6
LC–MS Applications in Environmental and Food Analysis

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6.1
Introduction

Over the past few years, the number of applications of LC–MS has increased considerably in many areas of chemistry, pharmaceutical sciences, and biochemistry because of the rapid advances in LC–MS technology. The volume and quality of knowledge acquired in these fields depend directly on such developments. Triple-quadrupole (QqQ) mass spectrometers are still preserving their market leadership due to their competitive price and unsurpassed performances in quantitative analysis. Hybrid instruments such as quadrupole time-of-flight (QqTOF) and Q-linear ion traps (QqQLIT) have already been affirmed for their peculiar advantages: QqQLIT for its capability to combine the selective scanning modes of QqQ with LIT experiments providing improved performance and enhanced sensitivity such as in enhanced full scan and product ion modes, and QqTOF for its high scanning speed, accurate mass measurement, and MS^2 operations. First, Fourier transform ion cyclotron resonance (FT ICR) and, more recently, orbitrap detectors have gained position in several application areas due to their superior resolving power. At the same time, newly introduced improvements in LC [1], involving monolith technology, fused core columns, high-temperature LC (HTLC), and ultrahigh-performance LC (UHPLC), make the LC–MS technology more attractive and powerful. The improved separation and detection capabilities of LC–MS instruments, as well as sophisticated software tools for data acquisition and processing, have played a central role in the development of new analytical strategies to perform high-throughput screening of contaminants, fingerprint analysis of natural products, quantitative analysis with enhanced identification power, and characterization of biomolecules such as peptides, proteins, oligosaccharides, lipids, and oligonucleotides. Environmental and food fields are two of the most important application areas of LC–MS technology. In this chapter, the practical aspects, advantages, and weaknesses of the

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several analytical approaches will be discussed along with trends and future potential developments within each of these application areas.

6.2 Environmental Applications

Environmental analysis is a very important application area of LC–MS mainly related to the study about the occurrence and fate of organic micropollutants in wastewater, sludge, natural waters, drinking water, sediments, soil, and aquatic biota. The term "organic micropollutants" is meant to include any organic contaminant – pesticides, pharmaceuticals, personal care products, industrial chemicals, hormones, flame-retardants, plasticizers, and others – which enters the environment during its production, consumption, and disposal at ppm or lower level. Among all the known organic contaminants, pesticides are the most relevant and investigated since approximately 900 approved active ingredients, belonging to more than 100 different classes, are still being used worldwide. The same applies to legal and illegal drugs: at present, about 4000 pharmaceuticals are in use in the European Union (EU) [2]. Growing concern and awareness about the potential toxicity to living organisms or/and eco-toxicity of these contaminants is the driving force for developing fast and sensitive multicomponent methods in order to expand monitoring strategies and to investigate the micropollutant fate in the treatment processes and environment. Nevertheless, the wide variety of chemicals occurring in the several environmental compartments exhibits significant differences in physicochemical properties (pKa, log P, etc.) causing serious problems in the development of a "universal" residue analytical method. Recently, much effort has been made in order to reduce time, complexity, and cost of analysis by developing multiclass, multiresidue methods both for screening and for confirmation purposes.

6.2.1 Last Trends in Sample Preparation for LC–MS Analysis

Most of the analytical methods for the determination of micropollutants in water samples include at least one step for the simultaneous sample enrichment and sample cleanup with solid-phase extraction (SPE) cartridges, SPE extraction disks, or LC–LC column switching [3,4]. These steps, allowing typical enrichment factors varying between 20 and 1000, constitute the most time- and labor-consuming parts of the analytical process. In the past few years, the most recent tendencies are toward the development of online sample preparation units: SPE cartridges, turbulent flow chromatography (TFC), and direct sample injection into an LC–MS system without any sample treatment. The latter approach, uniquely allowed by the high sensitivity and selectivity of LC–MS instrumentations, can be applied to isolate many different families of contaminants simultaneously and quickly. Nevertheless, the coextraction of interferences is

responsible for low sensitivity, poor selectivity, and stress of analytical systems (chromatographic column and/or MS) [5].

6.2.2 Advances and Trends in Liquid Chromatography

Over the past decade, advances on the LC side have also contributed to the development of high-throughput methods. UHPLC columns with sub-2 μm diameter porous particles have been widely and successfully employed to speed up the analysis of drugs [6], UV filters [7], pesticides [8], and perfluorinated compounds [9] maintaining similar or even better efficiency than the classical HPLC columns. The extremely narrow peaks produced by such columns have required the use of mass spectrometers with elevated acquisition rates in order to collect enough data points across the LC peak for accurate and reproducible data [10]. Other solutions to reduce the chromatographic run times are based on the use of monolithic and HTLC columns. Monolithic supports consist of a unique piece of porous material that offers a lower solvent resistance and enables ultrafast separations down to only a few seconds. Stationary phases of HTLC columns are based on materials with high temperature stability such as graphitized carbon, zirconium oxide, and polystyrene/divinylbenzene; application of temperatures between 90 and 200 °C is able to reduce column back pressure, to improve selectivity, to allow the use of high percentage of water (superheated water at 200 °C has a similar eluting power as methanol at ambient temperature), and to increase the separation speed by a factor of 3–20. However, both approaches present clear disadvantages that make their use in environmental analysis very limited [11]: stationary phases of monolithic columns have compositions and efficiencies unsuitable for the separation of a significant number of compounds of environmental concern. On the other hand, the high temperatures applied during HTLC separations can affect the stability of both the analytes and the packing materials [12]. Finally, the separation on fused core columns (superficially porous particles also called core–shell or porous shell particles) is gaining attention in this research field. Such columns, characterized by a smaller van Deemter *A* term, provide more than twice the speed and efficiency of columns with sub-2 μm totally porous particles at half the back pressure. This performance enhancement is also applicable to all HPLC instruments by increasing the column temperature between 30 and 40 °C in order to further decrease the back pressure. Wode *et al.* [13] separated 72 micropollutants (industrial chemicals, analgesics, anticonvulsants, antihypertensive, psychoactive substances, flame-retardants, and neutral and acidic pesticides) on a C_{18} core–shell column (2.6 μm) kept at 30 °C, by using a UHPLC coupled to a single-stage orbitrap.

6.2.3 Advances and Trends in Mass Spectrometry

Up to now, electrospray (ESI) has been the most applied source in this research area, initially together with atmospheric pressure chemical ionization (APCI)

that is nowadays less used [14,15]. More recently, atmospheric pressure photoionization (APPI) has been emerging as an alternative for less polar pollutants such as steroids [16], even if it has not yet found versatile applications, whereas direct electron ionization has been valued for the detection of organochlorinated pesticides, which are not amenable to LC–API–MS [17]. Multimode ionization source is another option offered by the latest generation of instruments, which are able to simultaneously carry out both ESI and APCI experiments. Even if dual sources appeared to be a promising solution to increase screening productivity, they always compromise the sampling rate and sensitivity relative to a single ionization mode. Moreover, due to temperature constraint, the APCI mode is clearly not equal to a dedicated APCI source, and most apolar compounds could not be as efficiently ionized. In the past few years, environmental analysis has been performed by employing both low-resolution (LR) and high-resolution (HR) mass spectrometers. The recent advances in mass analyzer technology have favored the development of fast large-scale methods to determine a huge number of targeted and untargeted micropollutants and their transformation products. QqQ mass analyzers operating under selected reaction monitoring (SRM) have still been the most applied MS detectors because of their unsurpassed selectivity and wide linear dynamic range even if different solutions had to be adopted to overcome the limited number of SRM transitions monitored in a single chromatographic run. Greulich and Alder [18] conducted the determination of 300 pesticides in mineral water by direct sample injection into the LC–QqQ system. Two SRM transitions per analyte did not allow a sensitive detection of all analytes within a cycle time of 2 or 3 s; therefore, data acquisition was performed in two runs using time windows. Huntscha *et al.* [19] developed an automated multiresidue method consisting of an SPE–HPLC-QqQ system able to determine 88 polar organic micropollutants (pharmaceuticals, pesticides, biocides, corrosion inhibitors, an artificial sweetener, and their transformation products) with a broad range of physicochemical properties in groundwater, surface water, and wastewater. The global analysis took 36 min per sample. Of late, there is a growing use of QqQLIT, an LR hybrid mass spectrometer that combines fully functional QqQ and LIT within the same instrument. In addition to classical QqQ scan modes that make it suitable for sensitive and selective quantitative analysis, QqQLIT can perform MS^3 operations useful for the identification of transformation products. Reemtsma *et al.* [20] succeeded to develop a multimethod for the determination of 150 metabolites of pesticides in ground- and surface water by using direct injection LC–QqLIT. The analysis was performed in two analytical runs for positive or negative ESI, monitoring two SRM transitions for the analyte. The cycle time was 1.8 s, resulting in variable dwell times for each transition with a minimum of 30 ms. Gros *et al.* [21] described the development of a fast and robust analytical method, based on an automated offline SPE followed by UHPLC–QqLIT analysis, for the determination of 53 antibiotics and their metabolites in environmental matrices such as hospital wastewaters, urban wastewaters, and river waters. The use of HR mass spectrometers in environmental field began in 1999 with pesticide analysis [22], but

it has been in the recent years that their use has been increasing noticeably due to two major advantages: the accurate mass full-spectrum acquisition that allows the screening of thousands of compounds both target or unknown [23] and the improved selectivity able to distinguish between target ions and quasi-isobaric interfering ions. Although in comparison with LR mass spectrometers, HR mass analyzers have lower linear dynamic ranges and sensitivity, TOF, QqTOF, and orbitrap are endowed with insuperable identification power and a great versatility in performing a variety of tasks: pre-target analysis, post-target analysis, and nontarget analysis [23]. A *pre-target* approach requires compound-specific information before measurement, as typically occurs in LC–LRMS-based methods. In *post-target* analysis, the search for compounds is conducted after accurate mass full-spectrum acquisition and can be done even without using reference standards. The m/z values of the target analytes are extracted from the full-scan total ion current (TIC) chromatogram after its acquisition using a narrow mass window ($\pm$10 mDa); the identification is based on the retention time window, the measurement of the exact mass (accuracy < 5 ppm for orbitrap, 5–20 ppm for TOF), and the isotopic pattern. Finally, *non-target screening* methods evaluate the occurrence of compounds after the acquisition of the full-scan chromatogram by searching in-house spectral libraries, to identify unknown compounds without having any previous information. Martínez-Bueno *et al.* performed the analysis of 56 organic pollutants in wastewater by applying an efficient LC–MS strategy based on QqQLIT in combination with TOF-MS [24]. Quantification was performed by LC–QqQLIT operating in SRM mode in both positive and negative ion. Unequivocal identification was provided by the acquisition of three SRM transitions per compound and by LC-TOF analysis, which allowed achieving accurate mass measurements of the identified compounds with errors lower than 2 ppm. The flexibility of QqQLIT instruments allowed improving confirmatory information by the application of additional operation modes based on the use of the LIT mode (see Figure 6.1). Furthermore, the acquisition of full spectra at all times permitted a post-target analysis of non-target compounds, metabolites, or degradation products, thus avoiding additional cost and time.

Another LC-TOF method providing the advantages of this technique is described in the work of Lara-Martin, González-Mazo, and Brownawell [25]. The comprehensive analysis of the most commonly used anionic and nonionic surfactants and their main degradation intermediates in aqueous and solid environmental matrices was carried out. Apart from the identification of the target compounds, the recorded full-scan spectra permitted to identify non-target metabolites such as alkyl sulfates and alkyl ether sulfates. Pesticides and their degradation products have also been studied by LC–TOF-MS. A multiclass method for the chromatographic separation and accurate mass identification of 101 pesticides and their metabolites using LC–TOF-MS was reported by Ferrer and Thurman [4]. Hybrid tandem instruments, such as QqTOF, provide HR data and structural information by performing product ion scan (PIS) experiments. This instrument can collect full-scan spectra and PIS spectra in a single injection by planning data-dependent acquisition (DDA) or information-dependent

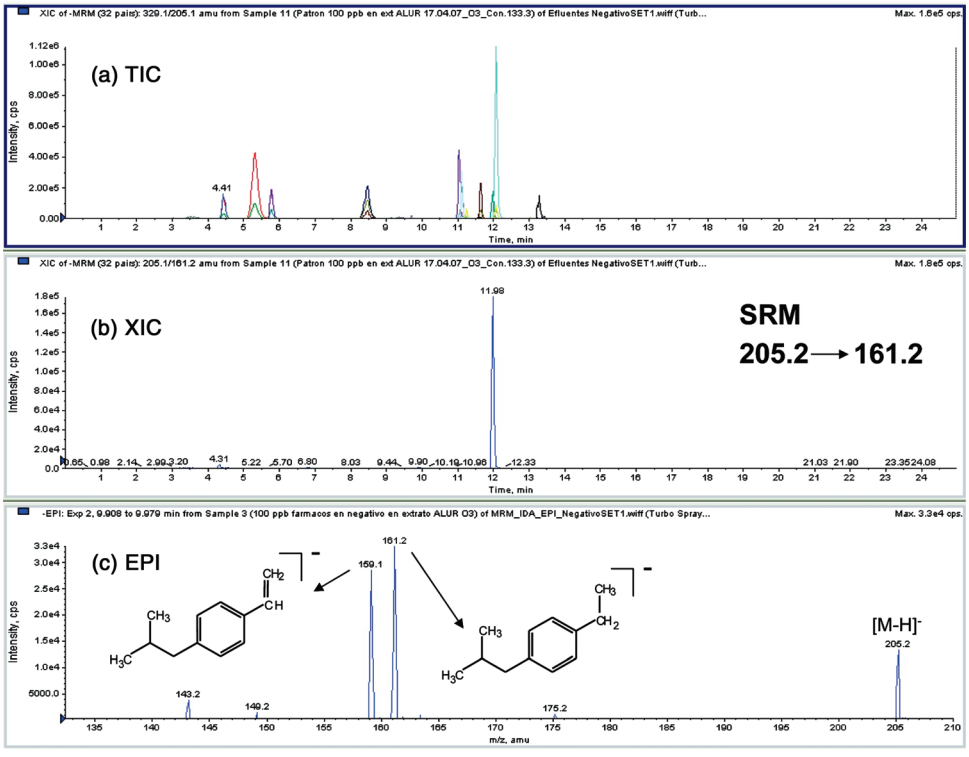

Figure 6.1 SRM total ion chromatogram (a), extracted ion chromatogram (b), and enhanced product ion spectrum (c) of ibuprofen simultaneously obtained by IDA experiments. (Reproduced with permission from Ref. [24]. Copyright 2007 American Chemical Society.)

acquisition (IDA) experiments in which TOF-MS full scan is the survey scan, while PIS scan is the dependent scan [23]. Hernández *et al.* [26] used QqTOF and a specialized processing data application manager to perform the retrospective analysis of pharmaceutical metabolites in urban wastewater without the need of an additional injection of sample extracts. Around 160 metabolites were investigated in wastewater samples using LC–QqTOF under MS^E mode, that is, the simultaneous recording of two acquisition functions, at low and high collision energy, to maximize the number of structural information. Finally, orbitrap mass analyzer is an alternative to QqTOF instruments for identification of transformation products and metabolites or screening over a large mass range. Advantages of the LIT-orbitrap over QqTOF are high ion transmission resulting in higher MS^n sensitivity and detection limits and a higher intensity range over that accurate mass data can be acquired. Helbling *et al.* [27] identified a variety of previously reported and unreported microbial transformation products of organic micropollutants by using LIT-orbitrap MS. Pharmaceuticals and pesticides were spiked into batch reactors seeded with activated sludge, and the

candidate transformation products were preliminarily identified with an innovative postacquisition data processing method based on target and nontarget screenings of the full-scan MS data. Structures were proposed following the interpretation of MS spectra and MS/MS fragments. The results showed that the complementary use of both approaches allowed for a more comprehensive interpretation than either would have provided individually. However, the application of orbitrap in the environmental field is still rare [28] because of the two main drawbacks: a low scanning speed that makes it difficult to couple to UHPLC systems, where very narrow peaks are obtained, and a phenomenon of postinterface signal suppression that affects the sensitivity in detecting small molecular weight compounds.

6.3 Food Toxicant Applications

Food toxicants are mainly small molecules (100–1000 Da) ranging from environmental contaminants, such as pesticides, heavy metals, dyes, and mycotoxins, to registered veterinary drugs and banned substances. Both the parent compound and its metabolites can occur in foodstuffs individually or as multicomponent mixtures with enhanced adverse effects on public health. For these reasons, in the past few years, the most relevant LC–MS applications have especially focused on the development of multiclass residue determinations. The regulatory agencies of many countries have been establishing restrictive food control measures to protect consumer health. In this respect, the EU has pursued a very tough policy and issued several regulations and directives, and maximum residue limits (MRLs) have been set both for the allowed veterinary drugs and for mycotoxins and other contaminants in foodstuffs (Commission Regulation (EC) No 1881/2006), whereas the use of hormones and other performance enhancers for animal fattening have been prohibited (Commission Regulation (EU) No. 37/2010). Instead of the standardized methods, criteria and procedures to develop novel analytical methods (Commission Decision 657/2002/EC and its implementation) have been laid down so as to ensure flexibility and ready adaptation to technical developments, useful to face new emerging problems efficiently.

6.3.1 Recent Trends in Sample Preparation for LC–MS Analysis

The latest trends in preparing food samples for LC–MS analysis are geared for generic and nonselective sample preparation procedures in order to maximize the number of analytes belonging to different toxicant families. Recovery efficiency can be low, but these multicomponent protocols have to be time-/cost-effective, simple, and with high sample throughput. Basically, procedures with these characteristics can be categorized in three groups: solvent extraction (SE), solid-phase extraction, and the QuEChERS methodology. SE has been applied to

isolate more than 100 substances (up to 350 toxicants), among them antibiotics, food additives, mycotoxins, pesticides, sedatives, and growth promoters, from different kinds of foods with a minimum sample preparation without further purification [29,30]. Conventional SPE is the most used technique, but it is less suitable than SE for comprehensive multiresidue methods (up to 50 veterinary drugs) [31]. The latest trends show a growing use of the online SPE–LC–MS to save time and solvents [32]. Dispersive SPE (dSPE) is a recent variant based on the uniform dispersion of the sorbent in a sample solution/suspension that has been applied for single-class [33] and multiclass determinations of more than 100 veterinary drugs [34]. Molecularly imprinted SPE (MISPE) is a selective technique that can be compared with synthetic antibodies assays [35]. For this reason, these materials are mainly suitable for the single-class determination of a limited number of toxicants [36]. QuEChERS (quick, easy, cheap, effective, rugged, and safe) is a two-stage process, combining SE with dispersive SPE. This extractive methodology allows preparing many samples in few minutes and extracting more than 200 different compounds (above all pesticides and veterinary drugs) with good efficiencies and repeatability [37–39] Matrix solid-phase dispersion (MSPD) is a flexible one-step sample treatment useful for extracting/purifying contaminants from a variety of solid, semisolid, viscous, and liquid foodstuffs. It has been applied for the LC–MS analysis of pesticides, veterinary drugs, persistent environmental chemicals, naturally occurring toxicants, and surfactants in several food matrices [40]. To achieve a faster and more efficient extraction of target compounds, MSPD with heated and/or pressurized solvents has also been proposed with pressurized liquid extraction (PLE) [41]. TFC is a compromise between size exclusion and chromatographic adsorption that has been devised as online automated system for achieving high sample throughput. Notwithstanding its potentialities, its use in food analysis is rare and so far has been limited to the analysis of veterinary drugs (up to 40) [42].

6.3.2 Recent Trends in LC–MS Screening Analysis

LC coupled to LR- or HR-MS has been emerging as an alternative technique to microbiological inhibition assay and immunoassay due to its capability to perform large-scale screening of more than 100 residues in a single chromatographic run (up to 300–500 substances in ~5 min). QqQ instruments operating in neutral loss scan (NLS), precursor ion scan (PrIS), or SRM have been used to carry out pretarget screening methods, employing authentic standards for the preselection of ion currents [34]. Martínez Vidal *et al.* [43] developed two multiclass screening methods for the rapid identification of 21 veterinary drugs in milk. The method was based on NLS and PrIS and allowed a rapid identification of residue families in real samples but with high cutoff levels. The other method used one SRM transition per compound and was more suitable for screening purposes at low concentration levels. Although a limitation of QqQ mass spectrometers is the number of analytes that can be monitored per injection, their

ability to detect residues not known *a priori* is the main reason why full-scan HR mass spectrometers are becoming more and more attractive [44]. TOF and orbitrap are especially used to perform post-target and nontarget screening analysis. The highest resolving power of orbitrap (up to 100 000 FWHM) is important to avoid both false positives and false negatives, while the product ion data acquired by QqTOF hybrid instruments are useful to identify compounds belonging to a specific class. Gómez-Pérez *et al.* [29] have created a database for the simultaneous analysis of more than 350 pesticides, biopesticides, and veterinary drugs using UHPLC–orbitrap MS. The developed database includes exact masses and retention times of the target ions, allowing their automated search, and then, the quantification of the detected compounds within the same run (see Figure 6.2).

Generic chromatographic and MS conditions have been used, so that new compounds can be included and the database can be easily upgraded. In the similar way, but using a LC–QqTOF-MS, Turnipseed *et al.* [45] collected exact mass data for approximately 200 veterinary drugs. Farré, Picó, and Barceló [8] performed the screening and quantification of a large number of pesticides and the characterization of other several contaminants in a number of environmental and food samples by using UHPLC–LIT-orbitrap MS. The full-scan MS data

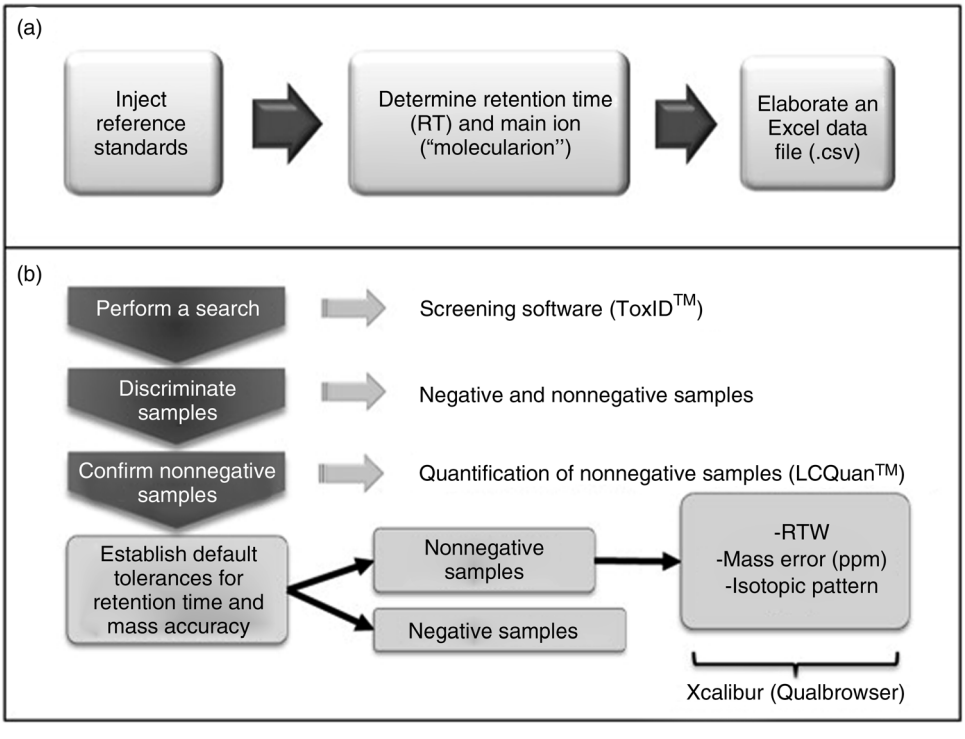

Figure 6.2 Workflow scheme used to create and apply the database to detect and identify the analytes in the samples. (Reproduced with permission from Ref. [29].

were acquired for quantification, while data on MS^2 and MS^3 product ion spectra were obtained for identification and/or confirmation. In addition to target analytes, this method enabled the simultaneous detection/identification of nontarget pesticides, pharmaceuticals, and drugs of abuse, mycotoxins, and their metabolites.

6.3.3 Recent Trends in LC–MS Confirmatory Analysis

According to the criteria of the Commission Decision 657/2002/EC, an indispensable requirement to perform confirmation analysis is to provide a clear identification of the target analytes. To date, the QqQ analyzer running in SRM mode is still the most used chromatographic detector to perform reliable quantitative multiresidue analysis due to its high duty cycle in this scan mode, high sensitivity and selectivity, robustness, extended linear dynamic range, and low cost. Since this analyzer does not record a full mass spectrum, at least two SRM transitions have to be selected in order to achieve a number of identification points (IPs), enough to confirm both group B (IPs ≥ 3) and group A (IPs ≥ 3) substances. Following the recent trends in LC–MS, several methods based on UHPLC and fast scanning mass spectrometers have been developed to separate different kinds of toxicants in short run times (less than 10 min) with high efficiency and sensitivity. A representative example is the UHPLC–QqQ method developed by Gómez Pérez *et al.* [39] for the determination of 17 veterinary drugs (macrolides, sulfonamides, and anthelmintics) in cheese in less than 9 min. In recent years, some methods have been developed using UHPLC coupled to polarity switching QqQ spectrometers to perform the simultaneous detection of positive and negative ions in a single chromatographic run. Leandro *et al.* [46] used an UHPLC–QqQ system for the determination of 52 pesticides in cereal-based baby foods, oranges, and potatoes. The UHPLC separated all of the pesticides, including the structural isomers butocarboxim sulfoxide and aldicarb sulfoxide, whereas the dual polarity detection enabled the determination of 44 compounds in the positive ionization mode and 8 compounds in the negative ionization mode in a single run. Whelan *et al.* [47] analyzed 38 anthelmintic drug residues (benzimidazoles, avermectins, and flukicides) in a 13 min run time by using an UHPLC–QqQ with fast polarity switching. Notwithstanding the high selectivity of the SRM scan mode, the unit resolution of the quadrupole analyzer cannot distinguish between the target analytes and the coeluting isobaric interferences, giving rise to potential false identifications and/or quantitation. This problem can be solved by increasing either the chromatographic or the MS resolution. The latter solution was adopted by Martínez-Villalba *et al.* [48] who carried out the selective LC–SRM analysis of a coccidiostatic drug and its metabolites in meat by using an enhanced resolution QqQ with 0.1 Da at FWHM. Comparing performances of QqQ, TOF, and orbitrap mass spectrometers, Kaufmann *et al.* [49] demonstrated that orbitrap can discern isobaric interferences better than TOF (12 000 FWHM but QqTOF can reach 50 000) due to

its superior mass resolution. Notwithstanding the potentialities of such instruments, LC–HR-MS still remains exceptional for target quantitative approaches. Vanhaecke *et al.* [50] have demonstrated that steroid analysis based on orbitrap, operating at a resolution of 50 000 FWHM, can compete with QqQ in terms of selectivity and linearity. Nevertheless, QqQ-MS has been proved superior in terms of precision (<20%) and sensitivity that is an indispensable requirement for the detection of the banned compound residues.

6.4 Foodomics as a Recent Approach Embracing Metabolomics, Proteomics, and Lipidomics

The past two decades have witnessed a remarkable progress of high-throughput, *omics* technologies. The information provided by this holistic approach has boosted the possibility of linking food knowledge with the optimization of human health and nutrition within a new "foodomics approach" [51,52]. This novel discipline, intending to provide a global outlook on food, which covers the characterization of its composition, food safety, quality, and traceability, the modifications induced by the (bio)technological processes, the alterations over time and the impact that food consumption has on human health, is considered as an affordable way to prevent future diseases. As a result, proteomics, metabolomics, and lipidomics supported by advanced analytical techniques such as LC–MS and bioinformatics are applied to investigate topics in food science and nutrition that were considered unthinkable only a few years ago [53]. The multiple areas covered by foodomics and the tools employed are depicted in Figure 6.3.

6.4.1 Food Proteomics

Proteins are active components of food giving its textural, functional, and sensory characteristics. For this reason, the analysis of food proteome [54], able to generate a systematic view of the protein composition and of the biological and chemical interactions among proteins, is steadily growing. The development of both high-resolving separation techniques and improved mass spectrometers with a better sensitivity and high mass accuracy and resolution is nowadays essential to solve the proteome complexity. Most of the proteomics studies are conducted on hybrid tandem mass spectrometers such as QTOF or TOF–TOF, often operated in DDA mode, where the survey scan spectrum is recorded, with a high mass accuracy (5 ppm), and then the most abundant ions are submitted to MS/MS fragmentation. FT ICR and orbitrap instruments, usually as LIT hybrids, offer the advantage of a fast scanning in the LIT, high mass accuracy (1–3 ppm), and high resolution (100 000–750 000, FWHM). In this case, the DDA mode can be performed at high resolution and the most abundant ions are fragmented in the LIT. An important issue for MS proteomics is the complex nature and the

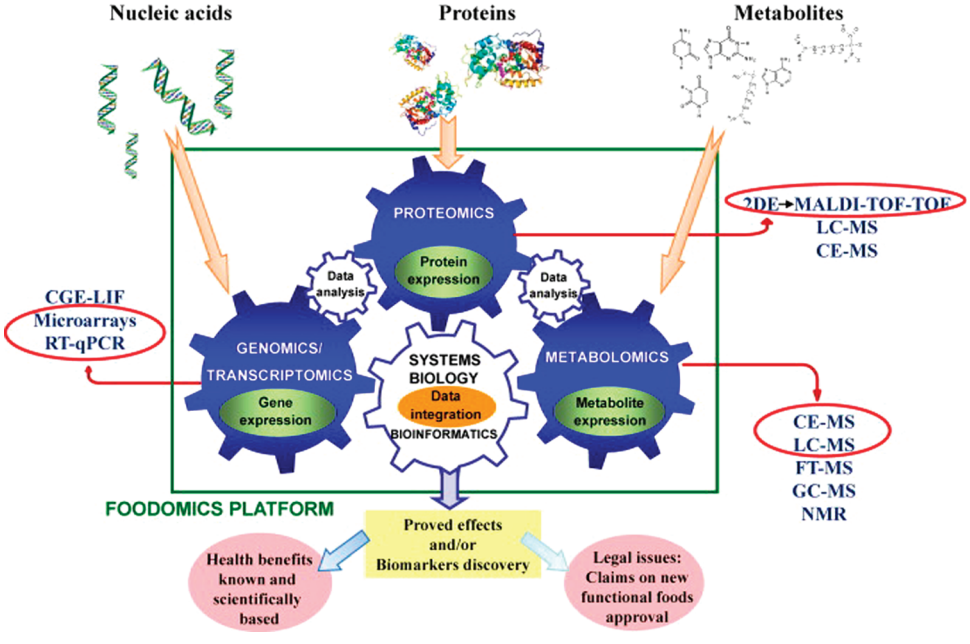

Figure 6.3 Ideal foodomics strategy to explore the health benefits from dietary constituents, including methodologies and expected outcomes. (Reproduced with permission from Ref. [53].)

huge dynamic concentration range of proteomes. For this reason, all MS-based proteomics approaches consist of several separation steps: isolation and (pre) fractionation, depletion of high-abundance proteins and subsequent enrichment forego the MS or MS/MS qualitative/quantitative analysis, and the comparison of MS spectra with calculated mass values through database searching. The proteomics workflow can be performed with two fundamental analytical strategies: the *bottom-up* and the *top-down* approaches. The most common protocol, the bottom-up or peptide-based strategy, involves the combination of two-dimensional gel electrophoresis (2DGE) followed by the identification of individual proteins excised from gel spot by MS and database searching. After image analysis of the 2DGE gels, the proteins of interest may also be converted into peptides by means of enzymatic digestion (i.e., trypsin) [55]. 2DGE provides the highest protein-resolution capacity with low costs, but it is time-consuming and suffers from low sensitivity. A gel-free alternative strategy is an advanced bottom-up approach named *shotgun* or multidimensional protein identification technology (MudPIT). In this methodology, the whole protein mixture is enzymatically digested without prior fractionaction. The resulting peptides undergo a multidimensional chromatographic separation according to their different acidity and hydrophobicity and an online LC–MS/MS analysis is done in a DDA manner.

Despite the huge number of peptides in the hydrolysate, they are by far the better analytical targets than the original protein and ESI is the interface of choice to ionize peptides. This represents the so-called "peptide-centric" approach [56], which is considered to be the most powerful. However, one of the major drawbacks of the gel-free approach is that the information on the intact protein is lost unless the complete protein sequence is covered. By contrast, the top-down approach bypasses the proteolytic digestion step, enabling the characterization of the amino acid sequence after the fragmentation of intact proteins directly inside the mass spectrometer. The main advantages with respect to the bottom-up strategy are the higher sequence protein coverage and the identification of post-translational modifications. FTICR and orbitrap instruments, due to their high resolution and mass accuracy, are the most effective for top-down proteomics [57]. The MS ability to provide structural protein characterization has allowed description of the entire panel of milk proteins both in ruminant species [58] and in humans [59]. Picariello *et al.* [60] developed a peptide-centric approach based on LC–ESI–MS/MS, able to distinguish a goat cheese produced with milk containing high α_{s1}-casein levels. Casein variants strongly influence the quality of milk and this strategy may provide a tool to help milk producers to decide in advance the destination of milk. One of the most notable fields of proteomics applications is that of wheat flour-derived products, since gluten proteins control the dough-making properties. In a recent work, Pompa *et al.* [61] compared the quality of performance of gluten proteome from three different durum Italian wheat cultivars by 2DGE and nano-HPLC–IT-MS/MS allowing the almost complete characterization of gluten complex sequence. In food authentication, the proteomics strategy applied for the identification of species-specific biomarkers consists of a primary discovery phase, typically performed using the bottom-up approach, where a proteome is comprehensively explored, followed by a target-driven phase [62]. Carrera *et al.* reported a faster strategy for monitoring species-specific peptides from parvalbumin [63] that have been demonstrated to be good markers for the identification of fish species. In the second phase, the diagnostic peptide biomarkers previously identified are monitored through a targeted proteomics approach. Thus, LC–MS/MS in MRM mode combined with the use of standard isotope-labeled (SIL) peptides has been evaluated for quantitative analysis of chicken-specific peptides in meat mixes [64]. Moreover, peptidome may be considered as a novel source of information for food traceability. The specific peptides generated from the low proteolysis that occurs in frozen foods are targets to distinguish the fresh from the cold-stored materials, and thus ascertain frauds [65]. While in raw materials the characterization of proteins can be considered quite standardized, proteomics of processed foods remains a challenging task [52,66,67]. Proteins may also act as toxins, antinutrients, or allergens; thus, the study of differentially expressed proteins in GMO foods has raised interest in safety assessment. The bottom-up approach is the most commonly used analytical methodology to profile differentially expressed proteins in GMOs [68]. For example, Koc *et al.* [69], using a LC–TOF-MS strategy, investigated the different profiles of the low molecular weight

protein fractions of both GMO and non-GMO maize, revealing a higher degree of divergence between GMO and conventional maize samples for the profile of the globulin fraction. Finally, from a nutritional standpoint, rather than simply protein degradation products, peptides have proven to be important bioactive food components. Food bioactive peptides act as hormone-like species explicating opioid, antihypertensive, antimicrobial, immunomodulatory, and other effects *in vivo*. MS is the elective technique to profile bioactive peptides [62].

6.4.2 Food Metabolomics

Metabolomics focuses on a comprehensive, simultaneous analysis of all endogenous or exogenous low molecular weight entities (typically less than 1500 Da) present in a given biological system. Food science is a research field in which metabolomics has found a large number of applications [52], since it might reveal attractive relationship between food consumption and variations in metabolic pathways, with interesting impact on food and health domains. There are three basic approaches that can be used in metabolomics [70]: *target analysis*, to quantify specific biomarkers or reaction products; *untargeted metabolic profiling*, which refers to the analysis of a set of (bio)chemically related metabolites; and *metabolic fingerprinting* that compares patterns of metabolites that change in response to a disease, a treatment, environmental or genetic alterations, and so on. The untargeted metabolomics approaches are used in the search for new biomarkers of quality and authenticity, and with the aim to better understand the mechanisms of action in food production processes. Given the large number of chemical entities and dynamic concentration range, a single analytical technology cannot cover the complete food metabolome profile. The advent of modern LC–MS-based metabolomics provides the tools to get a comprehensive profile of hundreds or even thousands of metabolites in food at trace levels in a single run proving to be a successful analytical platform for food quality control [71,72]. Hyphenated systems such as QTOF, ITTOF, and TOF–TOF [73] provide high-resolution (30 000–40 000 FWHM) with both high mass accuracy (<3 ppm) and acquisition rates. These characteristics along with MS/MS data might facilitate the identification of unknown metabolites. FTICR and orbitrap allow analyses with sub-ppm mass accuracy and, under certain conditions, a revolving power up to 1 000 000 [74] enabling (especially with compounds of up to 500 Da) unequivocal assignment of a molecular formula. Composition analyses by LC–MS-based metabolomics have been performed for a number of raw foods, including olive oil [75], processed foods [76,77], GMO crops [68], wine [78], and fruit juices [79]. Wine is a challenging food matrix containing a broad variety of polyphenolics. The combination of two or more separation steps in multidimensional configurations is used to improve the resolution of overlapping metabolites in complex samples. Dugo *et al.* [80] developed a comprehensive two-dimensional LC system using a microbore column in the first dimension at low flow rates (10 µl/min) and a fused core column in the second

dimension at high flow rates (4 ml/min). By means of a switching valve, the first dimension eluate can be simultaneously collected and injected into the second dimension. Thanks to the great resolution and accuracy of an IT-TOF detector, 18 different polyphenolic compounds from different families could be adequately separated and identified. There is a clear trend in nontargeted food metabolomics toward the use of UHPLC–MS in order to obtain higher peak capacity, improved resolution, increased sensitivity, and shorter run times [81,82]. Inoue *et al.* [78] presented an original metabolomics platform based on UHPLC fluorescence TOF-MS assay for specific thiol-containing compounds that are highly associated with quality characteristics of various wines. After fluorescence derivatization, the decrease in MS signals of unknown thiols was monitored and compared with nonderivatized thiols. The mass difference from the derivatization reagent was utilized for the identification of unknown thiols using the fluorescence peaks, the retention time, and the metabolomics databases. Jandric *et al.* [83] performed an untargeted metabolomics approach, based on UHPLC–QTOF-MS and multivariate statistical analysis to identify potential biomarkers to detect fruit juice adulteration. To achieve the separation of a range of compounds of different polarities, both RP C_{18} and HILIC columns were used. The MS^E experiments were used to simultaneously achieve the exact mass of both precursors and fragment ions, so enabling the elucidation of potential structure of unknown metabolites. Authentic fruit juices were differentiated from their adulterated mixtures down to 1% adulteration level.

In recent years, the use of LC–MS-based metabolomics approaches combined with statistical data has been of great help in food traceability and decisively helps the selection of appropriate markers to carry out geographical product certifications. For example, metabolomics studies of honeys, performed in order to identify floral markers and hence to certify the botanical origin, are of great relevance. Polyphenolic profiles of different varieties of unifloral honeys were determined for the first time by UHPLC coupled to hybrid LIT-orbitrap mass analyzer [84], enabling qualitative/quantitative MS/MS analysis and a total of 43 phenolic compounds detected in less than 5 min. Statistical data evaluation established potential floral markers of the botanical origin.

6.4.3
Food Lipidomics

Lipids include both structurally and functionally different classes of metabolites that fulfill multiple key biological roles: as structural constituents of cell membranes, as energy storage sources, and as intermediates in signaling pathways. Consequently, an imbalance of lipid metabolism can lead to many severe health problems including obesity, diabetes, cardiovascular diseases, and some cancers. Lipidomics is a subfield of metabolomics that focuses on the comprehensive identification and quantification of each distinct lipid species present within a biological system, including the understanding of biological activities, distribution of tissues, their interactions with neighboring lipids and proteins, and

response to environmental changes over time. Compared to quite standardized proteomics, the high degree of heterogeneity of lipids is a challenge in their qualitative/quantitative analysis that, however, has significantly gained importance in many fields of biosciences. In the majority of foods, the lipids and their interaction features contribute significantly to the nutritional and sensory values. For these reasons, lipidomics can also be applied in food research, such as in the development of food products, both to control quality, functionality, bioactivity, and toxicity and to detect frauds. The rapid technical progresses in MS combined with bioinformatics have contributed inarguably to push forward the knowledge in lipidomics [85]. Two major strategies are commonly applied, *targeted analysis* of a single or a lipid class and the *global lipidomic approach* that aims at a comprehensive, nontargeted profiling of the lipid components. The MS-based platforms for lipidomic investigations include the direct infusion of the sample extract in the so-called shotgun approach and the LC approach. Particularly effective for polar lipids, the shotgun methodology is based on "in-source" separation by means of precursor ion and constant NL scans, giving rise to a lipid class-specific fragmentation. Usually, HR mass spectrometers such as QTOF and orbitrap instruments are selected. Nevertheless its simplicity and speed, the major limitation of shotgun approach is the ion suppression, which hampers the sensitivity and quantitative robustness of the determination when nonpolar or low-abundance lipids are to be detected. LC–MS is among the most powerful tool for both targeted and nontargeted lipid analyses, using various types of mass spectrometers, ranging from a simple single quadrupole to hybrid instruments and to HR instruments. Targeted analyses, typically using HPLC or UHPLC–QqQ, are able to determine the lipid content of various foodstuffs with very high sensitivity. For global profiling and identification of novel lipids, the popular choices are combinations of UHPLC–QTOF that allow fast analysis and HR detection. The matrix effects are also an important challenge in the global profiling with UHPLC as it is not possible to use labeled standards for all compounds. The sensitivity is typically not as high as in targeted methods, both because the methodological conditions cannot be optimized for each compound separately and because lipids present at high concentrations may suppress the signals of minor metabolites. Due to its healthy properties and high value, adulteration of olive oil is one of the biggest sources of agricultural frauds in the EU. HPLC–MS/MS was successfully performed in discovering adulterations in vegetable oils [86]. Triglycerides (TGs) present in oils and fats are important constituents of the human diet. The nutritional value of TG mixtures are given by their fatty acid (FA) composition and degree of saturation. Two LC techniques are most widespread in the characterization of TGs in complex natural samples, nonaqueous reversed phase (NARP) and silver ion chromatography (SIC). In NARP-LC, the separation of TGs is based on their equivalent carbon number. SI-LC is based on the formation of weak reversible charge-transfer complexes between the π electrons of double bonds of unsaturated TGs and the silver ions immobilized on the stationary phase. The elution order follows the increase in the number of TG unsaturations. Coupling LC to APCI enables an unambiguous

identification of TG species and discrimination of regioisomers [87]. NARP-UHPLC based on the serial use of four shell-packed C_{18} columns was exploited by Dugo *et al.* [88] for the separation of TGs occurring in a complex fish oil and a total of 137 different TGs were positively identified by means of protonated molecules and fragment ions. The good orthogonality of SIC and NARP modes is also used in the comprehensive multidimensional LC approaches for the characterization of very complex TG mixtures. TG mixtures from marine oil, such as cod liver oil, have been characterized and profiled by LC–ESI-IT–MS/MS giving a TG fingerprint that can clearly distinguish the vegetable oil from the marine oil [89]. The sphingolipids composition of food has received interest due to their nutritional importance. Blass *et al.* [90] have recently profiled and quantified sphingomyelin in human milk using HILIC-ESI–MS/MS. The obtained data can help in understanding the nutritional aspects of this lipid and, in the case of breast milk, to design substitutive infant formula. HPLC–DAD with ESI or APCI has been exploited both to profile fat-soluble micronutrients and to determine geometric isomers of carotenoids and xanthophylls in plant foods [91] and in milk from different animal species [92].

6.5
Trends and Future Developments

Over the past 20 years, LC–MS techniques have advanced dramatically in terms of their sensitivity, specificity, and reliability. At present, there is a growing demand for new analytical methodologies to cope with the analysis of a large number of analytes in complex matrices, such as environmental, and food samples, in a short analysis time. Hence, the emphasis is on the achievement of maximum chromatographic resolution in a drastically reduced time by application of fast and HR-LC systems. Fused core columns and UHPLC technology seem to be the most convenient approaches for modern, high-throughput, efficient, economic, and ultrafast analysis. UHPLC appears to be slightly more advantageous, because chemically more stable stationary phases are available. On the other hand, fused core technology enables to easily achieve fast LC separations even with conventional LC instrumentation, proposing itself as a promising alternative to sub-2 μm particle size columns. The use of HILIC is becoming a complementary tool allowing better separation of highly polar compounds. Comprehensive multidimensional techniques, such as LC × LC, are also an improvement that allow for achieving an enhanced resolution and a large increase in the number of peaks, selectivity, and sensitivity. Regarding MS, the use of QqQ instruments in SRM mode remains the most common approach for targeted analysis. Nevertheless, the shift from LR to (ultra)HR tandem mass analyzers providing high mass accuracy (<1 ppm) such as TOF, FTICR, or orbitrap, as well as hybrid configurations (QqTOF, QqLIT, and LIT-orbitrap) are becoming more and more popular. The combination of a high resolving power with accurate mass measurements facilitates the identification of unknown

compounds that is essential both for environmental and for food applications in the analysis of metabolites and transformation products. In this way, orbitrap instruments working in AIF mode will become a powerful tool in the near future.

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