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Review

Overview of the applications of liquid chromatography–mass spectrometry interfacing systems in food analysis: naturally occurring substances in food

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Abstract

This paper reviews applications of different LC–MS techniques for the analysis of natural compounds in foods. Specific examples of substances discussed are lipids, oligosaccharides, vitamins, flavonoids and related substances, phenolic compounds, glucosinolates, and other miscellaneous naturally occurring compounds in food products. LC–MS is a powerful technique in food analysis and especially for analysis of complex mixtures, where additional analytical information is required to confirm positively the identity of the separated compounds or few separations are obtained. Among the interfacing systems used to couple LC with MS, the newly developed electrospray/ionspray mass spectrometric liquid interface offers undoubted advantages in terms of sensitivity and capability to analyze large, thermally labile and highly polar compounds; in addition, tandem MS techniques are useful for structural elucidation studies. © 1998 Elsevier Science B.V.

Keywords: Interfaces, LC–MS; Lipids; Oligosaccharides; Vitamins; Flavonoids; Phenolic compounds; Glucosinolates

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1. Introduction

The use of the hyphenated technique liquid chromatography–mass spectrometry (LC–MS) in the analysis of food extracts provides important advantages because of the combination of the separation capabilities of LC and the power of MS as an identification and confirmation method. The inability of gas chromatography–mass spectrometry (GC–MS) to analyze non-volatile, high-polar and/or thermally unstable substances has led to a growing interest in the development of LC–MS as a valuable analytical technique. In this context, liquid chromatography–mass spectrometry is a viable system for analyzing naturally occurring compounds in food. Analysis of complex mixtures, such as extracts of food products, requires highly selective analytical techniques to identify and quantify targeted components and to characterize unknown compounds. HPLC with its wide range of applicability offers the best choice of separation method. Recent developments in the coupling of HPLC and MS have overcome the main analytical problem, which is the limited information about identity given by the detectors usually associated with HPLC. Using the conventional detectors in LC analysis there is always some vagueness as to whether measured peaks are actually those of target compounds or others coeluted with them; in such cases, on-line LC–MS is useful giving positive identification of components of a complex mixture and an overall reduction in analysis time with respect to off-line techniques, even for unresolved peaks.

LC–MS has been extensively reviewed over the past years, focusing attention on instrumental aspects and developments [1–15] and applications in various fields [16–20]. There are different approaches to the hyphenation of HPLC with MS and some commercial interfaces are now available. In the 1980s, the thermospray (TSP) interface has been the most widely used. With the advent of atmospheric pressure ionization (API) techniques as a means for mass spectrometric sample introduction, LC–MS has increased in popularity; as API is a ‘soft’ and highly efficient ionization method, it is suitable for the analysis of polar, ionic, high-molecular-mass and thermally labile compounds which are amenable to LC. API-based interfacing systems are electrospray (ESP) and ionspray (ISP), which are liquid-based interfaces; a different process, i.e. a gas-phase ion-molecule reaction process leading to the ionization of analyte molecules under atmospheric pressure conditions, occurs in the heated nebulizer-atmospheric pressure chemical ionization (HN-APCI) technique. ESP/ISP and HN-APCI well complement one another as regards polarity and molecular-mass of analytes and of the LC eluent. Another soft ionization technique which has been applied in LC–MS coupling is the fast atom bombardment (FAB) technique with a direct ionization from the miniaturized effluent stream (continuous flow-FAB) or via a stainless-steel frit (frit-FAB). An alternative approach was the development of the particle beam (PB) interfacing system which has been the most successful among the solute-enrichment interfaces; the moving belt (MB) interface, which is based on the same principle, is no longer used. Also the so-called direct liquid introduction (DLI) interface is not generally applied nowadays.

Since the newly developed API-based methods produce mild ionization, for structural elucidation studies it can be complemented by invoking fragmentation-induced collisions in the interface itself or by recourse to LC-tandem MS as realized with the use of a triple quadrupole system. In addition, the mixture analysis capability of tandem MS could make the identification of components in unresolved LC peaks easier, since a collision-induced dissociation (CID) mass spectrum is characteristic of the structure of the parent compound. The reader may refer to recent review papers [9–13] for more information on the LC–MS interface technology.

The theme of this overview is the applications of the hyphenated LC–MS technique with different interfacing systems in food analysis. The results obtained using the oldest and currently unused interfacing systems are extensively discussed, since they represent important pioneering contributions to the use of LC–MS in the field of natural compounds. Topics covered include LC–MS analysis of lipids, carbohydrates, vitamins, flavonoids and related compounds, phenolic compounds, glucosinolates and other miscellaneous natural substances in foods, i.e. molecules not amenable to study by GC–MS. This survey will attempt to cover the state of the art up to 1996.

2. Applications

2.1. Lipids

The analysis of lipids in foods is an important evaluation in food-processing research and development. The evaluation involves quantitation of total fats but also determination of individual triacylglycerols to be related to their corresponding fat sources. The applicability of mass spectrometry by a direct inlet using electron ionization (EI) [21], chemical ionization (CI) [22], desorption chemical ionization (DCI) [23] and field desorption (FD) [24] has been evaluated for the analysis of mixtures of acylglycerols. However, since these ionization methods require volatilization of the triglycerides through application of heat, a fractionation effect where more volatile triacylglycerols volatilize first, is reported, making determination of triglyceride mixtures difficult. Electrospray as a soft ionization method has been demonstrated more suitable for the analysis of synthetic mixtures containing monoglycerides, diglycerides and triglycerides, providing a capability to differentiate species from one another [25]; recently, negative-ion ESP mass spectrometry and tandem mass spectrometry have been used to characterize saturated and unsaturated fatty acids [26].

If triglyceride analysis is of primary importance in lipid research, the characterization of the lipidic fraction comprising fatty acids, waxes, sterols, carotenoids and fat-soluble vitamins among the neutral lipids, and phospholipids among the polar lipids is also of great value in the food industry. In this review paper, fat-soluble vitamins will be discussed in the section dealing with the LC–MS analysis of vitamins.

As for phospholipids, mass spectrometry has proven useful for the analysis of the distribution of individual molecular species; direct analysis of intact phospholipid molecules has been a difficult task and methods involving DCI [27], FD [28] and FAB [29–31] mass spectrometry have been used to characterize phospholipid classes; nevertheless, laborious off-line procedures have hampered the efficient analysis of complex mixtures. Mass spectrometry coupled on-line with liquid chromatography appears to be a more useful technique particularly suited to studies of lipid molecules both in biochemistry and in food chemistry; by coupling the retention time and the spectral information, the structures of the components of a complex acylglycerol or phospholipid mixture can be successfully elucidated. Several fat and oil systems have been characterized by HPLC coupled with MS [32–49,51–67].

2.1.1. Triacylglycerols, fatty acids and carotenoids

The pioneering work of Erdahl and Privett demonstrated the use of a simple interface for coupling HPLC with chemical ionization mass spectrometry (CIMS) for lipid analysis [32,33]. By using the moving chain transport principle, this system was based on the continuous conversion of the acyl groups of the lipids to hydrocarbons by reduction with hydrogen prior to their introduction into the mass spectrometer operating in the CI mode. This system was applied to a standard mixture of tripalmitin, cholesteryl palmitate and cholesterol separated by adsorption chromatography and exhibited a sensitivity of about 1 ng/component injected [32]. Subsequently, this LC–MS methodology was applied to reference compounds representative of triacylglycerols, sterols, steryl esters, glycerophosphatides, sphingolipids and fatty acid methyl esters [33]; sensitivity studies on these lipid classes led to similar results as reported in their previous work [32]. A drawback of this method was the hydrogenation of some of the double bonds in the acyl groups of polyunsaturated fatty acids (PUFAs), making void the identification of the fatty acid constituents. The same authors devised an alternative method based on the use of an inert gas in place of hydrogen for determination of a profile of the lipid classes and molecular species of triacylglycerols [34]. By using an inert carrier gas in the reactor, the acyl groups of the lipid classes are split as free acids from the backbone structures of both triglycerides and phospholipids in the reactor and identified as the $[RCOOH+1]^+$ and $[RCO]^+$ ions; characterization of the fatty acid constituents of the parent compounds was accomplished on the basis of these ions.

Fatty acid analytes were converted into the corresponding *tert*-butyldiphenylsilyl derivatives in order to increase volatility for mass spectral analysis [35]. The derivatives, which were separated under reversed-phase conditions, were fed to the mass spectrometer via a moving belt interface and then ana-

lyzed in EI mode; the mass spectra were characterized by intense fragment ions at $[M-57]^+$ due to the release of the *tert*-butyl moiety and by a signal at m/z 199 corresponding to $[Ph_2SiOH]^+$.

Marai and co-workers investigated extensively the use of a direct liquid inlet (DLI) interface to couple HPLC with MS for the analysis of natural triglycerides [36–40]. In an initiating study, they applied this approach for the analysis of triglycerides in edible oils such as maize oil, peanut oil and soya-bean oil, and in stripped lard; the triacylglycerol elution profile was obtained from the total ion current in the chemical ionization mode [36]. For complete LC–MS analysis, about 5 μg of sample was required to give specific fragmentation because only 1% of the eluate is introduced into the mass spectrometer.

Even though in these works HPLC–DLI-MS was demonstrated to be well suited for the separation and identification of molecular species of triglycerides in natural oils and fats, appropriate calibration with standards appeared necessary for accurate determination of these lipids. In a quantitation study, the same authors determined calibration factors for various mixed triacylglycerols; the peak area ratios obtained by LC–MS were compared with the molar proportions of the mixed triglycerides, known to be present in a sample of well-characterized corn oil and in randomized peanut oil [37]. By means of the calibration factors obtained for these oil samples, the determination of the molecular species composition of selected natural oils was feasible. An interesting result appeared to be the influence of positional isomerism on the relative yields of the $[MH-RCOOH]^+$ fragment ions from a representative isomeric pair of triglycerides; as illustrated in Table 1, the positional distribution in the triacylglycerol of the 16:0 and 18:1 acyl chains affects the ion intensity much more than the nature itself of the fatty acids; thus an accurate quantification of triglyceride molecular species by the abundances of the $[MH-RCOOH]^+$ ions would require information on the positional distribution of the isomers.

The same authors reported the application of chloride attachment negative chemical ionization MS for the identification and quantitation of triglyceride molecular species in two complex natural oils by LC–MS [38]. By combining information of the diacylglycerol fragment ions obtained by PCI with the formation of pseudomolecular ions in the NCI mode, the reversed-phase LC–MS method was effective in establishing the elution profile of the short-chain saturated triacylglycerols present in the most volatile 2.5% distillate of butteroil, as well as the elution order of the complex polyunsaturated triacylglycerols of menhaden oil. When operating in NCI mode, analyte detectability was 100-fold better than that obtained using PCI detection; this enhanced sensitivity was described as an interesting feature of the methodology, particularly in the case of the use of the DLI interface which can introduce only 1% of the total LC effluent into the mass spectrometer.

Table 1
Effect of positional isomerism on the relative yields of the $[MH-RCOOH]^+$ ions[a] [37]

$[MH^+-RCOOH]$ ion		Triacylglycerol isomer	
m/z	Acyl pairing	16:0 18:1 18:1	18:1 16:0 18:1
577	16:0 18:1	1.1	7.6
603	18:1 18:1	1.0	1.0

[a] The yields observed for these two isomers are typical of all such isomers. The $[MH-RCOOH]^+$ ion that results from the loss of a fatty acid from either of the *sn*-1 or *sn*-3 positions is approximately four times more abundant than the equivalent ion that results from loss of a fatty acid from the *sn*-2 position (7.6:2=3.8).

The identification of the less common isologous short-chain triglycerides in the most volatile 2.5% molecular distillate of butter oil was the subject of further study by Myher et al. [39]. Both saturated and unsaturated triacylglycerols containing straight and branched-chain odd-carbon fatty acids in combination with short-chain acids were identified from the CI-LC–MS analytical data. After a reversed-phase chromatographic separation, the molecular species were identified by means of the $[M+H]^+$ and the $[MH-RCOOH]^+$ ions in PCI mode. With the aim of extensively characterizing butterfat composition, this research group investigated the reversed-phase LC–MS behaviour of randomized butterfat containing various isomeric triglycerides [40]. The molecular species were identified by on-line positive CI-MS. The use of the rearranged butteroil for the LC separations allowed the recognition of the elution times of numerous uncommon triglyceride species. Differently from that expected in the case of a reversed-phase system, within a given series the

triglycerides containing the longest chains were eluted first and the acetates were eluted the last of all. The lowest-molecular-mass triacylglycerol which was identified in the randomized butterfat was 4:0-4:0-2:0, followed by 4:0-4:0-4:0, 4:0-4:0-6:0, 4:0-4:0-8:0 (this 'shorthand description' used by the authors to denote fatty acyl residues in a triglyceride molecule indicates the number of carbon atoms in the acid chain and the number and positions of the double bonds). The retention behaviour of these species was found to be in accordance with that observed in the case of mixed saturated fatty acid isologous triglycerides of their higher-molecular-mass homologues.

The advantages of combining chromatographic fractionation with MS analysis of lipids are discussed by Laakso and Kallio, who investigated the configurational isomers of triglycerides of a complex natural mixture like winter butterfat [41,42]. In these studies, negative ion chemical ionization-MS was used to study the molecular species of differently unsaturated triacylglycerols of butterfat. Fig. 1 displays NCI mass spectra of the triglyceride fractions of winter butterfat obtained by silver ion HPLC. Further information on the composition of disaturated monoenoic and saturated dimonoenoic triglycerides was obtained by the MS/MS method, i.e. by collisional activation of each of the $[M-H]^-$ parent ions in the second stage of the MS analysis (Fig. 2).

A particle beam LC–MS approach was taken by Huang et al. for the separation and identification of individual triglycerides in fat samples obtained by supercritical fluid extraction (SFE); three food products (cookies, candies and ice cream) made with SALATRIM 23CA, an interesterification product of triacetin, tripropionin and hydrogenated canola oil, and a refined hydrogenated coconut oil were considered [43]. After a reversed-phase chromatography, which provided enough resolution to separate SFE samples into individual triglycerides, the mass spectrometer was operated in the PCI mode, using ammonia as the reagent gas. The PB PCI mass spectra proved to be useful for structural elucidation of the major components of the fat samples analyzed, allowing to positively correlate the individual triacylglycerols to their corresponding fat sources.

The potential of the LC—PB-MS technique for the separation and the unequivocal peak identification of

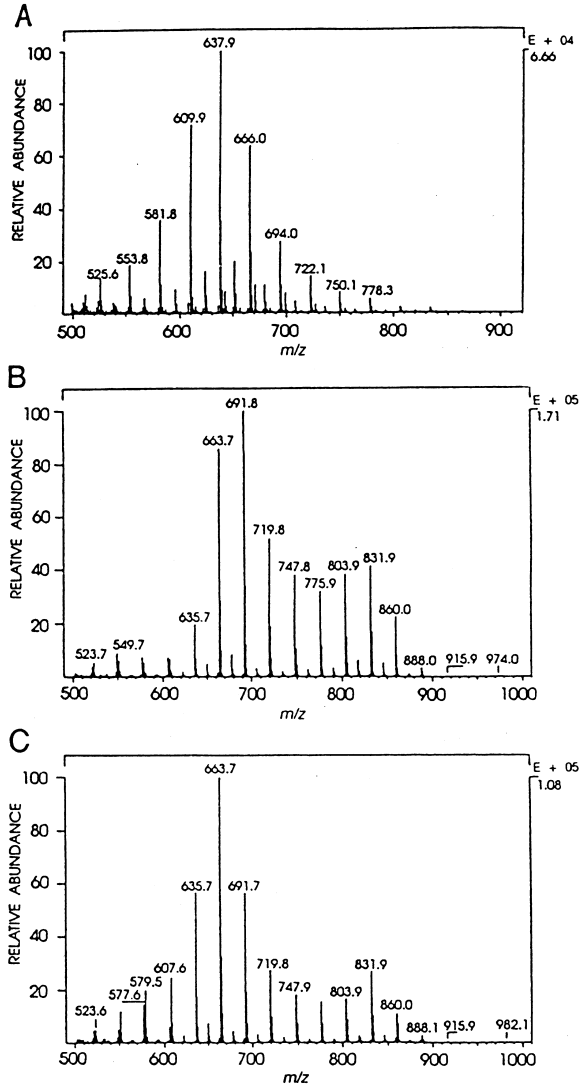


Fig. 1. Mass spectra of the negative ions produced by chemical ionization with ammonia of the triacylglycerol fractions of winter butterfat obtained by silver ion high-performance liquid chromatography (Ag-HPLC): (A) trisaturated Ag-HPLC fraction (1), (B) disaturated *trans*-monoenoic Ag-HPLC fraction (2) and (C) disaturated *cis*-monoenoic Ag-HPLC fraction (3) triacylglycerols. The ions of the displayed areas represent the deprotonated triacylglycerols, $[M-H]^-$ ions. Reprinted with permission from Ref. [41].

triglycerides in fats or oils has been demonstrated [44]. For confirmation purposes, ammonia CI detection was chosen. The authors concluded that adequate analysis is only obtained when results from

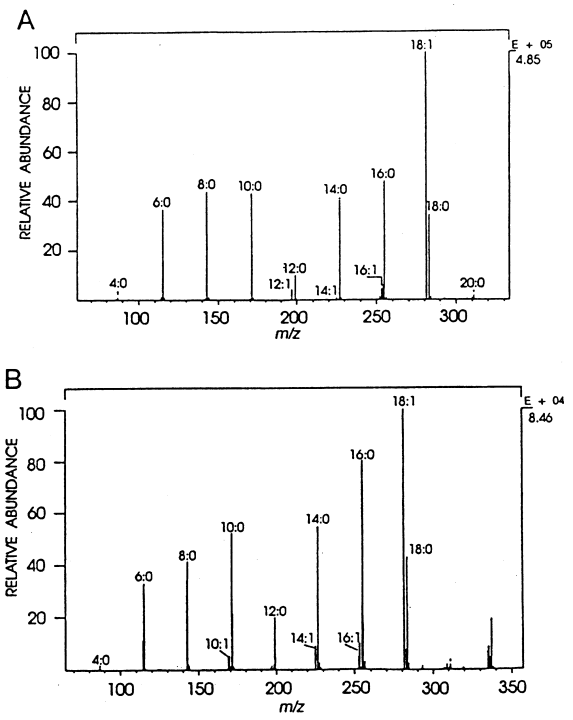


Fig. 2. Collision-activated daughter spectra of the parent ion 719.8 from disaturated *trans*-monoenoic (A) and disaturated *cis*-monoenoic (B). The ions of the displayed areas represent the deprotonated fatty acids, RCO_2^- ions. Experimental conditions: collision gas, Ar at a pressure of 0.4 mTorr; offset for the collision quadrupole, 13 eV. Reprinted with permission from Ref. [41].

LC-UV or LC with light scattering detector are combined with results from LC–MS methods, because of the complexity of the triglyceride composition.

Another technique for the analysis of lipids in food materials is thermospray LC–MS. A method for the determination of hydroxy and hydroperoxy polyunsaturated fatty acid acetyl derivatives has been developed by Yamane and co-workers by using HPLC combined with TSP-MS [45,46]. By operating in the positive-ion mode with selected ion monitoring (SIM) acquisition, the authors demonstrated the capability to detect simultaneously many hydroxy and hydroperoxy polyenoic acid derivatives within 30 min without gradient elution. The temperature of the TSP interface was optimized for maximum sensitivity of the acetyl derivatives; limits of detection of approximately 0.2 and 1 pmol were given for the hydroxy and hydroperoxy PUFA derivatives, respectively.

A TSP interface operating in the discharge mode, i.e. solute ionization performed by a discharge electrode with no salt in the eluent, has been used in LC–MS experiments aimed at characterizing methyl esters of fatty acids obtained by hydrolysis of pigments from bleached and deodorized canola oil [47]. The objective of this study was to identify the chromophores responsible for color in deodorized oils. The method enabled to reveal that the pigments were prevalently oxygenated C_{18} and C_{20} unsaturated fatty acids with one to five double bonds; these attributions were made on the basis of the molecular masses of the compounds, as well as the characteristic fragmentation pattern for oxygenated methyl esters.

HPLC-fast atom bombardment mass spectrometry with a frit-FAB interface has been proved valuable for the molecular species analysis of polar, labile and non-volatile oils containing polyunsaturated fatty acids, such as icosapentaenoic acid and docosahexaenoic acid [48,49]. *m*-Nitrobenzyl alcohol was used as the FAB matrix and introduced to the ion source by post-column addition for simultaneous UV detection. Ion species such as $[RCO]^+$, $[RCO+74]^+$ and $[MH-RCOOH]^+$ could be clearly detected and were thus useful for compositional analysis of natural oils. The authors underlined the difficulty in obtaining the structural characterization of molecular species in natural oils by using other methods such as GC–MS and HPLC.

The same group used a combination of HPLC–FAB-MS and the relative retention potential index theory [50] to study the polyunsaturated fatty acid-related molecular species in polyunsaturated fatty acid-enriched fish oil, a complex mixture of various triglycerides [51]. PUFA-enriched fish oil was obtained by lipase-catalyzed selective hydrolysis. Both positive- and negative-ion FAB mass spectra of triacylglycerols consisting of polyunsaturated fatty acids were recorded; in general, the negative ion FAB mass spectra were simple, carboxylate anions being the predominant peaks, whereas positive ion FAB spectra contained characteristic fragment ions corresponding to $[RCO]^+$, $[RCO+74]^+$, $[MH-RCOOH]^+$, as illustrated in Fig. 3 in the case of triicosapentaenoin (EPA-TG). The authors concluded

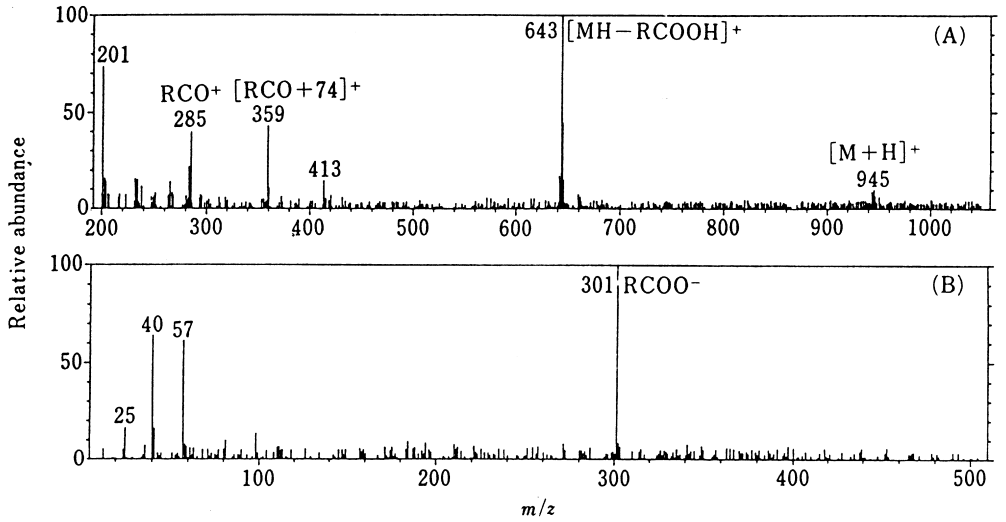


Fig. 3. Positive and negative FAB mass spectra of triicosapentaenoin. (A) Positive-ion FAB mass spectrum. (B) Negative-ion FAB mass spectrum. Reprinted with permission from Ref. [51].

that positive- and negative-ion FAB mass spectra provided indications of the possible combinations of fatty acids in each triacylglycerol component.

Methods using atmospheric pressure chemical ionization have successfully been applied by Kusaka and co-workers to the analysis of fatty acids [52–54]. A mixture of anilide derivatives of saturated ($C_{16:0}$–$C_{30:0}$) and unsaturated ($C_{16:1}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$) fatty acids were separated by reversed-phase HPLC and detected by APCI-MS; the mass spectra displayed predominantly the protonated molecules in each case [52]. This method seemed to have a good potential for the identification and determination of low levels of fatty acids including very long-chain fatty acids. In another work, a method for analyzing hydroxy and non-hydroxy fatty acids as amide derivatives by LC–APCI-MS was devised [53]. By monitoring the single ion $[M+H]^+$ of six types of palmitamide derivatives, a sensitivity decreasing in the following order was found: N-*n*−propylamide > anilide > N-N-diethylamide > amide > N-N-diphenylamide > N-1-naphthylamide. Individual fatty acids were identified from a mixture of amide derivatives of fatty acid standards from $C_{16:0}$ to $C_{30:0}$. The same authors further tried to quantitatively measure labile fatty acids such as polyunsaturated hydroxy and hydroperoxy fatty acids as their 3-methyl-7-methoxy-1,4-benzoxazin-2-one derivatives by LC–MS using the APCI technique [54]. First, LC–MS with spectrophotometry was used to analyze the derivatives of a mixture of eight authentic fatty acids (Fig. 4); peaks in the UV chromatogram were superimposable to those in the mass chromatogram. Sensitivity data obtained for the derivative of palmitic acid by monitoring a single ion $[M+H]^+$ showed that even 5 ng of this analyte could be detected. This work also described the analysis of hydroperoxy fatty acids split from photooxidized lecithin by phospholipase A_2; various hydroperoxy fatty acids were identified, thus demonstrating that the proposed method may be useful for experimental studies on lipid peroxidation in biomembranes.

Very recently, this research group successfully used the LC–APCI-MS system for stereospecific analysis of plant triacylglycerols and hydroperoxidized triacylglycerols [55]. Positive-ion APCI mass spectra displayed characteristic fragments which allowed to discriminate fatty acids between *sn*-1- (or -3-) and -2- positions of triacylglycerols. It is noted here that the method is very simple, not time consuming and that it requires nanomole levels of triglycerides without derivatization.

The applicability of the LC–APCI-MS technique to triacylglycerol analysis was also recently explored by Byrdwell and co-workers [56,57]. A mixture of triglyceride standards containing fatty acids having

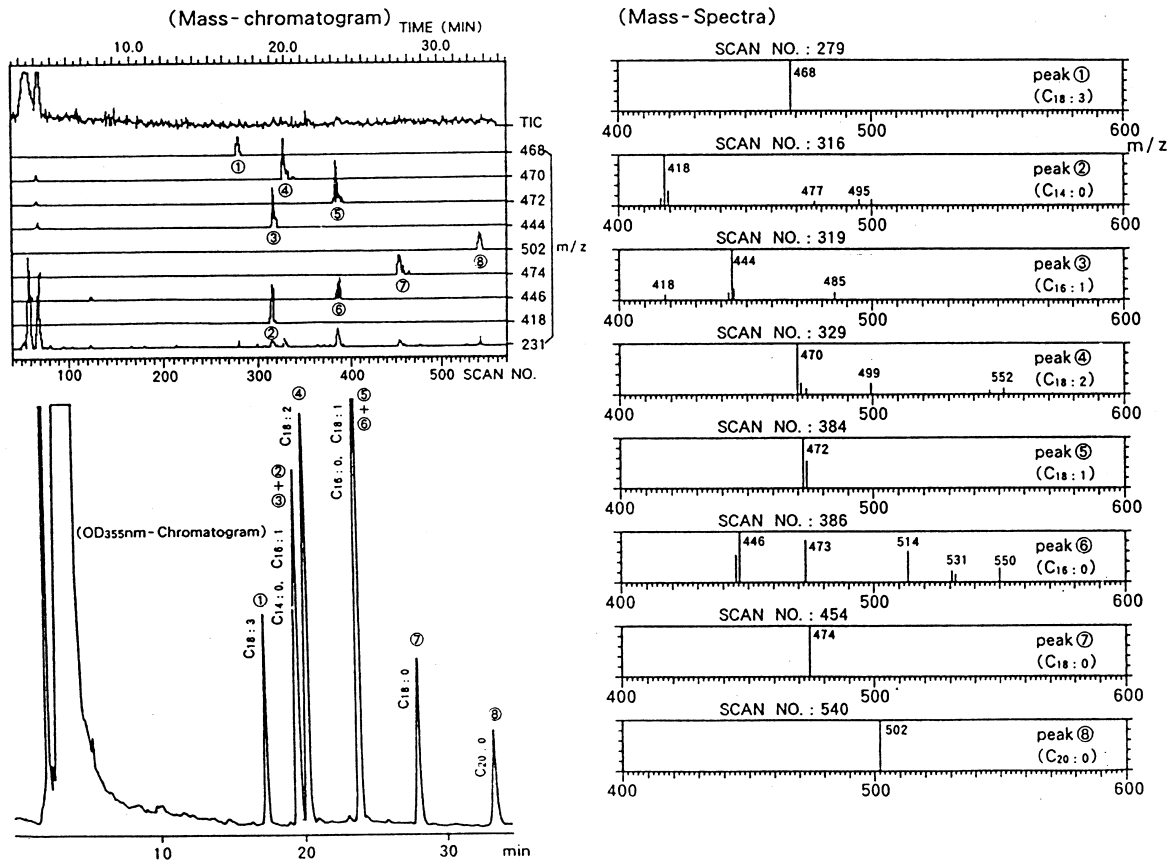


Fig. 4. LC–MS of the 3-methyl-7-methoxy-1,4-benzoxazin-2-one derivatives of standard fatty acids. A mixture of eight kinds of fatty acids (approximately 10 nmol each of $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$, $C_{20:0}$) was the starting material. The mobile phase was acetonitrile–water (80:20, v/v) with acetonitrile increasing linearly at the rate of 1% per min. Reprinted with permission from Ref. [54].

from zero to three degrees of unsaturation was separated by reversed-phase HPLC and then analyzed by APCI-MS [56]. An interesting aspect was the remarkable effect of the number of double bonds within the acyl chains on the proportions of the protonated molecules and the diglyceride $[M-RCOOH]^+$ ions formed in the APCI ion source; this fact allowed to distinguish triacylglycerols containing saturated fatty acids from those containing fatty acids with one or more double bonds. The proposed method was applied in another work for the analysis of soybean oil triglycerides [57]. Triacylglycerols were separated and identified in high-palmitic and high-stearic soybean varieties and were determined by reversed-phase LC with flame ionization detection.

The advantages of reversed-phase HPLC, DCI mass spectrometry and tandem mass spectrometry have been evaluated for the characterization of milk fat triacylglycerols [58]. An evaporative light-scattering detector was used to trace the peaks and the resolved fractions were subsequently analyzed by DCI-MS in positive-ion mode. Mass spectra were useful both for structure elucidation and mass weight determination; in the case of the presence of more than one triglyceride or of impurities in a fraction, tandem MS was successfully carried out by using high-energy collisional activation on the protonated molecules generated by DCI; the MS–MS approach was also able to distinguish odd-chain triacylglycerols.

Among neutral lipids, the class of carotenoids is

worth mentioning. Carotenoids, which are polyene hydrocarbons biosynthesized from eight isoprene units, are known to be responsible for the intensive yellow, orange or red color of a great number of foods of plant origin. Recently, great interest in the analysis of individual carotenoids has been evident, particularly in fruits and vegetables, because of the role of these substances as naturally occurring antioxidants and anticarcinogens. The fact that carotenoids comprise a high number of compounds, many of which are closely related structurally, makes it essential that new analytical methods can provide extensive information on the molecular structure of each carotenoid, thus positively identifying individual carotenoids. HPLC–MS overcomes the limitations of detection methods encountered in HPLC, including photodiode array detection (DAD), which allows continuous accumulation of spectrophotometric data during analysis but requires retention time measurement and complete chromatographic resolution of absorbing species. Very recently, the LC–MS analysis of carotenoids has been the subject of a comprehensive report [59]; various LC–MS interfacing systems including moving belt, particle beam, continuous flow-FAB, electrospray and APCI were thoroughly evaluated with regard to the applicability to these thermolabile substances. For this reason, this topic will be not included in the discussion in the present overview.

2.1.2. Phospholipids

Among the first LC–MS techniques used to analyze phospholipids from biological extracts were the DLI interface [60] and the MB interface with chemical ionization [34,61]. Sugnaux and Djerassi compared the on-line LC–DLI-CI-MS technique with an off-line method using desorption chemical ionization for the analysis of sterol peroxides and phospholipids in marine organisms [60]; they found similar results in terms of CI spectrum quality, reporting that the use of ammonia as desorption/chemical ionization reagent gas increased the abundance of the pseudo-molecular ions of phospholipids.

Jungalwala et al. observed specific fragment ions in the low mass range for each phospholipid in the PCI mass spectra using ammonia as the reagent gas; for complete LC–MS analysis, about 5 μg of an individual phospholipid had to be injected [61]. As already discussed, Erdahl and Privett obtained mass spectra of phospholipids containing acyl group-based ions such as $[RCOOH+1]^+$ and $[RCO]^+$ fragment ions, which were helpful for structural elucidation but not useful for molecular mass determinations of these lipids. These results show that these CI mass spectra differ significantly from both the qualitative and quantitative point of view, in dependence of the design of the interface and the operational conditions. In addition, limitation to phospholipid analysis by use of LC–MB-MS include loss of chromatographic resolution during transport of the sample into the ion chamber of the mass spectrometer.

Kim and Salem explored the possibility to detect and identify phosphatidylcholine and phosphatidylethanolamine molecular species by LC–MS with a TSP interface [62]. Their experiments were based upon the observation that this interfacing system allows on-line LC operations at conventional flow-rates and that it produces mass spectra of intact involatile molecules without derivatization. Chromatographic separations were carried out under reversed-phase conditions and filament-assisted thermospray was performed in the positive-ion mode; the use of an external ion source was necessary because of the high organic content of the mobile phase, which hampered an efficient ionization of the analytes. Reliable structural and molecular mass information for each molecular species was obtained, since TSP mass spectra contained protonated molecules and fragment ions derived from the polar head group and the mono- and diacylglyceride ions. Even for a complex mixture such as natural phosphatidylethanolamine from egg yolk, this approach enabled to achieve detailed structural information with short analysis times. In addition, good analyte detectability was claimed, detection limits ranging between 10 and 100 ng. This rapid and relatively sensitive LC–MS approach was then successfully applied by the same authors to other phospholipid major classes and related compounds such as triglycerides [63]. Five fast chromatographic systems were developed for the separation of molecular species of phosphatidylinositols, phosphatidylserines, sphingomyelins, triglycerides and platelet-activating factor, which is a potent mediator of cellular function. The sensitivity of the LC–TSP-MS technique was found to be analyte dependent, but the total sample amount

required by selected ion monitoring was usually in the low ng range.

Initial studies carried out by Sakairi and Kambara have shown the potential and limitations of the atmospheric-pressure spray (APS) technique for the LC–MS analysis of various thermolabile and involatile substances including phospholipids [64]. These substances, which require a high percentage of organic solvents for their dissolution, produced intense protonated and cationized molecules in APS ionization without external ionizing source, different from that reported in the case of TSP ionization [62,63].

Electrospray mass spectrometry in conjunction with reversed-phase LC separation of phospholipid molecular species has been proposed in more recent studies [65,66]. For neutral phospholipids, such as phosphatidylcholine, phosphatidylethanolamine, and for sphingomyelin, protonated molecules were the major ions displayed in the positive-ion spectra, whereas sodiated molecules were most prominent in the ESP spectra of acidic phospholipids (phosphatidylinositol and phosphatidic acid) [65]. Linear response curves were obtained over at least two orders of magnitude, thus allowing accurate determination of each molecular species using stable isotopes as internal standards. Remarkable sensitivity was obtained in comparison with the TSP method; phosphatidylcholine exhibited the greatest molar response, detection limit being in the 0.5–1 pmol (5–10 fmol after 1/100 split) range. In general, sensitivity was found to be more dependent on the nature of the phospho head group than the fatty acyl composition within a class, as illustrated in Fig. 5 for the LC–ESP-MS analysis of a standard phospholipid mixture (83 pmol of each molecular species).

Negative-ion ESP ionization tandem MS has been evaluated for the characterization of bacterial phospholipids [66]; specificity for characteristic ions of analytes allowed to use direct introduction MS–MS, without any chromatographic separation, for phospholipid analysis. All phospholipid classes except phosphatidylcholines produced a prominent deprotonated molecule using NI detection mode; the product ion mass spectra of the $[M-H]^-$ ion contained fragments useful for identification of the class of phospholipid and the characterization of the molecular masses of the two fatty acyl groups. Using this

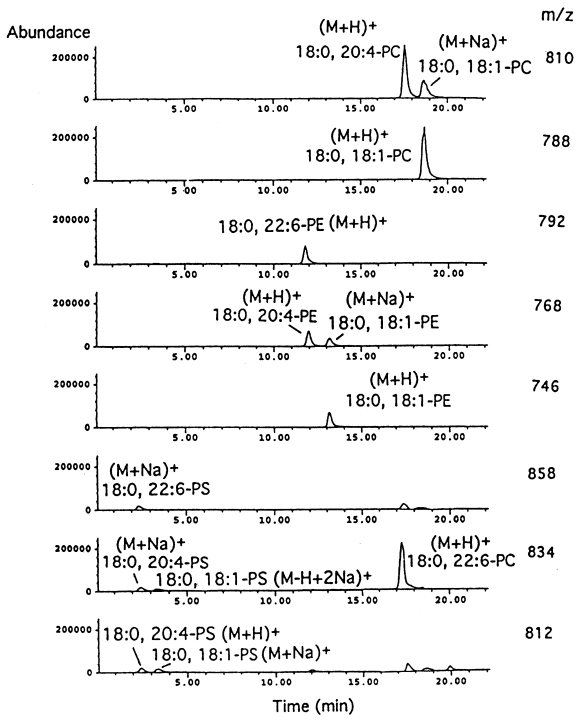


Fig. 5. Ion chromatograms obtained from standards (83 pmol each) by selectively monitoring molecular ions with the 200 ms dwell time. The capillary exit voltage was set at 200 V. The separation was achieved using a C_{18} column (5 μm, 2.1 mm×15 cm) and the mobile phase consisting 0.5% ammonium hydroxide in water–methanol–hexane changing from 12:88:0 to 0:88:12 in 17 min after holding at the initial solvent composition for 3 min. One hundredth of the HPLC flow was introduced to the mass spectrometer. Reprinted with permission from Ref. [65].

technique, different phospholipid profiles were obtained for four bacterial species, indicating the possibility of identifying bacteria on the basis of their fatty acid distribution.

LC–MS techniques with PB and ISP interfaces have been used to identify phospholipids from soybean products [67]. Using the normal-phase LC–PB-MS system and operating in CI mode, positive- and negative-ion spectra did not provide useful fragment ions for characterizing different classes of these compounds; in addition, the authors observed instrument contamination after injection of crude soybean product extracts. In contrast, valuable results were obtained by applying the ionspray approach. Detection of the positive ions generated by ISP was more successful for phosphatidylcholine and phos-

phatidylethanolamine, whereas the ionization method of choice for the acidic compound phosphatidylinositol was found to be negative-ion ISP. LC–ISP-MS yielded molecular weight information of the individual homologues. In order to obtain a more complete characterization of the fatty acid chains and of the polar head group, ISP-MS–MS was used; the triple quadrupole MS–MS CID mass spectra of the phospholipid molecular species investigated were found useful for structural confirmation, containing peaks corresponding to mono- and diacylglycerol ions besides acylium ions $[R–C\equiv O]^+$. In addition, the MS–MS selected-reaction monitoring (SRM) technique was used for increased sensitivity and selectivity; the higher signal-to-noise ratio allowed to detect the phospholipids at 40 ng level, whereas using SIM for the same amount of phospholipids, phosphatidylinositol was not detectable.

The examples reported demonstrate that coupling of MS with HPLC offers an important advance in analytical methodology in the research area of lipids. Using appropriate interfacing systems, LC–MS provides useful structural information not otherwise achievable on small quantities of compounds; quantitation of the various lipid species can be performed in complex mixture by monitoring specific ions of the analytes in order to enhance both selectivity and sensitivity.

2.2. Oligosaccharides

Oligosaccharide characterization has been of utmost interest in various areas such as medicine, biochemistry, and food chemistry. These biologically relevant molecules are ideally suited for mass spectrometric investigation, because of the capability of this technique of offering structural and mass molecular information. Mass spectrometry has played an important role in the structure elucidation of sugar oligomers; different ionization modes, such as FAB [68], DCI [69], matrix-assisted laser desorption mass spectrometry [70] and ESP mass spectrometry [71] have been shown to be useful for the structural analysis of neutral non-derivatized oligosaccharides.

The power of the hyphenated technique LC–MS in the characterization of oligosaccharides is attested by various reports of application of HPLC–MS in this field [5,64,72–90]. In the earliest studies, mono-, di- and trisaccharides have been used as test compounds to establish the absence of thermal degradation in DLI LC–MS interfaces [72–74] and in the MB interface [75]. The use of negative chemical ionization for coupled LC–MS using a MB interface has been described for the analysis of raffinose and stachyose [76]; the same interfacing system with FAB ionization has been successfully applied to the analysis of sugar oligomer standards and to a sample of high mannose oligosaccharides [77]. In a fundamental instrumental research, Sakairi and Kambara investigated the applicability of the APS system for LC–MS to various involatile compounds including saccharides [64]; the APS ion source also generated abundant cationized and/or protonated molecules for these analytes. The authors gave an overview of the use of water and 0.1 *M* ammonium acetate as the mobile phase and observed that intense $[M+Na]^+$ and $[M+K]^+$ can be detected without using buffers. Detection limits ($S/N=10$) using the SIM mode were estimated to be 30 and 75 pg for sucrose and raffinose, respectively, thus demonstrating high sensitivity of APS ionization.

The use of a TSP interface for LC–MS has been preliminary described by Rajakylä; after reversed-phase chromatography on C_{18} stationary phase and water as eluent, which was demonstrated a very good alternative to either ion-exchange or amino columns for the separation of sugars, MS analysis of a starch hydrolysate was performed in the presence of 0.05 *M* ammonium acetate as the ionization buffer [78]. Total ion current chromatograms were recorded in positive- and negative-ion operational modes. Deprotonated molecular anions and peaks due to the loss of water molecules or due to the ammonium addition were predominantly observed.

An extensive study of the application of LC–TSP-MS to monosaccharides and various derivatives has been performed by Liptak et al. [79]. To establish the optimum conditions for the ionization of the analytes, TSP-MS experiments were carried out using various highly polar model compounds including pentoses, hexoses, sugar alcohols, methyl glycosides and different derivatives used in preparative carbohydrate chemistry. The best results were achieved using a mobile phase made up of a methanol/acetonitrile mixture (1:1) containing 0.1 *M* aqueous ammonium acetate; the mass spectrometer was operated in

filament-off mode without discharge ionization. Noticeable mass spectral results were reported by the authors; the TSP mass spectra of most of the sugars displayed strong $[M+NH_4]^+$ ions which made possible unequivocal molecular mass determination. In addition, the presence or absence and the relative intensities of $[M+NH_4-H_2O]^+$, $[M+H]^+$ and $[M+NH_4]^+$ ions allowed to group and distinguish the underivatized and derivatized monosaccharides.

High-performance anion-exchange chromatography (HPAEC) is the technique of choice for the separation of oligosaccharides, but in the analysis of oligomers containing different sugar residues in one oligomer, the retention behaviour of the oligomers is unpredictable. In this context, on-line HPAEC–MS is a very powerful tool in the separation and rapid molecular mass determination of the various constituents of complex oligosaccharide samples without the use of standards. Simpson et al. demonstrated that the use of an anion micromembrane suppressor (AMMS) makes possible the coupling of HPAEC and MS via a thermospray interface; the applicability of this system was demonstrated for both isocratic and gradient separations of mono- and diaminosugars, but the results indicated that thermal degradation in the TSP interface occurs [80]. Sensitivity studies conducted on each of the model sugars showed minimum detectable amounts to be in the low μg range.

Arpino demonstrated that under conventional TSP conditions, i.e. in the presence of ammonium acetate in the eluent, sugar oligomers undergo gas-phase acetolysis to the constituent monomers; as a consequence, molecular mass information in the mass spectra is either weak or absent [5].

On this basis, Niessen et al. studied the LC–TSP-MS behaviour of oligosaccharides up to a degree of polymerization (DP) of 10 by evaluating the effect of various mobile phases, including pure water, ammonium acetate- and sodium acetate-containing mobile phases [81]. The aim of this study was to devise an LC–MS method capable of providing molecular mass and structural information of this class of substances; sucrose, cellobiose, maltose and raffinose were considered as test compounds. Oligosaccharide separation was performed by RP chromatography on a C_{18} stationary phase or ion-moderated partition chromatography on Aminex HPX-22H column; the TSP interface was operated in the discharge-on mode with all eluents, except for the ammonium acetate-containing mobile phases for which thermospray buffer ionization was used as well. Using mobile phases with ammonium acetate, a significant breakdown was observed and no molecular mass information was obtained for the intact dimers and trimers tested; the authors attributed the considerable fragmentation to a mechanism of thermally induced ammoniolysis instead of the mechanism based on gas-phase acetolysis proposed by Arpino [5]. In contrast, these analytes were characterized using aqueous eluents containing 10^{-4} *M* sodium acetate; in these conditions, sodiated ions $[M+Na]^+$ and, in the case of larger oligomers (DP> 4) disodiated molecules $[M+2Na]^{2+}$, were detected with no fragmentation. Fig. 6 shows the TSP mass spectrum of maltoheptaose (DP=7). By plotting the relative intensities of the monosodiated and disodiated ions against the degree of polymerization (Fig. 7), the authors note that at higher DP (DP>5) the doubly charged disodium ion is the base peak in the spectrum, so that the LC–MS analysis of a mixture of sugar oligomers can be performed by using a limited mass range for DP=2–10. In the same work, the researchers evaluated the applicability of the PB interface for the LC–MS analysis of oligosaccharides, but unsuccessful results were obtained both for small oligomers, for which weak

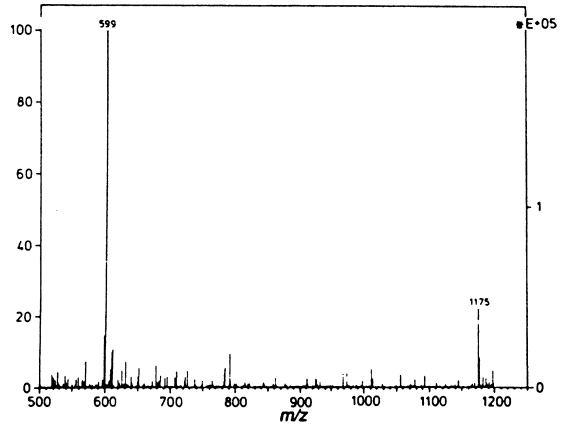


Fig. 6. Thermospray mass spectrum of maltoheptaose (DP=7), obtained with a 20-μl injection of a solution containing 20 μg in a mobile phase of 20% methanol in 10^{-4} mol/l aqueous sodium acetate. Reprinted with permission from Ref. [81].

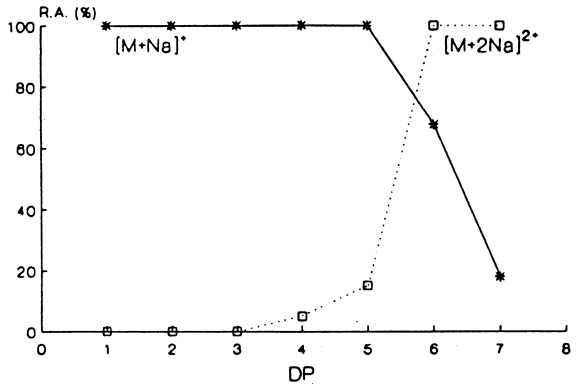


Fig. 7. Relative intensities of the $[M+Na]^+$ and $[M+2Na]^{2+}$ ions as a function of the degree of polymerization; 20 μg injected. Reprinted with permission from Ref. [81].

molecular-mass peaks were detected under ammonia CI, and for larger oligomers, for which an extensive fragmentation was observed. Similar considerations were given by the same authors in a previous work [82]; in this case, reversed-phase LC–MS was carried out with a mobile phase containing low concentrations of sodium acetate and intact sodiated molecules were observed in the PI mode, for maltodextrins up to a degree of polymerization of 10.

The characterization by HPAEC–TSP-MS of neutral and acidic oligosaccharides obtained by enzymic degradation of plant cell-wall polysaccharides was the subject of other studies by Niessen and coworkers [83,84]. Because of the incompatibility of the eluents used in anion-exchange chromatography with on-line HPAEC–MS, a device similar to that proposed by Simpson was used to couple these techniques. In the first work, the authors observed that in the positive-ion TSP mass spectra of acidic oligosaccharides signals due to the sodiated molecules $[M+Na]^+$, the sodiated sodium salts, e.g. $[M-H+2Na]^+$ and a sodiated fragment ion due to the release of water from the sodiated acid $[M+Na-H_2O]^+$ were detectable [83]. For polyuronic acids, additional peaks due to sodiated sodium salts are observed, e.g. $[M-2H+3Na]^+$ and $[M-3H+4Na]^+$ for a uronic acid trimer. These observations allow to determine the number of acidic sugar residues in a oligosaccharide. However, if HPAEC–MS has the important feature of obtaining molecular mass information from an oligosaccharide mixture without the need for time-consuming fraction collection and peak purification, the approach has two major drawbacks, i.e. the lack of structural information and the low sensitivity, since about 1–10 μg of a mixture have to be injected for the analysis. In these experiments oligosaccharides with DP values of 10 could be analyzed. The range of oligomers (DP values) suitable for HPAEC–MS depends mainly on two effects, i.e. the high sodium concentration needed for the elution of larger oligomers and the lack of sensitivity generally observed for oligomers with higher DP values. Also the sugar composition affects the upper DP values amenable to HPAEC–MS; if with α-1,4-glucose and α-1,5-arabinose samples, oligomers could be detected up to DP=6 and DP=9, respectively, oligomers of β-1,4-xylose were observed up to DP=25, as a consequence of the elution of the latter at low sodium acetate concentrations [84].

An improvement to the potential of the coupled technique HPAEC–MS via an AMMS for the analysis of oligosaccharides obtained by enzymic digestion of plant cell-wall polysaccharides is described in a subsequent study of the same research group [85]. In order to extend the applicability of this approach to large sugar oligomers by overcoming the limitations observed [83,84], the use of a new column material, CarboPac PA100, which requires lower sodium acetate concentrations for the elution of higher oligomers, is proposed. In addition, improved chromatographic peak shapes and better signal-to-noise ratio were observed by using multiple-ion detection. Under these conditions, the authors performed a comparison between positive- and negative-ion detection of oligosaccharides with the aim of finding the conditions for the best sensitivity. NI TSP experiments were carried out on α-1,4-glucose and arabinogalactose oligomers as test compounds. The formation of numerous adduct ions result in negative-ion spectra unexpectedly complex, as indicated by the various peaks detected for α-1,4-glucose oligomers with DP=4 and 8 (Table 2). The most abundant peaks in the mass spectra of oligomers with DP<5 are attributable to $[M-H]^-$, $[M+OAc]^-$ and $[M+HSO_4]^-$, whereas for larger oligosaccharides, various doubly charged ions are detected, with $[M+SO_4]^{2-}$ being the most intense. The authors predict a more extensive application of the NI mode in TSP

Table 2
Typical ions detected for α-1,4-glucose oligomers in the negative-ion mode with HPAEC–MS using a thermospray interface [85]

Charge	Ion identity	m/z value relative to M_r	m/z value for Glc_4	m/z value for Glc_8
−1	$[M-H]^-$	M_r-1	665	1313
−1	$[M+OAc]^-$	M_r+59	725	1373
−1	$[M+HSO_4]^-$	M_r+97	763	1411
−1	$[M+NaSO_4]^-$	M_r+119	785	1433
−1	$[M+HOAc+OAc]^-$	M_r+119	785	1433
−1	$[2M-H]^-$	$2M_r-1$	1331	n.d.[a]
−1	$[2M+HSO_4]^-$	$2M_r+97$	1429	n.d.
−1	$[2M+HOAc+OAc]^-$	$2M_r+119$	1451	n.d.
−1	$[2M+NaSO_4]^-$	$2M_r+119$	1451	n.d.
−2	$[M-2H]^{2-}$	$(M_r-2)/2$	n.d.	656
−2	$[M-H+OAc]^{2-}$	$(M_r+58)/2$	n.d.	686
−2	$[M+SO_4]^{2-}$	$(M_r+96)/2$	n.d.	705
−2	$[M+2OAc]^{2-}$	$(M_r+118)/2$	n.d.	716
−2	$[M+2HSO_4]^{2-}$	$(M_r+194)/2$	n.d.	754

[a] Not detected.

experiments because of the significant gain in sensitivity obtained especially for high oligomers in comparison with the PI mode.

An advantageous alternative to the TSP approach for the characterization of oligosaccharides is the LC–MS analysis performed via the ESP [82,86–88] and the ISP interfaces [89,90]. Preliminary experiments with an ESP interface have been performed by Niessen et al. who verified that sodium acetate-containing eluents were also suitable for an electrospray analysis of neutral sugar oligomers [82]. Mass spectrometric analysis of neutral and acidic oligosaccharides using ESP ionization in LC–MS was the subject of a qualitative study by Tinke et al. [86]. In a first step, various neutral and acidic oligomers were analyzed as test compounds in both positive- and negative-ion modes by performing the experiments in the constant-infusion mode. In the PI mode molecular mass information for neutral non-derivatized oligosaccharides could be achieved up to M_r 4000; under these conditions, the authors observed better sensitivity than that achieved in LC–TSP-MS experiments, especially for the higher-DP oligomers. The total ion intensity in the PI mode for both neutral and acidic compounds was 10 times higher than in NI mode, but with an increase of the degree of polymerization values this difference becomes less pronounced. The information obtained from this initial investigation was then applied to the characterization of methylated β-cyclodextrins and of unknown oligosaccharides enzymatically isolated from apple and pear fruit material. As for the analysis of unknown sugar oligomers, although structural information was lacking in the ESP mass spectra, the molecular mass determined allowed the calculation of possible sugar compositions in terms of monomeric units, e.g. of the number of pentose, hexose, deoxyhexose and uronic acids. This method used in conjunction with other information concerning the origin of the polysaccharide and the extraction procedure applied proved to be helpful in characterizing unknown oligosaccharides.

The advantage of on-line post-column addition of metal chlorides has been demonstrated for the LC–MS and LC–MS–MS analysis of carbohydrates using a triaxial electrospray probe. This probe consists of three coaxial stainless steel tubes; the sample, the metal chloride solution and the nebulizer gas flows through the innermost, the central and the outermost tubes, respectively [87]. After LC separation of two four-component carbohydrate test mixtures, the addition of a metal chloride solution to a sugar sample was accomplished directly within the ion source of the mass spectrometer, and metal-coordinated oligosaccharides were subsequently analyzed by MS and tandem MS. Enhanced sensitivity was primarily observed, since LC–MS with post-column addition of LiCl was capable of analyzing 1.7 pmol of oligosaccharide. In addition, in the case of the carbohydrate–cobalt complex, on-line LC–

MS–MS was effective in achieving relevant structural elucidation. The usefulness of this method should stimulate further research also for metal coordination and analysis of other substances such as peptides.

The performance of ESP and of FAB ionization for LC–MS were compared in terms of sensitivities in a detailed study on various derivatives of oligosaccharides [88]. A large number of derivatives, such as 2-aminopyridine (AP), 4-aminobenzoic acid ethyl ester (ABEE), 1-phenyl-3-methyl-5-pyrazolone (PMP) as well as 4-methoxyphenyl analogue, 2-aminoethanethiol (AET) and 2-aminobenzenethiol (ABT) derivatives, were considered. A previous study reported that the ABEE and the AP derivatives were sensitively detected by liquid secondary ion MS and electrospray MS techniques, respectively [91,92]. In frit-FAB-MS under flow injection analysis (FIA) conditions, maltopentaose labeled with ABEE provided the most intense $[M+H]^+$ ions in PI mode when ionized in glycerol or in thioglycerol matrix supplied in aqueous methanol. The lowest detection limit corresponding to 6 pmol of ABEE derivative of maltopentaose was achieved for this derivative in these matrices in water. Taking into accounts these preliminary results, the authors analyzed derivatives of isomaltooligosaccharides (DP= 2–20) by micro-LC coupled on-line with frit-FAB-MS. It was found that efficiency of separation seriously affects sensitivity in LC–MS, as exemplified by the negative-ion LC–frit-FAB-MS separation of an isomaltooligosaccharide mixture in SIM mode (Fig. 8). As for ESP ionization, a PMP derivative of maltopentaose gave the most abundant $[M+H]^+$ signals in PI mode (Table 3). Finally, glycoprotein-derived oligosaccharides were analyzed by LC–MS using both interfaces; LC–ESP-MS of oligosaccharides derived from thyroglobulin enabled the separation and the identification of high-mannose-type oligosaccharides having six to nine mannose residues.

On-line HPLC–pneumatically assisted electrospray-MS proved to be an attractive approach for the characterization of sugar oligomers derived from glycoproteins [89,90]. Henion's group investigated the experimental conditions for ionspray and MS–MS characterization of complex, underivatized N-linked oligosaccharides, which are usually found in biological systems [89]. The PI mode was well suited for oligosaccharides which did not contain sialic acid residues, and the addition of 10 m*M* sodium acetate or ammonium acetate to the carbohydrate solutions significantly enhanced the analyte response in this detection mode; sugar oligomers which contained sialic acid were best analyzed in the NI mode after deprotonation. Tandem MS characterization of these oligosaccharides produced fragment ions deriving from cleavages of glycosidic bonds. In addition, the analysis of the heterogeneous mixture of N-linked oligosaccharides obtained from ovalbumin was carried out; ISP mass analysis of this glycoprotein allowed differentiation of six oligosaccharides of different molecular mass which were attached at the N-glycosylation site (Fig. 9). The positive-ion electrospray mass spectrum of the oligosaccharides digested from ovalbumin with N-glycanase allowed characterization of more than 15 oligosaccharides with different molecular masses. A limitation of the proposed LC–ISP-MS method is the inability of mass spectrometry to differentiate isomeric sugars, so that chromatographic selectivity is needed to fully characterize the carbohydrate mixture.

On-line coupling of HPAEC and ISP-MS through the use of a membrane suppressor for desalting has been demonstrated by Conboy and Henion [90]. The method was applied to the determination of high-mannose oligosaccharides derived by the treatment of RNase B with endoglycosidase. Sugar oligomers of the type GlcNAc–$(Man)_n$ with n=5–9 were observed in these experiments.

Use of LC–MS proved to have considerable advantages in this research area, both as a technique for the confirmation of identity of known oligosaccharides and as an aid in the characterization of unusual oligosaccharides; in particular, on-line LC–MS makes rapid identification of unknown carbohydrates feasible without the need for laborious and time-consuming fractionation and purification, taking into account that well-defined sugar oligomers are produced in the degradation of polysaccharides by specific enzymes. The electrospray/ionspray approach has been demonstrated more useful than the thermospray strategy, especially for the LC–MS analysis of the largest oligosaccharides. In spite of the potential of this technique, the number of studies performed using ESP/ISP ionization in this field is still too limited, but the published results are promising particularly using tandem mass spectrometry.

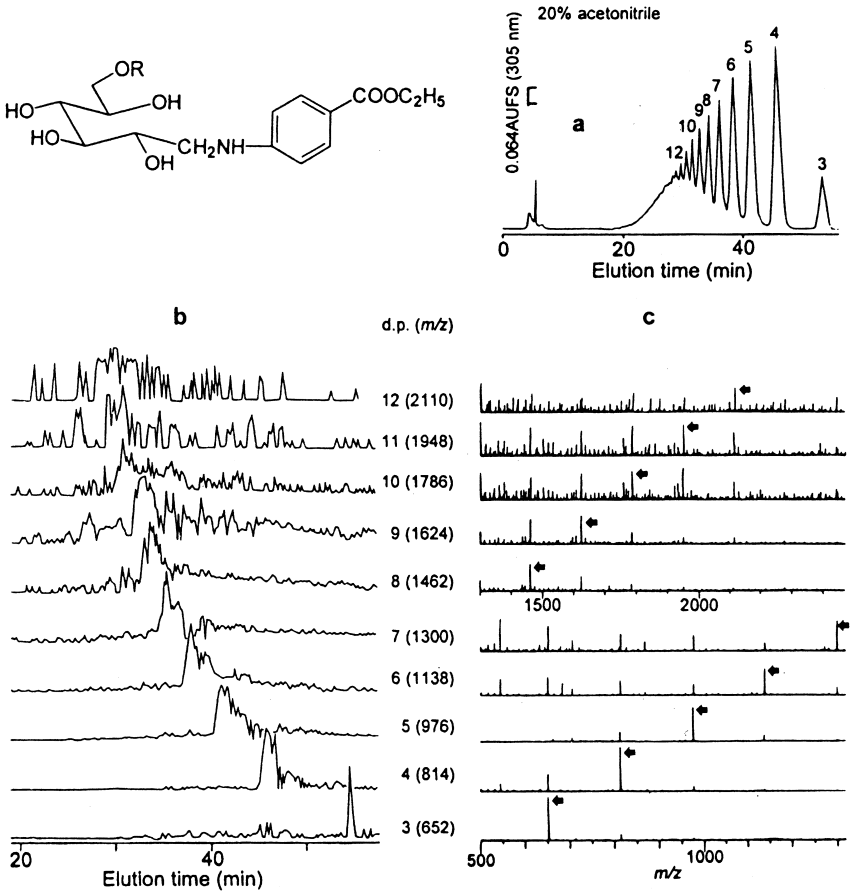


Fig. 8. Negative-ion LC–frit-FAB-MS of an isomaltooligosaccharide mixture as ABEE derivatives. (a) Elution profile obtained by UV detection at 305 nm. (b) Selected ion chromatograms. (c) Mass spectra at the peak positions. Column, Develosil ODS (0.5 mm I.D., 15 cm); eluent, 50 m*M* acetic acid (pH 5.2 with triethylamine) containing 0.8% (w/v) glycerol–acetonitrile (linear gradient of 2–4% in 5 min then to 8% in 40 min); flow-rate, 5 μl/min; amount of sample, 100 μg as an isomaltooligosaccharide mixture. Adapted from Ref. [88].

2.3. Vitamins

The few studies on the LC–MS analysis of fat-soluble and water-soluble vitamins have revealed that this coupled technique has considerable potential in the characterization of these thermally labile

Table 3
Relative intensities of the $[M+H]^+$ signals of various derivatives of maltopentaose in ESP-MS[a] [88]

Sample concentration (pmol/μl)	Derivative				
	AP	ABEE	PMP	PMPMP	ABT
1000	5.80	4.00	100	105	2.80
100	12.2	14.1	100	115	6.80
10	9.70	14.5	100	80.5	9.90
1	46.6	21.9	100	77.9	12.1
0.2	49.6	52.7	100	79.9	21.6

[a]The data were expressed as the values relative to those of the PMP derivative.

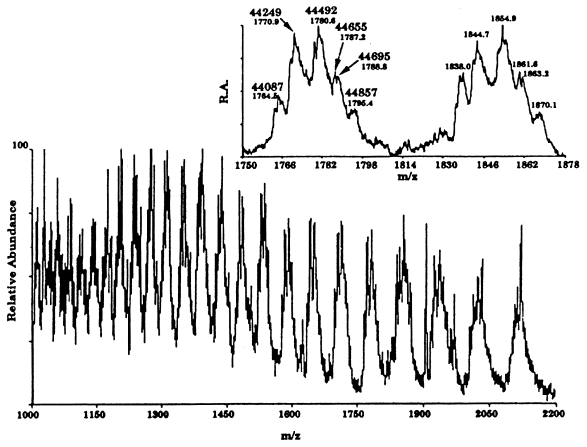


Fig. 9. Positive-ion electrospray mass spectrum of ovalbumin, a glycoprotein with one N-glycosylation site. Within each charge state of the glycoprotein at least six different molecular mass glycoforms (labeled) that result from heterogeneity of oligosaccharide composition can be distinguished. Ovalbumin $(M+24H)^{24+}$ and $(M+25H)^{25+}$ are pictured in the figure inset. Reprinted with permission from Ref. [89].

compounds [93–108]. Accurate determination of vitamins is very important in both food and pharmaceutical areas, but in most cases conventional detection methods for HPLC suffer from a lack of sensitivity and/or selectivity. On the other hand, mass spectrometry possesses outstanding characteristics for the identification of isolated substances with extremely high sensitivity.

Cyanocobalamin (vitamin B_{12}) has been used as model compound to test the potential of the direct liquid introduction LC–MS system under CI conditions [93]. A good quality negative-ion mass spectrum was obtained by introducing 92 ng of the analyte into the CI source; an intense deprotonated molecule at m/z 1353 and an adduct ion at m/z 1371 attributable to $[M-H+H_2O]^-$ were observed. Instead, DLI-LC–MS analysis did not provide a useful informative positive-ion mass spectrum of vitamin B_{12}, since an extensive fragmentation at low mass was observed; these results were not in accordance with those reported by the group of Vestal, who obtained a good quality positive-ion mass spectrum under thermospray conditions [94].

Vitamin B_{12} has been also analyzed by Sakairi and Kambara in a study aimed to evaluating the characteristics of the atmospheric pressure spray ionization method for LC–MS analysis of thermolabile and involatile substances [64]. The authors were able to detect significant protonated and cationized ions with minor fragmentation, the base peak for this analyte being the sodiated molecule at m/z 1377.

Another fundamental study discusses the evaluation of laser desorption atmospheric pressure ionization for interfacing LC in mass spectrometry for the analysis of nonvolatile biological species; among these compounds, water-soluble vitamins such as niacin, pyridoxine hydrochloride, riboflavin and thiamine hydrochloride were considered [95]. In neutral desorption followed by soft ionization at atmospheric pressure one or two fragment peaks dependent on the analyte were detected. The compounds which are in the form of hydrochlorides exhibited intense peaks due to the elimination of HCl, which could be released in either the desorption or ionization step.

The DLI approach for LC–MS analysis of vitamins has been taken by Azoulay et al. for the quantitative assay of biotin and dethiobiotin methyl esters in complex biological samples [96]. When operating in the PCI mode with SIM, the detection of biotin and dethiobiotin methyl esters at levels of 10 ng was feasible, and the calibration graphs were linear up to 300 ng. Using the trideuteromethyl esters of the compounds as internal standards, linearity over the 10 ng–1 μg range of biotin and dethiobiotin esters injected was observed. Good results were reported by applying the LC–MS method to the quantitation of the conversion dethiobiotin→biotin by *Bacillus sphaericus*.

An LC–MS method for the assay of the E vitamers, i.e. α-, β-, γ- and δ-tocopherol and α-tocotrienol in maize-germ oil has been developed [97]. After dissolution of the sample in *n*-hexane and direct injection of an aliquot into the LC–MS system, separation was carried out in normal-phase chromatography and the eluates were fed to the mass spectrometer via the MB interface; it is noted here that this system was chosen because it is particularly appropriate for normal-phase LC experiments. Both EI and CI mode were investigated, but electron impact proved to be the most sensitive method for the determination of these substances in seed oil samples.

TSP interfaces have been proposed for the LC–MS analysis of both water-soluble [98,99] and fat-soluble vitamins [100,101]. The behaviour of a buffer consisting of formic acid and ammonium formate was investigated as a new acidic eluent for the thermospray ionization of vitamins such as thiamine, nicotinic acid, nicotinamide, pyridoxine and pantothenic acid [98]. The new buffer significantly repressed peak tailing of amines, leading to an improvement in detection limits of these analytes; an improvement in separation due to a larger retention than that obtained using the common TSP buffers was also observed for the acidic species, such as nicotinic and pantothenic acid. This method was then successfully applied to the assay of the above-mentioned vitamins in a commercial health drink and in the fermented soybean paste *miso* [99]. Mass spectra of all the compounds tested provided the $[M+H]^+$ ion as the quasimolecular ion, whereas no signals of the ammoniated molecules were observed as a consequence of the difference in the proton affinity between the analytes and the mobile phase. By using the SIM acquisition mode, linearity, detection limit and reproducibility were evaluated for the five compounds; calibration graphs were linear from 0.5 to 50 ppb of the analytes and detection limits ($S/N>$ 10) were 10–100 pg. The assay of the target vitamins in a commercial health drink was feasible without any treatment, as is shown in Fig. 10, which depicts the TIC and the mass chromatograms of the characteristic ions of the vitamins in this sample. Mass chromatograms were free from peak tailings and allowed to overcome the complexity of the TIC signal due to the presence of co-eluting interfering compounds. The specificity of the LC–TSP-MS method allowed to determine trace amounts of thiamine in the *miso* sample.

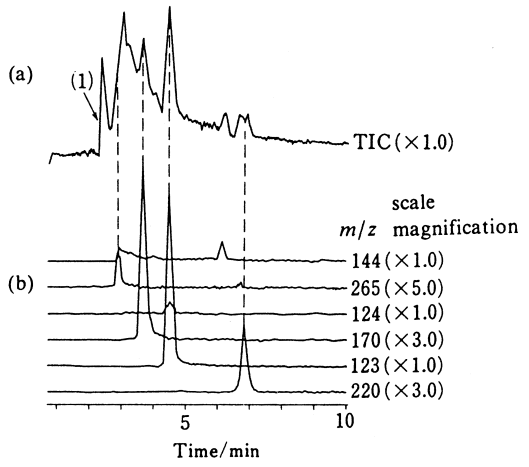


Fig. 10. (a) Total ion chromatograms of a commercial health drink and (b) mass chromatograms of characteristic ions of water-soluble vitamins. m/z 144 is basepeak of thiamine; m/z 265 is MH^+ ion of thiamine; m/z 124 is MH^+ ion of nicotinic acid; m/z 170 is MH^+ ion of pyridoxine hydrochloride; m/z 123 is MH^+ ion of nicotinamide; m/z 220 is MH^+ ion of calcium panthotenate. LC conditions: Shim-pack CLC-ODS(M) column (5 μm, 4.6 mm I.D.×150 mm); mobile phase, acetonitrile–water containing 0.1 *M* formic acid and 0.1 *M* ammonium formate (pH 3.5) (7:93) (flow-rate 1 ml/min); temperature, 50°C; TSP interface. Reprinted with permission from Ref. [99].

TSP mass spectrometry provided reliable identification of vitamin D_2 in shiitake mushrooms after isolation of the compound by TLC and HPLC [100]; the TSP mass spectrum indicated that no thermal decomposition of vitamin D_2 had occurred, which is a typical problem in the GC–MS of this thermolabile substance.

HPLC–TSP-MS has been successfully used by Vreeken et al. to quantify vitamin D_3 and some of its metabolites, such as 1α(OH) vitamin D_3, 25(OH) vitamin D_3, 1α25$(OH)_2$ vitamin D_3, 24,25$(OH)_2$ vitamin D_3 [101]. On-line post-column Diels-Alder derivatization, which requires 1 min at room temperature, was performed, and TSP detection was carried out in PI and NI modes. The two ionization techniques are complementary for the compounds investigated; in fact, using these two modes, it was possible to distinguish positional isomers. NI detection yielded the best results in terms of sensitivity; discharge ionization in NI mode led to detection limits down to 0.12 pmol injected.

An interesting approach based on the use of the phase-system switching technique has been applied by Asakawa et al. to overcome the problems imposed by the use of involatile buffers in the eluents [102]. An LC–MS system with a frit-FAB interface was used for the determination of tocopherol and riboflavin. Valuable spectral data were given in the case of riboflavin, for which the FAB spectrum obtained with LC–frit-FAB-MS showed an intense protonated molecule and a series of interpretable fragment ions. The column-switching technique allowed the use of optimum mobile phases containing

involatile buffers to separate the target analytes in the LC–frit-FAB-MS system, without incurring the problem of inhibition of ionization.

Recently, HPLC coupled with APCI-MS has been used for the assay of vitamin D_3 and D_2 in multivitamin tablets [103] and for the analysis of thiamine in dried yeast [104]. After a reversed-phase separation of vitamin D_3 and its analogs, the eluate was fed into the APCI interface and vitamins were determined in the selected ion monitoring mode [103]. Under SIM conditions, analytes could be detected at the 0.5-ng level at a signal-to-noise ratio of 2.

As for the analysis of thiamine, the APCI-MS mass spectrum displayed an abundant molecular ion and characteristic fragment ions useful for structural analysis of thiamine, when injecting 50 ng of this compound [104]. The LC–APCI-MS detection limit ($S/N=3$) obtained in the SIM mode was estimated to be 2 ng, and the linearity of the peak intensity versus the injected amount of thiamine was established in the 2–50 ng range. The vitamin was then identified in an ion-pair extract of dried yeast and the results were compared with those obtained by the thiochrome method showing an excellent agreement.

The PB interfacing system has been extensively evaluated for the analysis of both fat-soluble [105,106] and water-soluble vitamins [107]. After investigating the proper ionization method for vitamin A, A acetate, A palmitate, D_3, E and E acetate, a LC–PB-MS system under EI conditions was applied to the identification of vitamin A, D and E in foods and in multivitamin preparations [105]. The applicability of the LC–PB-MS system was verified for the analysis of these thermally labile substances in complex matrices with the simplest treatment of the samples. Detection limits for the fat-soluble vitamins analyzed were typically in the 0.6–25 ng range under SIM conditions. A similar approach was taken for the determination of vitamin K_1 in vegetables by HPLC with PB-MS detection [106]. LC–MS experiments were performed in both EI and CI modes, the negative-ion CI mode being the most valuable approach both in terms of sensitivity and selectivity. A detection limit ($S/N=3$) of 2 ng was calculated when using the single-ion monitoring mode in NI operation. The proposed LC–PB-NCI-MS method allows analysis of vitamin K_1 at low levels (0.1 μg/g) with high selectivity, as demonstrated in the case of tomato samples, for which the conventional UV detection provided inaccurate data. Co-elution of vitamin K_1 with lycopene is clearly evident in the LC–PB-NCI-MS mass chromatograms of a crude tomato extract (Fig. 11). In addition to reliable identification and quantitation of the target analyte, the advantage of using the LC–PB-MS method is that the on-line characterization of the matrix can also be performed by obtaining spectral information concerning the other compounds eluting with the analytes considered.

Very recently, the same research group explored the applicability of the LC–PB-MS system for the analysis of 11 water-soluble vitamins [107]. The possibility of overcoming the problem of the use of aqueous eluents with the particle beam interface by using a narrow-bore column was successfully verified, in spite of the inherent scarce compatibility of

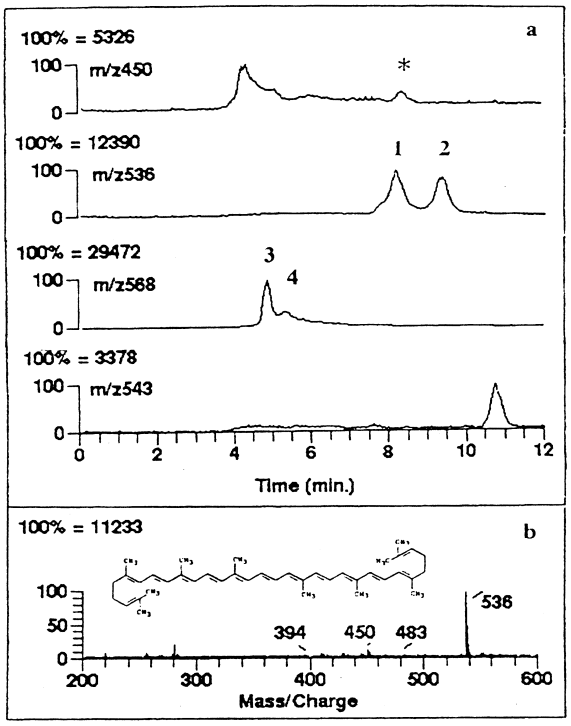


Fig. 11. Analysis of the crude tomato extract. (a) Selective display at m/z 450 (vitamin K_1), m/z 536 (1=lycopene; 2=β-carotene), m/z 568 (3=lutein; 4=zeaxanthin), m/z 543 (phytoene). (b) PB NCI mass spectrum of lycopene. Reprinted with permission from Ref. [106].

the PB interface with a water-rich eluents. Among the ionization modes explored, generally the best sensitivity was obtained in PCI mode. Under these conditions and operating in SIM mode, linearity, detection limits and precision of the analysis was evaluated. Promising results were obtained for nicotinamide and nicotinic acid in PCI mode and for pyridoxal in NCI mode, detection limits being at the low-ng level.

This class of vitamins has also been studied by using a gas-phase ionization techniques, i.e. APCI, and a liquid-based ionization technique, i.e. ISP [108]. The two API-based techniques provided comparable results in terms of structural information, even though in the APCI mass spectra the abundances of the quasi-molecular ions were higher for all the compounds investigated. To enhance the identification potential, the APCI-MS–MS approach was applied and, in particular, valuable spectral data were obtained for the three vitamers of vitamin B_6, i.e. pyridoxal, pyridoxine and pyridoxamine. The use of structure-specific fragmentation in the triple-stage quadrupole CID spectra of the vitamins permitted the fast screening of a vitamin mixture without the need for LC separation.

The above applications show that LC–MS with PB and HN-APCI interfacing systems can be used successfully in the identification and determination of vitamins. Even though so far very few applications of APCI to LC–MS analysis of these substances have been reported, this technique is expected to be more exploited in this area as a consequence of its versatility, high eluent flow-rate capability and sensitivity. In addition, it is known that APCI is suited to the analysis of low-molecular-mass compounds [20] and most of the vitamins present this feature.

2.4. Miscellaneous natural substances in food

In the past years, there has been a notable increase in the amount of literature pertaining to the LC–MS analysis of non-nutritive food chemicals for their potential antioxidative and anticarcinogenic effects, many of them being low-volatility thermolabile or ionic compounds. Other natural components of foods are recognized as responsible agents for the multiple organoleptic properties of food products. In this context, methodologies involving different LC–MS interfacing systems have been developed to detect, identify and quantify flavonoids and related compounds, glucosinolates, phenolic compounds and other various naturally occurring substances in food extracts. The use of LC–MS in the analysis of food materials provides important structural information on target or unknown substances directly in their matrices; in some cases the coupled technique can afford a full on-line structural analysis involving no time-consuming isolation process.

2.4.1. Flavonoids and related compounds

The flavonoids, which are glycosides with a benzopyrone nucleus, represent one of the large groups of secondary metabolites occurring widely in plants; they are found particularly in the leaves and petals and are widely distributed in edible parts of food plants. Although some occur in free form (Fig. 12), exhibiting in this case phenolic properties, more compounds exist in nature as the glycoside form. D-Glucose is the most frequent sugar residue but the sugar part can also consist of D-galactose, L-rhamnose, L-arabinose and D-xylose, as well as some uronic acids. Structural variation among flavonoids is considerable, since changes in the number of sugar residues, in the binding site on the aglycones and in acylation in sugar residues provide different varieties. Potential reactivities with active oxygen species are the main characteristic of flavonoids; lipid antioxidant features of quercetin and other flavonoids, which are believed to be due to their reactivity with free radicals participating in the peroxidation reaction, have been known for many years [109].

The analyses of flavonoids are usually carried out by TLC and HPLC using refraction index or UV detection, which have the drawbacks of not being sufficiently selective for the unambiguous identification of targeted flavonoids. On the other hand, electron impact mass spectrometry is a powerful tool for elucidating flavonoid structures [110]. Since flavonoids are usually found as complex mixtures in plants, the particular mixture varying according to the plant examined, hyphenated techniques are needed; among these, LC–MS represents a rapid and reliable technique to analyze these involatile substances. Flavonoids have been analyzed both in food

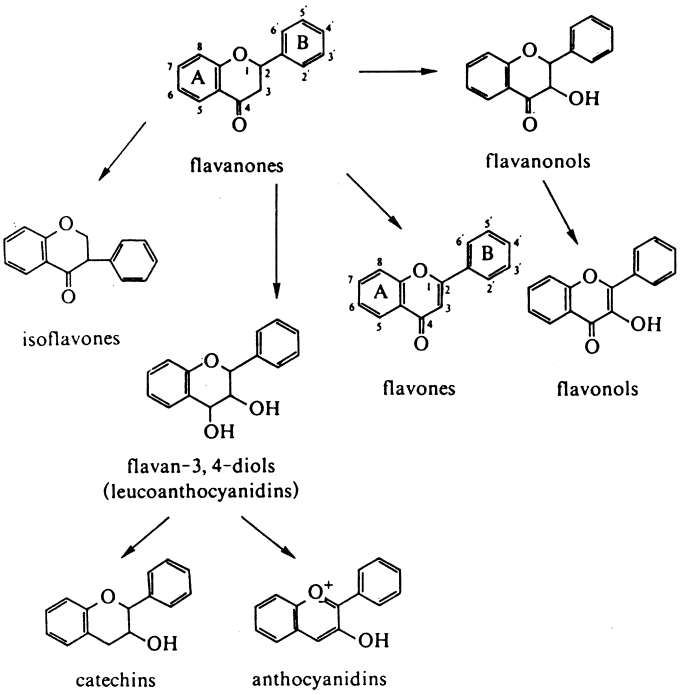


Fig. 12. Sub-classes of flavonoids. Adapted from Ref. [109].

and in plant extracts using on-line HPLC–MS; the present overview also discusses applications of LC–MS in the analysis of these substances in food-related plant materials [111–113,115–121,123,125].

Analysis of polymethoxylated flavones in Valencia orange peel oil and juice was carried out by direct liquid introduction LC–MS and LC-UV [111]. After separation of flavones on a C_{18} column using a water–acetonitrile mixture (3:2) as eluent, mass data provided useful structural information on the different compounds present in the extracts. Seven polymethoxylated flavones were identified in Valencia orange juice and eight in the peel oil; their structures were established as tangeritin, tetra-O-methylscutellarein, heptamethoxyflavone, nobiletin, sinensetin, dimethyltangeretin, 5,7,8,3′,4′-pentamethoxyflavone, and 3,5,6,7,3′,4′-hexamethoxyflavone.

A moving belt LC–MS method has been proposed to study flavonoids under both EI and CI conditions [112]; for this purpose, representative substances of four flavonoid aglycone classes, i.e. flavones, flavonols, flavanones and flavanonols, covering an extensive range of polarities were examined. If good quality EI and CI mass spectra were obtained for the low- and medium-polar flavonoids considered, the interface used proved unsuitable for the mass spectral analysis of high-polarity and involatile compounds. In addition, the comparison between the detection limit of a low-polar flavonoid and of a medium–high polar compound obtained by directly spotting on the belt and by injecting into the LC column showed a dramatic loss in sensitivity for the high-polarity and involatile flavonoids. The authors predict that HPLC–FAB-MS using the MB interface may give improved detection performance for the more polar and non-volatile derivatives.

LC–MS of two isoflavonoids and one triterpenoid in a licorice root extract powder using a PB interface has been described [113]. Many naturally occurring compounds in food have been recognized as anticarcinogenic agents because of their inhibitor effect on various initiating and promoting mechanisms of chemical carcinogenesis; among these, isoliquiritigenin contained in licorice root is known to inhibit the formation of skin papilloma [114]. The LC–PB-MS approach was demonstrated to be

adequate for the identification of the isoflavonoids formononetin and isoliquiritigenin and the triterpenoid glycyrrhetinic acid, which were identified in the LC eluates on the basis of the corresponding PB-EI mass spectra. In the case of the GC–MS method, it was necessary to obtain the corresponding mono- or disilylated derivatives, since the involatility means that these substances are not amenable to direct analysis by GC–MS (Fig. 13).

Analytical methods utilizing TSP-LC–MS for the analysis of flavonoids have been extensively applied [115–121]. An HPLC–TSP-MS method proved useful in analyzing phytoestrogens in various soya protein preparations [115]. Best separations of the phytoestrogens daidzein, genistein, coumestrol, formononetin and biochanin A were obtained in reversed-phase mode using a methanol–0.1 *M* ammonium acetate buffer of pH 4.6 (3:2) containing 0.25 m*M* EDTA as mobile phase. After extraction of soybean products with aqueous 80% ethanol to obtain isoflavones, their polar conjugates and related substances, the extracts were submitted to enzymic hydrolysis before LC analysis. The compounds were positively identified in the chromatographic effluent by performing MS analysis in the range m/z 110–300; daidzein and genistein were detected in high

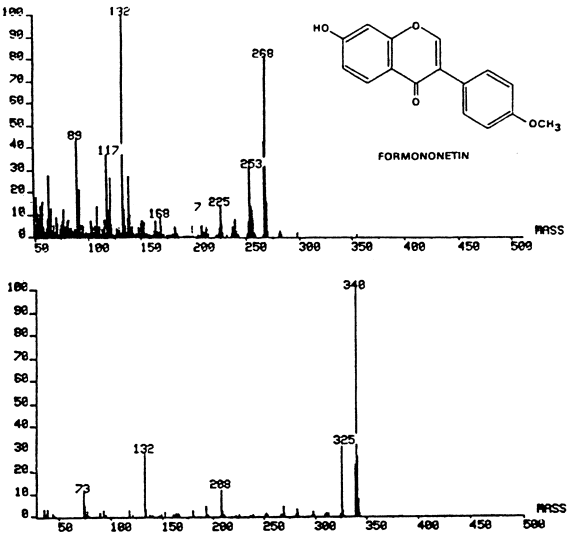


Fig. 13. Mass spectra of (upper) component identified as formononetin, obtained by LC–MS, and (lower) component identified as monosilylated formononetin, obtained by GC–MS. Reprinted with permission from Ref. [113].

concentrations in soybean milk formulas, soybean flakes and textured soybean protein. In another study of the same group, thermospray MS–MS was shown to be a useful technique yielding valuable structural information, which was lacking in the mass spectra of the phytoestrogens recorded under TSP-MS conditions [116]. The resultant product ion spectra contained fragments characteristic of each phytoestrogen subclass, allowing the use of tandem MS to confirm identification and propose structures for unknown compounds. TSP-MS–MS analysis of these substances was also carried out in the neutral loss operating mode by monitoring the neutral loss of 56 (due to consecutive releases of CO) which is common to all members of this family. Several soy protein preparations were investigated to confirm the presence or absence of phytoestrogens; in one soybean product analyzed, daidzein, genistein and an unknown phytoestrogen of the Biochanin A subclass were found; this unknown was tentatively identified as 6,7-dihydroxy-4′-methoxyisoflavone using its product ion spectrum.

HPLC coupled with thermospray mass spectrometry was investigated as a reliable method for analyzing acetylated derivatives of the most abundant apigenin-7-O-β-glucoside from *Chamomilla recutita* [117]. The LC–MS technique allowed to identify four isomeric monoacetates and two diacetates; except for apigenin-7-(6″-O-acetyl)-glucoside all acetylated derivatives were found to undergo fast ester hydrolysis.

Coupling of a TSP interface with tandem MS enhances the identification potential; by using a thermospray LC–MS–MS strategy, catechins and other flavonoids were successfully characterized in complex mixtures (Fig. 14) [118]. Four major catechins, (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin 3-O-gallate and (−)-epigallocatechin 3-O-gallate were identified in a mixture extracted from the tea plant *Camellia sinensis* by their protonated molecules. Collision-induced dissociation spectra of the $[M+H]^+$ ions gave simple yet structurally informative patterns, which allowed assignment of the substituents and the ring structures of the molecules. In the same work, the authors studied TSP-MS and CID mass spectra of representative flavonoids, i.e. one flavanone, one flavone and three flavonols; CID spectra were able to discriminate these flavo-

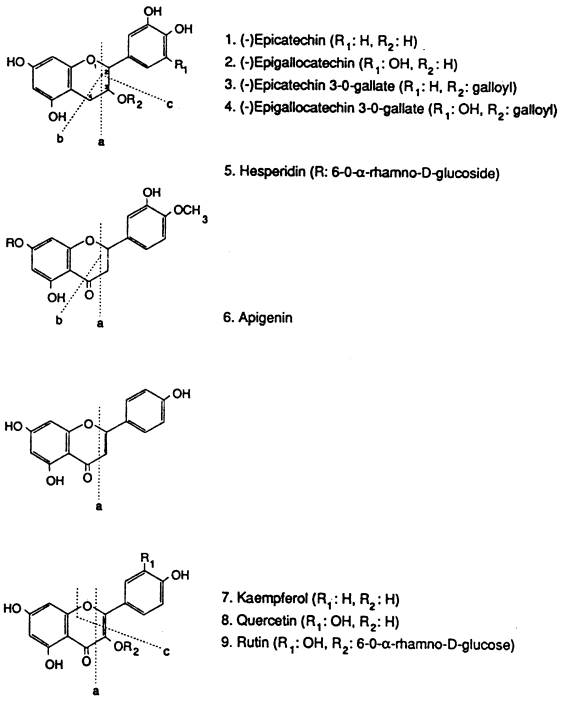


Fig. 14. Structures of catechins and flavonoids studied by thermospray LC–MS and thermospray LC–MS–MS. Reprinted with permission from Ref. [118].

noid classes on the basis of three types of ring cleavages in the pyran ring of the molecules, as is shown in Fig. 14. TSP-MS and CID mass spectra of the catechins and flavonoids investigated are reported in Table 4. From the relevant structural information obtained by using the thermospray-LC–MS–MS, it can be inferred that this approach appears to be the method of choice for the study of polar and scarcely volatile substances, such as catechins and flavonoids, including their glycosides, in complex mixtures.

Using the LC–TSP-MS technique, flavonol glycosides, flavanols, flavanol gallates, 4-coumarylquinic acid, and chlorogenic acids were analyzed in black tea liquor [119]. Bailey et al. used the TSP interface in the discharge mode, the so-called plasmaspray, which provides more fragmentation than thermospray and can analyze analytes belonging to different classes with less difficulty without ammonium acetate in the eluent [4]; in fact, a complex matrix, such as a black tea liquor, contains several classes of substances which would require different conditions for optimal optimization. LC–MS with the plasmaspray interface was demonstrated to be useful for the analysis of this sample; ion-current chromatograms were obtained for the pseudo-molecular ions of kaempferol and quercetin, i.e. the aglycones of the main flavonol glycosides, and for the pseudo-molecular ions of the flavanols and flavanol gallates, 4-coumarylquinic acid and chlorogenic acid (Fig. 15). The authors claimed unsuccessful results for myricetin and its glycosides, as a consequence of the instability of this flavonol in the plasmaspray interface.

In a research program aimed at evaluating power-

Table 4
Thermospray MS and CID spectra of catechins and flavonoids [118]

Compound	Thermospray MS m/z (rel.%)[a]		CID spectra m/z (rel.%)[a]			
	$[M+H]^+$	(Others)	A)	(B)	(C)	(Others)
1	*291*(100)	275(6)	139(100)	165(10)	123(58)	207(18), 147(13)
2	*307*(100)		139(100)	181(10)	139(100)	223(4), 163(7)
3	*443*(100)	291(40),275(100),273(24)	139(24)	165(5)	123(100)	291(47), 273(18), 153(14), 151(22)
4	*459*(100)	307(13),291(100),285(25)	139(100)	181(5)	139(100)	307(17), 289(34), 153(14), 151(18)
5	611(100)	465(8)				
		303(100)	153(100)	177(68)	—	
			149(19)			
6	*271*(100)		153(89)	—	—	
			119(100)			
7	*287*(100)		153(100)	—	121(70)	
8	*303*(100)		153(100)	—	137(60)	
9	*611*(8)	465(3)	—	—	—	465(27), 303(100), 165(7), 147(24), 129(45)
		303(100)	153(100)	—	137(70)	

[a]CID spectra were obtained with the italicized MS ions.

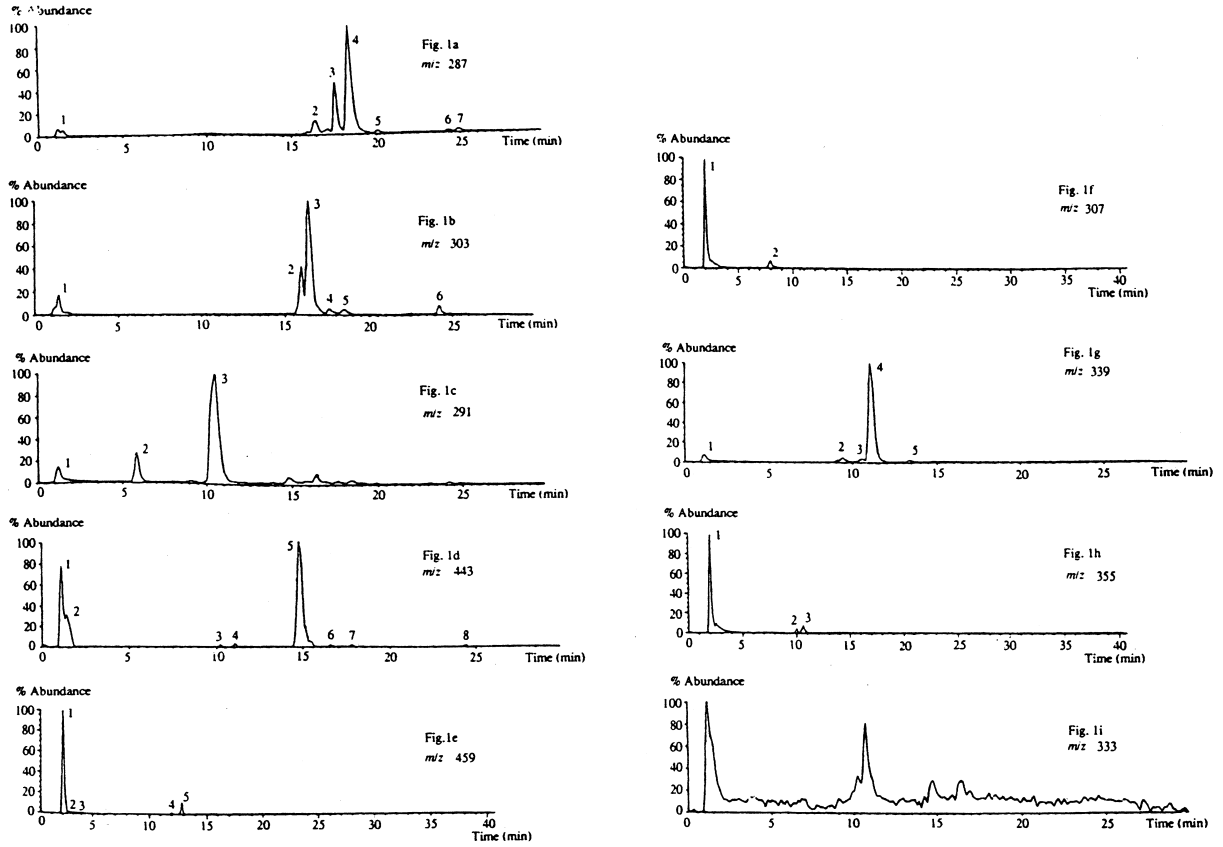


Fig. 15. Ion current chromatograms extracted from the plasmaspray LC–MS of a black tea liquor. (a) *m/z* 287, kaempferol+1; (b) *m/z* 303, quercetin+1; (c) *m/z* 291, (+)-catechin+1 or (−)-epicatechin+1; (d) *m/z* 443, (−)-epicatechin gallate+1; (e) *m/z* 459, (−)-epigallocatechin gallate+1; (f) *m/z* 307, (+)-gallocatechin or (−)-epigallocatechin; (g) *m/z* 339, *p*-coumarylquinic acid+1; (h) *m/z* 355, chlorogenic acid+1; *m/z* 333, (i) fragment of an unresolved polymer. Reprinted with permission from Ref. [119].

ful methods for plant extract screening, conditions have been established for the LC–TSP-MS analysis of flavonol glycosides [120]. Because of the thermolability of these compounds, good optimization of the temperature of the TSP vaporizer was crucial for the observation of their molecular ions. The mass spectra recorded under TSP buffer conditions provided very valuable structural information on molecular ions, sugar sequence and glycosylation site.

The application of TSP-MS and DAD coupled with HPLC has proved very useful in the analysis of lemon peel extract [121]. After a reversed-phase separation of the different fractions of the sample extract, the mass spectrometer was operated in the negative-ion discharge-assisted thermospray mode; mass spectra displayed molecular ions and diagnostic fragment ions (Table 5). The combined data from LC-UV diode array detection and LC–TSP-MS permitted the identification of various compounds belonging to four different classes of flavonoids, i.e. flavones-O-glycosides, flavones-C-glycosides, flavonols and flavanones, together with limonoids, coumarins and a phenyl propanoid glycoside. Among these constituents, limonoids are of interest because they are responsible of the bitterness of the citrus juices. The described methodology is quite simple because there is no need for isolation of the individual substances prior to LC–MS characterization of the products.

As for coumarins, a thorough study on the applicability of LC–MS using a micro particle beam interface [122] to a large number of these substances

Table 5
List of identified compounds and MS fragments in the negative-ion mode using a thermospray interface [121]

Compounds	Observed masses
Limonoids	
(1) Nomilinic acid	$[M-1]^-=531$
(2) Limonin	$[M]^-=470$
(3) Limonoic acid	$[M-1]^-=487$
(4) Limonin 17-β-D-glucoside	$[M-1]^-=649$
Phenyl propanoids	
Coumarin	
(5) Isoimperatorin	$[M]^-=282$
(6) Bergamottin	$[M]^-=328$
(7) Bergaptol	$[M]^-=196$
(8) Limettin derivative a	$[M]^-=260$
(9) Limettin derivative b	$[M]^-=328$
Phenyl propanoid glycosides	
(10) Citrusin A	$[M]^-=520$; $[M-162]^-=358$
Flavonoids	
Flavones-C-glucosides	
(11) 6,8-Di-C-glucopyranosyl-luteolin	$[M]^-=642$
(12) 6,8-Di-C-glucopyranosyl-apigenin	$[M]^-=626$
Flavonols	
(13) Limocitrol	$[M]^-=376$; $[M-30]^-=346$; $[M-60]^-=316$
(14) Isolimocitrol	$[M]^-=376$; $[M-30]^-=346$; $[M-60]^-=316$
(15) Limocitrin	$[M]^-=346$; $[M-30]^-=316$
(16) Rutin	$[M]^-=610$; $[M-146]^-=464$; $[M-308]^-=302$
Flavones-O-glucosides	
(17) Diosmetin-7-rutinoside	$[M]^-=608$; $[M-308]^-=300$
Flavanones	
(18) Hesperitin-7-rutinoside (hesperidin)	$[M]^-=610$; $[M-146]^-=464$; $[M-308]^-=302$
(19) Eriodictyol-7-rutinoside (eriocitrin)	$[M]^-=596$; $[M-146]^-=450$; $[M-308]^-=288$
(20) Naringenin-7-rutinoside	$[M]^-=580$; $[M-146]^-=434$; $[M-308]^-=272$

has been carried out by Cappiello et al. [123]. These natural products, which can be isolated from plants and essential oils, are widely studied because of their important pharmacological properties and because of their toxicological risks to human health [124]. After optimization of the interface parameters, reproducible and structurally informative PB-EI mass spectra were obtained from the 18 coumarins investigated. Instrument detection limits for these analytes ranged from about 1 to 70 ng at a signal-to-noise ratio of 5 by operating in SIM mode without the LC column. The proposed method was then applied to identify and determine several coumarins extracted from a sample of *Smyrnium perfoliatum* L. (Umbelliferae) as a typical example of coumarin-containing plants.

Recently, HPLC with APCI-MS and ISP-MS has been investigated for the first time for the analysis of isoflavones and their conjugates in soy food products [125]. These naturally occurring compounds, which are found in concentrations ranging from 0.1 to 5 mg/g in soybeans and in soy-derived foods, play a role in reducing risks of certain cancers [126]; for this reason, it is of particular interest to qualitatively and quantitatively analyze the composition of isoflavones in soy foods. The analytes were detected in both PI and NI mode using the heated nebulizer-APCI and the ISP interfaces. The more valuable spectral data about each isoflavone conjugate were obtained using the HN-APCI system in positive-ion mode. Enhanced sensitivity was also observed for the positive isoflavone aglucone ions produced in the HN-APCI interface; these ions were about 1.5–3 times more intense than the protonated molecules generated in the ISP interface. The effect of mobile phases containing 0.1% acetic acid or 10 m*M* ammonium acetate was also described as for the major ions detected in the HN-APCI negative-ion mass spectra of the isoflavone conjugates (Table 6).

Table 6
Ions observed for isoflavone conjugates by atmospheric pressure chemical ionization-mass spectrometry in the negative mode[a] [125]

Isoflavone[b]	*m/z*	Relative abundance	
		10 m*M* NH_4OAc	0.1% Acetic acid
Daidzein 6-OMalGlc	517 $[M-COOH+CH_3COOH]^-$	36.5	66.7
	457 $[M-COOH]^-$	14.2	32.6
	253 $[M-H-MalGlc]^-$	100.0	100.0
Daidzein 6-OAcGlc	517 $[M-H+CH_3COOH]^-$	40.7	63.8
	457 $[M-H]^-$	16.4	30.8
	253 $[M-H-AcGlc]^-$	100.0	100.0
Daidzin	475 $[M-H+CH_3COOH]^-$	24.1	100.0
	415 $[M-H]^-$	8.5	39.5
	253 $[M-Glc]^-$	100.0	100.0
Genistein 6-OMalGlc	533 $[M-COOH+CH_3COOH]^-$	40.1	67.8
	473 $[M-COOH]^-$	46.0	70.3
	269 $[M-H-MalGlc]^-$	100.0	100.0
Genistein 6-OAcGlc	533 $[M-H+CH_3COOH]^-$	9.1	85.5
	473 $[M-H]^-$	25.2	100.0
	269 $[M-H-AcGlc]^-$	100.0	27.5
Genistein	491 $[M-H+CH_3COOH]^-$	25.5	69.2
	431 $[M-H]^-$	28.1	100.0
	269 $[M-H-Glc]^-$	100.0	94.2
Glycitein 6-OMalGlc	547 $[M-COOH+CH_3COOH]^-$	2.0	13.5
	487 $[M-COOH]^-$	9.3	29.2
	283 $[M-H-MalGlc]^-$	100.0	100.0
Glycitein 6-OAcGlc	547 $[M-H+CH_3COOH]^-$	17.5	100.0
	487 $[M-H]^-$	7.7	49.3
	283 $[M-H-AcGlc]^-$	100.0	34.8
Glycitin	505 $[M-H+CH_3COOH]^-$	5.1	82.6
	445 $[M-H]^-$	1.8	29.7
	283 $[M-H-Glc]^-$	100.0	100.0

[a]Mass spectra obtained during HPLC analysis (in a background of 10 m*M* ammonium acetate or 0.1% acetic acid) of an 80% aqueous methanol extract of toasted soy flour using the HN-APCI interface. Orifice potential was −60 V.
[b] 6-OMalGlc, 6″-O-malonylglucoside; 6-OAcGlc, 6″-O-acetylglucoside.

2.4.2. Phenolic compounds

Phenolic compounds are present in both plants and fruits and in their derived products such as beers, wines, juices and plant extracts; they are important for the quality of foods and feeds, such as vegetables, dietary fiber supplements, wine and alcoholic beverages. In fact, they play a fundamental role in determining the bitterness and astringency of alcoholic beverages and in the ageing of wines; in addition, these substances are the building blocks of lignin, and the so-called dietary fiber is known to determine the texture and nutritional value of vegetable foods [127]. Understandably, evaluation of the phenolic composition of vegetable fibers and of wines and alcoholic beverages is of considerable interest and various HPLC methods have been proposed for the determination of the separated compounds. The specificity of MS detection for the HPLC analysis of this class of compounds have opened a new perspective, allowing to unambiguously characterize both target and unknown phenolic compounds in complex matrices.

A pioneering study on LC–MS analysis of phenolic acids has been described by Alborn and Stenhagen [128]. Micro-HPLC–MS was carried out by using fused-silica packed columns directly coupled to a standard EI ion source. Because of the electrostatic field between the column tip and the ion source block, electrostatic nebulization occurs in high vacuum and the spray is focused into the MS source, where ionization of the molecules takes place. Application of this system was described for three phenolic acid reference compounds, i.e. caffeic acid, *o*-coumaric acid and sinapic acid; EI mass

spectra provided both molecular mass and diagnostic fragment ions. In addition, since phenolic acids easily thermally decarboxylate, the effect of the temperature ion source on the fragmentation pattern of these compounds was studied. The major drawback of this system is its difficulty to be implemented routinely.

Different approaches were used by other research groups for the LC–MS analysis of phenolic compounds [129–131].

An HPLC–MS method using a TSP interface and discharge-assisted ionization has been devised for the analysis of low-molecular-mass phenolics in plant material [129]. On the basis of the observation that the LC conditions in terms of mobile phases and flow-rates used for the determination of these phenolic compounds are amenable to the TSP interfacing system, the authors developed a fast and reliable method for obtaining a qualitative profile of these useful marker compounds; in fact, the phenolics investigated are indicative of the degree of lignification during plant growth. Useful analytical information on complex mixtures of plant phenolics extracted from a wheat straw acid hydrolysate sample was obtained (Fig. 16). Poor analyte detectability was claimed, since the minimum detectable amounts of the phenolic compounds were on the order of 1 μg; it is noted here that improved sensitivity could be achieved under NI TSP detection mode.

In another work the same research group described an improvement in the LC–MS analysis of plant phenolics by using ESP ionization [130]. After ion-pairing separation on a phenyl column, phenolic acids and aldehydes were detected in the NI mode, in which only intact deprotonated molecules are formed. As regards the sensitivity of detection, ESP showed a 1- to 2-fold better detection limit than positive-ion TSP, limits of detection ranging from 1 to 6 pg/inj. of the phenolics considered. The developed LC–ESP-MS method was successfully applied to the assay of phenolic compounds extracted from natural alimentary fiber sources; high-fiber dietary supplements and cornmeal were analyzed after alkaline hydrolysis of the products. Ferulic acid and *p*-coumaric acid were the most abundant phenolic compounds found in the cornmeal and oat bran extracts; gallic acid was the only phenolic acid present in the pectin sample analyzed.

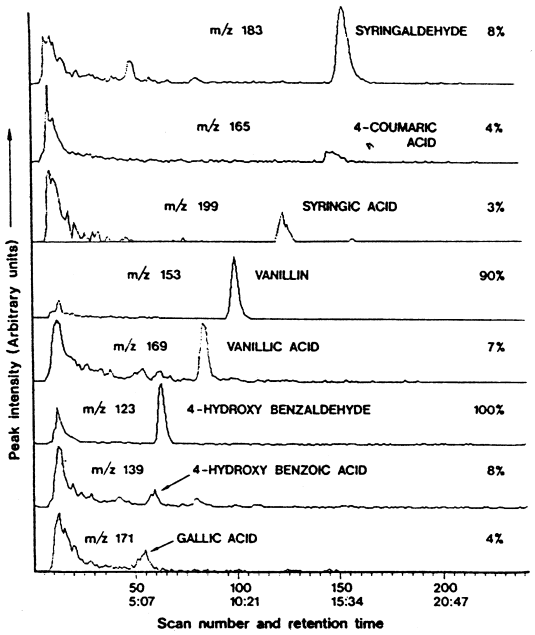


Fig. 16. Mass chromatogram of phenolic compounds detected in a wheat straw hydrolysate by thermospray high-pressure liquid chromatography–mass spectrometry. Reprinted with permission from Ref. [129].

In a recent study, the performance of the PB interface has been explored for the LC–MS analysis of phenolic acids with the aim of devising a reliable and sensitive method capable of detecting these analytes at low concentration levels in complex matrices, such as wines and alcoholic beverages [131]. PB-EI-MS detection was compared with UV and coulometric LC detection systems as regards the linearity, precision and sensitivity of response. A normal-phase chromatography method especially developed for PB-MS detection allowed to obtain the separation of the 15 phenolic acids considered resulting in resolved sharp peaks for most of the analytes and greater sensitivity. PB-EI library-searchable mass spectra were obtained by injecting low amounts (5–10 ng) of phenolic acids in FIA mode. The minimum detectable amounts were in the low-ng range and thus adequate for the assay of phenolic acids in matrices like wine or alcoholic beverages. Unsatisfactory results were obtained for caffeic acid, which gave a poorly shaped peak and consequently low sensitivity. PB-EI mass spectra provided valuable structural information for the

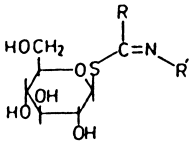
identification of some phenolic acids in a brandy sample analyzed; for the same acids, quantification data were reported, which were in accordance with those obtained by electrochemical detection.

2.4.3. Glucosinolates

Particular attention has been devoted to the identification and determination of glucosinolates, which are natural substances found in many plants and vegetables, such as Cruciferae. This class of compounds is of special interest in food research as a consequence of their activity as precursors of pungency in products like mustard and horseradish and as antinutrients in foods such as rapeseed. Their physiological properties are imputable to their enzymatically released aglycones, which are known to determine the flavour and odour of many plant products [132]. Table 7 shows the general structures of glucosinolates and desulphoglucosinolates. Separation of both classes of these compounds can be performed by gas chromatography using FID or MS detection or liquid chromatography using UV detection; LC–MS methods could overcome the limitations of GC methods, especially for those glucosinolates and desulphoglucosinolates containing sulphinyl or indolic groups which are not amenable to GC–MS analysis. FAB-MS and collision activation of deprotonated molecular anions have been used to directly examine glucosinolate mixtures without prior separation; the product-ion spectra after CID of the $[M–H]^-$ ions yielded useful structure information allowing to determine the nature of the nonpolar side chain of the glucosinolates [133]. HPLC coupled to frit-FAB-MS and to frit-FAB tandem MS was used by Kokkonen et al. to separate and characterize intact glucosinolates in standard mixtures and in plant extracts [134,135]. In both studies a standard-bore C_{18} column with an aqueous ammonium acetate–glycerol–acetonitrile gradient was used to obtain the separation of individual glucosinolates; a pneumatic splitter reduced the flow-rate into the mass spectrometer to 4 μl/min. FAB spectra recorded in NI mode displayed various fragment ions; in the case of K salts of sinigrin and progoitrin, the $[M–K]^-$ anions were detected [134]. Detection limits were estimated to be <20 μg/g when the method was applied to methanol extracts of Brussel sprouts [134]. In another study of the same group, glucosinolates were examined by collisional activation of $[M–H]^-$ ions produced by FAB [135]. Both group- and compound-specific fragments and group-characteristic neutral losses were observed; the authors also indicated strategies for identification of unknown glucosinolates in plant extracts from their NI tandem mass spectra.

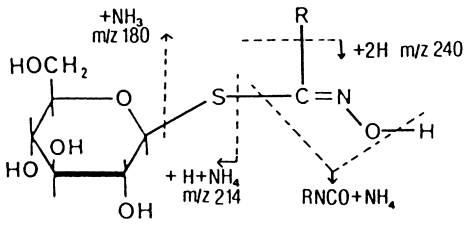
On line HPLC–MS approaches using a TSP interface were evaluated by different research groups

Table 7
General structures of glucosinolates and desulphoglucosinolates and the composition of the R group of the compounds analyzed [138]



$R'=SO_4$, glucosinolates; $R'=OH$, desulphoglucosinolates

Desulphoglucosinolate	Side-chain (R)	Mass	Molecular mass
Glucobrassicin	CH_3-	15	253
Sinigrin	$CH_2=CHCH_2-$	41	279
Gluconapin	$CH_2=CH(CH_2)_2-$	55	293
Progoitrin	$CH_2=CHCHOHCH_2-$	71	309
Glucotropaeolin	$C_6H_5CH_2-$	91	329
Glucoiberin	$CH_3SO(CH_2)_3-$	105	343
Gluconasturtiin	$C_6H_5(CH_2)_2-$	105	343
Glucobrassicin	Indole-3-CH_2-	130	368
Neo-glucobrassicin	4-Methoxyindole-CH_2-	160	398



Scheme 1. Fragmentation scheme for TSP mass spectra of desulphoglucosinolates. Reprinted with permission from Ref. [136].

[136–138]. In all cases, thermospray LC–MS analyses of desulphoglucosinolates were performed in TSP buffer ionization mode after a reversed-phase separation in gradient elution mode. TSP mass spectra of these substances displayed weakly or even non-detectable protonated molecules; many diagnostic fragment ions attributable to the glucosyl and thioglucosyl moieties of the molecules were also observed. A fragmentation pattern for PI TSP mass spectra of desulphoglucosinolates is reported in Scheme 1 [136].

Hogge et al. investigated the potential of TSP-LC–MS, by studying also the negative-ion spectra of various desulphoglucosinolates; fragment ions indicating the presence of the glucosyl and thioglucosyl parts were observed in all the spectra together with diagnostic ions of general formula $[R–C(=NOH)–S]^-$, which were common in all the NI spectra of the compounds considered [137]. A scarce sensitivity was claimed by these authors as a drawback of the TSP approach, since 500–2000 ng of sample had to be injected in order to obtain good-quality mass spectra. The coupling of the HPLC-TSP system with tandem MS was also explored by Heeremans et al. to detect desulphoglucosinolates [138]; since all the compounds analyzed showed a similar fragmentation of the protonated molecule, i.e. a loss of 162 u corresponding to a release of the sugar ring $C_6H_{10}O_5$, CID mass spectra were recorded in the neutral scan loss mode. The

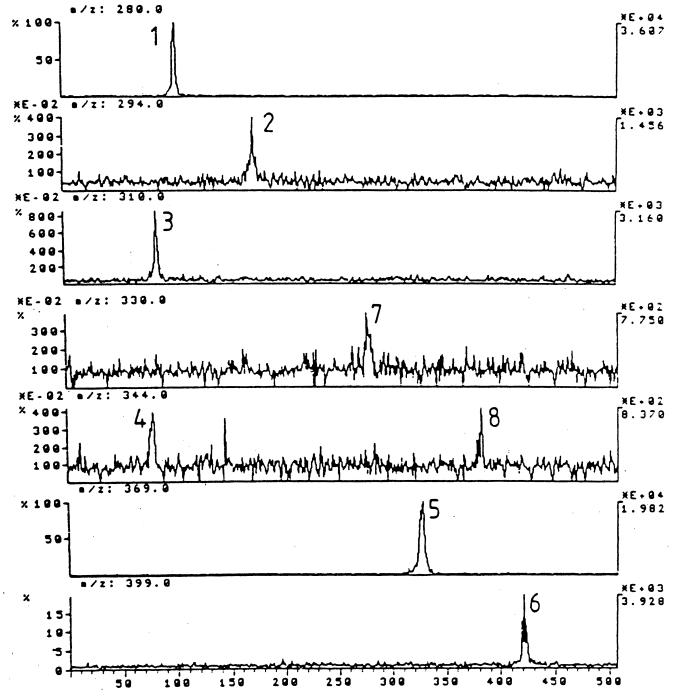


Fig. 17. Mass chromatograms of the deprotonated molecules of desulphoglucosinolates in a sprout extract obtained in the neutral loss (162 a.m.u.) scanning mode. Peaks: 1, sinigrin; 2, gluconapin; 3, progoitrin; 4, glucoiberin; 5, glucobrassicin; 6, neo-glucobrassicin; 7, glucotropaeolin; 8, gluconasturtiin. Collision gas, on; collision energy, 30 eV. Reprinted with permission from Ref. [138].

results obtained for a sprout extract sample by performing LC–MS–MS experiments are illustrated in Fig. 17. The enhanced specificity typical of tandem MS allowed to selectively detect desulphoglucosinolates by direct analysis in the flow-injection mode, either scanning or using the SRM mode.

2.4.4. Others

Organic acids are naturally occurring compounds of concern in foods, since they are responsible for the taste and the stability of several products such as wines and juice drinks [139]. A mixture of four monoprotic organic acids has been studied using HPLC with TSP-MS by operating in thermospray and in discharge ionization modes [140]. Low ionization efficiency for the carboxylic acids considered was observed under TSP ionization, with detection limits in the 500–2000-ppm range; in contrast, discharge-assisted ionization provided an increase in sensitivity for all analytes by a factor of 20–200; the mass spectra generally displayed a dominant $[M+H]^+$ ion signal followed by the $[M+H+H_2O]^+$ ion peak and no fragmentation. In addition to these monoprotic carboxylic acids, the authors analyzed, unsuccessfully, lactic, succinic, malonic and oxalic acids; neither thermospray nor discharge ionization modes were able to give useful mass spectral data in PI acquisition mode; these analytes are thus expected to be detectable using NI ionization.

In a more recent work, PB has been proved useful for coupling ion chromatography to mass spectrometry for the analysis of dicarboxylic acids in juice and wine samples [141]. Analyses were carried out under EI and CI conditions, by monitoring positive and negative ions. structurally informative EI spectra were obtained, whereas PCI provided only the protonated molecule for each analyte and NCI experiments produced molecular ions with no fragmentation. Grape juice and red wine samples were then subjected to the LC–PB-MS analysis after a purification step using solid-phase extraction. The combinations of the EI and PCI analyses allowed to detect and identify the organic acids under investigation in the beverages, even in the case of co-eluting acids.

Studies of the oleoresins of black pepper and capsicum using both on-line HPLC–MS and FD-MS have been carried out by Games et al. [142]. Investigations aimed at determining the composition of capsicum oleoresins and analyzing the pungent principles of pepper have been performed by a large number of research groups, as attested by the numerous papers and books published [143–146]. The LC–MS analysis using the MB interface and both EI and CI ionization modes proved useful in providing structural identification of nordihydrocapsaicin, capsaicin, dihydrocapsaicin and homocapsaicin in capsicum oleoresins; a series of N-isobutyltrienamides and dienamides, together with piperettine and piperine isomers, piperoleine A and piperoleine B, piperanine and piperyline were found in the oleoresin of black pepper.

Among the natural non-nutritive food chemicals, diketopiperazines are worthy of consideration; these cyclic dipeptides, which have been the subject of many studies, play an important role in determining the bitterness of food products, such as roasted malts for brewing and roasted cocoa [147,148]. Diketopiperazines have been usually analyzed by thin-layer chromatography [148], and by gas chromatography with and without derivatization [149,150]. Although these low-volatility compounds are amenable to HPLC analysis, their detection is difficult with this technique. The need for a rapid screening method for diketopiperazines led to the development of an HPLC–MS method based on the use of the MB interface for the identification and quantitative assay of diketopiperazines in cocoa powder [151]. As demonstrated by the same research group for the analysis of the composition of pepper and capsicum oleoresins [142], moving belt produces library-searchable EI mass spectra and solvent-independent CI spectra, useful for structure elucidation and molecular mass information. Typically the protonated and the ammoniated molecules dominate the CI spectrum, so that CI was preferred for a rapid profiling of diketopiperazines and their quantitation, even though EI has great potential for identification purposes. Under CI conditions the on-line method applied to cocoa powder extracts was able to identify 11 diketopiperazines from reconstructed multiple ion traces.

Saffron components have been determined in crude plant extract by HPLC–UV-Vis-DAD-MS using both TSP and ESP interfacing systems [152].

Three classes of substances were characterized, i.e. picrocrocin and its derivatives, crocins and flavonoid derivatives. Picrocrocin [4-(β-D-glucopyranosyloxy)-2,6,6-trimethyl-1-cyclohexen-1-carboxaldehyde] and crocins, which are glucosyl esters of crocetin (8,8′-diapocarotene-8,8′-dioic acid), are recognized to determine the organoleptic characteristics of saffron; in particular, picrocrocin is the bitter agent of this plant. Concerning the LC–MS analysis of crocins, the mass spectra acquired on-line allowed to differentiate *cis* and *trans* isomers and to establish the sequence of glucoses. TSP-MS was found suitable for the unequivocal molecular mass determination of mono-and diglycosides of crocetin, but it failed in analyzing higher glycosides. In contrast, LC–ESP-MS appears to have greater potential than LC–TSP-MS as an identification technique for glycosidic carotenoids bearing up to five glucoses. The two interfacing systems showed similar behaviour for the small molecules (safranal, picrocrocin derivatives and flavonoids), TSP and ESP mass spectra displaying predominantly protonated and sodiated molecular species.

LC–MS with TSP and PB interfaces has been used complementary to GC–MS for the identification of curcuminoids in five commercial powdered turmeric samples [153]. Turmeric powder is used as a food colouring in various products and as a component in curry. The antioxidative and anticarcinogenic activities of curcumin, an important involatile component of turmeric, are well known [154,155]; in addition, curcumin determines the characteristic yellow–orange colour of turmeric, along with two other curcuminoids. After a reversed-phase separation of curcumin, demethoxycurcumin, bisdemethoxycurcumin and other major and minor components, PB-EI-MS mass spectra were recorded for the nonvolatile curcuminoids; PB failed in analyzing volatile compounds, which were lost in the momentum separator of the interface, as already observed for other compounds [156,157]. Mass spectra of volatile components of turmeric powder were obtained by direct thermal desorption-GC–MS. In contrast, when operating under TSP conditions, the LC–MS method was able to produce mass spectra of all the substances, even though with low fragmentation.

Very recently, various HPLC–MS techniques (thermabeam, ESP and APCI) and other hyphenated methods (GC–MS and solid-phase microextraction (SPME)-GC–MS) have been investigated for the analysis of the true *Allium* odour [158]. The aim of this work was that of verifying whether rearrangement products of sulphenic acids (cepaenes, cepaene homologues, zwiebelanes, ajoenes) are actually present in *Allium* odour or if they are artifacts of extraction. For this purpose garlic (*Allium sativum* L.), leek (*Allium porrum* L.) and onion (*Allium cepa* L.) samples were analyzed. Analysis of *Allium* odour indicates the presence of thiopropanal *S*-oxide, thiosulphinates and related substances (zwiebelanes, cepaenes), whereas disulphides or other rearrangement products were not detected. ESP and APCI mass spectra of thiosulphinates displayed the protonated molecules and dimers, which were characteristic of each compound (Fig. 18). The geometry and the type of interface proved to affect the formation of dimers of thiosulphinates; the authors observed that

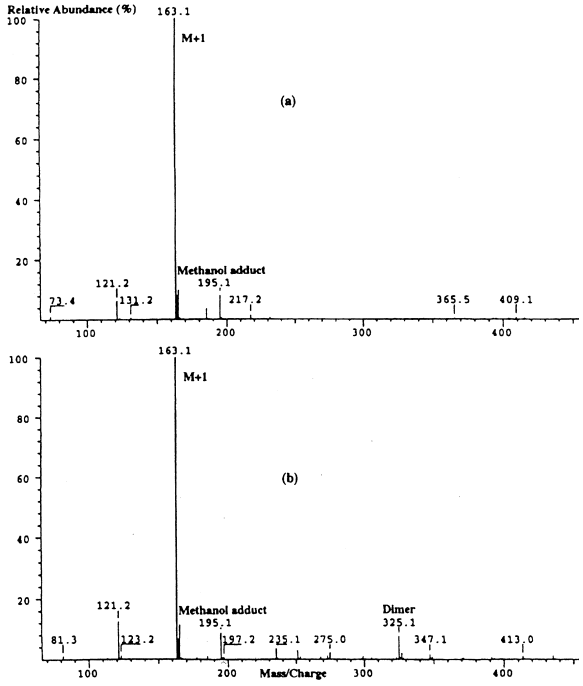


Fig. 18. Mass spectra of: (a) bis(2-propenyl) thiosulphinate and (b) unresolved isomers; 2-propenyl, 1-propenyl thiosulphinate and 1-propenyl, 2-propenyl thiosulphinate (Finnigan electrospray interface). Observation of dimers characteristic of 1-propenyl thiosulphinates. Reprinted with permission from Ref. [158].

the PE Sciex APCI system produced dimers to a lesser extent than the ESP Finnigan interface, because in the former clusters are broken by curtain gas. In the same work the thermabeam interface, an evolution of the PB interface in which the nebulization of the LC effluent is thermally aided, was used; by applying this system, detection of thiopropanal *S*-oxide and 2-hexenal in leek and onion extracts was feasible, components which were not identified using the HPLC–ESP-MS and HPLC–APCI-MS techniques.

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

3. Conclusions

The rapidly growing number of papers dealing with the applications in the analysis of natural substances in food attests that LC–MS has become a mature and robust technique. The development of the last generation of API interface/ionization systems has overcome many of the problems deriving from the low sensitivity and the crucial experimental conditions of the previous systems. The successful coupling of HPLC with the new electrospray and APCI systems has been demonstrated for tandem MS experiments; in this way, both molecular mass determination and valuable structural information can be achieved for a full characterization of natural products. Moreover, it is predictable that the standardization of the analytical procedure and of the experimental conditions will make it possible to create customized databases of the mass spectra obtained by means of CID or tandem MS, thus overcoming the current impossibility of an automatic identification of unknown substances.

It is expected that the now commercially available benchtop instrumentation based on the atmospheric pressure ionization principle, together with the automation of the entire LC–MS system inclusive of on-line sampling treatment, will favour the diffusion of LC–MS for routine analysis in various application areas, including natural and xenobiotic substances in food.

4. Abbreviations

AMMS	anion micromembrane suppressor
APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
APS	atmospheric pressure spray
CF-FAB	continuous-flow FAB
CI	chemical ionization
CID	collision induced dissociation
DAD	diode-array detection
DCI	desorption chemical ionization
DLI	direct liquid inlet
EI	electron ionization
ESP	electrospray
FD	field desorption
FIA	flow injection analysis
HN	heated nebulizer
HPAEC	high-performance anion-exchange chromatography
IC	ion chromatography
ISP	ionspray
NI	negative-ion
PB	particle Beam
PI	positive-ion
PSP	plasmaspray
PUFA	polyunsaturated fatty acid
SFE	supercritical fluid extraction
SIM	selected ion monitoring
SPME	solid-phase microextraction
SRM	selected reaction monitoring
TIC	total ion current
TSP	thermospray.

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