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Review

Recent advances in the application of mass spectrometry in food-related analysis

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Abstract

A review is presented on recent applications of mass spectrometry (MS)-based techniques for the analysis of compounds of food concern. Substances discussed are naturally occurring compounds in food products such as lipids, oligosaccharides, proteins, vitamins, flavonoids and related substances, phenolic compounds and aroma compounds. Among xenobiotics, applications of MS techniques for the analysis of pesticides, drug residues, toxins, amines and migrants from packaging are overviewed. Advances in the analysis of trace metals of nutritional and toxicological interest by MS with inductively coupled plasma (ICP) source are presented. The main features of mass spectrometry combined with separation instruments are discussed in food-related analysis. Examples of mass spectrometry and tandem MS (MS–MS) are provided. The development and application of matrix-assisted laser desorption ionization (MALDI) and electrospray (ESI) to the analysis of peptides and proteins in food is discussed. This survey will attempt to cover the state-of-the-art up from 1999 to 2001.
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1. Introduction

The importance of mass spectrometry (MS) to the future of food research is now well established. In the last few years the role of mass spectrometry and related techniques is increasingly built up as an enabling tool in food analysis for quality control. Improvements in instrumentation, advances in on-line separation techniques and in data processing have contributed to determine this great expansion in the role of MS also in food-related analysis. Liquid chromatography–mass spectrometry (LC–MS) coupling has led to the development of new interfaces, extending the possibilities and automation of various procedures even more [1,2]. Undoubtedly, significant advances in ionization techniques having a broad range of applicability and high sensitivity for the analysis of high-polar and high-molecular mass compounds of food concern have been the key of this development in the last years. The impact of new ionization techniques such as electrospray (ESI) and matrix-assisted laser desorption ionization (MALDI) [3] on quadrupole, magnetic sector or time-of-flight (TOF) instruments, or coupled with instruments with tandem MS (MS–MS) capabilities has been fundamental also for food applications. Among atmospheric pressure ionization (API)-based interfacing systems are ESI, that is a liquid-based interface, and heated nebulizer-atmospheric pressure chemical ionization (HN-APCI), in which a gas-phase ion-molecule reaction process leads to the ionization of analyte molecules under atmospheric pressure conditions. The reader may refer to the recent book by Niessen for instrumental aspects of LC–MS coupling [2]. ESI and APCI well complement one another as regards to polarity and molecular mass of analytes and of chromatographic conditions. Although use of APCI is not yet as widespread as ESI, the number of reported applications of APCI-MS is rapidly increasing.

Separation techniques such as gas chromatography (GC), liquid chromatography (HPLC) and capillary

electrophoresis (CE) have become analytical techniques with many applications in study of substances of food concern, ranging from naturally occurring compounds to xenobiotics. Analysis of complex food extracts requires highly selective analytical techniques to characterize and determine targeted compounds and to characterize unknown compounds. The coupling of chromatographic techniques and MS has overcome the main analytical problem, which is the scarce information about identity given by the detectors usually associated with GC and HPLC. High analytical power of GC–MS and on-line LC–MS have been convincingly proved. As for GC–MS, principles, instrumentation and analytical strategies using this techniques have been extensively discussed in a very recent book [4].

Liquid chromatography coupled to MS–MS offers a powerful tool also in food chemistry due to its selectivity, which enables the use of fast chromatography with low separation efficiency. However, in the analysis of real samples the existence of coeluting undetected components can lead to scarcely accurate method as a consequence of problems with the MS response, due to ion suppression and other effects. Thus the need for an efficient sample purification and chromatographic separation should not be undervalued. MS–MS can be accomplished using triple–quadrupole systems, which realize a tandem-in-space instrument, or by performing in-source collision-induced dissociation (CID) or using ion trap (IT) instruments [5]. The use of GC with tandem MS is one method of obtaining confirmation of the identity of xenobiotics at a sensitivity similar to those of typical GC detectors.

Mass spectrometry is now considered to be a significant aid in peptide and protein characterization. In particular, rapid and sensitive characterization of polypeptides and proteins combined with sequence-related information can be directly obtained from MS–MS experiments of proteins.

In recent years, inductively coupled plasma-mass spectrometry (ICP-MS) has become an analytical technique with many applications in the study of inorganic compounds also in food analysis [6]. The use of ICP as ion source for MS has great potential especially when combined with liquid chromatography, since LC–ICP-MS combines the identification and the detection capabilities of ICP-MS in the elemental analysis, with the possibilities of LC in the speciation of inorganic and organometallic compounds [7]. This is of particular interest in nutritional and toxicological research studies, where the goal is the identification and determination of the chemical form of an element, including metals and nonmetals, i.e., elemental speciation.

Further, there are a number of innovative MS techniques reported in recent literature on food-related analysis. A new approach is known as biomolecular interaction analysis-mass spectrometry (BIA-MS) [8], which is a combination of surface plasmon resonance–biomolecular interaction analysis (SPR–BIA) [8] and MALDI-TOF-MS. Alkali metal ion attachment mass spectrometry (IAMS) has been recently introduced with the aim to obtaining selective ionization of compounds in a mass spectrometer source [9]. Mass spectra recorded using this system consist only of quasimolecular ions formed by addition of alkali ions to analyte molecules. A specially made ion attachment mass spectrometer consists of a quadrupole mass spectrometer coupled with an alkali ion emitter [10]. Discussion of the few applications of these newly developed techniques will be reported below.

The purpose of present overview is to acquaint the reader with some of the existing recent applications of MS-based techniques in food analysis. Topics covered include MS analysis of both GC- and LC-amenable naturally occurring substances and xenobiotics. Advances in MS methods for the analysis of metals of food concern from the nutritional and toxicological point of view are overviewed. The major developments in the application of MS to solve different problems in food technology, such as the assessment of technological processes, quality, and authenticity control of animal foods, are considered. This survey will attempt to cover the state-of-the-art up from 1999 to at least 2001.

2. Natural substances in food

2.1. Lipids

Lipid analysis is of primary importance in food-processing research and development. The evaluation involves the characterization of the triglycerides,

fatty acids, waxes, sterols, carotenoids and fat-soluble vitamins among the neutral lipids, and phospholipids among the polar lipids.

A literature survey revealed that in the last years most investigators have preferred HPLC–MS techniques as the methods of choice for the analysis of different fat and oil systems [11,12,19,20,22,24–30]. Applications of GC–MS to lipid analysis [12–16,26,27] have mainly focused on the characterization of the acylglycerol and sterol fraction [12–16].

2.1.1. LC–MS and GC–MS

As reported in a recent review paper, the applicability of mass spectrometry by APCI-MS has been successfully evaluated for the analysis of mixtures of neutral lipids, such as triacylglycerols (TAGs) and sterols [11]; the reader may refer to this review paper for information on the APCI-MS analysis of lipids from a variety of classes over the past years. The author underlined the difficulty in obtaining the structural characterization of large neutral molecules such as TAGs and carotenoids by using other methods such as GC–MS and ESI ionization. Most lipids, including TAGs, carotenoids and phospholipids, are non-volatile large molecules, and therefore not amenable to GC or gas-phase ionization processes. In addition, even though ESI-MS and ESI-MS–MS are the methods of choice for analysis of polar lipids, APCI-MS can provide useful and complementary structural information on phospholipids as well [11].

The use of HPLC–APCI-MS to characterize 120 fat triacylglycerols in bovine milk has been demonstrated in a very recent paper [12]. In this report the composition of bovine milk fat was investigated performing a prefractionation by thin-layer chromatography (TLC) on silica and gel permeation chromatography (GPC), followed by a more detailed molecular species analysis carried out by HPLC–APCI-MS and high-temperature GC–MS. TAGs eluted by TLC gave two fractions which were analyzed by RP-HPLC–APCI-MS. The resulting chromatogram of the first fraction was shown to contain the majority of the TAG species present, but the elution profile was too complex to allow identification of peaks. The chromatogram obtained eluting the second fraction contained fewer peaks, which appeared to be better resolved, allowing the identification of 28 TAG species. Prefractionation with GPC gave 16 fractions which were collected and subsequently analyzed by RP-HPLC–APCI-MS. To elucidate the TAGs structures, fraction 1–12 were also analyzed by high-temperature GC–MS using a capillary column coated with a polarizable stationary phase specifically designed for the analysis of TAGs. The last four fractions (13–16) were not analyzed by GC–MS, since the TAGs present in those fractions were fully elucidated by HPLC–APCI-MS (Fig. 1). Analysis by RP-HPLC produced separations by increasing equivalent carbon number (ECN), which is the number of carbons in the acyl chains minus two times the number of double bonds (acyl carbon number, 2*n*). Using the data obtained from GC–MS

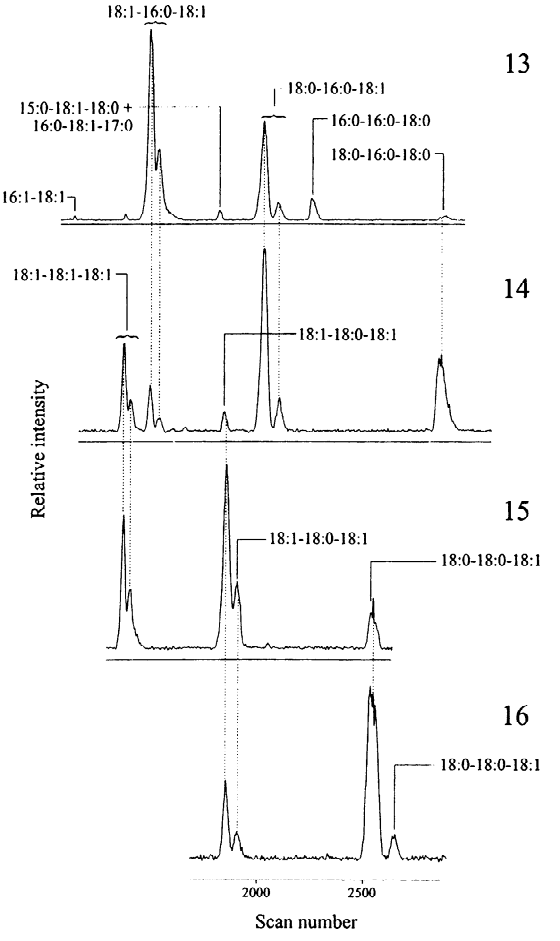

Fig. 1. The HPLC–APCI-MS profiles of GPC fractions 13–16 of milk fat. Reprinted with permission from Ref. [12].

and RP-HPLC–APCI-MS of the fractions, the authors were able to identify 120 TAGs in the whole milk fat.

A different approach based on the use of a combination of silver ion adsorption-TLC and GC–MS has been taken by Fontecha et al. for the determination of triglycerides in goat's milk fat [13]. The $AgNO_3$–TLC technique was used to separate in four distinct fractions the triglycerides contained in goat's milk fat and then each fraction was analyzed by GC and GC–MS. The proposed method was able to determine the distribution of the triglycerides according to their carbon number and degree of unsaturation.

The separation and analysis of monoacylglycerols (MAGs) derived from butter oil by fungal degradation has been proposed by Liu and Kinderlerer [14]. They developed a preparative TLC separation of acylglycerols formed from butter oil degraded by *Penicillium roquefortii*. Monoacylglycerols, lactones, 1,2- and 2,3-diacylglycerols, long-chain 2,3-diacylglycerols and free fatty acids were completely separated. MAGs were silylated with *N,O*-bis-(trimethylsilyl)acetamide (BSA) and separated by GC as trimethylsilyl (TMS) esters (MAG-TMS). Structures and composition of MAG-TMS esters were confirmed by GC–MS.

Other recent GC–MS applications on lipids concern characterization of the non-polar fraction of a series of fats used as cocoa butter supplements [15] and the identification of steryl esters in cocoa butter [16].

Sterols are the major components of the unsaponifiable fraction of lipids in most fats. Plant fat and oils contain phytosterols, which are present in pure or esterified form, or conjugated as glycosides, their composition being characteristic of the plant species. These nutritionally significant lipids have been shown to exhibit anti-inflammatory and anti-neoplastic activity, so that it is of interest to develop reliable methods for their determination in food matrices. An extensive review on the chromatographic methods for the analysis of plant sterols in various sample matrices and vegetable oils has been recently published [17]. In this article the potential of GC–MS for the analysis of sterols in vegetable oils and plant samples is discussed with emphasis on the use of GC–MS-SIM for trace determination of sterols in plant samples because of increased detection sensitivity and analyte specificity of MS detection. In addition, the author points out that in spite of the capabilities of HPLC–MS for the simultaneous separation, quantitation and structural elucidation of sterols, few publications on the practical application of HPLC–MS in sterol analysis have appeared in the literature [17].

A method based on the use of GC–MS for the identification of the non-polar fraction of a series of fats used for cocoa butter equivalent has been devised by Crews et al. [15]. Steradienes, which are the main components of the non polar fraction of the refined fats obtained from palm, illipe, kokum, mango, sal and shea can be used as markers to identify and quantify cocoa butter supplements in chocolate. In this work steradienes in the investigated samples were isolated by a silica column and directly analyzed by GC connected to a quadrupole MS.

More recently, Kamm et al. reported the separation of steryl esters in cocoa butter by an on-line LC–GC method involving silylation of the sample, so that it could be extended to the simultaneous determination of free sterols [16]. The analytes, which were separated by a LC–GC fully automated apparatus, were identified by GC–MS using positive chemical ionization (CI) with ammonia as the reagent gas. All spectra displayed abundant $[M+NH_4]^+$ adduct ions, which in general were base peaks in the spectra and two fragment ions corresponding to the loss of the fatty acid moiety $[M+NH_4-RCO]^+$ and of one water molecule ($[M+NH_4-RCO-H_2O]^+$).

ESI tandem-MS has been demonstrated suitable for the characterization of free and esterified sterols in fat and oil [18]. The method was based on the separation of sterol ester, triacylglycerol, and free sterol fraction by flash chromatography. MS measurements were performed with a triple quadrupole mass spectrometer equipped with an ESI ion source. Direct inlet ESI mass spectra of cholesterol ester mixture, butterfat and vegetable oil dissolved in 10 m*M* ammonium acetate in chloroform–methanol (1:1) were recorded. All spectra showed abundant sterol ester ammonium adducts $[M+NH_4]^+$ ions and characteristic sterol fragment ions. Performing precursor ion ESI-MS–MS experiments of sterol frag-

ment ions in sterol ester fraction, sterol ester proportions were determined in butterfat and vegetable oil samples.

According to that observed by Byrdwell [11], APCI-MS detection in positive ion (PI) mode has been successfully applied by Careri et al. for the identification of phytosterols in soybean oil extract analyzed by HPLC [19]. APCI mass spectra of sterols were characterized by the protonated molecules of the analytes and an abundant peak corresponding to fragment-ion due to the release of a water molecule. Applying this technique, it was possible to successfully characterize the phytosterol fraction in soybean oil.

Polyglycerol fatty acid esters (PGE) are non-ionic surfactants which are of interest in food industry as fat replacers, food preservatives or to stabilize emulsions. They are constituted of complex mixtures which differ on the basis of their alkyl chain length, degree of polymerization and the linear, branched or cyclic structure of the oligoglycerol moiety. A method which combined GC, LC and LC–MS has been proposed by De Meulenaer et al. to characterize PGE [20]. To elucidate the polyglycerol composition of various PGE, samples were effectively separated in three different fractions by GC, following a procedure previously described consisting of saponifying the esters without influencing the degree of polymerization of the polyglycerol [21]. In all analyzed fractions, peak identity was verified and confirmed by LC–ESI-MS.

More recently, the degree of polymerization and characterization of PGEs and fatty ethers were carried out by HPLC coupled with mass spectrometry [22]. To enhance chromatographic selectivity requested for a complete separation of isomers and diasteroisomers present in the analyzed samples, a porous graphitic carbon column (PGC) was used to separate diasteroisomeric lauryl esters and lauryl ethers, whereas silica-based octadecyl columns were successfully used to separate polyethoxylated alcohols. Structure elucidation of each compound was performed by NMR and by a triple quadrupole mass spectrometer with an ESI source.

Carotenoids are tetraterpenes which are present in a variety of foods and food products showing many advantageous biological and nutritional effects. The use of ESI ionization with the turboionspray system [23] for LC–MS analysis of carotenoids was proposed by Careri et al. [24]. Previously, the same group proposed the determination of carotenes and xanthophylls by a HPLC–MS method based on the use of the particle beam interface under electron-capture negative ion (NI) conditions [25]. Coupling RP-HPLC with MS via the ESI interface different carotenoids were investigated. The method was validated in terms of dynamic range, sensitivity, limit of detection and precision. LC–MS determinations were performed by operating the mass spectrometer in the positive ion mode. Quantitative analyses were carried out using single ion monitoring (SIM) of the molecular ion peaks of carotenoids at m/z 568 (lutein, zeaxanthin), m/z 564 (canthaxanthin), m/z 552 (β-cryptoxanthin), m/z 536 (β-carotene). The positive ESI mass spectra obtained for all carotenoids except that of astaxanthin, were characterized by the molecular ion M^+, which was due to the loss of one electron and formation of a stable radical system. In contrast with that reported by other authors, who described unsuccessful application of ESI ionization for the analysis of β-carotene unless solution-phase oxidants were post-column added to the LC eluent, the results obtained in this work showed that ESI detection in PI mode is well amenable also for this carotene. In general, better sensitivity under PI than NI conditions was proved. The developed method was applied to the qualitative and quantitative analysis of carotenoids in a microalgae sample.

An interesting study has also been carried out by Robinson et al. in order to evaluate β-carotene degradation products generated by lipoxygenase (LOX) catalyzed co-oxidation in soybean and pea seeds [26]. It is known that in order to reduce lipid autoxidation, β-carotene is used as an antioxidant due to its free radical scavenger properties. On the other hand, a pro-oxidant character has been recognized to this molecule as a consequence of the extensive system of double conjugated bonds. To elucidate this behaviour of β-carotene, GC–MS and HPLC–MS were applied. Information obtained from LC–APCI-MS and GC–MS mass spectra allowed the identification of a great number of primary and secondary oxidation products, such as apo-products, epoxyproducts and dicarbonyl compounds, thus suggesting that LOX oxidation could occur randomly

both along the hydrocarbon chain of β-carotene and in the cyclohexene ring.

Dachtler et al. explored the possibility to identify carotenoid stereoisomers in spinach, as well as from biological tissues, by on-line coupled HPLC–APCI-MS and HPLC–NMR techniques [27]. Chromatographic separation of stereoisomers of lutein and zeaxanthin was carried out on a C_{30} reversed-phase (RP) column, which was more suitable to resolve carotenoid stereoisomers then the commonly used C_{18} phases. Detection in PI mode was able to distinguish stereoisomers of the xanthophylls lutein and zeaxanthin, which differ only in the location of one double bond in one ionone ring system, because of the characteristic loss of water from the protonated molecule. Furthermore, the unambiguous structural elucidation of carotenoid stereoisomers was performed by on-line HPLC–NMR coupling.

All fat and oils contain a number of phospholipids. The lowest amounts of phospholipid are present in pure animal fats; in some crude vegetable oils, such as corn and soybean oils, phospholipids occur at levels of 2–3%. Phospholipids have been successfully analyzed using ESI-MS [11]. However, ESI mass spectra of phospholipids exhibit only molecular ions at conditions that do not cause CID (low cone or orifice voltages), so that methods based on in-source fragmentation or triple–quadrupole tandem MS have been evaluated to obtain structural information on these polar molecules. It is to be noticed that in general ESI ionization yields fragments corresponding to head group ions of phospholipids in PI mode, whereas more diagnostic fragment ions are produced by APCI-MS. In this respect, as above discussed ESI-MS and APCI-MS are to be considered complementary for phospholipid characterization. The potential of APCI-MS coupled with RP-LC has been recently exploited for the qualitative and quantitative analysis of phospholipid molecular species as nicotinate derivatives [28]. Phosphatidylcholines from soybean, egg yolk and bovine liver were converted to UV-absorbing diacylglycerol derivatives and separated isocratically by reversed-phase chromatographic methods. The diacylglycerol derivatives were able to be quantified by HPLC with UV detection and identified by LC–MS. A half quantitative estimation of the molecular species composition of complex samples was also obtained by LC–MS on the basis of the area from the TIC and from the areas of selected ions. In particular, operating the mass spectrometer in the positive APCI mode, the pseudo-molecular ion $[MH-123]^+$ was the most useful ion for estimating the amounts of many overlapping species.

The effect of a post-column modifier for negative ion detection of phosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine has been evaluated by performing ESI-MS experiments compatible with reversed-phase separation of phospholipids [29]. The chromatographic separation was performed on a C_{18} column by isocratic elution with a mobile phase consisting of a mixture of methanol containing 0.002% piperidine–water (95:5, v/v). ESI mass spectra were obtained using both a single and triple quadrupole MS. Post-column addition of a solution of 0.02% of piperidine in methanol was proved to be effective in the enhancement of phospholipid detection in the NI mode. Furthermore, piperidine in the eluent avoided interactions between analytes and silanol groups of the silica-based stationary phase and peak tailing of phosphatidylethanolamine was less noticeable. Sharper peaks increased the signal-to-noise ratio resulting in lower limits of detection (LOD) and quantitation (LOQ). The LOD and LOQ values were experimentally determined with both single and triple quadrupole instrument and the obtained results compared. As for phosphatidylglycerol, LOD and LOQ were found to be 20 and 60 fmol/μl, respectively, when monitoring three ions in the SIM mode with a single quadrupole MS. When operating with MS–MS technique improved analyte detectability for this compound was claimed, detection and quantitation limits being 446 amol/μl and 1.3 fmol/μl, respectively.

Normal-phase HPLC coupled with NI ESI-MS–MS was used to determine 68 phospholipid molecular species from atlantic salmon head kidney [30]. This study was aimed to study the effect of different diets based on soybean oil and fish oil on the relative distribution of these molecular species. The relative distribution of the molecular species was analyzed by selected reaction monitoring (SRM) of the carboxylate ion peak area from the *sn*-1 position. The highest number of molecular species (22 species) was found in the phosphatidylethanolamine class compared to the other phospholipid classes, the major molecular

species being the 16:0/22:6 species. An interesting feature of the method was the feasibility to assay the phospholipid species directly on-line without a previous purification of the phospholipid classes.

It can be concluded that, in the case of complex mixtures of volatile, semi-volatile and non-involatile compounds, combinations of different techniques which analyze compounds on a different basis are most advantageous to obtain both molecular mass and structural information.

2.2. Peptides and proteins

Proteins play a major role in determining nutritional and functional properties of food products. Further a biological function is ascribed to these important food components, owing to the presence of biologically active peptides in their primary sequences, making them potential health-promoting ingredients. Research on structural and physicochemical characteristics of food proteins aimed at elucidating their molecular structure responsible for their functionality is of utmost interest and studies of the structure–property relationships of proteins are needed to understand their functionality in food systems. The dominant state-of-the-art analytical approach applicable to protein food research is MALDI-TOF-MS and LC–ESI-MS with quadrupole mass spectrometers. Applications of mass spectrometry to the analysis of food peptides and proteins have been extensively reviewed in recent years [31–33]. Léonil et al. overviewed applications based on off-line MS and on-line MS coupled with liquid chromatography with ESI and continuous flow-fast atom bombardment (FAB) concerning peptides and proteins of milk, eggs and cereals besides to food protein hydrolyzates [31].

In the review paper by Alomirah et al. the capability of ESI-MS and MALDI-TOF-MS for the structural characterization of proteins is discussed with particular regard to their advantages in terms of sensitivity, mass accuracy and short analysis time [32]. In this article, the potential of these techniques for protein sequence determination, post-translational modifications, protein folding/unfolding and protein–protein or protein–ligand interactions are also discussed.

The reader may refer to these overviews and to the articles cited therein for information on mass spectrometry applications in food peptides and proteins up to 1999. Advances in the application of MS to food protein research in the 2000–2001 are a suject of discussion in the present article.

A more recent review paper outlines the research performed on the modern chemical methods for characterization and identification of post-translational modifications of allergenic proteins [33]. MS determinations are described as the commonly used methods for analysis of allergenic and non-allergenic proteins. With the final goal to elucidate physicochemical properties responsible for protein allergenicity, the authors underline the importance of identification of the allergenic epitopes and of determination of all characteristics of the molecular structure of an allergenic protein.

2.2.1. MALDI-TOF-MS

The development of MALDI-MS techniques for analyzing complex protein mixtures is an important area of research. In the past, MALDI has been demonstrated highly effective in determining fingerprint of the protein composition of various food samples [34–37].

The capability of MALDI-TOF-MS for the rapid and accurate evaluation of the authenticity of milk has been recently tested [38]. MALDI-TOF-MS proved helpful in evaluating the presence of cow milk in raw ewe and buffalo milk samples and detecting the addition of powdered milk to fresh raw milk samples. Rapidity and accuracy were the main features of the method proposed. Mass accuracy was always within the range 0.5–1%, whereas the speed of MALDI analysis makes it possible to perform a total of 100 analysis of milk samples in a time as low as 1 h. The authors discussed recent advantages in resolution of MALDI analysis obtainable with the use of delay extraction technique. The resolution achieved with the instruments equipped with this system is high enough to separate peaks differing by a few Da in a mass range up to 20–30 kDa. A result worth of mentioning was obtained when analyzing mixtures with different percentages by weight (5, 10, 20, 30 and 50 wt.%) of cow milk added to ewe milk. MALDI-MS was able to detect even small quantities of cow milk added to ewe milk on the basis of the variants A and B of cow and ewe β-lactoglobulins.

Lysozyme has been the subject of MS investigations on the characterization of the nonenzymatic reactions of food proteins with secondary plant metabolites such as chlorogenic acid [39] and phenolic acids [40]. Lysozyme derivatives with selected phenols, i.e., *o*-, *m*-, *p*-dihydroxybenzene, gallic and ferulic acid, were characterized by MALDI-MS with the final goal to evaluate modifications in selected physicochemical properties and effects on their digestion with the main proteolytic enzymes of the gastrointestinal tract [40]. Molecular weights of unmodified lysozyme control and phenol derivatives were determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis and MALDI-TOF-MS using sinapic acid as matrix. In accordance with that observed by SDS–PAGE, in contrast with other derivatives, the product deriving from the reaction of lysozyme with ferulic acid showed no apparent modifications in molecular composition if compared with underivatized lysozyme (curves 1 and 5, Fig. 2). In general, MALDI–MS was useful in illustrating the formation of products deriving from the first primary addition of a reacting compound. SDS–PAGE methodology gave further indications about the formation of polymerized products resulting from the reactions of phenols with lysozyme. The formation of these products was found to proceed following the order: *p*-DHB > *o*-DHB > gallic acid > *m*-DHB > ferulic acid.

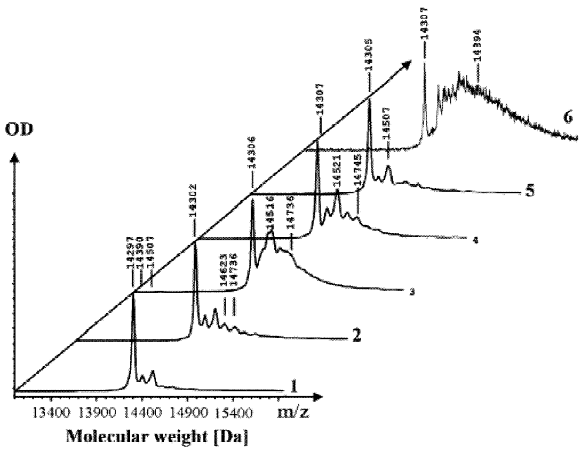

Fig. 2. MALDI-TOF-MS of lysozyme derivatives. Code: (1) unmodified lysozyme control; (2) *m*-DHB derivative; (3) *o*-DHB derivative; (4) *p*-DHB derivative; (5) ferulic acid derivative; (6) gallic acid derivative. Reprinted with permission from Ref. [40].

The applicability of MALDI-TOF-MS to the analysis of trypsin-digested peptides of lectin has been recently explored by Martínez-Cruz et al. [41]. Lectins are a group of proteins which are known to bind specifically sugars. In this work a galactose-specific lectin from corn (*Zea mays*) coleoptyle was studied and characterized. MS analysis of amino acid sequence in the digests indicated a 18% homology of the lectin investigated with a putative calcium-dependent Ser/Thr protein kinase from *Arabidopsis thaliana* and a 39% homology with NADPH-dependent reductase from *Zea mays*.

2.2.2. LC–MS

Recent ESI-MS and ESI-MS–MS studies demonstrate the potential of these mass spectrometric techniques in the research of food proteins. In particular, ESI-MS with quadrupole instruments has been demonstrated a powerful tool for mass determination of food proteins and for detection of protein modifications induced by processing. In the last years there is increasing interest in using LC coupled to ESI ionization on a time-of-flight instrument as a tool for accurate mass determination of proteins. Other advances in the field of MS analysis of biomolecules of food concern include the use of collision-cell CID of proteins in ESI-MS–MS experiments for elucidation purposes.

Characterization of low-molecular mass peptides using ESI ionization has been the subject of two studies [42,43]. A very effective method for peptide sequencing has been devised by Gaucheron et al. [42]. Liquid chromatography–tandem mass spectrometry with ESI ionization was successfully applied for the separation and identification of low-molecular-mass peptides released during milk sterilization. Using MS–MS under PI conditions, 10 peptides were identified, three of which derived from α-casein, two from β-casein and five α_{S1}-casein. Instead, no low-molecular-mass peptide coming from α_{S2}-casein and whey proteins was detected. Further, ESI-MS–MS methodology allowed to identify three peptides produced by the oxidation of methionine residues, corresponding to a molecular mass increase of 16 Da, and by a lactosylation, a chemical modification involving β-casein associated with an increase of the molecular mass of 324 Da. These

oxidized or lactosylated peptides were characterized during milk sterilization after a heating time of 30 min.

An approach based on the use of HPLC–ESI-MS has been applied for identification of oligopeptides in Parma hams of known cathepsin B activity [43]. The combination of free amino acid and oligopeptide LC–MS determination allowed the identification of substances involved in the development of bitter taste in dry-cured hams. In particular, the dipeptides Gly–Phe, Gly–Leu(Ile) and Leu(Ile)–Leu(Ile) proved to be correlated with bitter taste.

ESI-MS has been demonstrated a powerful tool for mass determination of milk proteins, detection of modifications in proteins caused by insertion, deletion or modification of amino acids, identification of genetic variants and post-translational modifications like deamidation, oxidation, glycosylation, phosphorylation, sulfation [32]. The characterization by RPLC–ESI-MS and flow-injection (FIA)-ESI-MS of ovine milk proteins was the subject of a study by Trujillo et al. [44]. The molecular mass of major ewe milk proteins was determined by HPLC–ESI-MS (Table 1), whereas the molecular mass of some whey milk proteins which were not resolved by LC–ESI-MS were measured in ESI-MS under FIA conditions. In particular, after purification by fast protein LC (FPLC), β-lactoglobulin B, α-lactalbumin and serum albumin were resolved from purified protein solutions by FIA-ESI-MS. Under these conditions, a M_r 66 322.23±35.40 was determined for serum albumin by the FIA-ESI-MS spectrum (Fig. 3). Further, the HPLC–ESI-MS allowed to evidence the great heterogeneity of ovine milk proteins mainly due to

Table 1
M_r determination of major ewe milk proteins [44]

Proteins	Exp. M_r[a]	Calc. M_r
κ-CN 3P	19373	19373
α_{S2}-CN 10P	25616	25622
α_{S1}-CN C-8P	23411	23401
β-CN 5P	23750	23751
β-Lg A	18170	18171
β-Lg B	18148	18145
α-La A	14152	14158
Serum albumin	66322	66327

[a] The average of relative molecular mass (M_r) from five milk samples.

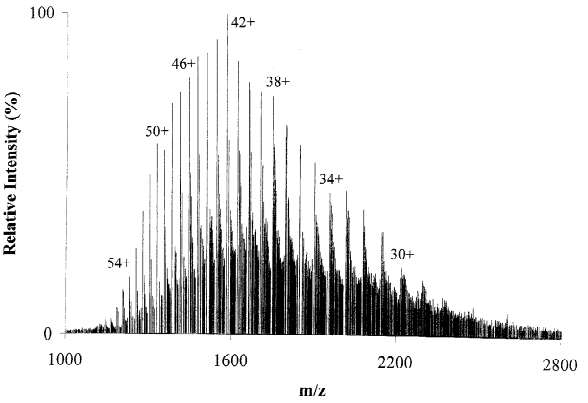

Fig. 3. Electrospray mass spectrum of isolated ovine serum albumin obtained by flow injection analysis. m/z, mass-to-charge ratio. Reprinted with permission from Ref. [44].

post-translational modifications, genetic polymorphism and the presence of multiple forms of proteins.

The power of accurate mass determination by using LC coupled to ESI ionization on a time-of-flight instrument has been proved by researchers of Nestlé Research Center in a problem of modified whey protein identification [45]. Whey proteins play an important role as ingredients in the food industry for their nutritional and functional properties, which are related to the native/denatured state of proteins [46]. On the other hand, if contribution of whey protein fraction to the quality of milk and dairy products is well established, knowledge about the relationship between structure and functional properties are of significance for the understanding of different industrial processing treatments. With the aim to characterizing the protein composition of a number of commercial whey samples, an analytical method based on the LC–ESI-TOF-MS technique was devised. A RP chromatographic method suitable both for separation of peptides and ESI-MS detection was developed on a PLRP-S column (150×4.6 mm) using a mobile phase made up of a 5% formic acid aqueous solution and a mixture of acetonitrile, water and formic acid in gradient mode. The use of formic acid instead of trifluoroacetic acid, which is normally used to separate peptides in ion-pair reversed-phase mode, allowed to improve both MS detectability and chromatographic behaviour. Higher resolution of the TOF analyzer provided very valuable results in terms

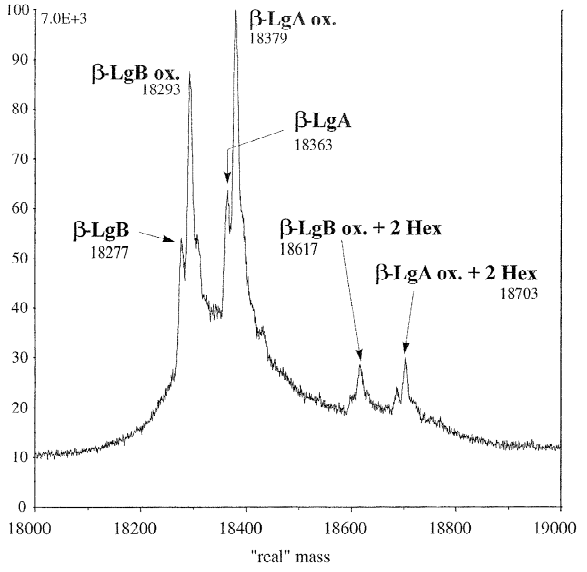

Fig. 4. Averaged and deconvoluted mass spectrum over the lactoglobulin region from the Lacprodan DI-9224 sample. The unmodified protein is present in minor quantities, while the most abundant peaks are attributed to oxidised proteins (+16 mass units) as well as to lactosylated/glycosylated and oxidised species. Reprinted with permission from Ref. [45].

of accuracy, which was better than 0.01% of the value calculated from the sequence, thus demonstrating superior performance of TOF instruments to any other technique used for molecular mass measurement of proteins. Besides unmodified α-lactalbumin, two genetic variants of β-lactoglobulin (A and B) and a number of modifications were characterized in almost all of the commercial whey samples under investigation. Lactosylation, oxidation and a combination of them were the major modifications identified by LC–ESI-MS, as illustrated in Fig. 4.

In contrast to the numerous reports of MS on milk proteins in the past, meat proteins have received much less attention [32]. In a recent report, ESI-CID-MS–MS proved helpful in analyzing myoglobins in different meat products [47]. ESI-MS was able to partially distinguish myoglobins from sheep, horse, beef and pork. In fact, the interpretation of ESI mass spectra was complicated by the presence of a series of natriated adducts of myoglobin, which made more difficult to differentiate myoglobins, particularly those for sheep (M_r 16 923) and beef (M_r 16 946) having a mass difference equivalent to that of sodium. A more successful approach was based on the collision-cell CID of myoglobins. Precursor ions for MS–MS experiments were thoroughly selected on the basis of the intensity in the ESI spectrum and a number of charges sufficiently high for partial fragmentation to occur. The ESI-MS–MS spectra from the four myoglobins were compared with the amino acid sequence of the intact protein. According to that reported by Loo et al. [48], fragmentation was found to occur at the amide bond of the proline residues. As reported in Table 2, valuable results obtained with ESI-MS–MS allowed to differentiate sheep and beef myoglobins from each other and from horse and pork. This example shows that ESI-MS–MS has great potential to provide supporting into elucidation studies involving biomolecules in real samples.

2.3. Carbohydrates

The knowledge of the qualitative and quantitative distribution of carbohydrates is of utmost interest in food chemistry. These compounds occur in a variety of food products and their composition is characteristic of quality, authenticity, ripeness, storage con-

Table 2
ESI-CID-MS–MS predicted and observed m/z values and number of charged sites for Y_{34}^{n+} fragment [47]

Species	Charged sites	Fragment Y_{34}^{5+}		Fragment Y_{34}^{5+}	
		Predicted m/z	Observed from $[M+17H]^{17+}$	Predicted m/z	Observed from $[M+17H]^{17+}$
Sheep	3	728.0	–	909.8	Yes
Beef	4	732.6	Yes	915.5	Yes
Horse	4	727.2	Yes	908.8	Yes
Pork	4	728.0	Yes	909.8	Yes

ditions, etc. As attested in a review paper, already in the past, MS techniques with different ionization modes and the hyphenated technique LC–MS have played an important role in the structure elucidation of sugars [6]. Among desorption techniques, FAB-MS and related techniques such as continuous flow-FAB and frit-FAB have contributed in the past to the mass determination of sugars [6]

In recent years, MALDI-TOF-MS has been demonstrated to be a powerful tool for the characterization of carbohydrates [49,50,52,54,55]. Coupling HPLC to isotope-ratio MS has been proved valuable for precise isotopic measurements for non-volatile species such as carbohydrates [60]. On the other hand, the combination of great sensitivity with high resolution of GC-based techniques make GC–MS suitable for studies on the characterization of oligosaccharides, as is attested by various reports of application of GC–MS in this field [62–68].

2.3.1. MALDI-TOF-MS, FAB-MS, LC–MS and CE–MS

MALDI has been successfully used by Wang et al. to develop a MS methodology for both qualitative and quantitative analysis of fructooligosaccharides in selected food samples [49]. In order to optimize the method, matrices, alkali–metal adducts, response intensity and sample preparation were examined individually. A series of experiments were carried out by the authors to study the analyte incorporation into matrix. The selection of matrix was based on a comparison of spot-to-spot or sample-to-sample repeatability and ability to reach a good quality spectrum with reasonable signal-to-noise ratio and the best resolution. In a first step of experiments, maltohexanose and γ-cyclodextrin were used as reference samples to verify the suitability of 2,5-dihydroxybenzoic acid (DHB), 3-aminoquinoline (3-AQ), 4-hydroxy-α-cyanocinnamic acid (HCCA), and 2,5-dihydroxybenzoic acid (DHB)–1-hydroxy-isoquinoline (HIC) (1:1) to be selected as the matrix. Table 3 illustrates the preparation of matrices and samples and performances of matrices. All these compounds, which were recommended as matrices for carbohydrate analysis using MALDI technique, presented some disadvantages. Good quality spectra and acceptable repeatability were achieved with DHB, whereas low repeatability was observed with 3-AQ, HCCA and DHB/HIC (Table 3). When using DHB, many matrix peaks were observed in the low mass region, which could interfere with low-molecular-mass analytes of interest, such the trisaccharide kestose with a mass of 504. Sharper peak and consequently better resolution compared to DHB matrix were achieved using 3-AQ. The authors achieved the best results using a 2,4,6-trihydroxy-acetophenone monohydrate (THAP) matrix which was first crystallized from acetone and then the aqueous sample was applied on the top of the formed crystals. The authors exploited the high solubility of THAP in acetone and the fast evaporation of the latter giving fine crystals and homogeneous incorporation of sample. Using this matrix, fructans from inulin with a degree of polymerization (DP) up to 55 corresponding to a mass of 9000 were resolved as sharp peaks (Fig. 5). Alkali–metal adducts, as well as laser strength were analyzed in detail.

Zeleny et al. reported the use of MALDI-TOF-MS to elucidate the primary structures of the N-linked oligosaccharides from tomato fruit, with the aim of giving structural information useful in immunological investigations regarding the involvement of glycans in food allergic reactions [50]. The study was carried out on two varieties of tomato fruit and both were investigated at two different stages of ripening. The low protein fraction was isolated from tomato fruit having high amount of free sugars and polysaccharides by low-temperature acetone powder method [51], which proved to be successful to remove salts and small hydrophobic compounds such as flavonoids and carotenoids. After peptic digestion, the glycopeptides were separated on an ion-exchange column and then deglycosylated by *N*-glycosylase A. The oligosaccharides were separated from peptide and residual glycopeptides by RP-chromatography and fluorescently labelled with 2-aminopyridine. Structural characterization was accomplished by means of two-dimensional HPLC in combination with exoglycosidase digestions and MALDI-TOF-MS, which was operating in the positive-ion linear mode. In the examined tomato varieties the same 16 *N*-glycosidic structures were detected, without any significant variation regarding the state of ripening. The authors of this work found that the two most abundant glycans showed identical properties to those of the major N-linked oligosaccharides of

Table 3
Performance of matrices for desorption and ionization of maltohexaose and γ-cyclodextrin [49]

Matrix	Matrix concentration	Preparation of matrix and sample	Spectra quality	Repeatability	Matrix peaks	Analyte molar ratio
2,5-Dihydroxybenzoic acid (DHB)	(1) 12.3 mg/ml in ethanol–water (1:1)	Mix matrix and sample together in a ratio of 1:1	Excellent	Good	Medium	Normal
	(2) 10.1 mg/ml in double deionized water	As above	Good	Good	Medium	Normal
2,4,6-Trihydroxyaceto-phenone mono-hydrate (THAP)	(1) Saturated in acetone	Put matrix on the probe first and then sample on top of matrix	Excellent	Excellent	Few	Normal
	(2) 12.5 mg/ml in acetonitrile–water (1:1)	Mix matrix and sample together in a ratio of 1:1	Excellent	Excellent	Few	Normal
3-Aminoquinoline (3-AQ)	10.1 mg/ml in 10% ethanol	As above	Good	Poor	Few	Normal
4-Hydroxy-α-cyano-cinnamic acid (HCCA)	13.3 mg/ml in 50% ethanol or matrix saturated in ethanol	As above	Good	Poor	Medium	Normal
Sinapinic acid	14.2 mg/ml in acetonitrile–water (1:1)	As above	No	No	Medium	
2-(4-Hydroxyphenylazo)-benzoic acid (HABA)	Saturated in acetone	Put matrix on the probe first and then sample on top of matrix	Poor	Good	Lots	Abnormal
2,5-Dihydroxybenzoic acid (DHB)/1-hydroxy-isoquinoline (HIC)	0.2 *M* DHB/0.6 *M* HIC in acetonitrile–water (1:1)	Mix matrix and sample together in a ratio of 1:1	Good	Poor	Few	Normal

horseradish peroxidase and both pineapple stem bromelain and barley peroxidase.

High-performance size-exclusion chromatography (HPSEC), high-performance anion-exchange chromatography (HPAEC) and MALDI-TOF-MS analyses were conducted by Vierhuis et al. to elucidate the structure of the xyloglucans and xylans present in olive fruit cell walls [52]. Hemicellulose-rich fractions obtained from olive fruit were fractionated by anion-exchange chromatography on a DEAE Sepharose fast flow column following a method previously described [53]. All fractions contained a xyloglucan-rich pool and four xylan-rich pools, which were submitted to analysis by HPSEC, HPAEC and MALDI-TOF-MS after degradation with specific enzymes. Furthermore, preparative HPAEC chromatography and MALDI-TOF-MS have been applied by the same group to purify and characterize the two most abundant oligosaccharides present in the endo-glucanase digest of xyloglucan [54].

The hyphenation of capillary electrophoresis (CE) with a mass spectrometric detector is a rapidly growing powerful analytical method. Moreover, the intrinsic high resolving power of CE is particularly suitable for the separation of carbohydrates which encompass a wide spectrum of compounds, many of which are isomers or slightly different from each other. Only few applications have been reported on CE–MS applied as analytical tool to determine carbohydrates of food interest.

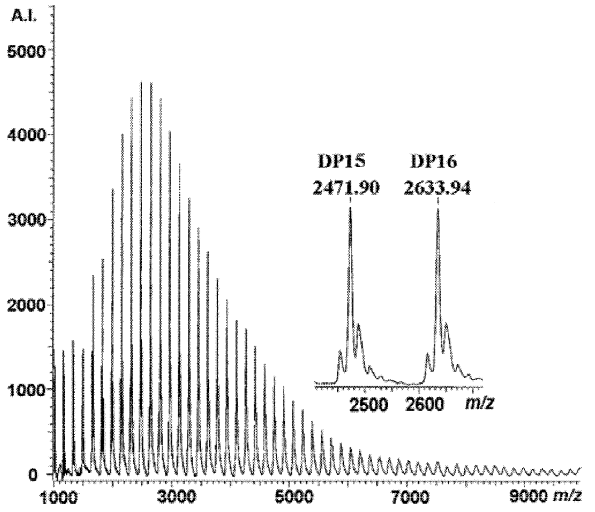

Fig. 5. MALDI-MS positive ion spectrum of inulin from Jerusalem artichokes. Inulin was dissolved in double deionized water to give a final concentration of 4 mg/ml. Saturated THAP (0.3-μl) in acetone was first placed on the probe and a 0.5-μl inulin sample was put on top of the crystallized matrix to dry. Sixty laser pulses at an attenuation of 22 were accumulated for the final spectrum. Reprinted with permission from Ref. [49].

Generally carbohydrates are separated by CE following two different approaches: the analytes may be separated in their underivatized form or after pre-column derivatization with an organic tag to enhance both separation and detection. Larsson et al. proposed an on-line CE method coupled with mass spectrometry detection for the analysis of carbohydrates after derivatization with 8-aminonaphthalene-1,2,3,-trisulfonic acid (ANTS) [55]. Derivatization was achieved to introduce negative charge to the analytes, enhancing both zone electrophoresis separation and UV detection. A tri-coaxial sheath-liquid configuration was selected to connect the CE system to a single quadrupole mass spectrometer. Electrolyte in the sheath-liquid was optimized in order to obtain high sensitivity detection in the ESI-MS negative ion mode. Low-pH conditions with isopropanol as the organic modifier were found to be stable and the most favourable in the CE–MS analysis of ANTS derivatized maize starch oligosaccharides on an in-house coated fused-silica capillary.

CE analysis of underivatized carbohydrates can be achieved using alkaline background electrolytes (BGEs), which allows the separation of carbohydrates as their oxyanions. Recently, Klampfl and Buchberger developed a CE–MS method for the determination of underivatized carbohydrates using strongly alkaline carrier electrolytes [56]. Optimization of BGE was performed with respect to its pH, ionic strength and the addition of an organic modifier. To maintain compatibility with ESI-MS detection, only volatile organic bases like diethylamine (DEA) were used in BGE composition. Changes in selectivity were observed adding acetonitrile to the BGE, whereas the effect of methanol was only a decreasing of the electroosmotic flow with the consequent increase of migration time of all analyzed sugars. ESI-MS sensitivity was influenced by composition and flow-rate of the sheath-liquid used. In any case it was observed that sensitivity was enhanced adding DEA to the sheath-liquid composition, explaining this behaviour by the increased pH which improved ionization of the carbohydrates in the ESI interface. The suitability of the proposed method was verified analyzing the carbohydrate profile of white and red wines. Carbohydrate content of sugars such as arabinose, ribose, xylose, galactose, glucose, fructose and the alditols inositol and mannitol were readily determined.

Other mass spectrometry techniques have been used to study structural aspects of some oligosaccharides. FAB-MS has been proposed for the characterization of a rare pentasaccharide isolated from the buffalo milk oligosaccharide fraction with the aim to analyzing buffalo milk for its oligosaccharide contents having immunostimulant activity [57]. Gel filtration chromatography and RP-HPLC were chosen to isolate this rare pentasaccharide. In particular, the oligosaccharides from buffalo milk were purified by gel filtration on a Sephadex G-25 column and the homogeneity of oligosaccharides eluted in two distinct fractions was confirmed by HPLC using a RP column under gradient elution. The oligosaccharides were then acetylated and separated over silica gel. After further treatment, the structure of the isolated pentasaccharide was assigned by ^{1}H NMR and FAB mass spectrometry. The FAB mass spectrum showed the highest ion peak at m/z 949 which was attributable to $[M+K]^{+}$. The other prominent peaks in the higher mass region were detected at m/z 933 and m/z 911, which were assigned to $[M+Na]^{+}$ and $[M+H]^{+}$ species, respectively. The mass spec-

trum confirmed the molecular formula, i.e., $C_{34}H_{58}N_2O_{26}$, obtained by elemental analysis and gave complete information to understand the characteristic fragmentation of the sugar moieties.

In the last 3 years surprisingly only few works have been published on food carbohydrate separations by LC–MS, and most published work has been concerned GC–MS applications.

HPLC–ESI-MS has been shown to be effective for reliable identification of two limonoid glucosides, which could be used as food additives [58]. After methanol extraction of the peel of *Citrus tangerina* (Tanaka) Tseng, obacunone glucoside (OG) and nomilin glucoside (NG) were separated on a RP column using a mixture of acetonitrile–water acidified with trifluoroacetic acid to avoid peak tailing of the two analytes. ESI-MS detection was performed in both positive-ion and negative-ion modes. Under PI conditions, natriated ions $[M+Na]^+$ were detected at very low intensity. In NI mode, $[M-H]^-$ ions were monitored as the most abundant signals, which were often detected as base peaks without further fragments; the dimer $[2M-H]^-$ was also detected. Both DAD and Total ion current chromatograms showed only two peaks, indicating the presence of limonoid glucosides without the necessity of isolating the individual compounds. The structures of OG and NG were assigned by ESI spectra and further confirmed by NMR.

Interfacing isotope-ratio mass spectrometry (IRMS) to HPLC has been demonstrated a powerful tool for analyzing very low alterations in ^{13}C abundance from carbohydrates as not volatile compounds requiring derivatization procedure. Coupling HPLC to IRMS was realized using a microwave-powered chemical reaction interface (CRI) [59]. In this type of coupling, the column effluent of the LC column is introduced into a microwave-powdered reaction cell where the molecular species are broken down to elements. Formation of small gaseous products which are characteristic of the atoms in the original molecule is obtained due to the continuous addition of a reactant gas to the microwave-induced plasma. Electron ionization (EI)-MS is then used to detect elements or isotopes in these products. This type of selective detection capability coupled with HPLC has been exploited in a recent study aimed to detecting possible adulteration of honey by corn syrup. NPLC using an amino polymer-based column was used to separate fructose, glucose and sucrose and coupled with a CRIMS system [60]. Carbohydrates in honey were eluted isocratically with a mobile phase containing water–acetonitrile (25:75, v/v), which was suitable for the mass spectrometer. Addition of 25% corn syrup to honey (HFCS) resulted in a remarkable alteration of the isotope ratio of honey, since high fructose corn syrup has a markedly different natural abundance of ^{13}C. $\delta^{13}C$ (‰) values were -0.06 ± 0.42 for fructose and 2.44 ± 0.72 glucose in honey added with 25% HFCS, whereas the corresponding values in honey were -2.93 ± 0.41 and -2.84 ± 0.51, respectively. These isotope ratios (means$\pm$SD, $n=6$ or 7 for each observation) were calculated as the differences between the observed values and an internal isotopic standard of rhamnose having a $\delta^{13}C$ of 23.95‰.

The potential of ^{13}C IRMS to detect fruit juice adulteration has been demonstrated in an inter-comparison trial [61]. In particular, the study involving 17 laboratories was performed in order to measure the repeatability (r) and reproducibility (R) of the ($^{13}C/^{12}C$) determination in fruit sugars.

2.3.2. GC–MS

Sugar analyses using GC–MS have been carried out after derivatization procedures, so that sugars have been detected as methyl, acetyl, TMS ethers and oxime-TMS ethers.

An analytical protocol involving GC–MS analysis of TMS or TMS-oxime derivatives of mono- and disaccharides in D-fructose, D-glucose and sucrose caramel has been proposed to prove the identity and authenticity of caramel, i.e., a product resulting from the controlled heat treatment of food-grade sugars for use as food additives [62]. Owing to the sequential oximation-trimethylsilylation followed by GC–MS assay of the resulting derivatives, the described methodology does not require preliminary fractionation of the caramel sample and is suitable for routine qualitative and quantitative analysis of major caramelization products. Structural differences in the disaccharide/pseudodisaccharide distribution of D-fructose, D-glucose and sucrose caramels suggested the use of non-reducing pseudodisaccharides, namely di-D-fructose dianhydrides (DFAs) (Fig. 6), as possible markers for caramel authenticity and identity.

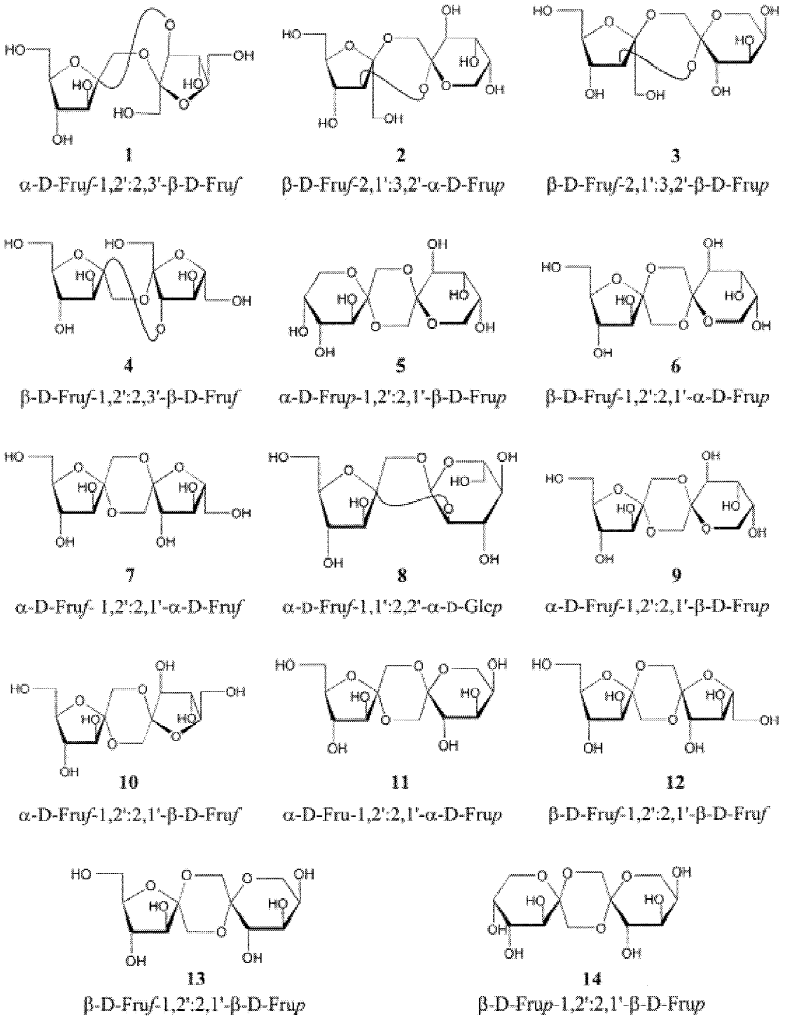

Fig. 6. Structural drawings for difructose dianhydrides found in fructose, glucose and sucrose caramels. Reprinted with permission from Ref. [62].

Trimethylsilyl methyl glycoside derivatives of red wine, apple, carrot and grape saccharides have been recently characterized by GC–MS [63]. Derivatization procedures were performed after methanolic–HCl treatment of plant polysaccharides. Information on glycoside-residue composition in the matrices under investigation was obtained by determination of the TMS methyl derivatives of usual sugars, such as pentoses, hexoses, 6-deoxy hexoses and uronic acids, together with unusual sugars, such as aceric acid, 2-keto-3-deoxy sugars (Dha and Kdo). In contrast with the alditol acetate procedure, which is time-consuming and requires large quantities of polysaccharides (100 μg), the method described is rapid, simple and useful for analyzing the neutral and acidic glycosyl-residue compositions of polysaccharides which are available in limited amounts (10 μg).

Changes in sugar composition of olive fruits have been studied by GC and GC–MS in order to optimize ripening and processing techniques [64]. TMS ethers of sugars from olive pulp were analyzed by GC–MS. Identification obtained by comparison of retention times with those of authentic substances

was confirmed by MS data. Qualitative and quantitative modifications depending on the variety and according to different industrial technologies were evidenced.

Analogously, modifications of sugars occurring during infant cereal production have been the object of a recent investigation [65]. Oxime trimethylsilyl ethers of carbohydrates were assayed in rice-based, wheat-based and oats infant cereal samples by GC and GC–MS.

An innovative approach based on a direct derivatization procedure of sugars performed in the presence of the matrix has been evaluated by Katona et al. [66]. Mono-, di- and trisaccharides, measured as TMS derivatives, were analyzed by GC–MS in apricot samples simultaneously to other constituents of the fruit, such as carboxylic acids, sugar alcohols and amino acids. Reliable determination was achieved without any pretreatment and without the use of internal standards in the 10^{-3}–≥40% concentration range. The final goal of the work was the study of the modifications in the sugar/sugar alcohol/carboxylic acid/amino acid composition of two apricot cultivars, as a function of their harvesting dates and storage conditions. Full scan and SIM acquisition modes were used in order to identify and quantitate, over the time, variation of glucose, fructose and raffinose concentrations due to sucrose and other saccharides hydrolysis. Mass spectrometric detection was also applied to better understand oligosaccharides structures.

Another application of GC–MS dealt with sugar composition related to brewing processes. With the aim to better understand and improve these processes, structures of non-starch polysaccharides, arabinoxylan and β-glucan in barley, malt and beer were elucidated by EIMS data [67]. Identification of partially *O*-methylated, partially *O*-acetylated alditols (PMAAs) derivatives allowed to determine sugar linkages revealing the presence of *t*-Glu-p, 4-Xyl-p, 4-Glu-p and 2,3,4-Xyl-p as major components and the existence of branched regions consisting of unsubstituted and double arabinofuransylated xylose residues.

A method based on HPAEC and on gas chromatography–mass spectrometry has been recently developed by Farine et al. for the separation and characterization of sugar oligomers deriving from enzymatic hydrolysis of sucrose (baker's yeast invertase) [68]. Differently linked fructoses and glucoses like 1-kestose, 6-β-fructofuranosylglucose, inulobiose, 6-kestose and neokestose contained in eluted fractions from CarboPac PA-100 preparative anion-exchange columns, were successfully identified by GC–MS analysis after suitable derivatization procedures. The unknown fructans collected from the preparative CarboPac PA-100 column, neutralized with HCl and then passed through a desalting column, were also submitted to ESI-MS analysis after permethylation. The authors evidenced the limited capability of single MS in giving information only about molecular mass of carbohydrates. In contrast, as reported in a recent investigation [69], ESI ionization and ion trap mass spectrometry having MS^n capabilities can provide information also on the stereochemistry of sugars useful for their complete structural elucidation.

2.4. Vitamins

Accurate determination of vitamins is very important in both food and pharmaceutical areas, but many HPLC-based methods using conventional detectors lack selectivity and/or sensitivity. The coupled technique LC–MS enables the separation of these non-volatile thermally labile substances for introduction into the mass spectrometer for reliable identification. Although the assay of vitamins is important in food analysis because of their important biological activity in humans, a few papers dealing with the LC–MS analysis of fat-soluble and water-soluble vitamins has been recently published. Nevertheless, the few studies on the LC–MS analysis of these natural compounds have revealed that this coupled technique has considerable potential in their characterization and determination [71,72]. In-source collision-induced dissociation experiments have been proved effective in achieving relevant structural elucidation of vitamins.

A study aimed to elucidating the metabolism of vitamin D_3 in plants and its regulation has been reported by Skliar et al. who applied electron ionization MS with magnetic sector analyzer for the identification of 7-dehydrocholesterol, vitamin D_3 and hydroxylated metabolites in the HPLC fractions of a plant species (*Nicotiana glauca*) [70]. The

presence of 7-dehydrocholesterol, vitamin D_3, 25(OH)-vitamin D_3 and 1α,25$(OH)_2$-vitamin D_3, the latter being a hormonally active D_3 metabolite, was established. This finding may lead to the use of plant culture systems to produce this biologically active compound. Even though this is not strictly an application of mass spectrometry in food-related analysis, an approach based on the study of structurally informative EI mass spectra may be successfully applied for characterization purposes of fat-soluble vitamins such as vitamin D_3 in food analysis as well.

2.4.1. LC–MS

Total folate content in food is traditionally determined by microbiological assay. When individual folate concentration is required, HPLC with UV or fluorescence detection is the most common analytical approach used to monitor the various forms of folate compounds. A method for the qualitative and quantitative analysis of the four main folate compounds using HPLC coupled with ESI ionization mass spectrometry has been devised by Stokes and Webb [71]. Separation of the analytes of interest was carried out under RP conditions. Mobile phase composition was optimized in order to obtain both good separation in a reasonable time and mass spectrometer compatibility. A mixture of 2.5 m*M* acetic acid–acetonitrile (88:12, v/v) was suitable to elute isocratically folic acid (PGA), 5-methyltetrahydropholic acid (5-MeTHF), folinic acid (CHOTHF), and tetrahydrofolic acid (H_4Folate) in less then 10 min. Acetic acid was added to the mobile phase to suppress the ionization of the folate compounds, and to enhance partitioning into the non-polar stationary phase. By operating in the negative mode with SIM acquisition, it was demonstrated the capability to detect simultaneously the four forms of folate compounds. Quantitation was performed using the abundance of the $[M–H]^-$ ion for each compound. The HPLC–ESI-MS method was applied to analyze food samples such as breakfast cereal and a concentrated beef and vegetable extract.

In a very recent paper, the potential of normal-phase and reversed-phase HPLC coupled to MS–MS detection for qualitative and quantitative analysis of tocopherols in food and phytopharmaceutical preparations has been demonstrated [72]. Under these optimized conditions, NP-HPLC and RP-HPLC were coupled to a quadrupole ion-trap mass spectrometer using an APCI interface. In-source collision-induced dissociation experiments were effective in achieving relevant structural elucidation of tocopherols, α-tocopheryl acetate and α-tocopheryl nicotinate in food (almond, peanut, spinach, spelt grain bran). By performing NP-HPLC–APCI-MS–MS was performed using on a silica narrow-bore column (100× 2.1 mm I.D.) using a mixture of isooctane-diisopropylether. Under the selected chromatographic and ionization conditions all investigated analytes were efficiently separated and mass spectra were characterized by protonated molecules $[M+H]^+$ and radical cations $[M]^+$. More sensitive detection was achieved by RP-HPLC–APCI-MS when chromatographic separation was on a C_{18} microbore column. In this case, the low flow-rate of the eluent methanol at 80 μl/min allowed more sensitive detection by more efficient ionization. Calibration, limit of detection and quantitation were also investigated. In particular, RP-HPLC–APCI-MS method enabled detection of 50 pg α-tocopherol, 1 ng β- and γ-tocopherol and 5.4 ng δ-tocopherol. The usefulness of this method should stimulate further research also for analysis of other fat-soluble vitamins.

2.5. Antioxidants: flavonoids, phenolics and related compounds

Polyphenolic compounds play an important role as natural potent antioxidants exhibiting various physiological and biological activities, such as anti-inflammatory, anti-allergic and anti-carcinogenic activities, in the human metabolism. Owing to recognized beneficial properties toward human health, identification of antioxidants, phenolic compounds and their degradation products has been regarded as an important target successfully reached by using HPLC–MS [73–79,81–83,87–95], MALDI-TOF-MS [84–86] and GC–MS [96–102,104,105]. In fact, since polyphenolic compounds are usually found as complex mixtures in plants, the composition of which changes according to the plant examined, hyphenated techniques are needed. Among these, LC–MS with different ionization modes represents a rapid and reliable technique to analyze these involatile substances. In some cases the coupled technique can

afford a full on-line structural analysis involving no time-consuming isolation process. Innovative results have been demonstrated for accurate mass determination of phenolics and related compounds using MALDI-TOF-MS.

2.5.1. MALDI-TOF-MS and LC-MS

The increasing interest in the characterization of phenolic compounds in food products has created new demands for the development of rapid, sensitive, and specific analytical methodologies for the identification and quantification of this class of chemical compounds in fresh and processed foods. As described in recent overview papers [73,74], over the past few years, various liquid chromatographic methods with UV–Vis absorption or DAD-UV, fluorescence and more recently with MS detection have been developed for the analysis of these naturally occurring antioxidants in foods. The authors evidence that using traditional approaches based on HPLC–DAD, UV spectra of phenolic compounds are often very similar and the possibility of unambiguous identification does not exist. With the introduction of bench-top instrumentation, mass spectrometry coupled to HPLC has evolved into a routine technique that enables collection of significant data on the structures of these compounds that show similar UV–Vis spectra [75].

Phenolic compounds in olive have been characterized by reversed-phase liquid chromatography using ESI-MS detection [76,77]. Extracts from several olive fruit samples were examined by LC–MS using ESI in the positive and negative ion modes to generate total ion current (TIC) chromatograms [76]. The elution of oleuropein, which was the major phenolic in olive fruit, was confirmed by the presence of very clean and distinct peaks in both the PI and NI mass chromatograms at m/z 541 and 539, respectively, with a sodium adduct at m/z 563 in the positive ion mass chromatogram. Compounds identified in olive fruit using the proposed LC–MS method included tyrosol, syringic, ferulic and homovanillic acids, quercetin-3-rhamnoside, oleuropein, ligstroside and isomers of verbascoside. With the aim to analyze phenolic compounds in olive fruit, the same authors performed semipreparative HPLC analyses to isolate the analytes of interest into distinct fractions and then the selected fractions were analyzed by LC–ESI-MS in both NI and PI modes [77]. Although positive and negative analysis were complementary, the latter showed better sensitivity and selectivity for the acidic and phenolic compounds. Structural information on oleuropein, which is the major phenolic compound in olive fruit, was achieved by tandem mass spectrometry which was performed on the m/z 539 and 541 ions in both negative and positive mode. Furthermore, the proposed method was able to allow the identification of isomers of verbascoside in olive fruit which had been not previously reported. More recently, the composition of simple phenolic compounds in olive oil residues has been evaluated by RP-HPLC analysis and the identity of phenolic compounds was confirmed by LC–MS equipped an ESI ionization source [78].

HPLC coupled with ESI-MS was investigated as a reliable method for analyzing wine polymeric tannins [79]. Separation was carried out on a narrow-bore RP column using a two-step linear gradient at a flow-rate of 200 μl/min. The apparatus coupled to the chromatographic system was a simple quadrupole mass spectrometer with a mass range of 2400 mass units. It was observed that the response of polyphenols (except for anthocyanins) was better in NI mode than in PI. Further, signal intensity decreased as the polymerization degree increased. Various series of ion peaks containing a variable number of trihydroxylated units were detected as monocharged ions $[M-H]^-$ from dimers to pentamers. The largest mass detected in the analyzed wine fraction corresponded to the mass of heptamers, which were found as doubly charged ions.

Both APCI and ESI interfacing systems have been explored for coupling with HPLC for determination of low-molecular-mass phenols and flavan-3-ols in wine [80]. Two different RPLC separation methods were optimized using APCI and ESI as the ion sources either in positive or negative ion mode. Data reported in this paper showed that ESI coupled with HPLC provided to be the method of choice for the analysis of low-molecular-mass phenols under NI mode, whereas flavan-3-ol compounds were well detected under both positive and negative ion modes.

Phenolic acids and aldehydes in red wine sample have been successfully analyzed by a capillary-scale particle beam interface [81]. This miniaturized sys-

tem combines high sensitivity with the advantages offered by EI/CI source [82]. The validation of the proposed method was based on the evaluation of instrument detection limits in the SIM mode, sensitivity, response linearity and intra-day precision. The chromatographic separation was performed under reversed-phase conditions using a 250-μm I.D. packed capillary column at an eluent flow-rate of 1 μl/min. Detection limits were in the low picogram range for most of the analyzed compounds. The proposed method was applied to detect 18 phenolic compounds in red wine samples.

HPLC–tandem mass spectrometry has been successfully applied to the quantitation of xanthohumol and five other prenylflavonoids in hops and beer [83]. After HPLC separation under reversed-phase conditions, prenylflavonoids were detected by APCI in PI mode. Quantitative MS–MS data were obtained by multiple-reaction monitoring using a triple–quadrupole mass spectrometer equipped with an APCI source. Attention was paid to accuracy and precision of the method, which were evaluated on spiked samples. A total of 13 commercial beers and two herb tea samples were assayed with the method.

Besides phenolic acids and aldehydes, phenolic compounds include flavonoids. This class of polyphenols have been found to be an important part of the human diet and have become an intense focus of research interest because of their perceived health-beneficial effects. Anthocyanidins are an important class of flavonoid compounds which are widely distributed in nature.

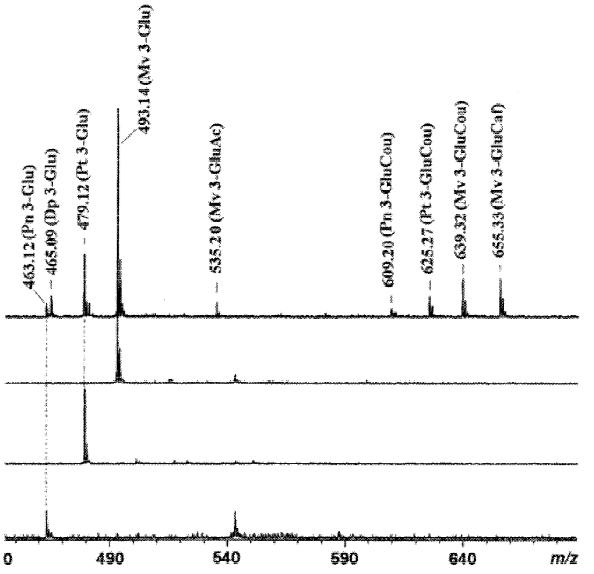

Fig. 7. MALDI-MS natural cation spectra of anthocyanins from preparative HPLC fractions. The spectra from top to bottom are from Zinfandel '98 red wine extracts, HPLC the third fraction (malvidin 3-glucoside), the first fraction (petunidin 3-glucoside), and the second fraction (peonidin 3-glucoside). Pn 3-Glu, peonidin 3-glucoside; Dp 3-Glu, delphinidin 3-glucoside; Pt 3-Glu, petunidin 3-glucoside; Mv 3-Glu, malvidin 3-glucoside; Mv 3-GluAc, malvidin 3-glucoside-acetate; Pn 3-GluCou, peonidin 3-glucoside-coumarate; Pt 3-GluCou, petunidin 3-glucoside-coumarate; Mv 3-GluCou, malvidin 3-glucoside-coumarate; Mv 3-GluCaf, malvidin 3-glucoside-caffeoate. Reprinted with permission of Ref. [84].

Considerable effort was put in polyphenol analysis by the group of Sporns [84–86]. The applicability of MALDI-MS for both qualitative and quantitative analysis of anthocyanins was first demonstrated in wine and fruit juice samples [84]. Anthocyanins under acidic conditions were predominantly in the aromatic oxonium ion form and easily ionized in MALDI-TOF-MS to form molecular cations $[M]^+$ in the positive ion mode. As illustrated in Fig. 7, three major anthocyanins were isolated as preparative HPLC fractions from a wine extract and then submitted to MALDI-TOF-MS analysis giving the correct masses for peonidin 3-glucoside, petunidin 3-glucoside and malvidin 3-glucoside. In this way the exact masses of various anthocyanins were measured in food and quantification was performed with the use of an appropriate internal standard. The same authors proposed the use of MALDI-TOF-MS to identify and study processing changes of isoflavones in soy products [85]. The matrix was selected in order to obtain good signal-to-noise ratios and spot-to-spot repeatability. Both THAP and DHB were found to be matrices producing good quality spectra of isoflavones in MALDI-TOF-MS. However, when DHB and THAP were used with crude isoflavone extracts, DHB exhibited better performance than THAP. By MALDI-TOF-MS, isoflavones exhibited only fragmentation corresponding to loss of their carbohydrate residues. The fragment ions, formed by glucosidic cleavage of isoflavones, provided characteristic information for structural elucidation. Following similar analytical procedures, a MALDI-

TOF-MS method was applied to identify flavonol glycosides in yellow onion and green tea [86].

Anthocyanins are the glycosidic form of anthocyanidins, which are widely used as natural colorants added to food products. Recently, the interest in these phenolic compounds has increased significantly due to their potential health benefits as antioxidants and antiinflammatory agents. In addition, anthocyanin composition of fruits and vegetables can be used as a fingerprint to monitor the authenticity of juices. A LC–MS method for the identification and quantification of individual anthocyanins in botanical raw materials used in the herbal supplement industry has been recently devised by Chandra et al. [87]. Anthocyanins from Balaton Tart cherry, elderberry, chokeberry and bilberry fruits were separated and identified on the basis of their respective $[M]^+$ ions using LC–ESI-MS. RPLC separations were performed eluting by a multi-step gradient with a mobile phase made up of 0.5% (v/v) aqueous phosphoric acid and a mixture of water–acetonitrile–glacial acetic acid–phosphoric acid (50:48.5:1.0:0.5, v/v). A low pH was required for maintaining the stability of anthocyanins in solution in the form of flavylium cation. The positive charge of anthocyanins at low pH values permits their easy detection using low voltages since other potentially interfering compounds are usually not ionized.

Many other applications of HPLC–ESI-MS for the determination of anthocyanins in fruit extracts have been recently reported [88–90].

The application of LC–MS with an ESI interface has been evaluated for the analysis of flavanones, flavones and flavonols [91]. Chromatographic separations were performed under reversed-phase conditions using a narrow-bore column and a flow-rate of 200 μl/min, which was compatible with the ESI interface used without post-column splitting. Flavonoid detection was performed by operating the mass spectrometer in NI mode. NI mass spectra of the analyzed flavonoids exhibited the $[M–H]^-$ ion as the base peak, allowing confirmation of the molecular mass. NI mass spectra of flavone and flavonol aglycones showed only one signal which corresponded to the deprotonated molecules. A valuable result was the feasibility of characterization of flavanone isomers differing in the glycosylation on the basis of different mass spectra. The applicability of the proposed analytical method was verified by identification and determination of flavonoids in an orange juice sample.

Naturally occurring flavonoid aglycones were separated by RP HPLC and analyzed on-line with ESI ion trap MS with the aim to obtaining structural information [92]. The selected chromatographic and spectrometric conditions permitted a good separation of 11 flavonoid aglycones (Fig. 8). The same figure (b–d) shows the LC–MS–MS spectra obtained from the $[M–H]^-$ ions for the three aglycones luteolin, quercetin, and isosakurametin. The negative ion mode was chosen to elucidate their mass spectrometric behaviour because it appeared more selective and more sensitive for further LC–MS analysis of flavonoids in plants. CID-MS–MS experiments caused the cleavage of the flavonoid molecules into a number of fragments according to strictly fixed pathways.

Recently, much attention has been focused on the protective biochemical function of naturally occurring antioxidants in biological systems and on the mechanisms of their action. Most phenolic compounds, which occur widely in plants, are present in red wines.

Some recent studies have determined polyphenolic compounds in wines principally using chromatographic techniques with UV spectrophotometric detection. More recently, LC–MS methods have been described to evaluate resveratrol content in wine [93,94]. A HPLC–MS method for the simultaneous determination of resveratrol in all its forms (free isomers and glycosylates) in fruit products and wine has been proposed by Wang et al. [95]. Samples were extracted and then analyzed by RP-HPLC. Positive APCI-MS was used for the detection and quantification of resveratrol without solvent spitting. The authors reported that a satisfactory ionization of resveratrol was achieved in both positive and negative ion APCI. In the PI and NI APCI mass spectra of resveratrol, protonated and deprotonated molecules formed the base peak at m/z 229 and m/z 227, respectively. The proposed method was validated in terms of linearity of the response factor, limit of quantitation, and recovery.

2.5.2. GC–MS

Analysis of antioxidants and phenolic compounds

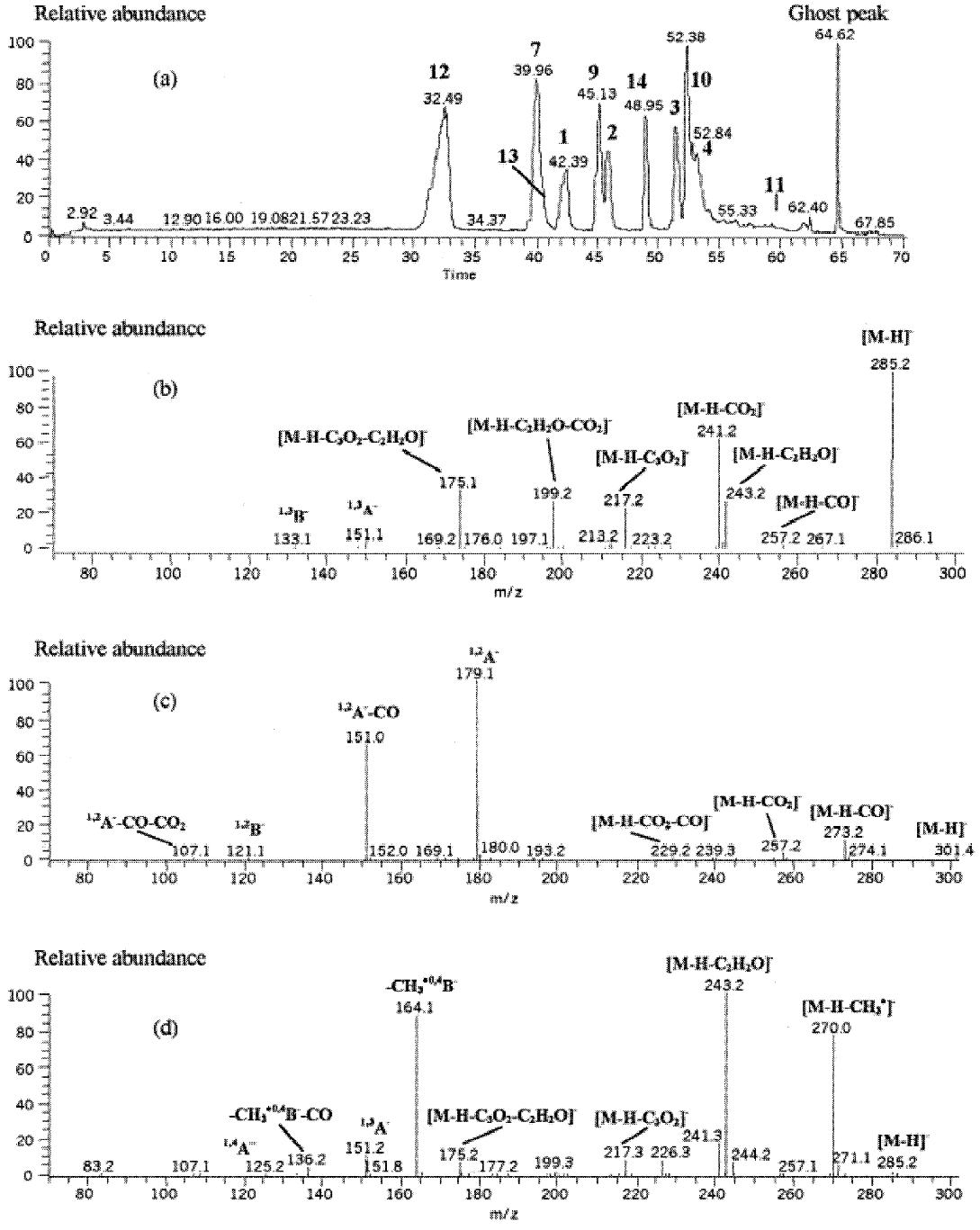

Fig. 8. LC-negative ion ESI MS total ion current of flavonoid aglycones (a). (1) luteolin, (2) apigenin, (3) genkwanin, (4) chrysin, (7) quercetin, (9) kaempferol, (10) galengin, (11) kaempferid, (12) eriodictyol, (13) naringenin, (14) isosakurametin. Negative ion ESI-MS–MS spectra obtained for luteolin (b), quercetin (c), and isosakurametin (d). Reprinted with permission of Ref. [92].

in food by means of traditional extraction techniques like steam distillation or Soxhlet extraction requires time-consuming and labour-intensive procedures as well as pre-concentration steps often resulting in the loss or degradation of target analytes. An interesting approach based on supercritical fluid extraction (SFE)-GC–MS has been developed by Scalia et al. for the analysis of different phenolic compounds including flavonoids in chamomile flowers [96]. Supercritical CO_2 at 90 atm and 40 °C (extraction time, 30 min) allowed 4.4 times higher extraction yields than those obtained by using conventional steam distillation for longer analysis times. By operating the mass spectrometer in the full scan mode, thermolabile compounds like matricina were detected in their natural form avoiding degradation processes usually occurring by applying conventional extraction methods.

Owing to the diffusion of antioxidants and preservatives in fatty foods, clean-up procedures before gas chromatographic analyses have been requested. A method based on continuous solid-phase extraction (SPE) system using XAD-2 cartridges, preceded by appropriate solvent extraction procedures, has been described by the group of Valcarcel to clean-up margarine, oil, fresh cheese, mayonnaise and pate sample extracts containing antioxidants [97]. GC–MS analyses of the final extract, containing only 0.03% of the total lipidic material were directly accomplished without prior sample derivatization. Good separation except for the overlapped peaks of 2,6-di-tert.-butyl-p-hydroxytoluene and ethyl p-hydroxy-benzoic acid was demonstrated. Analytical characteristics of the method were detection limits ranging from 0.8 to 2.0 μg/l, good linearity (verified over two orders of magnitude for each compound) and high precision with RSD%$<$4.2%.

GC–MS has been used to confirm identity of phenolic acid in a work aimed to study changes in phenolic acid content of winter oilseed rape leaves in relation to different growing conditions [98]. The results of a 28-days long study evidenced significant accumulations of phenolic acids (caffeic, p-coumaric, ferulic and sinapic) and marked promotions of phenolic esterification produced by freezing and cold acclimation.

Analytical methods for the characterization of phenolic compounds profile have been represented an useful tool also for forensic investigations in order to evaluate food authenticity [99–102]. In this context, phenolic acid and lignan content in cultivated mushrooms have been recently determined by developing a method based on enzymatic-, base- and acid hydrolysis followed by GC–MS in SIM mode [99]. No lignans and flavonoids were detected in the samples assayed. Cultivated mushrooms were characterized by very low levels of phenolic substances and higher amounts of vitamins, minerals and trace elements, which were quantitated by using mostly ICP-MS and HPLC methods.

Gas chromatographic separation of natural compound mixtures in food often requires time-consuming experiments in order to optimize temperature programme conditions. A new approach based on computer simulation of gas chromatograms for the determination of flavonoids in propolis has been devised by Maciejewicz et al. [100]. Computer-predicted operative conditions, based on the linear dependence of the logarithm of the retention factor on the reciprocal value of absolute temperature, allowed a perfect separation and a rapid determination of all the analytes directly injected into the gas chromatographic system without prior derivatization. Among the flavonoid compounds identified by GC–MS analyses (pinostrobin chalcone, pinocembrin, tectochrysin, galangin, 5-hydroxy-4′,7-dimethoxyflavone, apigenin), pilloin detection was observed in propolis samples for the first time.

A SPE method based on an anion-exchange mechanism followed by in vial elution and silylation has been applied for sampling a wide variety of acids and phenols in alcohol beverages at mg/l levels [101]. GC–MS analyses of the derivatized extracts showed that the method was characterized by good precision and long-term reproducibility with RSD values lower than 6 and 20%, respectively. Hydrolyzable tannins, competing for the exchange sites of the SPE cartridges, strongly influenced analyte recoveries. Mono acids, diacids, hydroxypolyacids, phenolic aldehydes and phenolic acids were detected and quantitated on the GC–MS extracted ion chromatograms using suitable target ions.

Very recently, GC–MS capabilities have been successfully explored by Marsilio et al. to study transformations of phenolic compounds in olives during californian-style processing [102]. Solvent

extraction and derivatization procedures with hexamethyldisilazane and trimethylchlorosilane were carried out before GC–MS analysis. Disappearance of vanillic acid and flavonoids coupled with an increase of oleuropein derivative aglycones (Fig. 9) were observed during brine storage. Mechanisms concerning browning of olives based both on phenols polymerization and on o-diphenols oxidation with o-quinone production followed by nucleophilic addition reactions of protein functional groups were proposed.

Isoflavonoids are polar phenolic compounds, the chemical structure of which is correlated both to their oestrogenic properties and to their antioxidant activity against peroxyl and hydroxyl radicals, with particular regard to prooxidant properties in the presence of Cu^{2+} [103]. Owing to negative health impact that phytooestrogens are able to cause, investigations of oestrogenic activity of different foods is an argument of great interest. In order to analyze isoflavonoids (equol, daidzein, genistein, lignan, enterodiolan and enterolactone) in different soya-based foodstuffs—i.e., soya flour, soya beans, coarse soya grits—by GC, a method based on the production of trimethylsilyl derivatives has been devised [104]. By operating the mass spectrometer in SIM mode, higher levels of genistein than other isoflavonoids were generally detected in the soya foodstuffs analyzed.

Beer oestrogen-active substances have been recently characterized by using both biological and chemical means [105]. Detection limits lower than those obtained by HPLC–DAD (5–1000 ng/l vs. 10 μg/l) and good recoveries, i.e., 80 and 90% for phytooestrogens and oestrogens, respectively, were the analytical parameters calculated by GC–MS. The mass spectrometer was operated in the selected ion monitoring mode and GC–MS determinations were performed after derivatization with bis-(trimethylsilyl)trifluoroacetamide.

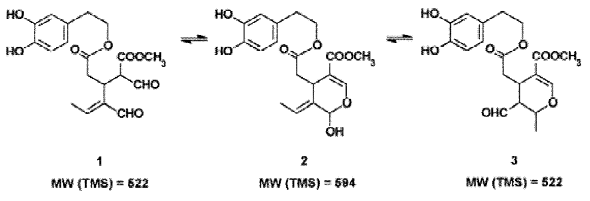

Fig. 9. Oleuropein aglycone interconversions and their molecular mass as trimethylsilylderivatives. Reprinted with permission from Ref. [102].

2.6. Aroma compounds

Gas chromatography coupled with mass spectrometry has considerable potential in the separation and characterization of food aroma compounds. As reported by Careri and Mangia in a recently published book [106], a great deal of information on flavour compounds has been obtained in the last year for various foodstuffs using GC–MS equipped with various analyzers. Generally the mass spectrometers designed for GC–MS analysis in flavour research use magnetic sector or quadrupole design. Ion trap mass spectrometers are also useful to obtain structure-specific fragmentation of volatile aroma compounds in the multiple-stage of MS–MS experiments. Generally, electron ionization is used for the characterization of the volatile fraction, chemical ionization being used mostly to confirm molecular mass of compounds.

The power of GC–MS in the characterization of volatile components is attested by various reports of application of this technique in food analysis in a period as short as that between 1999 and 2001 [107–131]. Other newly developed approaches for the analysis of aroma substances are also discussed [132–135]. Among innovative analytical methodologies in the research area of aroma compounds, API mass spectrometry-based approaches have been proved effective in characterizing volatile fractions of food and beverages. In this context, the potential of APCI-TOF-MS analysis has been also demonstrated for accurate mass measurements. Further, advances in MS in food concerns the application of a new approach based on lithium ion attachment mass spectrometry for the on-line continuous analysis of volatiles in air.

2.6.1. GC–MS

Combined with GC–MS analysis, a number of extraction techniques are used for the characterization of the aromatic profiles of different products such as solid-phase microextraction (SPME) [107,113,119,123,129], dynamic headspace and purge-and-trap techniques [108–110,117,118,121,

125,126,128], steam distillation [111,114–116, 124,127], solvent extraction [112,120] and solid-phase extraction [122,130,131].

A two-fiber SPME–GC–MS method for the analysis of volatile compounds in cooked pork has been reported [107]. Volatiles were extracted and desorbed into a silica retention gap cooled in solid carbon dioxide to allow cryofocusing of low-boiling components, and connected to the analytical column. SPME was carried out by using together two fibers coated with different stationary phases, i.e., 75 μm Carboxen-polydimethylsiloxane and 50/30 μm divinylbenzene-Carboxen on polydimethylsiloxane. Ninety-five substances were detected and identified, 36 of which for the first time in this food product. As illustrated in Fig. 10, different results were obtained using the two fibers. In fact, five compounds were absent and 10 were at trace level when using divinylbenzene-Carboxen on polydimethylsiloxane, whereas four compounds were not detected and one was at trace levels from the other fiber. An interesting feature of the two-fiber method was that the use of the two stationary phases simultaneously allows to achieve more information on the aroma of boiled pork than using either separately. In the chromatogram recorded using combined fibers (Fig. 10), only one out of 95 peaks was not detected and all of the peaks found at trace levels using separately the stationary phases were found at levels above trace detection.

Flavour was studied on a large number of virgin olive oils of good quality by Angerosa et al. [108]: sensory evaluation data and aroma compounds, produced through lipoxygenase pathways and extracted by dynamic headspace (HS), were correlated demonstrating the basic role of hexanal in the formation of most attributes. In a more recent study the same authors applied dynamic HS-GC–MS to study the effect of malaxation temperature and time on the quality of virgin olive oils [109]. Results revealed that changing in the aromatic profile of virgin olive oils could be influenced by operative conditions adopted during malaxation of olive pastes. Optimal malaxation conditions were found on the basis of studies regarding the modification of the concentrations of C_6 and C_5 compounds, extracted by a dynamic HS technique and identified by comparison of their mass spectra with those of authentic reference compounds.

Dynamic HS-GC–MS analysis of virgin olive oil samples has been also performed by Koprivnjak et al. in a study aimed to evaluating the influence of different methods of storage (air and aqueous media) on the oil aromatic profile [110]. Compounds (i.e.,

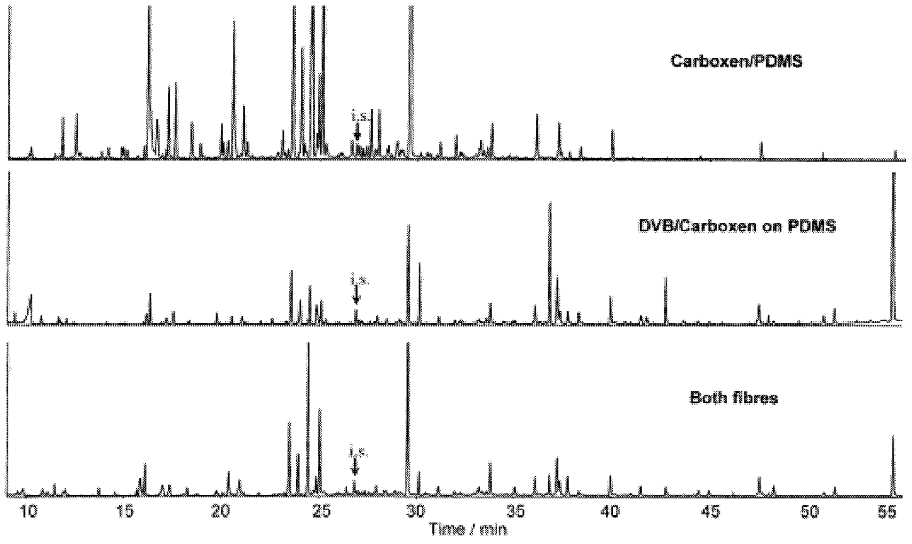

Fig. 10. Gas chromatographic traces of the aroma volatiles of cooked pork, using solid-phase microextraction with two different stationary phases, separately and combined. I.S., internal standard. Reprinted with permission from Ref. [107].

2-pentenal, hexanal, ethyl acetate and pentanal) responsible both of negative and positive odours were detected and identified by recording EI mass spectra. Storage of olives in aqueous media always revealed a prevalence of negative aroma volatiles; by contrast, storage in wooden boxes always showed a higher presence of positive compounds, even though modifications of their relative concentration occurred over the time.

GC–MS has been proved to be a powerful tool in the characterization of essential oils. Steam distillation technique has been investigated for the GC–MS analysis of essential oils of five Sideritis plants [111]. Ninety-nine compounds, mainly monoterpene hydrocarbons, were characterized in these samples by GC–MS in EI mode. Chemical ionization with methane as the reagent gas was then used to confirm molecular mass of each compound. In addition, structure elucidations were best provided by EIMS–MS analysis of selected GC eluting compounds using a GC-ITMS system.

Flavour analyses were also performed in order to obtain information about food processing by evaluating the possible modifications of sensory and nutritional quality during specific treatments or during the shelf-life of commercial samples. Lambert et al. compared the effect of both high pressure and sterilization treatment on the aromatic volatile composition of strawberry coulis [112]. By recording GC–MS chromatograms in the full scan mode, 46 compounds were identified showing that the alterations of the aromatic profile were caused by pressure treatments over 800 MPa or by sterilization (120 °C, 20 min) with the generation of volatiles like 3,4-dimethoxy 2-methyl furan, γ-lactone, vanillin and geraniol.

An approach based on the use of HS-SPME for the extraction of volatile compounds has been taken up with the aim of evaluating the influence of thermal treatments on tomato juice [113]. GC–MS was able to detect 191 aroma compounds, 102 of which identified by the mass spectra. The thermal treatment was found to modify mainly saturated and unsaturated C_6 alcohols and aldehydes, esters and ketones. The optimization of the blanching thermal treatment was then performed choosing several volatiles including pentanal, 2,4-hexadienal and 2-methylfuran as markers according to their contribution to the fresh or to the cooked flavour of tomato juice.

Knowledge of changing in the volatile fraction of cheeses under controlled ripening conditions was considered by some authors an aspect of technological relevance in order to afford a standardization of cheese manufacturing process. Using GC–MS, Rehaman et al. [114] and Mulet et al. [115] demonstrated that ripening was related to the perception of different aromas and that many volatiles characterizing the aromatic profile could allow a distinction among cheeses from different ripening times. In order to direct the biotechnical processes towards typical cheese aroma characters for the production of enzyme modified cheese flavours, other authors studied GC–MS profiles of extracts obtained by applying simultaneous steam distillation-extraction technique [116]. Characterization of Gouda cheese samples from different producers and of Emmenthal cheeses from different origins was followed by a statistical treatment based on principal component analysis, which allowed the interpretation of the complex semiquantitative data matrix and the classification of the different cheeses.

Flavour forming abilities of wild Lactococcus lactis strains compared to industrial cultures in cheese production have been also evaluated by GC–MS data [117]. Relatively high levels of unusual flavour compounds, namely methyl alcohols and methyl aldehydes most likely derived from branched-chain amino acids were extracted by purge-and-trap technique and identified by means of GC–MS. The good correlation found between GC–MS data and sensorial evaluation allowed to propose several wild strains promising starters for the development of new flavoured cheeses.

In a study aimed to characterizing cheese flavour for authenticity purposes, aroma substances were extracted and identified from Roncal Protected Denomination of Origin cheese by means of purge-and-trap-GC–MS [118]. Up to 68 volatile compounds in different Roncal cheeses were identified, revealing the existence of different productions among different lactation seasons.

The advantages of combined cryo-trapping/solid-phase microextraction (SPME)/GC–MS technique has been described for the analysis of Camembert cheese aroma [119]. Thirty-one compounds includ-

ing aldehydes, alcohols and ketones were identified by mass spectrometry and acceptable variations (C.V.$<$24%) were obtained by performing several replicated analyses. The method was described as simple, automated and fast.

Solid phase extraction has been evaluated for the analysis of polar flavour compounds at trace levels in butter [120]. SPE extracts obtained using a copolymer sorbent were submitted to GC-EIMS. Both full-scan acquisition and SIM were used for detection of flavour substances. The method was tested with respect to linearity, recovery and limit of detection in two different types of fresh butters using five polar model compounds. LODs values ranging from 100 pg to 1 ng and from 2 to 10 pg were calculated for diacetyl, furaneol, sotolon, maltol and vanillin under full scan and SIM conditions, respectively. Changes in the aromatic profile of heated butter were also observed with a 500–1000-fold increase of the analytes responses.

Purge-and-trap coupled to GC–MS has been proven to be effective in isolating volatile components derived from milk [121]. The evolution of flavour substances during shelf-life of whole and skimmed milk was investigated. A total of 40 volatiles were quantitated including methyl ketones with three, four, five and seven carbon atoms and furanic compounds produced by Maillard reactions and related to a decrease of the sensory quality also revealed by a panel test.

By means of GC–MS analyses, Vendramini and Trugo studied the variation of volatile components in acerola fruit in relation with different stages of maturation [122]. The chemical characterization of these volatiles allowed to control the maturation process both in the plant and during storage simply following the formation of typical compounds like ethyl acetate, (*Z*)-3-hexenyl acetate and 2-methyl propyl acetate.

Off-flavour chemistry has been also studied by means of gas chromatography–mass spectrometry. Generation of off-flavours like hexanal, dimethyl sulfide and acetone during heating of spice paprika powder was followed by Cremer et al. by means of static headspace GC and solid-phase microextraction coupled with GC–MS [123]. Changes in the concentration of 4-methoxy-2-methyl-2-butanethiol, responsible for the undesired blackcurrant-like odour in virgin olive oils, were determined by mass spectrometry [124]. A stable isotope dilution assay was carried out spiking the oil samples with labelled 4-methoxy-2-methyl-2-butanethiol. Spiked samples were submitted to multi-dimensional GC analysis for purification purposes and thiols were determined by GC–MS recording mass chromatograms in CI mode with methanol as the reagent gas.

In order to avoid the negative effects of microbiological activity on the quality of cork stoppers and consequently on the quality of wine, the effect of electron-beam irradiation treatment on cork volatiles has been studied at different doses (25, 100 and 1000 kGy) by Careri et al. [125]. A total of 70 volatile compounds including hydrocarbons, carbonyl compounds and alcohols were identified by means of dynamic HS-GC–MS both in non-irradiated and irradiated corks revealing significant quantitative differences starting from dose levels of 100 kGy. Qualitative and quantitative analysis of 2,4,6-trichloroanisole (TCA) breakdown products generated from electron beam irradiation treatment on TCA solutions at 25 and 50 kGy dose levels was also performed by the same authors [126]. Since mono- and dichloroanisoles, identified as the principal TCA degradation products does not significantly contribute to off-flavour appearance and TCA content considerably decreased, irradiation of cork contaminated by TCA was proposed as a valid alternative to the chemical sterilization based mainly on the use of chlorine or hydrogen peroxide.

Volatile compounds can be considered also as markers both for the characterization of botanical and geographical origin of foods and for the detection of illicit products. Floreal origin markers of heather (*Calluna vulgaris* and *Erica arborea*) honey have been found by Collin and co-workers by extracting the volatile compounds of 24 samples via dichloromethane solubilization followed by a Likens-Nickerson simultaneous steam-distillation/solvent extraction [127]. A total of 48 aroma substances were identified, but only phenylacetic acid, dehydrovomifoliol and 4-(3-oxo-1-butynyl)-3,5,5-trimethylcyclohexan-2-en-1-one were recognized as specific markers of *Calluna vulgaris*, whereas 4-methoxybenzaldehyde, 4-methoxybenzoic acid and methyl vanillate proved the *Erica arborea* floreal origin. Authenticity assessment of honey has been the object of

a very recent work dealing with the analysis of 43 honey samples of different botanical and geographical origin [128]. Volatiles were extracted by means of dynamic headspace technique and analyzed by GC–MS. Linear and branched aldehydes, ketones and short-chain alcohols were found in most or all of the honeys assayed. Analyzing GC–MS data from a qualitative point of view, it was possible to identify marker compounds typical for nine different botanical origins and for two geographical origins (Table 4). The study proved that aroma compounds play an important role in assessing the origin of honey.

A number of publications can be found in the field of alcoholic beverages in relation with the detection of illicit spirits. Aromatic profile of whiskeys has been studied for the purpose of identifying and authenticating a selection of Irish and Scotch whiskeys and of quantitatively determining the concentrations of volatiles in these samples [129]. A SPME method combined with GC–MS analysis was developed and optimized for the characterization of 17 congeners in whiskey samples including fuel alcohols, acetates and esters. Improved detectability for the determination of ester congeners was demonstrated, LODs values ranging from 0.1 mg/l (ethyl caproate) to 12 mg/l (methyl acetate). GC–MS has been further applied for the characterization of a number of reddish alcoholic beverages on the basis of aroma compounds as indicators [130]. This study was described to be useful for criminological purposes, since these beverages are often used to dissolve stimulant drugs.

Research on glycosidically bound volatile compounds present in fruit [131] and wine [132] have been recently performed. GC–MS proved helpful in identification of aglycones released by acid and enzymatic hydrolysis of heterosidic compounds in cupuacu, an amazonian fruit [131]. A total of 47

Table 4
Marker compounds typical for certain floral and geographical origins [128]

Botanical/geographical origin	Marker compound	
	Presence of	Absence of
Acacia	Both cis-linalool oxide and heptanal	Both phenylacetaldehyde and dimethyl disulphide
Chestnut	2-Methyldihydrofuranone or α-methylbenzyl alcohol or both 3-hexen-1-ol and dimethylstyrene	–
Eucalyptus	1-Octene or 2, 3-pentanedione or a cyclic ether or N.I.[a] (m/z 43, 55, 73, 114) or N.I.[a] (m/z 43, 71, 114) or N.I.[a] (m/z 43, 57, 85) or N.I.[a] (m/z 57, 69, 85)	–
Heather	N.I.[a] (m/z 70, 154) or N.I.[a] (m/z 67, 91, 110) or bicyclo-2,2,2-octan-1-ol-4-methyl or 4-ethylphenyl acetate	–
Lime	2 Methylfuran or N.I.[a] (m/z 91, 119) or α-terpinene or α-pinene oxide or α-terpene (m/z 91, 119, 134) or terpene (m/z 93,121,136) or bicyclo-3,2,1-octane-2,3 bis (methylene) or methyl isopropyl benzene or an aromatic hydrocarbon	3-Methyl-1-butanol
Lavander	Heptanal	4-Oxoisophorone
Rape	Dimethyl disulphide	2-Methyl-1-propanol
Rosemary	–	2-Acetylfuran
Sunflower	α-Pinene or 3-methyl-2-butanol	Both heptanal and 4-Oxoisophorone
Denmark	–	3-Methylbutanal
UK	1-Penten-3-ol	–

[a] N.I., not identified (characteristic fragment ions in parenthesis).

volatile substances encompassing aromatic compounds, aliphatic alcohols, oxygenated terpenic compounds, esters, acids and lactones were identified. Further, identification of methylated alditol acetates and trifluoroacetylated glycosides suggested that glucose was involved in glucosidic and glycosidic structures. The research carried out in wine [132] is not an application of GC–MS, so that it will be discussed below.

2.6.2. Other MS techniques

Recently, new approaches have been proposed in flavour-related studies. API mass spectrometry has been used to investigate carbohydrate-flavour conjugates in wine [132]. The aim of the study was to verify if glycosides could contribute to the flavour of wine during consumption. The hydrolysis of hexyl β-glucopyranoside in mouth was tested by placing a solution of the glucoside in the mouth and monitoring expired air from the nose for hexanol. Air was sampled from the nose of the subject through a custom-built interface and the process was studied by API-MS which was operated in positive ion mode to detect the $[M+H-H_2O]^+$ ion of hexanol (*m/z* 84.9).

A novel approach based on lithium ion attachment mass spectrometry has been successfully developed and applied for the on-line continuous analysis of volatiles in air by Fujii et al. [133]. Strawberries were chosen as a source of volatile compounds with the aim of applying the method also to food control. After headspace volatiles sampling, direct IAMS analysis was carried out. Mass spectral peaks, consisting of adducts formed by addition of Li^+ ions to sample molecules, were mainly identified on mass number allowing both the univocal detection of volatile compounds most of which were short-chain alcohols and the evaluation of the ripening by recording their signal variation over the time.

A paper dealing with a direct MS analysis has been recently published [134]. A mass spectrometer-based electronic nose was used to differentiate several grapefruit juice samples on the basis of a single volatile compound extracted by dynamic headspace. An ion trap mass spectrometer was transformed in a MS-sensor by connecting directly the sample vials to the mass spectrometer. The MS-based system was proved to be able to obtain signal from low-concentration compounds. In the field of aroma research, this is a worth mentioning result, since the instrument can determine the aroma-active substance instead of a compound present in higher concentration that correlates with the aroma-active substance.

In a valuable research, Taylor et al. investigated the applicability of the APCI-MS system for in vivo analysis of volatile flavour release [135]. An APCI interface and a quadrupole mass spectrometer allowed to ionize and detect volatiles from expired air from nose of people during eating. A schematic view of the device is depicted in Fig. 11. In order to assign ions to compounds unambiguously, an increase of sensitivity was achieved by optimizing the sampling cone voltages to obtain the highest ion intensity for the $[M+H]^+$ ions. Cone voltage modification, isotopic ratio analysis and subtraction of specific signals represented other helpful tools for confirmatory purposes. Detection limits ranging from 10 to 100 μg/l were calculated, whereas linearity was verified over three orders of magnitude for many compounds. Further, the authors demonstrated the potential of APCI interface connected to a TOF-MS instrument for accurate mass measurements in order to differentiate compounds with the same nominal mass but with different elemental compositions. Analyses of coffee headspace were carried out for this purpose. In the spectrum a peak around *m/z* 155 was identified which appeared to contain two compounds. By varying the cone voltage and using *p*-cymene as lock ion, accurate mass measurements were obtained and two peaks were clearly distinguished allowing to determine the contribution of different compounds to a specific ion intensity (Fig. 12).

The examples reported demonstrate that these MS approaches alternative to GC–MS analysis offer an

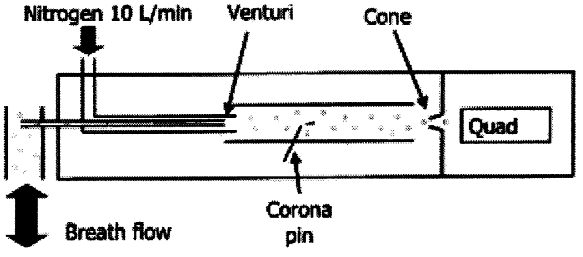

Fig. 11. Schematic diagram of the API source. Reprinted with permission from Ref. [135].

Fig. 12. (a) Time of flight mass spectrometry of coffee headspace. Each of the three traces shows the spectrum at a different cone voltage, demonstrating that there are two compounds around m/z 155; (b) time of flight mass spectrometry of coffee headspace. Using p-cymene as lock ion, accurate mass data for the two components around m/z 155 can be obtained. Each trace was obtained at a different cone voltage. Reprinted with permission from Ref. [135].

important advance in analytical methodology in the research area of flavour compounds, even though some of these, particularly lithium ion attachment mass spectrometry, necessitates further studies and validation of the methodology.

3. Xenobiotic substances in food

3.1. Pesticides

Pesticides are of interest in food analysis because of their widespread use in a variety of crops, such as fruits and vegetables, for field- and post-harvest protection. Pesticides and their degradation products could be of potential health hazards because of their toxicity or carcinogenicity. Consequently, strict regulation of maximum residue limits (MRLs) has been set for most food products.

The analysis of pesticides poses special problems for the chemist, since the pesticides belong to different groups of chemical substances having a broad range of polarity and acidic characteristics. Most pesticides are volatile and thermally stable, and therefore are amenable to gas chromatography in combination with element-specific detectors or with mass spectrometry. In contrast to GC, liquid chromatographic methods have the advantage of being suitable for thermally unstable and polar/ionic pesticides, as these compounds require derivatization prior to GC analysis.

In order to supervise MRLs, multiresidue methods

Table 5
GC–MS monitoring parameters for pesticides analyzed [159]

Compound	Retention time (min)	Target ion (m/z)	Qualifier ions (m/z)
Dicloran	12.70	206	176, 208
Dimethoate	12.85	87	93, 125
Diazinon	13.50	179	199, 304
Chlorpyrifos-methyl	14.42	286	125, 288
Vinclozolin	14.51	285	212, 287
Carbaryl	14.79	144	115, 116
Methiocarb	15.09	168	109, 153
Dichlofluanid	15.19	123	224, 226
Parathion-ethyl	15.52	109	139, 291
Triadimefon	15.59	208	210, 293
Procymidone	16.39	96	283, 285
Myclobutanil	17.40	179	181, 288
Iprodione	19.52	314	187, 316
Imidan	19.64	160	161, 317
Dicofol	19.89	139	111, 1414
Phosalone	20.31	182	184, 367
Azinphos-methyl	20.38	160	77, 132

traction followed by liquid desorption (LD) and analysis by LC–APCI-MS. Analytical results were comparable with those obtained using the stir bar sorptive extraction-TD-GC–MS technique. The negative APCI mass spectra of vinclozolin and procimydone were characterized by fragment ion at $[M+CH_3OH-H]^-$, whereas iprodione gave the $[M-CONHCH(CH_3)_2]^-$ ion, confirming its thermolabile character because of the degradation under CI conditions.

The capabilities of ASE with water as extraction solvent followed by sorptive enrichment techniques (SPME and stir bar sorptive extraction) and subsequent TD-GC–MS have been explored by Wennrich et al. with the aim to analyze organochlorine pesticides and chlorobenzenes in fruit and vegetable samples [161]. The ASE-SPME and ASE stir bar sorptive extraction procedures were developed and optimized for strawberry, but they could be applied successfully to other fruits and vegetables such as apples, lettuce and tomatoes. Detection limits in the 0.5–6-μg/kg range with the exception of the DDX compounds (detection limits up to 40 μg/kg in strawberry) were calculated for the ASE-SPME–GC–MS technique. In general, higher detectability was claimed when applying the ASE stir bar sorptive extraction procedure. The methods developed were applied to the assay of these pesticides in fruits and vegetables taken from a potentially contaminated area in East Germany.

An extensive study reporting the ‘‘ultimate’’ analysis scheme for pesticide residue screening analysis which has evolved over the last 20 years in author’s laboratory has been performed by Stan [162]. The method was based on a modified miniaturized DFG S19 extraction method applying acetone for the extraction followed by a further liquid–liquid extraction step with ethyl acetate–cyclohexane and gel permeation chromatography. Three parallel operating gas chromatographic systems using effluent splitting to ECD, NPD and MS were used for the chromatographic determination. More than 400 pesticides could be monitored and identified mainly by full scan GC–MS down to the 0.01 mg/l concentration level in plant foodstuffs.

Determination of pesticide residues in food is often complicated by the presence of fats and requires time-consuming clean up and preconcentration procedures before analysis. Off-line coupling of SFE and ASE-GC–MS for the determination of pesticides in a large number of baby food and adult duplicate-diet food samples were evaluated by Chuang et al. [163]. Atrazine, carbofuran, chlorpyrifos and metolachlor were the pesticides evaluated by SFE-GC–MS; chlorpyrifos, malathion, 4,4′-DDE, 4,4′-DDD and 4,4′-DDT were analyzed by ASE-GC–MS. ASE extracts were also submitted to enzyme-linked immunosorbent assay (ELISA) to assay chlorpyrifos; previous study had reported a comparison of conventional analytical methods (i.e., GC–MS) for the analysis of pesticides with immunochemical methods like ELISA [164]. The ASE-GC–MS technique provided satisfactory recoveries of the target pesticides, in particular by selecting acetonitrile as ASE-solvent and ENVI-Carb SPE columns under different eluting conditions for the clean up procedure. Good agreement was also obtained between the ASE-ELISA and ASE-GC–MS methods for the recovery of chlorpyrifos both in the spiked baby food and in the adult duplicate-diet food samples, whereas SFE-GC–MS technique showed always recoveries lower than 25%.

Very recently, a SPME–GC-ITD-MS method for the rapid screening of several pesticide residues in honey has been devised, the approach being developed independently by two public control lab-

oratories [165]: the extraction conditions were optimised by evaluating the exposure time of the fiber and the addition of concentrated ammonia (30%) to the aqueous solution showing that the determination of cymiazole and coumaphos was possible only with the ammonia addition. Quality parameters of the method such as precision, accuracy, linearity, limits of detection and limits of quantitation are presented.

GC–MS with selected ion monitoring has been investigated as a simple and highly sensitive method for the rapid screening of organophosphate pesticides (dichlorphos, heptenophos, dimethoate, fonofos, etrimfos and fenthion) in different food items such as baby foods, soft drink and instant soups [166]. Sample treatment was based on a simple one-step extraction procedure with organic solvent followed by direct analysis by GC–MS without prior derivatization. The procedure was demonstrated to be an useful tool for forensic investigations.

An approach based on direct sample introduction technique (DSI) coupled with GC–ITD-MS–MS has been taken by Lehotay et al. for the analysis of 43 pesticides in a fatty matrix such as eggs [167]. No sample clean up or solvent evaporation steps were required; acetonitrile was chosen as the most selective extraction solvent among those tested, being able to minimize the co-extraction of interferences like lipids, proteins and carbohydrates. A large volume injection was performed by analyzing 10 μl of the extract; the analytes were then monitored with 23 MS–MS segments, obtaining detection limit values lower than 10 ng/g for 25 of 43 pesticides. In a subsequent study, the same author and his coworkers reported a method for the fast analysis of 20 pesticide residues in carrots based on low-pressure (LP) GC–MS [168]. Sub-atmospheric pressure conditions occurred throughout the 10-m×0.53-mm I.D., 1 μm film thickness column, coupled with a 3-m×0.15-mm I.D restriction capillary at the inlet end, whereas a common quadrupole mass spectrometer operated in SIM mode was used for the analysis. Larger volumes than those obtained with a traditional GC–MS system could be injected; detection limits of pesticides like thiabendazole, acephate or methamidophos resulted to be improved because of the better chromatographic peak shape normally tailed using conventional GC–MS systems (Fig. 14). For comparison purposes, Fig. 14 shows also peak shape of procymidone as an example of non-problematic pesticide. Responses of thermally stable compounds were similar using both the traditional GC–MS and LP-GC–MS systems, whereas thermolabile

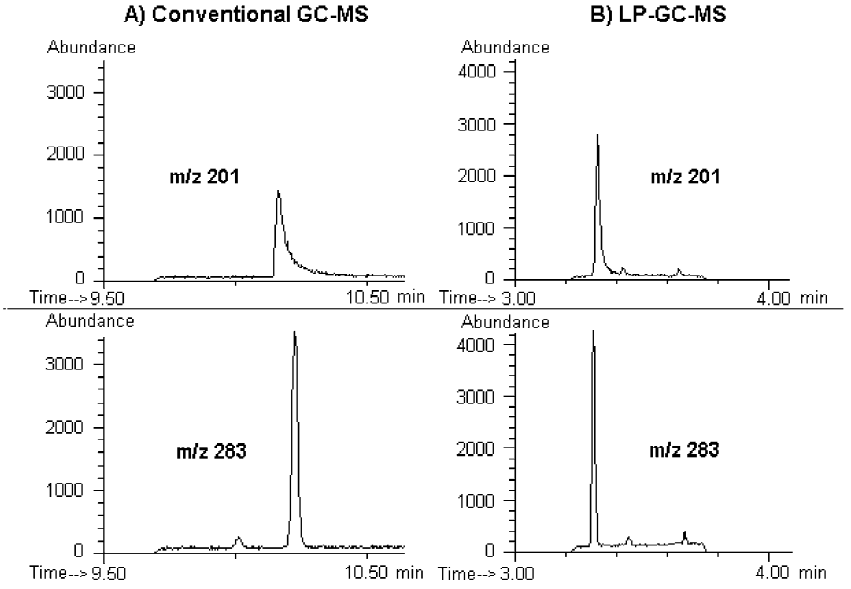

Fig. 14. Comparison of peak shapes of thiabendazole (m/z 201) and procymidone (m/z 283) obtained by (A) conventional GC–MS and (B) LP-GC–MS (1-μl injection). Reprinted with permission from Ref. [168].

compounds such as carbamates, carbaryl and methiocarb were better detected by applying the innovative technique due to the more rapid time of analysis (lower time spent in the liner and in the column).

More recently, GC–MS has been successfully applied to the detection of chlordimeform and its degradation products in honey samples after conventional solvent extraction [169]. Detection limits obtained in electron impact ionization were 1 mg/l in scan mode and 20 μg/l in SIM mode. Positive (PCI) and negative (NCI) chemical ionization was performed by using methane as the reagent gas; under SIM conditions, NCI experiments proved helpful in obtaining detection limit as low as 1 μg/l. Two chlordimeform degradation products were identified in honey by means of GC-EIMS spectra and their structures were confirmed by recording PCI spectra.

The above applications show that automated and innovative sample pretreatment techniques are of utmost importance in analytical GC–MS methods for the accurate determination of pesticide residue in complex matrices such as foodstuffs.

3.2. Drug residues

A wide range of licensed veterinary medicines is administered to food-producing animals for the purposes of treatment and prevention of disease and to promote growth. The administration of any pharmacologically active chemical to a food-producing animal inevitably leads to the occurrence of residues in food. Owing to the widespread use of drugs in veterinary medicine as therapeutic or growth-promoting agents, the development of analytical methods for the determination of drug residues in meat and other animal by-products (milk and egg) has become an important task of toxicological and regulatory concern. The use of these substances is regulated because of the concerns about their possible effects on human health [170–172]. In recent years, LC–MS-based strategies have been widely applied for confirmation of veterinary drug residues [173,174,176–186,188–190]. In particular, advances lie in the combination of novel sample treatment procedures, e.g., matrix solid-phase dispersion (MSPD) coupled with selective molecularly imprinted solid-phase extraction (MISPE), with HPLC–MS. Further, ion trap multiple-stage mass spectrometry as a powerful tool for confirmation of drug residues and approaches based on the analysis of incurred samples for accurate measurements of drug residues in food are worth of mentioning.

Concerning GC–MS, MS methods preceded by gas chromatographic separation are less diffuse owing to the need of extensive clean up and time-consuming derivatization procedures prior to capillary GC [192,194–196].

3.2.1. LC–MS

The illegal use of β-agonists as growth promoting substances in cattle raised for human consumption underlines the necessity of developing proper control systems which include highly sensitive techniques and adequate sampling procedures. The European Union Commission has adopted a maximum residue limit of 0.05 μg/kg for the residues of β-agonists [170]. A rapid method for the identification and determination of four β-agonists based on the use of packed-column supercritical fluid chromatography (pSFC) coupled with APCI detection has been developed [173]. It is to be noticed that SFC technique was applied to the separation of β-agonists for the first time in this work. pSFC can provide faster analysis times than HPLC with good resolution and without the need for derivatization as in the case of GC. The authors demonstrated that the pSFC-APCI-MS hyphenated system was a promising approach for the rapid separation and identification of these analytes. Separation of clenbuterol, salbutamol, terbutaline and fenoterol was performed using a cyano column maintained at temperature of 50 °C and eluting with a mobile phase made up of CO_2 modified with methanol containing trifluoracetic acid and triethylamine as additional additives. β-Agonist detection was performed by operating the mass spectrometer in PI mode. The APCI mass spectra gave the $[M+H]^+$ ion of clenbuterol at m/z 277, terbutaline at m/z 226, salbutamol at m/z 240 and fenoterol at m/z 304. More characteristic fragmentations were obtained increasing sampling cone voltage. To verify the optimized method milk samples fortified with β-agonists were assayed. Results showed that the matrix caused no interference, detection limits in SIM mode being approximately

500 μg/l in the milk sample. The method showed good intra- and between-day precision.

To support regulatory action a method has to be able to identify a drug with high specificity. An interesting approach based on the use of an innovative sample pretreatment technique and ESI-MS–MS detection has been applied by Crescenzi et al. to overcome the problems imposed by tedious and time-consuming procedures and obtain unambiguous identification of drugs [174]. The suitability of combining MSPD with MISPE followed by HPLC coupled with ESI IT multiple-stage mass spectrometry has been investigated to identify the β-agonist clenbuterol in bovine liver. MSPD is a new extraction technique suitable for solid samples that combines homogenization, analyte extraction and purification in one step [175]. Tandem extraction by coupling MSPD cartridge with MISPE cartridge containing a molecularly imprinted polymer allowed selective adsorption of the analyte. The so-purified extract was directly analyzed by LC–ESI-IT-MS. Detection limit of the method was <0.1 μg/kg, thus proving to fulfill EU criteria [170]. Further, on-line LC–IT-MS^n was effective in achieving relevant structural elucidation for selective detection of clenbuterol.

Horie and Nakazawa developed a selective and sensitive HPLC–ESI-MS method for the analysis of residues of growth promoters, zeranol (ZER) and trenbolone acetate (TBA), in bovine muscle and liver [176]. MRLs for hormonal anabolic hormones were established in the Codex Alimentarius [171]. ZER, TBA and its metabolites 17α-trenbolone (α-TBOH) and 17β-trenbolone (β-TBOH) were separated by HPLC under RP conditions and detected by ESI-MS. ESI in negative ion mode was particularly suitable to ionize the weak acidic compound ZER, whereas positive ionization produced typical $[M+H]^+$ molecular ion of α-TBOH and β-TBOH. Detection limits were estimated 0.5 ng/g in meat sample for these hormones, making the method suitable to determine the recommended MRLs in the foods under investigation.

Residues of the nitroimidazole, dimetridazole (DMZ), which is used to control protozoal infections in poultry, have been determined in poultry meat by a LC–MS method [177]. DMZ and its metabolites were separated under isocratic conditions using a reversed-phase C_{18} column. The HPLC was coupled to a quadrupole mass spectrometer with an ESI interface. DMZ and all the separated metabolites were detected in PI mode, and the full scan spectra exhibited an intense protonated molecule $[M+H]^+$ for each compound. Furthermore, the acetonitrile adduct $[M+CH_3CN+H]^+$ was observed for each compound. The method was validated in terms of linearity, intra-day and inter-day repeatability, accuracy and detection limit. It was capable to reliably measure DMZ residues in muscle at levels below 5 μg/kg.

A selective LC–APCI-MS–MS method for the measurement of anabolic hormone residues and their metabolites in bovine biological samples has been developed by Draisci et al. [178]. The formation of the protonated molecule, $[M+H]^+$, of 17β-19-nortestosterone, 17β-testosterone and progesterone and their major metabolites, was observed with negligible fragmentation. Tandem mass spectrometry was used in order to obtain additional structural information by detecting diagnostic product ions by CID of the precursor ion. The proposed analytical method is particularly valuable for routine control of the illegal use of anabolic hormones and their potentially toxic metabolites in cattle production.

Antibiotics are the most important bioactive and chemotherapeutic compounds made by microbiological synthesis. Structures of antibiotics vary widely, so that they have been classified in many groups. Furthermore, their origin and instability leads to a situation in which small amounts of structurally related compounds and by-products may be present together.

Tetracycline antibiotics (TCs) are widely used all over the world as veterinary medicines and feed additives. HPLC and hyphenated HPLC techniques have been commonly used for the determination of TC residues in food, as recently reported in two exhaustive reviews [179,180]. In these review papers recent developments in MS analysis of TCs in foods are discussed with emphasis on advantages and limitations of each MS technique. Methods combining a simple chromatographic separation with an appropriate mass spectrometric technique are described to provide a significant advantage for reliable confirmation of the residual TCs with high sensitivity and selectivity. LC–MS appears to best suited for

this purpose, even though LC conditions in terms of the mobile phases required for the separation of these antibiotics are not amenable to the LC–MS interfacing systems [180]. In this context, the results recently obtained by Nakazawa et al. are noteworthy, since they demonstrated that an eluent containing involatile compounds can be applied to HPLC–APCI-MS without causing clogging at the interface [181]. HPLC–APCI-MS–MS was applied to the simultaneous determination of residual TCs in food of animal origin [181]. After a RP chromatography, using a C_8 column and a mobile phase containing oxalic acid, which provided the best separation between TCs and co-eluting substances, APCI tandem MS detection was performed. Operating the APCI interface at a temperature of 475 °C, clogging of the interface with oxalic acid of the LC eluent was not observed, since oxalic acid decomposes to carbon dioxide and water at high-temperature. TC antibiotics, such as oxytetracycline (OTC), chlortetracycline (CTC), and doxycycline (DC) were determined in various food items including animal tissues, honey, milk, eggs and fish. The recovery of antibiotics from these samples fortified at 0.05, 0.10, and 0.50 ppm ranged from about 60 to 89% with a relative standard deviation of 1.2–8.7%.

Sulphonamides (SAs), N-derivatives of 4-aminobenzenesulphonamide, comprise a large group of synthetic antibacterial compounds. A method for the determination of residual sulphonamide antibacterials in animal liver and kidney has been developed using HPLC with ESI mass spectrometric detection after a clean-up procedure using a ion-exchange cartridge [182]. Ten different kinds of SAs were separated using a C_{18} column, eluting with a mixture of acetonitrile, methanol and aqueous formic acid which was applicable with ESI-MS–MS detection without any modification. To investigate the applicability of the method to LC–MS–MS analysis, a swine kidney sample and a bovine kidney sample were analyzed, both of which were previously found to be positive for SAs by microbiological assay. Operating in PI mode, product ion scan provided $[M+H]^+$ ions at m/z 279 and m/z 282 corresponding to sulfadimidine (SDD) and to sulfamonomethoxine (SMMX), respectively. The MS–MS spectra confirmed both the identity of SDD and of SMMX.

ESI tandem MS has been applied for the quantitative determination of a number of sulfonamides in meat [183]. Two adduct ions, $[M+H]^+$ and $[M+Na]^+$, were the major ions detected in PI mode (Fig. 15). Detection limits in the 0.02–0.05-μg/ml range were claimed, even though determined by analysis of standard solutions. SAs were isolated from some pork, beef and chicken samples using a solvent extraction procedure and then determined by LC–MS–MS.

Gentamicin and neomycin belong to aminoglycosides, a family of broad spectrum antibiotics widely used to treat infections in dairy cattle. Their occurrence in bovine meat and by-products such as milk makes necessary the development of reliable confirmation methods for their residues in order to provide safe products for consumers. Heller et al. developed a LC–MS–MS method for the confirmation of aminoglycosides gentamicin and neomycin in milk [184]. The two aminoglycosides were extracted from milk by a weak cation-exchange extraction procedure. The extracted analytes were separated by ion-pair HPLC and detected by ESI-tandem-MS on a benchtop IT mass spectrometer. Protonated molecules were selectively stored in the IT-MS and then collisionally dissociated to yield diagnostic product ion spectra.

Antibiotics of the β-lactam group are represented by several classes of compounds, among which the cephalosporins and the penicillins are the most important. The intense use of these drugs has led to problems with residues in the livestock products and to an increasingly potential risk for human health. European Union has established MRLs of 50 μg/kg for ampicillin, benzylpenicillin and amoxicillin in food-producing animal muscle [172]. Recently, methods based on the use of LC–ESI-MS [185] and LC–ESI-MS–MS [186] have been devised with the aim to determining ampicillin residues in muscle tissues and β-lactam antibiotics in bovine milk, respectively. Incurred muscle samples from a pig treated with ampicillin were assayed at the residue level of the antibiotic in muscle tissue during storage using HPLC–ESI-MS [185]. The effect of different conditions of storage on the stability of ampicillin antibiotic residues in meat was monitored by testing two factors by these technique. In particular, two factors were tested: storage temperature (−20 and

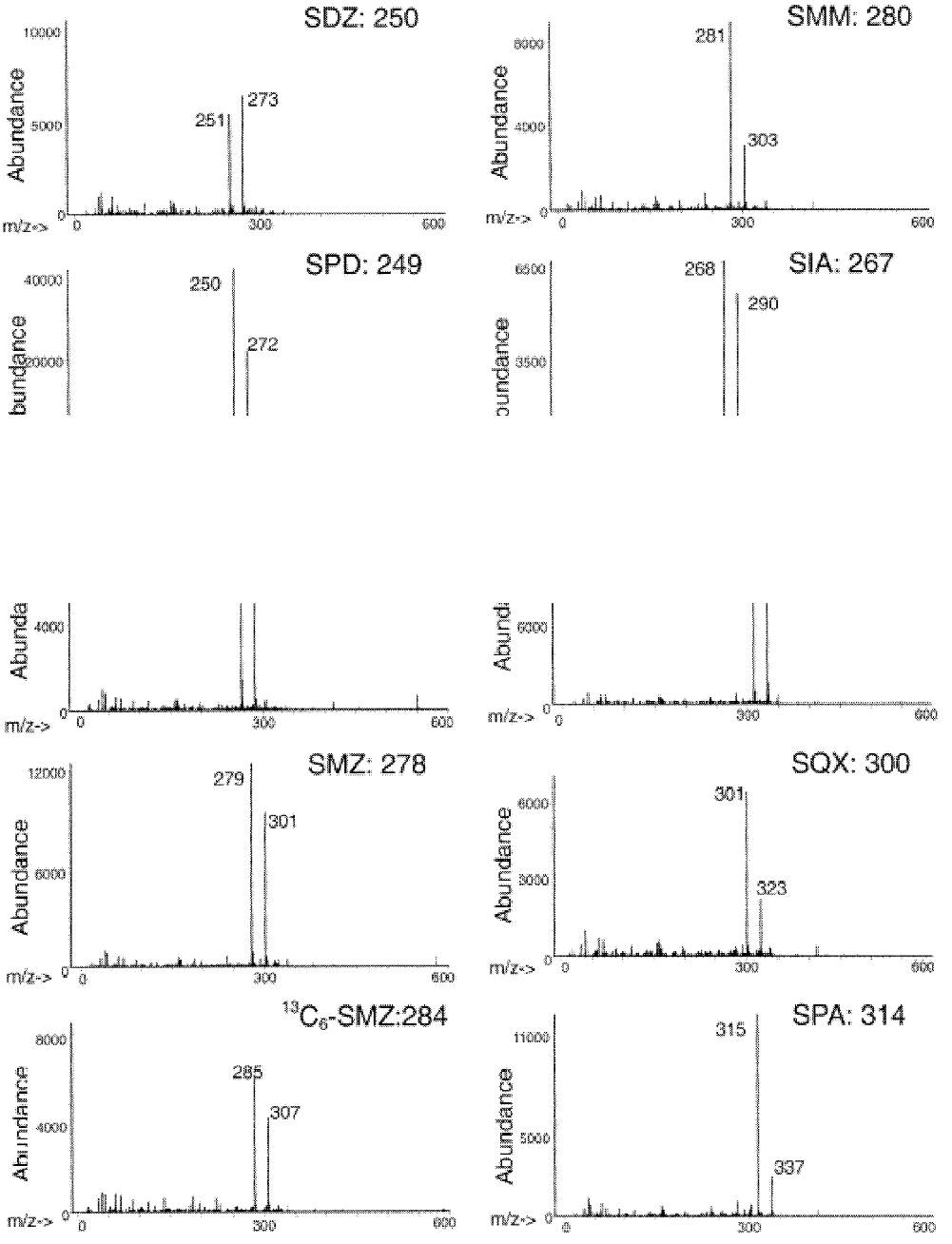

Fig. 15. Positive ES ionization spectra of sulfonamides at 100 V. The data were generated by introducing standard solution (10 μg ml^{-1}) directly using a syringe (50 μl min^{-1}). Reprinted with permission from Ref. [183].

−75 °C) and storage packaging (ground meat and bulk meat). The HPLC–ESI-MS method was developed and applied to confirm ampicillin residue identity. After proper pretreatment, meat samples were purified by C_{18} SPE cartridges and then analyzed for ampicillin residues. Chromatographic

separations were obtained under RP gradient mode. ESI MS measurements were conducted in SIM mode, monitoring positive ions at m/z 350, 351, 372, 373 and 374 for ampicillin and ion at m/z 348 for cephalexin which was selected as the internal standard. Good agreement was observed using the three independent techniques: the antibiotic concentration was found to be affected by the storage packaging, whereas no significant differences were observed as a function of the storage temperature. It can be concluded that the approach used based on the analysis of incurred samples has to be considered of great worth for accurate measurements of drug residues in muscle tissue.

In order to evaluate possible improper and illegal drug uses, a multiresidue method for the detection of five important β-lactam antibiotics in milk by LC–ESI-MS–MS has been recently proposed by Riediker and Stadler [186]. Amoxicillin, ampicillin, cloxacillin, oxacillin, and penicillin G were determined in fresh milk. The analytes, after extraction and clean up step using C_{18} SPE cartridges, were separated under RP conditions and detected using tandem MS after ESI ionization in the PI mode. MS–MS experiments were performed to generate product ion fragments which were used for confirmatory purposes. All analytes gave origin to the class-specific fragment at m/z 160 and compound-specific product ions $[M+H+159]^+$ (Fig. 16). Quantitation was carried out using a stable isotope-labeled internal standard in SRM mode by monitoring the SRM transition showing maximum signal response. With the aim to adding confidence to the identity of the analyte, two or more fragmentation transitions per compound were monitored, thus complying with MS confirmation criteria as established by 1999/333/EG

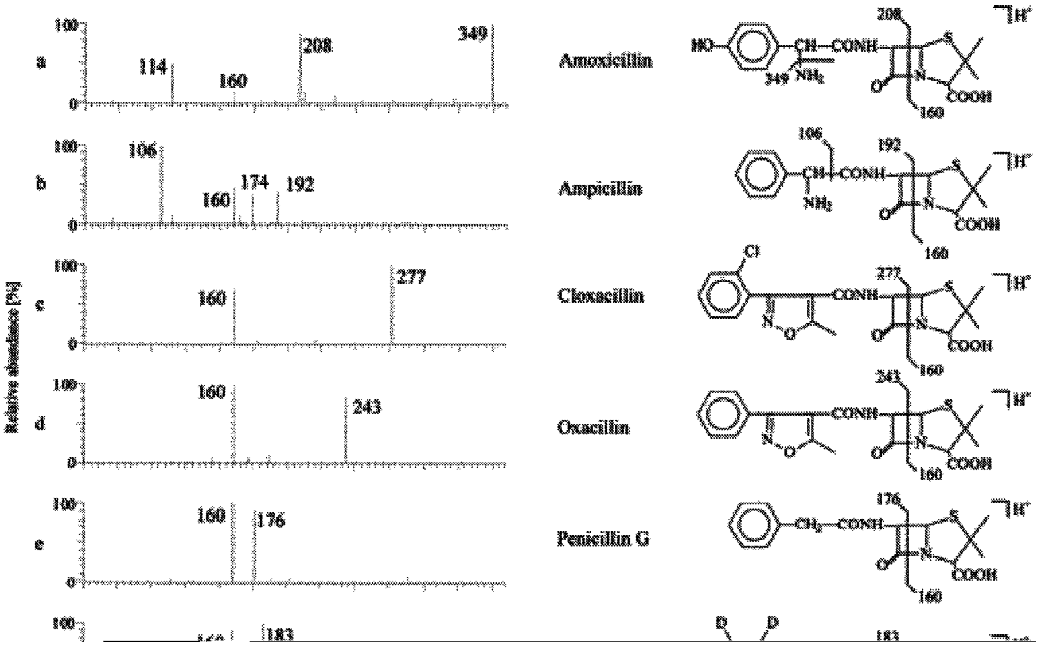

Fig. 16. ESI-MS–MS daughter ion spectra, molecular structures, and major fragment ions of amoxicillin ($[M+H]^+$ 366), ampicillin ($[M+H]^+$ 350), cloxacillin ($[M+H]^+$ 436), oxacillin ($[M+H]^+$ 402), penicillin G ($[M+H]^+$ 335), and penicillin-d_7 G ($[M+H]^+$ 342). Spectra were recorded in positive ion mode by scanning a mass range from m/z 50 to $[M-1]$ at a scan time of 1 s. Reprinted with permission from Ref. [186].

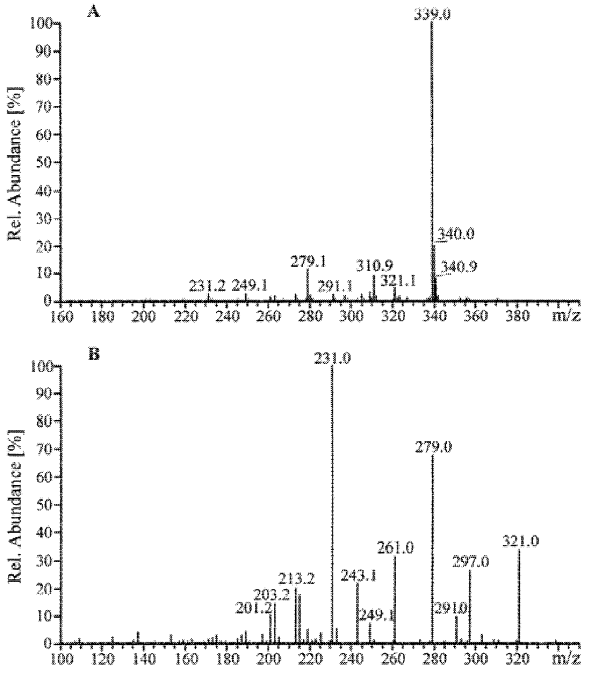

Fig. 19. MS spectrum of 3-ADON (A) and the corresponding MS^2 spectrum of m/z 339 (B). Reprinted with permission from Ref. [198].

dard methods) by means of ESI-MS–MS detection [199]. Due to its higher sensitivity in comparison with APCI, ESI-MS–MS detection was proposed not only to elucidate OTA fragmentation pathways, but also for quantitative analyses at low and sub ppb levels. By monitoring the most abundant fragments from the $[M+H]^+$ ion in the SRM mode, OTA quantitation was feasible at 0.14 and 0.15 μg/l levels in two contaminated coffee samples. Analogously, LC–ESI-MS–MS determination in combination with C_{18} SPE has been shown to be a valid alternative to liquid chromatography-fluorescence protocols for the detection of OTA in wine samples at trace levels [200]. Although lower detection limits were reached by means of fluorescence both with SPE clean up and with immunoaffinity procedures, the less expensive SPE clean up coupled with tandem MS was demonstrate suitable to guarantee unambiguous analyte identification and accurate OTA quantitation within the European Community guidelines. Linearity of the method was demonstrated over about two orders of magnitude in the ppt to low ppb range.

LC coupled with APCI-MS detection has been used for confirmatory purposes by Howard et al. with the aim to elucidating the role of fusarin C in DNA adducts formation by Fusarium metabolites [201]. Mass spectrometric detection, providing additional informations to those obtained by performing other spectroscopic experiments, allowed to characterize fusarin C as an isolated compound thus revealing the lack of role of this analyte in DNA adduct formation.

An interesting method based on LC–ESI-MS technique has also been successfully developed to determine the significance of the fumonisin metabolite N-(carboxymethyl)fumonisin B1 (NCM-FB1) presence during extrusion processes of corn products [202]. By using SPE C_{18} clean up procedures, isotopically labeled fumonisin as internal standard and by operating the mass spectrometer in SIM mode, NCM-FB1 was quantitated at a level of 10 ng/g, recoveries ranging from 50 to 60%. Simultaneous determination of NCM-FB1, HFB1 and FB1 in corn samples spiked with FB-1, D-glucose or sucrose revealed that high-temperature treatments were related to higher amounts (maximum value 97 ng/g) of NCM-FB1 mainly in D-glucose-spiked samples, even though from these data it could be concluded that the significance of NCM-FB1 in food seems to be a minor one.

LC–MS has been revealed a powerful tool also for the selective determination of shellfish poisoning toxins in marine foods both offering the advantage of rapid analysis time and avoiding the use of different reagents needed for both derivatization and simultaneous detection of various analytes by means of fluorescence detection. Recently, Piñeiro et al. have optimized chromatographic and mass spectrometric conditions for the analysis of amnesic shellfish poisoning (ASPs) toxins in clams by using an ESI interface [203]. Efficiency, chromatographic resolution, selectivity and retention factors were the analytical parameters evaluated in this work. Eight percent of acetonitrile and 0.05% of formic acid added as ion suppressor was considered the optimum mobile phase composition, whereas optimized MS conditions, achieved by flow injection analysis allowed quantitative detection of ASP toxins in SIM mode after their purification on SPE SAX cartridges. More recently, four diarrhetic shellfish poisoning

toxins—okadaic acid, dinophysistoxin-1, pectenotoxin-6 and yessotoxin—in scallops were successfully analysed by a novel approach based on LC–MS technique with sonic spray interface [204]. DSP quantitation problems, related to the presence of coeluting substances causing signal suppressions because of the degradation of ionization efficiency, were solved by applying the standard addition method. By operating the mass spectrometer in the negative ion mode and recording the current of selected ions, good agreement between quantitative results and theoretical values as well as low detection limits, good linearity in the 20–500-μg/l range, good accuracy and precision with RSD% values ranging from 4.4 to 9.4% were claimed.

The potential of ESI LC–MS and LC–MS–MS for the determination of azaspiracid in mussel samples has been explored for the first time by Draisci et al. [205]. Azaspiracid is a marine toxin responsible for a new toxic intoxication known as azaspiracid poisoning. Owing to the lack of routine methods for the analysis of this toxin in seafood, selective and accurate analytical methods are needed. A μLC–MS–MS system was set-up for the first time in this work for univocal detection of azaspiracid in mussels sampled from two regions of Ireland. Positive-ion mass spectra provided protonated molecule without any evidence of fragmentation. CID-MS–MS experiments carried out on $[M+H]^+$ as the precursor ion gave evidence of three product ions $[M+H-nH_2O]^+$ with $n=1–3$, useful for identification purposes. The authors claimed good detectability of the toxin at 20 pg level in selected reaction monitoring mode.

3.3.2. GC–MS

Detection of toxic substances such as mycotoxins, phycotoxins and cyanobacterial toxins in foods usually requires time- and solvent-consuming sample preparation steps before chromatographic analysis. A new and simple procedure based on diphasic dialysis extraction coupled with gas chromatographic–mass spectrometric detection has been demonstrated effective in reducing solvent consumption for the determination of patulin in apple juice [206]. By using a semipermeable membrane, patulin was extracted from fortified apple juice samples into a methane chloride organic phase. An improvement in in situ derivatization and higher extraction recoveries were obtained by means of the addition of 4-N,N-dimethylaminopyridine as derivatization activator and of acetic anhydride as derivatizing agent to the sample and to the dialyzing solvent, respectively. By operating the mass spectrometer both in full scan and SIM mode, detection limits of 1 and 20 μg/l were calculated. Other analytical characteristics were a value of 10 μg/l, arbitrarily considered as limit of quantification to keep a desirable recovery and acceptable reproducibility, good linearity in the 10–250 μg/l range and good recoveries from 50 to 92%, according to 5–100 μg/l patulin fortified samples.

Recently, SPE clean-up with Florisil cartridge columns followed by a derivatizing procedure to prepare trimethylsilyl derivatives has been successfully used by Tanaka et al. for the simultaneous determination of seven trichothecene mycotoxins (deoxynivalenol, 3-acetyldeoxynivalenol, diacetoxyscirpenol, fusarenon-X, nivalenol, neosolaniol, T-2 toxin) and zearalenone in cereals [207]. Detection limits ranging from 5 to 10 μg/kg and recoveries up to 80% from wheat and corn spiked at 50 μg/kg concentration level were obtained by converting analytes in trimethysilyl derivatives and by operating the mass spectrometer in SIM mode. A worthy and fast method for the screening of trichothecenes in fungal cultures based on GC–IT-MS, although not yet applied to food analysis has been proposed by Nielsen and Thrane [208]. By performing MS–MS experiments, hydrolyzing and derivatizing extracts of *Fusarium*, *Stachybotrys*, *Trichoderma* and *Memnoniella* species cultivated on agar substrate, low detection limits for 16 trichothecenes, ranging from 10 to 120 pg were obtained even with a relatively dirty matrix. Confirmation purposes and molecular mass determination were also evaluated using negative chemical ionization with methane as the reagent gas. The method was demonstrated to be suitable for food analyses.

The possibility of using fungal volatile metabolites as indicators of ochratoxin A (OTA) and deoxynivalenol (DON) mycotoxins in order to determine grain quality has been investigated by Olson et al. by using both electronic nose and gas chromatographic–mass spectrometric detection [209]. By applying principal component and partial least-squares analyses, volatiles extracted from 40 barley samples by means of dynamic headspace technique were related

to OTA and DON content. Higher concentrations of aldehydes (nonanal and 2-hexenal) as well as alcohols (1-penten-3-ol and 1-octanol) and higher concentrations of ketones (2-hexanone and 3-octanone) were related to OTA levels below and over 5 μg/kg, respectively, whereas pentane, 3-pentanone, 3-octene-2-ol, methylpyrazine and isooctylacetate showed positive correlation with DON presence.

Owing to the high toxicity of aflatoxins towards human and animal health, studies regarding the determination and identification of chemical compounds associated with resistance to aflatoxin production are needed in order to control food contamination problems. Very recently, Brown et al. conducted an interesting study aimed to the characterization of the chemical composition of corn kernel pericarp wax both of the resistant population GT-MAS:gk and of other susceptible genotypes [210]. By performing GC–MS analyses on the whole wax extract and on the wax contained in TLC bands after development with a benzene–chloroform mixture and dissolution in hexane, an higher level of phenolic compounds in GT-MAS:gk was revealed and related with resistance to aflatoxins. The study was demonstrated to be an important tool for further research.

3.3.3. Other MS techniques

Owing to their dominant role in toxic shock syndrome resulting from consumption of contaminated food, detection of staphylococcal toxins at very low concentration levels may be potential for both clinical and food safety analyses. Recent advances in toxin determination concern the application of an approach based on biomolecular interaction analysis-mass spectrometry (BIA-MS) for the simultaneous detection of staphylococcal enterotoxin B (SEB) and toxic shock syndrome toxin-1 (TSST-1) in foods [211]. Capabilities of surface plasmon resonance-biomolecular interaction analysis (SPR-BIA) coupled with MALDI-TOF-MS (Fig. 20) allowed real time measurements and unambiguous detection of SEB and TSST-1 at 100, 10, 1 ng/ml level in spiked milk and mushroom samples without previous purification. By performing BIA analyses and recording sensorgrams after antibodies (receptors) immobilization on the sensor chip surface and sample injection, the amount of captured sample material (analytes plus other matrix components) was detected. By knowing the molecular masses of the target toxins, subsequent MALDI-TOF-MS analysis allowed both to confirm analyte presence and to identify other compounds retained on the sensor chip (Fig. 21). Fig. 21a,b illustrates the MALDI-TOF mass spectra recorded from the anti-SEB-derivatized flow cell following injection of the 1 ng/ml SEB-in-milk sample and the SEB-free milk sample, respectively. The presence of the enterotoxin is indicated by signals corresponding to the singly, doubly and triply charged ions of SEB at m/z 28338, 14187 and 9451.9, respectively. Non-SEB signals attributable to milk proteins were also detected at m/z 7100 (doubly charged at m/z 3550). In the milk sample obtained by direct MALDI-TOF analysis, a complex spectral pattern was observed as expected for a complex matrix such as milk (Fig. 21c). In the milk sample added with 100 ng/ml SEB (Fig. 21d), a very weak signal at m/z 28336 corresponding to singly charged ion of the toxin indicated that this was the lowest concentration of SEB detectable in milk by direct MALDI-TOF-MS analysis. Thus BIA-MS approach was demonstrated a useful tool for qualitative and quantitative toxins assessments in different foodstuffs as well as a potential method for quality food control providing a rapid detection of low toxin amounts.

3.4. Miscellaneous natural and xenobiotic substances in food

Other substances of great concern for food safety are heterocyclic amines, β-carbolines and packaging residues. Heterocyclic amines are recognized as highly mutagenic compounds, which have been isolated mostly from various protein-rich heated foods, such as cooked meat and fish. β-Carbolines are naturally occurring alkaloids having a tricyclic pyrido[3,4-*b*]indole ring structure. Comutagenic and genotoxic properties have been attributed to these compounds, which have been detected in foodstuffs including smoked meat, cheese, soy sauce and in alcoholic fermentation products such as beer, wine and sake. As for packaging residues, interest is devoted to monitor food contamination from compounds used for food contact materials or from reaction products of these compounds with food

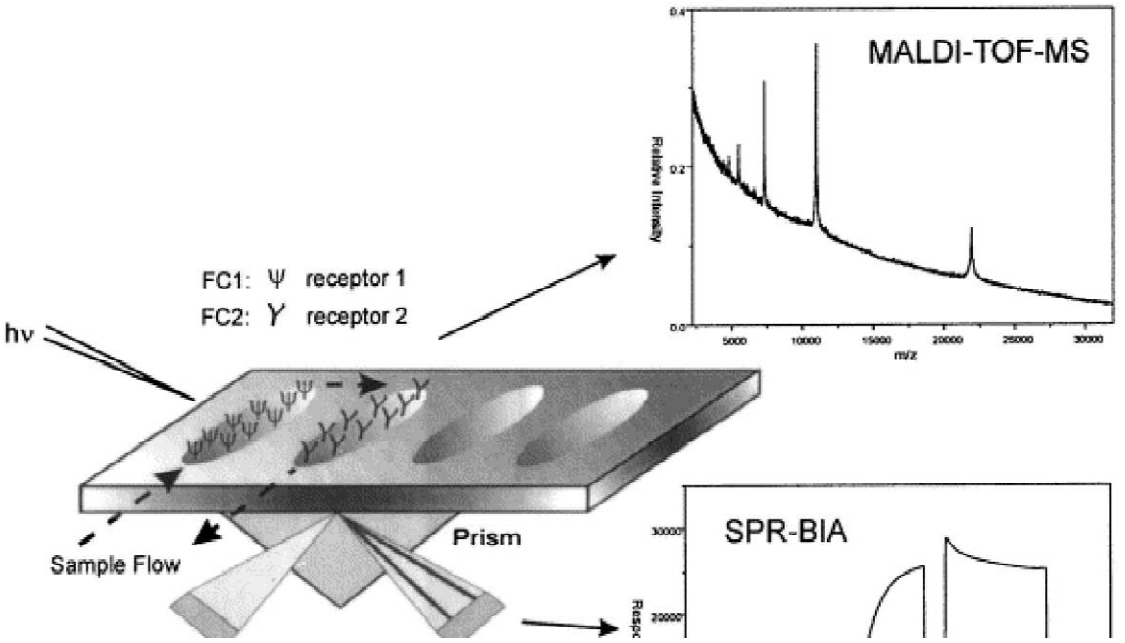

Fig. 20. Schematics of the BIA-MS approach. Receptors are covalently immobilized on the surface of the flow cells (FC) present on an SPR-active sensor chip. Samples of interest are passed over the flow cells where analyte(s) are retrieved via the immobilized receptor(s). SPR is used during capture to monitor and accurately quantitate the amount of material retrieved from solution. The sensor chip is then removed from the biosensor, addressed with a MALDI matrix and the chip subjected to MALDI-TOF analysis. The signals observed in the resulting MALDI-TOF spectrum are indicative of the components captured on the chip. Reprinted with permission from Ref. [211].

components. This is the case for example of chlorohydrins of bisphenol A diglycidyl ether (BADGE) and bisphenol F diglycidyl ether (BFDGE). For determination of these substances of toxicological concern, both GC–MS and LC–MS techniques have been proposed [212–216]. Also for these involatile toxic compounds, advantages of LC–MS-based techniques over GC–MS in terms of simplicity and rapidity have to be underlined. In addition, in the last years as in the case of other xenobiotic substances attention has been paid on the evaluation of proper purification and preconcentration techniques before chromatographic analysis and MS detection.

3.4.1. Amines and β-carbolines

Determination of mutagenic and carcinogenic substances such as diamines, polyamines, β-carbolines and aromatic amines usually present at trace levels in complex matrices requires both the development of analytical methods able to detect low amounts of toxic substances and the evaluation of long-term exposure in order to assess the risk for human health.

An overview dealing with the analysis of bioactive alkaloids tetrahydro-β-carbolines (THβCs) and β-carbolines (βCs) in food has been recently published by Herraiz [212]. Liquid–liquid extraction and SPE

under cationic-exchange and reversed-phase mechanisms were described as the most widely used methods for THβC and βC isolation and clean-up, whereas gas chromatography and liquid chromatography coupled with mass spectrometry were cited as useful and increasingly applied techniques for separation and confirmation purposes.

3.4.1.1. LC–MS and GC-MS

Mutagenicity and arylamine content of fumes from heated cooking oils (sunflowers, vegetables and refined lard) have been evaluated during a 10-year period in order to study women exposure in chinese kitchens [213]. GC–MS analyses allowed to confirm the presence of 2-naphthylamine and 4-aminobiphenyl at concentration levels ranging from 23 to 43 $\mu g/m^3$ evidencing high risks coupled with long exposure time.

A sensitive GC method based on MS detection has been successfully applied for the simultaneous determination of diamines (1,3-diaminopropane, putrescine and cadaverine), polyamines (spermidine and spermine) and aromatic amines (β-phenylethylamine and tyramine) in port wine and grape juices [214]. Bis-2-ethylhexylphosphate dissolved in chloroform was proved to be a powerful and very efficient ion-pair reagent allowing enhanced purification of the sample extract as well as high recoveries. By operating the mass spectrometer in SIM mode and by using five different internal standards, detection limits below 10 ng/g were obtained for all the derivatized amines. A target ion and two qualifying ions for each compounds were always chosen for quantitative assay. Analytical characteristics of the method were a good linearity over at least three orders of magnitude, good precision with RSD% ranging from 0.7 to 16.2% and high recoveries in the 74–119% range.

The applicability of LC–MS with both APCI and ESI ionization techniques has been investigated for both toxic amine and β-carboline determination in food products [215,216]. LC–MS-based techniques allow to avoid time-consuming derivatization procedures needed prior to GC–MS determination

Analytical methods utilizing LC–MS for the assay of heterocyclic amines (HAs) in meat have been developed [215]. Because of the complexity of many food matrices, clean-up and preconcentration procedures before analysis are usually performed. In this study, the comparison of different commercial SPE cartridges for HA extraction from lyophilized meat extract has been realized. After the establishment of the optimal time necessary both to achieve a complete release of HA from the matrix by performing an alkaline treatment and to reproduce binding effects, detection limits in the 0.4–11.7-ng/g range were calculated. Using LC–APCI-ITD-MS, different tandem extraction procedures coupling cationic exchanger (PRS) and C_{18} SPE cartridges were investigated. For this purpose, recoveries achieved testing the use of different commercial PRS and C_{18} sorbents were evaluated. Good recoveries greater than 50% were obtained for all the analytes using the IST 200 mg cartridges, whereas in the case of C_{18} sorbents, higher recoveries were obtained when monofunctional Isolute C_{18} were used. On the basis of the observed differences between trademarks and structures, screening studies prior to analyze unknown samples were recommended in order to select the suitable sorbents.

Owing to the confirmatory power of LC–MS detection, characterization studies have been carried out by Diem and Herderich in order to identify novel β-carboline reaction products of tryptophan with carbohydrates [216]. In previous research performed by the same authors the production of N-glycosides, C-glycosyl conjugates, and glyco-tetrahydro-β-carbolines together with additional unknown substances had been observed. LC–ESI-MS analysis allowed to determine the protonated molecules of the new carbohydrate-derived-β-carbolines at m/z 303, 287 and 269, respectively, whereas tandem mass spectrometry information coupled with UV and NMR data demonstrated the presence of β-carboline alkaloids with glucose-derived side chains (Fig. 22). By using LC–ESI-MS–MS in the selected reaction monitoring mode, several food samples including ketchup, soy sauce, fruit juices and red wine were assayed and the analytes detected in almost all the matrices analyzed. Relating β-carboline presence to different reaction conditions applied during food production, analytes were also demonstrated potential markers for monitoring food processing procedures.

3.4.2. Migrants from packagings

Determination of packaging residues in food is of primary importance in order to ensure consumer

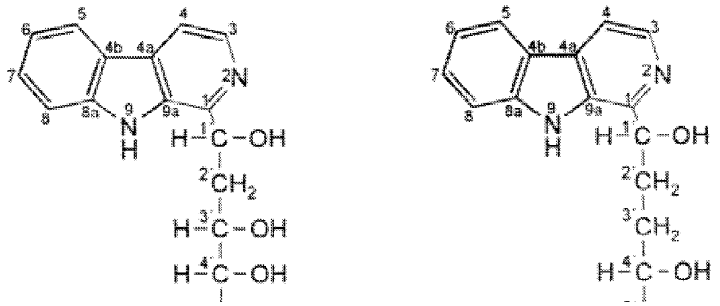

Fig. 22. Carbohydrate-derived β-carbolines. Reprinted with permission from Ref. [216].

protection and to assess the risk of potential contamination. Harmonization of legislation is of utmost importance since regulations governing the use of plasticisers in food-contact applications vary from country to country and foods rejected by countries with stricter regulations can be easily accepted to others having different laws. Owing to its identification capabilities and confirmatory power, in recent years the use of MS techniques is increased for packaging residues detection [217–220].

3.4.2.1. LC–MS and GC-MS

A method based on Soxhlet extraction followed by GC–MS analysis has been developed by Balafas et al. for the determination of phthalate and adipate esters in Australian packaging materials [217]. By operating the mass spectrometer in SIM mode a total of 136 samples such as dairy, baked goods, bread, breakfast cereal, beverage, confectionery, pasta and miscellaneous packaging materials were analysed. All of the samples were found to contain at least two phthalates above the detection limits of 0.01 μg/kg, di-2-ethylhexyl phthalate, dibutyl phthalate, benzyl butyl phthalate and di-2-ethylhexyl adipate being the more predominant additives identified in printed polyethylene materials. The characterization of volatiles released from low-density polyethylene packaging has been also carried out by using a dynamic headspace GC–MS technique [218]. Desorption of volatile substances trapped on a Tenax cartridge