, 356, 475
 - citric acid 242
 - importance of 98
 - milk 80
 - natural 98, 105
 - nitrite 297
- Pressurised hot water extraction (PHWE) 449
- Pressurised liquid extraction (PLE) 448–449
- Pressurised microwave-assisted extraction (PMAE) 451
- Principal component analysis (PCA) 70, 405–406, 407, 409, 410*f*, 428
 - beer quality analysis 75
 - calibration 424, 425
 - classification 421
 - cork analysis 48, 49
 - data processing 79
 - flavour estimation 84
 - HS-SPME/GC-PCA 33–38
 - polysaccharide food additive analysis 13
 - polysaccharide food additive screening and analysis 20
 - for process monitoring and quality control 417
 - squid freshness 82
 - three-way 417–420
- Principal component regression 424
- Principal components 406, 407
- Process management, quality-oriented 5
- Process monitoring, and quality control 417
- Process organisation 1, 4, 5
- Process quality 5, 356
- Processing parameters 473–476
 - droplets 475
 - heat treatment 474
 - for high-pressure processing 475
 - temperature 474
 - water distribution 475
- Product composition 466–471
 - biological hazards 468
 - chemical hazards 468–470
 - cleaning and disinfection agents contamination 473
 - desired product constituents 470
 - influence of processing on 472–473
 - metabolites 470
 - physical hazards 466–468
 - reactions between naturally present substances in food 472
 - source of constituents 470–471

- Product structure 471–472
 - Propidium iodide 159
 - Propidium monoazide 194
 - Propyl gallate 296
 - Prostate specific antigen (PSA) 258, 260
 - Prostate specific membrane antigen (PSMA) 260
 - Protected denomination of origin
 - products 423
 - Protein corona 317
 - Protein standard for absolute quantification (PSAQ) 122
 - Proteins, nanotubes produced from 310
 - Proteomics 97, 116–117, 126
 - Pseudomonas* 170
 - Pulse field gel electrophoresis (PFGE) 195
 - Pulsed amperometric detection (PAD) 348–349
 - Pulsed electric field (PEF)
 - processing 475–476
 - Pulsifier 157
 - Purge and trap 34, 446
 - Putrescine 246
 - Pyridoxal 352
 - Pyridoxal 5'-phosphate (PLP) 145, 148
 - Pyridoxamine 352
 - Pyridoxine 352
- q**
- Q&S systems 5, 6–7
 - Quadratic discriminant analysis (QDA) 421, 422, 423
 - Quality
 - classical techniques for testing 65–75
 - communication layers 8
 - delivery concepts 4–5
 - guarantees 2
 - issues 1–2
 - sustainable delivery of 1
 - Quality control 13–61
 - cork-wine interactions 44–52
 - FT-IR spectroscopy 14–33
 - GC × GC-ToFMS 38–44
 - HS-SPME/GC-PCA 33–38
 - process monitoring and 417
 - Quality management systems *see* quality programs
 - Quality programs 5–7
 - examples focusing on food chains 5–6
 - IKB concept 6
 - Q&S systems 5, 6–7
 - trade initiatives 7
 - Quality systems *see* quality programs
 - Quantum dots 281
 - Quartz 170
 - Quartz crystal microbalance (QCM) 170, 171, 283, 287
 - Quercetin 292
 - Quercus suber* L. 44
 - Quinoline yellow 294, 295
 - Quinolone 360
- r**
- Ractopamine 288
 - Radio frequency identification tags (RFT) 85, 329
 - Radio immunoassay (RIA) method 252
 - RainDance Technologies 169
 - Raman spectroscopy 171, 284, 285
 - Rapid microbiological methods 153–185
 - automation and multi-pathogen detection 155–156
 - culture dependent vs independent assays 154–155
 - currently in the market 157–172
 - immunological/ serological based 163–164
 - luminescence-based 162
 - metabolism-based detection 161–162
 - microscopic-based 157–161
 - next-generation technologies 169–170
 - nucleic acid-based (molecular) 164–169
 - qualitative tests 154
 - quantitative tests 154
 - separation and concentration 156–157
 - Raspberries 64, 78
 - Ready-to-eat (RTE) food 99, 105
 - Reduced graphene oxide (RGO) 271
 - Reflectance spectroscopy 171
 - Refractive index 348, 349
 - Relative humidity 98, 469
 - Relative limits 218
 - Reliability 28, 115, 160, 196, 280

- Repeatability 160, 212
- Reproducibility 117, 120, 207, 275, 435
- Restriction enzymes 169, 195, 196, 208
- Restriction fragment length polymorphism (RFLP) 196
- Reverse transcriptase polymerase chain reaction 193–194
- Riboflavin 352, 451
- Riboprinting 169
- Ribose 188
- Ribosomal RNA 165, 189
- Rice 74, 104, 123, 442
- Ricin 123
- Ricketts 148
- RNA 188
 - based amplification assays 193
 - hybridisation methods 189–190
- RNAaseH 194
- Robustness 115, 187
- Roche NimbleGen 189
- Root mean square error in calibration (RMSEC) 425
- Root mean square error in prediction (RMSEP) 26, 28, 425
- Root mean square error of cross-validation (RMSECV) 30
- Rotavirus 63
- Roundup Ready soybean 216, 217, 219
- rRNA 165, 189
- s**
- Safety 3–4
 - controls, food monitoring and 329
 - issues 1–2
- Saffron spice 70
- Sage oil 316
- Salami 385*t*
- Salmon 221
- Salmonella* 63, 64, 103, 106, 124, 125, 256
 - cytotoxins 114
 - endo- and exotoxins 108
- Salmonella enterica* 101, 106, 108, 109, 120, 166, 193
 - amplified fragment length polymorphism (AFLP) 196
 - assessment of viable and dead cells of 194
 - control of 99
- Salmonella heidelberg* 108
- Salmonella infantis* 108
- Salmonella kentucky* 108
- Salmonella stanley* 108
- Salmonella typhimurium* 64, 83, 106, 110
- Salmonellosis 108
- Salt 102, 280, 394
- Sample preparation extraction
 - techniques 432–452
 - extraction from liquid samples 432–446
 - extraction from solid samples 446–452
- Saponins 353
- Sausages
 - analysis of amines using HPLC 359*t*
 - olfactometry studies 385*t*
 - see also* Meat
- Scanning electron microscopy (SEM) 45, 285, 322, 323
- Sea salt 41
- Seafoods 67*t*, 75, 99, 116
 - quality of 81–82
 - vitamin E in 351
- SecDF 123
- Seeds 84, 172, 307
- Sensitivity 43, 75, 423
- Sensors, nanoparticles-based 279–304
- Sensory evaluation 35, 82, 379, 382, 418
- Sequence polymorphisms 196
- Sequential injection analysis (SIA) 435, 436, 452
- Sequential injection single drop micro-extraction (SI-SDME)
 - system 437–438
- Serotyping 116, 117, 195
- Sesame oil 83
- Sheep 288
- Sheep milk 426
 - see also* Milk
- Shelf life 38, 76, 98, 105, 246, 317
 - of fish and seafood 81
 - food packaging and quality preservation 315, 316
 - fruit juices 475
 - of fruits and vegetables 83
 - of meat 79

- Shellfish 63, 273
- Sherry wine 395
- Shiga toxin 97, 109, 114, 121, 125
 - MALDI-TOF MS detection of 160
 - PCR assays for detection of 166–167, 193
- Shigella* 100, 106, 156, 160, 163, 167
- Shigella dysenteriae* 109
- Shigella flexneri* 101, 120
- Shrimp 82, 122, 440
- Signal-to-noise ratios 349
- Silica 315
- Silicon oxide 305
- Silver nanoparticles 280, 281, 305, 311, 312, 316
 - as antibacterial products 305
 - food packaging and quality preservation 315
 - oxidation to silver chloride 328
 - for pathogen detection 172
 - size of 327
 - synthesis of 307, 315
 - toxicity of 320
- SIMCA (soft independent model of class analogy) 423
- Simultaneous distillation extraction (SDE) 380
- Single-drop microextraction (SDME) 433, 434–435
 - continuous flow microextraction (CFME) 434, 437–438
 - direct immersion 434
 - directly suspended droplet microextraction (DSDME) 434, 436–437
 - drop-to-drop microextraction (DDME) 434
 - exposed-drop (in-needle) 434
 - headspace single drop microextraction (HS-SDME) 434, 435–436
 - liquid-liquid-liquid microextraction (LLLME) 434, 437
 - unexposed-drop (in-syringe) 434
- Sniffing ports 379
- Sodium chloride 469
- Sodium dodecyl benzene sulphonate (SDBS) 271
- Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) 123
- Sol-gel method 239
- Solid phase cytometry 161
- Solid-phase extraction (SPE) 345, 432, 440–442
- Solid-phase hybridisation 188
- Solid-phase microextraction (SPME) 13, 33–38, 380, 381*t*, 442–443, 444
 - beer 41–44
 - headspace 13, 78, 446
 - sample preparation 432
 - uses of 382, 388
- Solid samples, extraction from 446–452
 - matrix solid phase dispersion (MSPD) 446–448
 - pressurised liquid extraction (PLE) 448–449
 - super-heated water extraction (SHWE) 449
 - supercritical fluid extraction (SFE) 449–451
- Solubility 433
- Solvent assisted flavour evaporation (SAFE) 380, 381, 388
- Solvent extraction 380, 381, 381*t*
- Solvent microextraction (SME) 433
- Solvents, supercritical fluid extraction (SFE) 449
- Sorbic acid 436
- Southern blot method 188
- Soxhlet extraction 380
- Soy sauce 242, 386*t*, 436
- Soya 217, 353
- Soybean protein isolate (SPI) 319
- Soybeans 219, 220, 296, 315, 319, 356
 - Roundup Ready soybean 212, 216, 217, 219
 - transgenic 217
- Specificity 43, 75, 154, 231, 423
- Spectroscopy 70–74, 425
- SPI1 protein 125
- Spices 99, 100, 104, 444
- Spinach 64, 83, 99, 172, 315
- Spoilage 76, 81, 82, 98, 103
 - biogenic amines (BA) 246
 - dry cured ham 392

- indicators 154, 162
 - meat 329
 - microorganisms 468
 - Squid 82
 - Stacked GM events 220–221
 - Stainless steel 468–469
 - Standard deviation 409, 425
 - Staphylococcal enterotoxin A (SEA) 121, 122
 - Staphylococcal enterotoxin B (SEB) 121, 122, 123
 - Staphylococcus* 102, 103
 - Staphylococcus aureus* 63, 83, 103, 104, 121, 123
 - exotoxins 111–113
 - infections of dairy animals' mammary glands of 102
 - Starch 14, 15*f*, 19
 - Starter cultures 98
 - Statistical methods 221, 401
 - Statistical tests 401, 417
 - Stepwise injection (SWIA) 452
 - Sterilisation 475
 - Sterility testing 477
 - Sterols 405, 427
 - Stir-bar-sorptive extraction 382, 445–446
 - Stomacher 156–157
 - Storage conditions 41, 76, 98, 102
 - Strawberries 64, 74, 82, 295
 - Subcritical water extraction (SWE) 449
 - Sucrose 16, 17, 18, 33, 35, 246
 - biosensors for analysis of 239, 240
 - FT-IR analysis of 14
 - Sudan I 295
 - Sugars, biosensors for analysis of 239–241
 - Sulfonamides 447
 - Sulfur aroma compounds 390*t*
 - Sunset yellow 294, 295
 - Super-conducting quantum interference devices 256
 - Super-heated water extraction (SHWE) 449
 - Supercritical fluid extraction (SFE) 350, 449–451
 - Suppressed conductivity detection 355
 - Surface acoustic wave (SAW) 83, 170
 - Surface-enhanced Raman scattering (SERS) 171, 256, 284, 285, 290, 297
 - Surface-modified quantum dots 298
 - Surface plasmon resonance (SPR) 171, 252, 284, 285
 - Surfactants (emulsifiers) 318
 - Surveillance systems 115
 - Sweeteners, artificial 296
 - SYBR Green 156, 168, 191, 194
 - Synthetic hybrid amplicons 210
 - Synthetic phenolic antioxidants 295–296
- t**
- Tannins 140
 - Taq* polymerases 166, 190, 208
 - TaqMan probes 191, 208
 - Tartrazine 294, 295
 - Tea 45, 75, 307, 439, 470
 - black 84
 - green 307, 314, 317, 437
 - herbal 84
 - Temperature distribution 474
 - Terpenoids 82, 140
 - Texture 98, 280, 419, 471
 - Thermal desorption unit (TDU) 446
 - Thermal imaging (TI) 74
 - Thermus aquaticus* 190
 - Thiamine 352
 - Thiocyanate (SCN[−]) 292
 - Thyme oil 316
 - Thymine 188
 - Time temperature indicators (TTI) 329
 - Titanium dioxide 305, 311, 312, 315, 320, 326
 - Tocopherols 449
 - Tocotrienols 449
 - Tomatoes 64, 74
 - Total analysis system (TAS) 443
 - Total quality management 4
 - Total viable counts 80, 161
 - Total volatile basic nitrogen 80
 - Toxicity 317–321
 - Toxins 273–274, 469
 - see also* bacterial toxins
 - Trace elements 145, 352
 - Traceability 206, 431
 - Tracking and tracing schemes (TT schemes) 2–3

- Transducers 231, 235–238
 - amperometric 237
 - electrical 236*t*
 - electrochemical 236*t*
 - optical 236*t*
 - piezoelectric 236*t*
 - thermal 236*t*
- Transgene copies 219
- Transmission electron microscopy (TEM) 273, 285, 286, 322
- Tree nuts 444
- 2,4,6-trichloroanisole 45
- Trimethylamine 81, 246
- Trimethylamine-nitrogen (TMA-N) 436
- Tween-20 319
- Two-dimensional gas chromatography
 - combined with time-of-flight mass spectrometry (GC×GC-ToFMS) 13, 14
 - beer volatile profiling 41–44
 - principles and advantages 38–41
- Type III secretion systems (T3SS) 125
- Typhi* 108
- Tyrosinase 233*t*, 245
- u**
- Ultra-high temperature (UHT)
 - treatment 468
- Ultrasound-assisted extraction (UAE) 451–452
 - dynamic 452
- Ultrasound-assisted push/pull perfusion (UPP) 452
- Ultrasound imaging (UI) 74
- Ultraviolet-visible spectroscopy (UV-Vis) 70, 284, 285, 348, 349, 436
- UNEQ modelling technique 423
- United States 97
 - Department of Agriculture (USDA) 64
 - Environmental Protection Agency (USEPA) 297, 298
- Uracil 188
- Urea 272
- Urease 244
- v**
- Validation 217–218
- Variable-number tandem repeats (VNTR) 196
- Variable selection 426–428
- Variance 425
- Vegetable oil 83
- Vegetables 64, 66*t*, 72–73*t*, 82–83, 246, 292
- Velocity distribution 474
- Verocytotoxigenic (VTEC) *E. coli* 109, 191
- Veterinary drug residues 287–289, 359–360
- Vibrational spectroscopy 15
- Vibrio cholerae* 106, 120
- Vibrio parahaemolyticus* 116
- VIDAS system 159
- Viruses 100, 257
- Viscosity 471
- Vitamin A 350
- Vitamin B 351
- Vitamin B12 252–253
- Vitamin B5 252
- Vitamin B6 145–148, 291, 295
- Vitamin C 148, 291–292, 351
- Vitamin D 148, 350
- Vitamin E 148, 350, 351
- Vitamin K 350
- Vitamins 145, 350–352, 470
- VITEK MS 160
- Volatile compounds 77, 79–80, 380–382, 436, 443, 445
- Volatile organic compounds (VOCs) 76
- Voltammetry
 - cyclic 45–46, 47*f*, 48–52, 255, 282
 - differential pulse 282
 - square wave 46, 47*f*, 273, 282
- w**
- Watermelon 439
- Wheat
 - beers 395
 - flour 436
 - starch 419
- Whey protein isolate (WPI) 319
- Whiskey 407–414, 420
- Whole genome sequencing (WGS) 173, 197–198

Wine 84–85, 241, 242, 359*t*, 443, 445
 cork-wine interactions 44–52
 polymeric material extracts 29–33
World Health Organization (WHO) 63,
 106, 115, 297
World Trade Organization (WTO) 4

x

X-ray diffraction (XRD) 286
X-ray imaging (XRI) 74

X-ray photoelectron spectroscopy 286,
 322

y

Yeast 29, 80, 98, 243
Yersinia 100, 125, 156, 163
Yersinia enterocolitica 125, 171

z

Zinc oxide 311, 312, 315