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Review

Electron–capture mass spectrometry: recent advances

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Abstract

Electron-capture (EC) is a sensitive and selective ionization technique for mass spectrometry (MS). In the most familiar form of EC, a susceptible analyte (electrophore) is detected after eluting from a gas chromatography (GC) column, where a low attomole detection limit for standards is routine. High-performance liquid chromatography can facilitate sample cleanup prior to detection by GC–EC-MS, but carryover and shifts in retention time for the ''invisible'' analyte can be difficulties. Solid-phase extraction avoids these difficulties, but the degree of cleanup and recovery can be problems. Alternative electrophoric derivatizing reagents are available to help deal with interferences, and new reagents such as ''AMACE1'' are emerging. Releasable forms of electrophores can be used as tags for labeling macromolecules, motivated by the desire to multiplex ligand-type assays. The conventional, gas-phase ion source for EC is not well-understood, especially the role of wall reactions. Using an electron monochromator to tune the electron energy adds to the selectivity and information provided by EC-MS. High-resolution and tandem EC-MS measurements are emerging. Electron-capture dissociation is a new technique to sequence small- to medium-sized peptides, having the advantage of providing more extensive sequence information relative to other MS techniques. Particle-beam EC-MS tends to be less sensitive than GC–EC-MS, but not always. Recently it was demonstrated that EC-MS can be accomplished on an ordinary laser desorption time-of-flight mass spectrometer, and also by using atmospheric pressure chemical ionization. Two applications are discussed here in detail: bile acids and oxidized phenylalanine. EC-MS is well-established as a useful technique for trace analysis in special cases, and the scope of its usefulness is broadening (qualitative analysis and detection of more polar and larger molecules), based on advances in both the chemical and instrumental aspects of this technique.

Keywords: Reviews; Electron-capture mass spectrometry; Mass spectrometry; Instrumentation; Bile acids; Phenylalanine

Contents

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1. Introduction

1.1. Definition

In electron-capture mass spectrometry (EC-MS), at least one of the steps leading to an ion that is detected involves the capture of a relatively low energy electron by a precursor molecule (M), ion or radical. Classically, electron-capture takes place in one of two ways: nondissociative EC and dissociative EC, as indicated for M by reactions (1) and (2), respectively.

$$M + e^- \rightarrow M^{\cdot -} \quad (1)$$

$$M + e^- \rightarrow A^- + B^{\cdot} \quad (2)$$

Other ionization reactions can take place simultaneously with EC, or under similar conditions, such as ion pair formation (from more energetic electrons), and also positive and negative chemical ionization [1]. These other ionization processes will not be discussed here.

In the most common form of EC, the low-energy electrons are produced by bombarding a moderating gas (usually methane, about 1 Torr; 1 Torr = 133.322 Pa) in the ion source with high-energy electrons (e.g. 100 eV) derived from a filament. This gas mainly provides three functions for EC: source of the secondary electrons, collisional cooling of the latter to thermal electrons, and collisional cooling of energetic ions.

1.2. Beginning

The early development of EC-MS was gradual, as has been summarized [2], until Hunt and Crow in 1978 [3] boosted its performance significantly. By testing strong electrophores (intense EC response) on a GC–EC-MS system fitted with a conversion dynode, they demonstrated that EC-MS can be a practical, ultrasensitive technique. In Fig. 1 is shown their detection of an electrophoric derivative of dopamine at the low attomole level.

1.3. Selectivity and sensitivity

Primarily two features motivate the interest in EC as an ionization technique for MS: selectivity and sensitivity. The selectivity arises since few classes of compounds are strong electrophores. However, this selectivity is compromised when electrophoric derivatization is done, since background substances in the sample containing the functional group of interest then become strong electrophores too. Nevertheless, the selectivity is a real advantage for inherent electrophores, defined as compounds which either undergo EC without derivatization, or after nonelectrophoric derivatization to enhance their volatility. Polychlorinated, environmental pollutants such as polychlorobiphenyls, polychlorodioxins and toxaphene are familiar examples of inherent electrophores that have been measured extensively with high selectivity by GC–EC-MS, as has been reviewed [4]. Examples of inherent electrophores

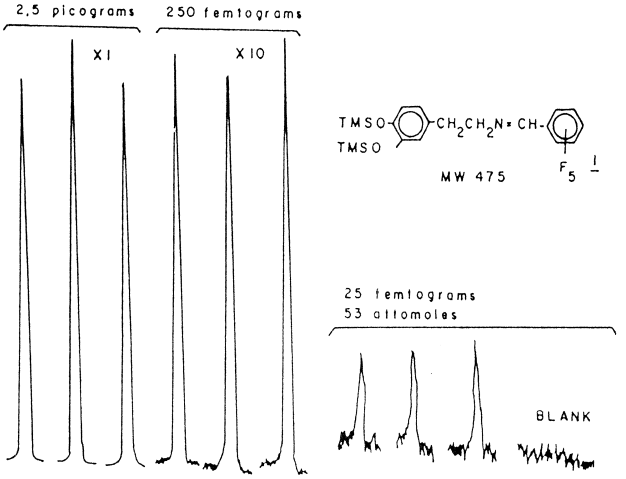


Fig. 1. Response obtained by monitoring the M^- ion (m/z 475) of the dopamine derivative shown under GC–EC-MS conditions with the instrument operating in the SIM mode. Signals correspond to three successive injections of 2.5 pg, 250 fg, and 25 fg samples, respectively. Reprinted with permission from [2].

which can be detected by EC-MS after nonelectrophoric derivatization are carotenoids [5] and flavonoids [6]. Because of extensive conjugation of double bonds in these molecules, there is a low-lying, empty molecular orbital that can capture the electron readily.

The extraordinary sensitivity of EC-MS for a strong electrophore basically derives from the high efficiency with which such a species captures a thermal or near-thermal electron and forms a dominant anion product (as opposed to multiple products that divide up the signal). Apparently this efficiency is near 100% [7], while other steps in the overall process such as loss of the anion product at the walls of the source and in the ion optics between the source and detector limit the observed detection limit. In some favorable cases, strong electrophores have been detected as diluted standards at the zeptomole level by GC–EC-MS [8,9].

Overall one can observe that EC-MS has grown slowly through the years, basically because MS instrumentation has been expensive, and both sample preparation and the instrumental aspects are always demanding for high sensitivity. However, benchtop GC–EC-MS equipment now is available which is less expensive and more rugged than the earlier instrumentation. As will be discussed, the knowledge base is growing about how to be efficient and successful in the chemical aspects of EC-MS, and the technique is being practiced in new ways beyond GC–EC-MS.

The remainder of this review is divided into three categories: sample preparation, instrumentation and applications. Elements of each category permeate the others, since these subtopics cannot be treated in isolation. Recent literature is emphasized and is reviewed selectively rather than comprehensively. Other reviews or partial reviews of EC-MS are available [1,4,7,10–12].

2. Sample preparation

2.1. Sample cleanup: HPLC vs. solid-phase extraction

Usually the analytes for EC-MS are at the trace level (because this is its forte), so significant sample cleanup is required. A high degree of sample cleanup is important not only to remove observable interferences (interfering peaks), but general matrix background as well. Electron depletion in an EC ion source can begin at the 1–10 ng level for a strong electrophore. Thus, significant consumption of the

electrons by matrix will result in a lower signal for the analyte. There may be no clue on the mass chromatogram for this problem since selected ion monitoring is often performed, so interferences may be present but not visualized. The problem can be monitored by using an internal standard to the degree to which it co-elutes and has identical physico–chemical properties as the analyte for EC. The method of standard additions can also be used to establish reliable results.

Two laboratories have recommended use of high-performance liquid chromatography (HPLC) for sample cleanup prior to GC–EC-MS [13,14]. HPLC is well known to provide high-resolution separations. However, two problems with HPLC for trace cleanup are carryover in the injector [15], and shifts in retention time for trace analytes from chaotic contamination and aging of the column. Of course these problems are enhanced since the trace analyte goes undetected until the final GC–EC-MS step on the proper eluent fraction. A low-cost, dedicated HPLC, in a technique called ''satellite HPLC'', provides a way to control ordinary sample carryover in the injector [13]. By building up experience with a method, one can mitigate the problem of shifting retention times, and also one can collect fractions before and after the proper one as a precaution.

Solid-phase extraction (SPE) avoids the problem of sample carryover, since each SPE device is only used once. However, use of SPE that is based on a general retention mechanism (such as nonpolar retention) only provides low resolution, and the lower quality of SPE materials (necessary to make them disposable) than in HPLC increases the likelihood of irreproducibility as different batches of SPE devices and reagents are used. This latter problem is enhanced by the fact that the sample is applied to SPE as a ''first injection,'' which is a problem even for HPLC [16]. Although affinity (e.g. immuno) SPE can be attractive for sample cleanup prior to GC–EC-MS [11], increasingly one will want to set up EC-MS procedures that detect multiple analytes simultaneously in order to justify the cost and labor of EC-MS techniques. Thus, HPLC and SPE each have their good and bad points for sample cleanup prior to EC-MS detection, and continued use and improvement of each for this purpose can be anticipated.

2.2. Derivatization

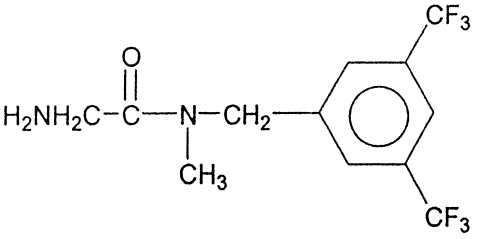
Derivatization is used to enhance the volatility and stability of the analyte for the GC stage, and usually its sensitivity as well for the EC stage. In order to minimize the buildup of interferences in the electrophoric derivatization step, freshly-purified, inert solvents and a low reaction temperature should be employed [14,17,18]. Pentafluorobenzylation is used commonly, since it readily replaces active hydrogens (e.g. phenol, carboxylic acid, heterocyclic NH), leading to a product that typically undergo facile, dissociative EC to form an analyte-characteristic anion. A good strategy for overcoming a persistent interference is to switch to a related derivatization reagent that permits the same reaction conditions (for convenience), while shifting the GC retention time, and perhaps changing the mass of the ion which is monitored [11]. As analogs of pentafluorobenzyl bromide, 4-(trifluoromethyl)-2,3,5,6-tetrafluorobenzyl bromide [19]bis-3,5-(trifluoromethyl)benzylbromide, and 2,3,6-trifluorobenzyl bromide are available commercially for this purpose. While the latter reagent has not been tested yet in this context, it should work well, at least in some cases, since even difluorobenzyl derivatives of 4-hydroxyacetophenone gave responses by GC with electron-capture detection that were only about two-fold lower than that of a corresponding pentafluorobenzyl derivative [19].

Naritsin et al. have lyophilized samples in the presence of a large excess of tetrabutylammonium hydrogensulfate (TBAS), an organic-soluble salt, in order to subsequently pentafluorobenzylate very polar, acidic analytes (in acetone or acetonitrile using *N,N*-diisopropylethylamine as the base) such as some of the tryptophan metabolites [20]. In this nice technique, the TBAS forms a lyophilized powder that enhances exposure of the analyte to the derivatizing reagent. Ordinarily the aqueous sample would be evaporated, and analyte could be lost at the wall or in an organic-insoluble residue. The latter problem has been studied by others and found to be intensified by evaporation after solid-phase extraction due to column bleed [21]. In another study, derivatization relying on K_2CO_3 rather than this lyophilization technique was selected, since the latter

procedure was found to offer no advantages [22]. However, as discussed in more detail later, Blount and Duncan set up an EC-MS method for measuring hydroxyphenylalanines by adopting Naritsin et al.'s technique [23].

Silylation derivatization reactions prior to GC–EC-MS, as a means to cap residual acidic sites on analytes which are inherent electrophores, or already have undergone electrophoric derivatization, should be considered with caution. Such reactions offer great convenience, since residual reagent can be removed by evaporation, or even injected into the GC–EC-MS system (the latter technique may lead to more frequent cleaning of the ion source). However, the price for this is a derivative that is less stable thermally and hydrolytically relative to other derivatives such as esters of OH sites [24]. The instability problem is minimized when phenolic [3] or secondary aliphatic hydroxyl sites [25] are derivatized, and when a more bulky silyl group such as dimethylethylsilyl [25] or *tert.*-butyldimethylsilyl [26] is employed.

Recently a new electrophoric derivatizing reagent named ''AMACE1'' (aminoacetamide electrophore) was introduced, possessing the following structure [27].



The reagent was developed in order to accomplish the electrophoric derivatization of oxidized sugar products, such as those formed when DNA undergoes oxidation damage on its deoxyribose sites. Such products tend to contain functional groups such as keto (aldehyde or ketone), carboxylic acid and lactone. All of these sites can be derivatized with AMACE1 under mild aqueous conditions, especially due to the fact that its functional, primary amine group has a relatively low pK_a of 8.2.

Residual OH sites on the AMACE1-labeled, oxidized sugars were derivatized with butyric anhydride, which was superior to acetylation (tailed peaks for products on GC–EC-MS) and pivalyation (hindered OH labeled poorly). Due to the built-in site which triggers dissociative electron-capture to yield an analyte-characteristic anion [from loss of the bis-3,5-(trifluoromethyl)benzyl moiety as a neutral radical], the products formed in this study from model, analyte standards all could be detected at the low attomole level by GC–EC-MS as shown in Fig. 2. Since keto or carboxylic acid moieties are often present in small organic analytes, or can be formed in them, the reagent is likely to find additional applications. However, to date it has only been tested on standards.

2.3. Chemical transformation

Beyond derivatization, one can consider reactions that more severely alter the structure of the analyte in order to bring it into the realm of EC-MS, sometimes in conjunction with electrophoric derivatization. An example is shown in Fig. 3, in which a DNA adduct of benzo[*a*]pyrene diolepoxide is chemically transformed by mild acid hydrolysis (releasing benzo[*a*]pyrene-7,8,9,10-tetrahydrotetraol) and oxidation (with KO_2/O_2) to form pyrene-1,2-dicarboxylic acid. The latter product then can be pentafluorobenzylated for detection by GC–EC-MS [28]. More recently the method has been extended to measure the polar (semi-oxidized) aryl compounds in air particulate samples [29]. These compounds, containing groups like keto and phenol, also are converted by KO_2/O_2 to corresponding carboxy-aryl products, and are of interest since they account for much of the mutagenicity that is present in an organic extract of air particulates [30]. In other examples of chemical transformation, nitro-aryl compounds were chemically transformed to amino-aryls [31], and a guanine adduct of ethylene oxide was transformed to a corresponding xanthine adduct with nitrous acid [32] prior to electrophore derivatization–GC–EC-MS.

2.4. Release tags

Since electrophores can be detected with high sensitivity and selectivity, and mass spectrometry

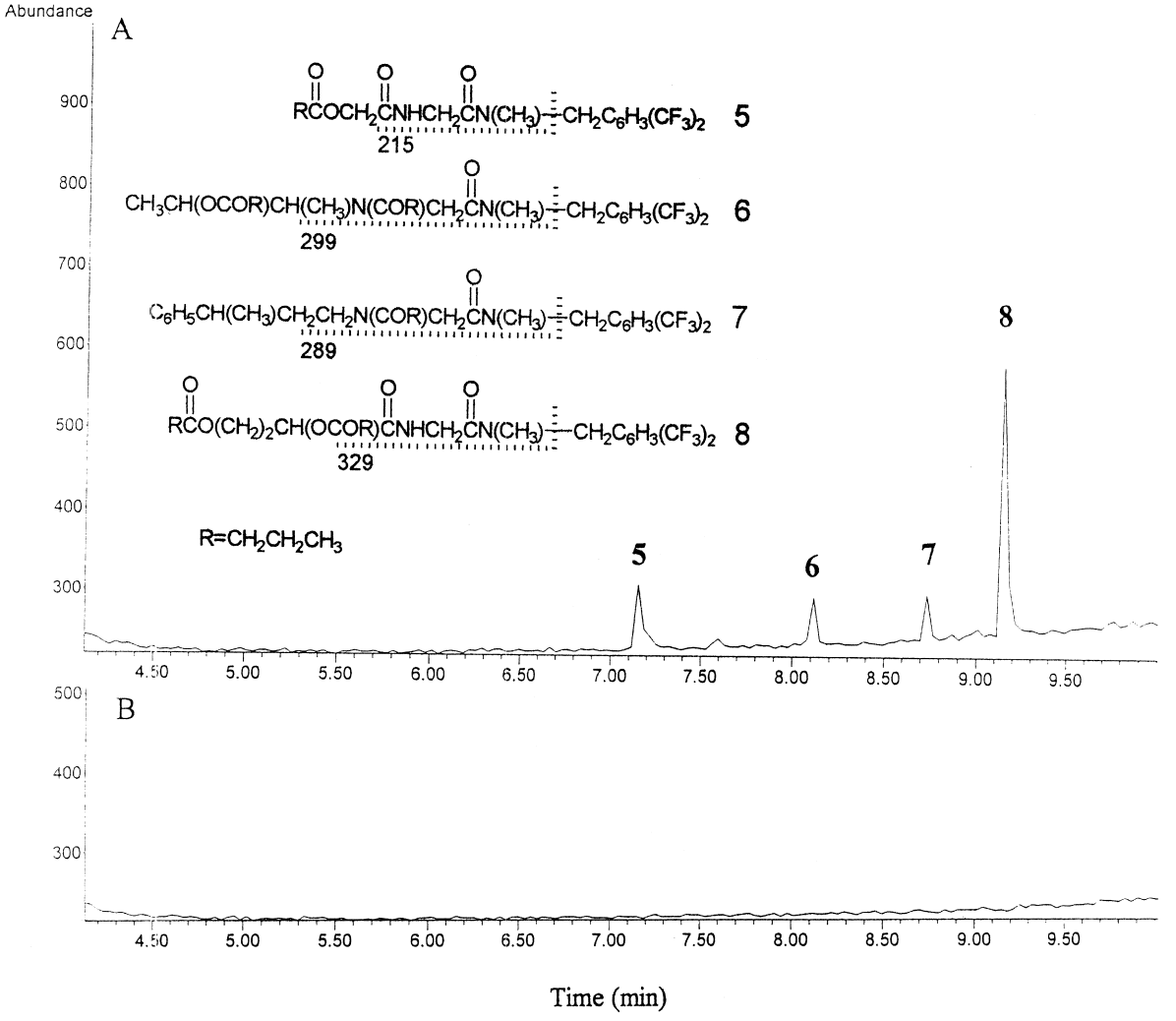


Fig. 2. (A) Structures and fragmentation by electron-capture of electrophoric AMACE1 derivatives, and mass chromatogram obtained by injecting 1 μl of ethyl acetate containing 10 fg each of these derivatives into a GC–EC-MS system. (B) Blank chromatogram obtained by injecting ethyl acetate. GC–EC-MS conditions: model 6890 GC system interfaced to a 5973 MS system (Hewlett-Packard, Palo Alto, CA, USA), Ultra 1 (cross-linked methyl siloxane) column, 50 m×0.2 mm I.D.×0.11 μm film thickness; inject with column at 100°C then immediately ramp at 20°C/min up to 300°C and hold for 10 min; multiple selected ion monitor *m/z* 215, 289, 299 and 329. Reprinted with permission from [27].

enables multiple ions to be detected simultaneously, there is interest in using electrophores as multiple, releasable tags so that they can be used to detect a multiplicity of macromolecules such as DNA fragments derived from dideoxy DNA sequencing reactions. The basic idea is to label each distinctive DNA primer or probe covalently with an electrophore that has a unique mass, using a common, cleavable molecular leash to make this attachment. This contrasts with the use of a more convenient tag such as a fluorescent dye that does not have to be released prior to detection. However, a large number (e.g. ≥100) of different electrophore tags can be employed simultaneously as a cocktail for high throughput, especially if a fast mass spectrometer such as a time-of-flight instrument is employed. This

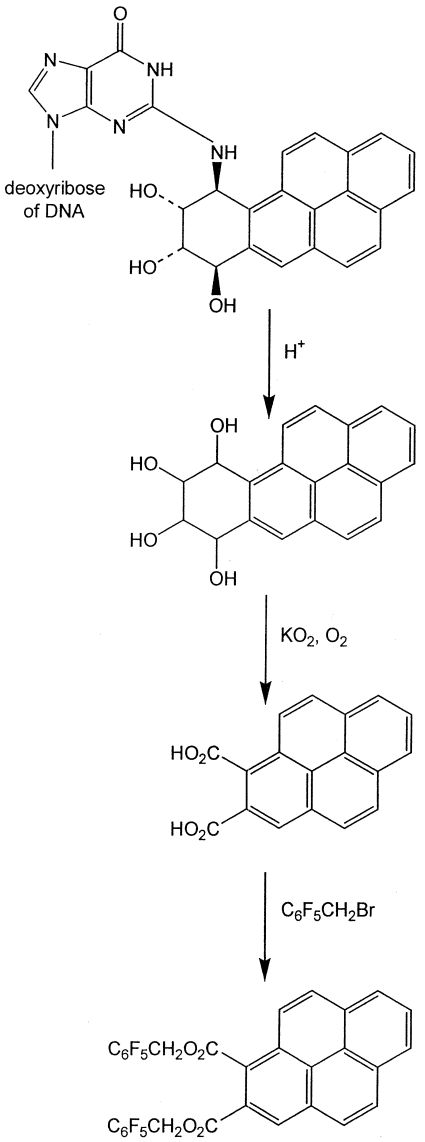


Fig. 3. Scheme for the chemical transformation and electrophoric derivatization of a benzo[*a*]pyrene diolepoxide DNA adduct.

concept is under development as has been described [33], and measurement of proteins in this way also is of interest [34]. Multiple electrophoric release tags are used currently to code the plastic beads employed in one strategy for combinatorial synthesis, although to date the detection of these tags is by GC with electron-capture detection (ECD) rather than an EC-MS technique [35]. Each of the tags possesses a unique alkyl component, along with a common electrophoric group, so the tags have different retention times but similar sensitivity by GC–ECD.

3. Instrumentation

3.1. Ion source

Conventionally the ion source for GC–EC-MS has been a small metal container with an internal volume of about 1 ml that contains small holes for the entry of the analyte and primary electrons, and for the anions to exit. Small holes are necessary in order to maintain a sufficient pressure of the moderating gas. The source is maintained at a temperature ranging from 125°C [36] to 300°C [5]. The overall residence time (as decay time $t_{1/e}$) for the electrophore is about 3 ms [7] allowing plenty of time for the electrophore to make multiple contacts with the wall. This makes it remarkable that some relatively polar, sizable electrophores (e.g. electrophore-derivatized DNA adducts; 11) ever emerge from it. Perhaps the wall is passivated towards most analytes by the moderating gas including its ionization products. In other cases wall reactions lead to losses, or form unusual ion products that continue on to the detector. For example, the use of methane as a moderating gas leads ions such as $[M+H-CN]^-$ and $[M+CH_3+CN]^-$ from tetracyanoethylene [37]. Wall reactions can also complicate the measurement of some chloroorganic compounds, explaining some of the difficulty in reproducing the analysis by EC-MS of these compounds on different instruments [4]. The complete story of the wall in the EC ion source remains to be told, including the stability and control of its properties with extended use.

In a recent effort to minimize the complications of wall reactions, Stemmler reduced the source volume for EC to much lower values down to 15 μl [38]. It was considered that the electrophore would sweep through the smaller cell more quickly and thereby have less time to contact the walls. Further, higher pressures in the small cells were tested as well, in order to reduce wall contact for the electrophore by slowing its diffusion. Indeed, this strategy was successful, with the highest ratio of to surface-altered

products (e.g. ionization products of $M + xH - xCl$) using octachloronaphthalene as a test compound in the smallest cell at the highest pressure tested (1.4 Torr).

While methane is the most popular moderating gas for the EC ion source (isobutane, a related gas, also is common), carbon dioxide and ammonia are employed occasionally. Carbon dioxide has the features of being nonflammable, and avoiding hydrogen reactions at the wall [39]. When CO_2 was compared against methane for the measurement of several chlorinated organics by GC–EC-MS, the results were similar but with some unexplained exceptions [40]. Ammonia was preferred over isobutane as a moderating gas to keep the ion source cleaner [41]. Overall one can only conclude that moderating gases other than methane have been studied relatively little, so our detailed knowledge about the trade-offs in using one vs. another is meager.

Ordinarily the carrier gas for GC, and the moderating gas for EC, are filtered through traps that remove traces of water and O_2, since these substances tend to interfere as by increasing chemical ionization as opposed to electron-capture reactions for the analyte in the ion source. Nevertheless, chemical ionization reactions involving these or other additives can be useful in their own right, e.g. as a means to discriminate isomers [4]. One way to test for oxygen contamination is to inject octafluoronaphthalene and monitor the O_2-dependent ion at m/z 238 [8,42]. This ion corresponds to $[M+O_2-F_2-CO]^{-\cdot}$, and may therefore arise according to a scheme such as that shown in Fig. 4.

3.2. Electron monochromator

In a conventional EC ion source, high energy,

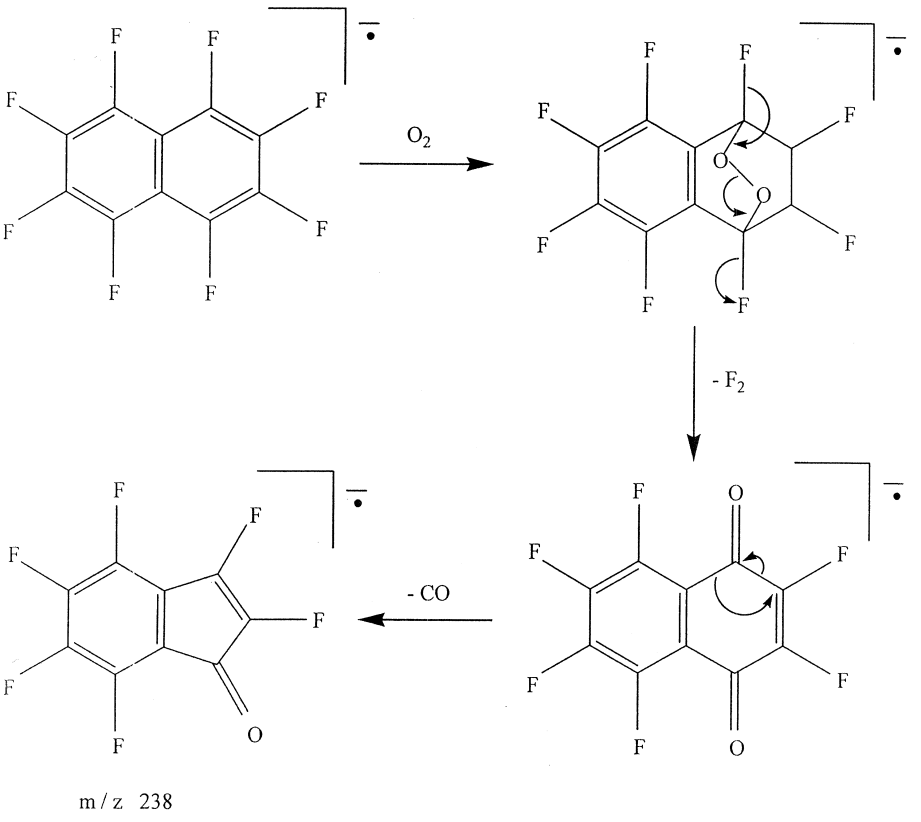


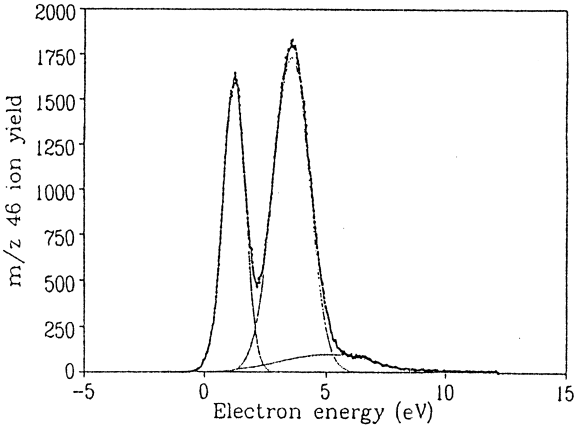
Fig. 4. Suggested fragmentation pathway for the O_2-dependent conversion of octafluoronaphthalene upon electron-capture to a product ion of m/z 238.

primary electrons are thermalized to near 0 eV by a moderating gas, and then captured primarily into the lowest unoccupied molecular orbital (LUMO) of the electrophore. The average energy and energy range of the thermal electrons depends on a number of variables such as the moderating gas, gas pressure and ion source temperature. Deinzer and co-workers, as summarized in a recent review [12], introduced the use of an electron monochromator for EC-MS. This device tunes the energy of the primary electrons down to a well-defined, sharp energy distribution before they enter the ion source, and thereby allows scanning of the electron energy throughout the range that permits EC (about 0–10 eV). Another dimension of information, providing increased selectivity (that can even discriminate geometric isomers), thereby is added to the EC-MS measurement, both one-dimensionally (monitor one anion) and multidimensionally (monitor multiple anions). Thus an electrophore can yield multiple peaks when abundance for one or more ions (individually or collectively) is measured as a function of the electron energy. The peaks along the electron–energy scale are the consequence of electrons undergoing capture into progressively higher unoccupied molecular orbitals. In Fig. 5 is shown an electron energy scan of nitrobenzene, based on monitoring the nitro group anion [12]. The ultimate sensitivity of EC-MS using an electron monochromator remains to be defined. Since a moderating gas is not needed, one can boost the sensitivity by opening up the exit slit on the ion source.

3.3. High-resolution and tandem MS

Both high-resolution and tandem MS are important for EC-MS since they can reduce sample cleanup. This can be quite important when electrophore derivatization is performed as part of sample preparation, since background substances become electrophoric as well and thereby interfering. For example, even when electrophore-derivatized N7-(2-hydroxyethyl)guanine, an ethylene oxide DNA adduct, was detected by GC–EC-MS in the selected ion monitoring mode at high resolution ($R_s = 10\,000$ using an electrostatic/magnetic MS system), several peaks surrounding that of the analyte are observed, as shown in Fig. 6 [43]. The detection limit for standard analyte was only 600 amol, reflecting a significant loss in sensitivity when a conventional sector instrument is used for high resolution, since the high resolution is achieved by trimming the ion beam. High resolution was helpful in minimizing interferences in the detection of environmental C_{10}–C_{13} polychloroalkanes by GC–EC-MS [44]. A variety of chlorinated compounds can be present in such samples.

A second strategy, not involving high-resolution measurements, for reliably measuring a given class of chloroorganics in the environment, in spite of the presence of several classes of such compounds, is to detect the chlorine isotope ratios for each of many compounds in the class to help certify the peaks. An automated form of this strategy was recently reported



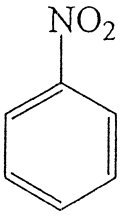


Fig. 5. Electron energy scan of the nitro group anion of nitrobenzene in an EC-MS system fitted with an electron monochromator. Reprinted with permission from [12] and [65].

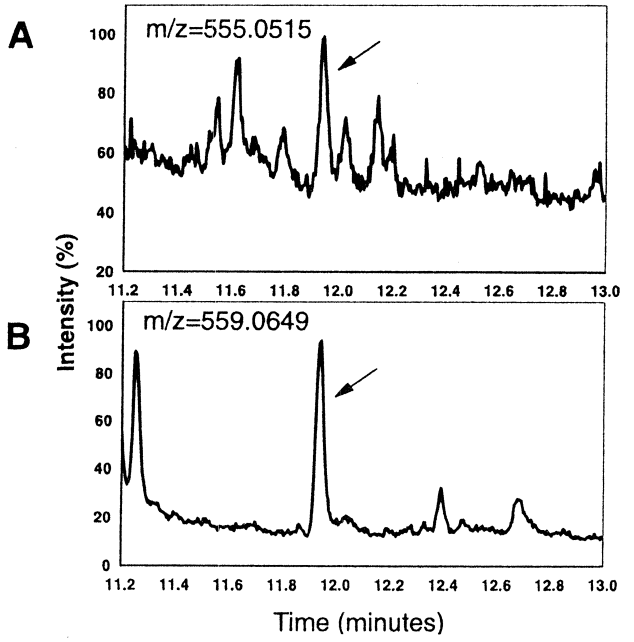


Fig. 6. Representative, selected ion mass chromatograms for sample (A) and internal standard (B) obtained from the following steps: (1) isolate 25 μg of DNA from the lymphocytes of a control human (no known exogenous exposure to ethylene oxide, the chemical of interest); (2) liberate the DNA adduct, N7-(2-hydroxyethyl)guanine, by neutral thermal hydrolysis and react with *tert*-butylnitrite followed by pentafluorobenzyl bromide; (3) purify by solid-phase extraction and detect by GC–EC-MS at high resolution. Reprinted with permission from [43].

for toxaphene measurement by GC–EC-MS [36]. Toxaphene, a mixture of at least 600 hexa-to decachlorinated bornanes and bornenes, persists in the environment from its use as an insecticide for about 30 years, up until the early 1980s.

Use of tandem techniques with EC-MS, e.g. for the measurement of nitro-substituted polyaromatic hydrocarbons [45], 8-isoprostaglandin $F_{2\alpha}$ [46], prostanoids [47] and anabolic steroids [48] similarly reduced sample cleanup while also compromising sensitivity for standards, relative to single stage, selected ion monitoring, by $\geq$10-fold. However, perhaps the highest sensitivity for detecting trace analytes in real samples (vs. standards) by EC-MS will come from the use of such high-resolution or tandem MS techniques. As about to be discussed here, it is particularly important that high-sensitivity EC is beginning to emerge from Fourier transform and time-of-flight mass spectrometers, which can provide ultra-high and high resolution, respectively, while maintaining high sensitivity.

3.4. Electron-capture fourier transform mass spectrometry

In a Fourier transform (FT) mass spectrometer, the ions are detected in an ion cyclotron resonance (ICR) cell based on an image current that is created in an external circuit as they orbit clustered together between two detection electrodes. Ions of different mass are distinguished by possessing different orbital frequencies. Very high resolution is the most distinguishing feature of FT-MS, as long as a very high vacuum can be set up when the ions cyclotron. It is also important that FT-MS acquires full mass spectra by measuring all of the ions simultaneously, and provides $(MS)^n$ measurements. EC-FT-MS was initiated in 1985 by Cody et al. when they reported the detection of 9,9-dicyanofluorene by GC–EC-FT-MS [49]. A dual cell was employed for detection, in which M^- was formed first in a source cell at a moderate pressure, and then diffused through a small hole to an adjacent analyzer cell kept at a much

lower pressure, where detection took place. The authors did not study the sensitivity of the technique.

More recently the potential of EC-FT-MS to provide high sensitivity has been demonstrated [50]. Shown in Fig. 7 is the detection of 480 amol of 4-(pentafluorobenzyloxy)acetophenone, as a diluted standard, by a GC–EC-FT-MS fitted with an external ion source. The signal/noise ratio is about 40, and the resolution is 270 000. Unfortunately the sensitivity on this prototype instrument falls off as electrophores of higher mass are injected, indicating a need to improve the heating in the transfer line that connects the GC system to the ion source (unpublished observations).

FT-MS also allows EC to detect electrophores with relatively low electron affinity such as pyrene (EA = 0.45 eV), as demonstrated by Li et al. [51]. This is accomplished by creating a cloud of electrons in an ICR cell, and allowing them to self-cool based on their cyclotron motion in the absence of a moderating gas. Ordinarily this moderating gas would strip an electron collisionally from M^- when M has a low electron affinity. However, residual, cold electrons in the ICR cell instead cool M^- sympathetically [52], allowing M^- to persist long enough for detection.

3.5. Electron capture dissociation

Mass spectrometry has become a powerful method for protein sequencing, based on sequencing analysis of the peptides obtained from the protein by specific proteolytic or chemical cleavage. (Although not discussed here, MS is now powerful as well for peptide mapping, which ordinarily is based instead on measuring the mass of the peptide, obtaining partial sequence information, or both.) While there are several strategies, the most convenient one, in principle, is to introduce an intact peptide into the mass spectrometer (as by electrospray), and use collisionally activated dissociation (CAD), or another dissociation technique, to form series of peptide fragmentation products for detection. If the peptide can be cleaved between every amino acid and the products detected, then its complete sequence can be determined (aside from the isomers leucine and isoleucine). Unfortunately, the energy deposited into the molecule by CAD and other,

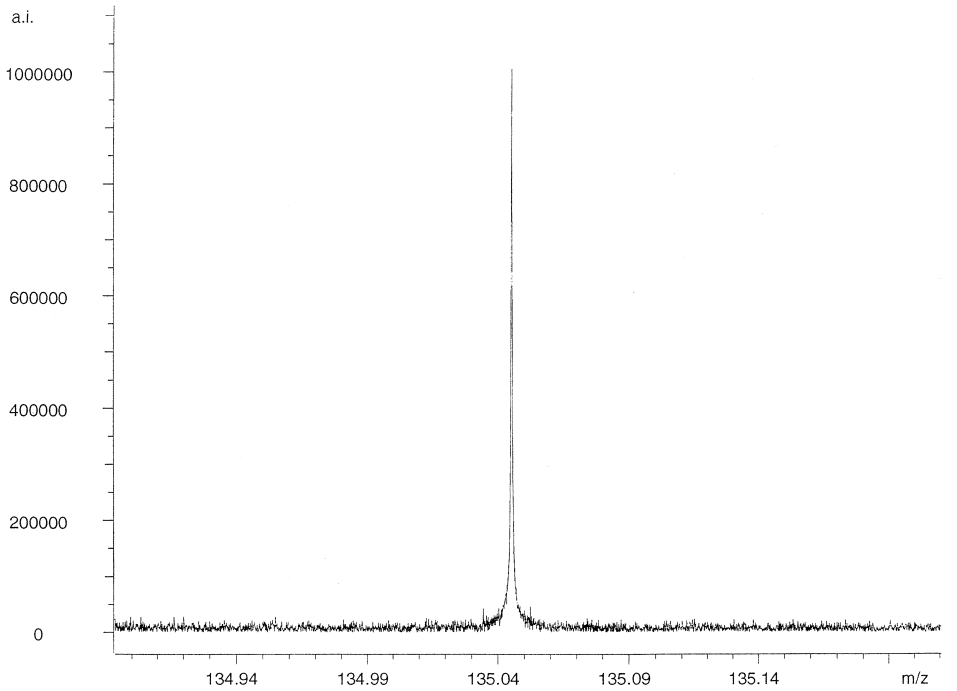


Fig. 7. Detection of 480 amol of 4-(pentafluorobenzyloxy)acetophenone by GC–EC-FT-MS (Rs = 270 000). Reprinted from [50].

conventional techniques distributes throughout the molecule rapidly, favoring cleavage of the weaker peptide bonds, which leaves the strong ones intact. Thus only partial sequence of the peptide is obtained.

Electron-capture dissociation helps significantly to overcome this problem. In this technique, introduced by Zubarev et al. [53], a multipli-charged peptide (containing two or more adducted protons, as from electrospray) is combined with a low energy electron in an ICR cell, which reduces the peptide cationic charge by 1, and results in a much more extensive cleavage between all of the amino acids. Predominantly the C^{α}–N bond is cleaved, and cleavage at the peptide bond is minor. In contrast, peptide bond cleavages are most important in CAD. Apparently the mechanism for such electron-capture dissociation is as follows [54]: (1) the electron is captured at a cationic site, releasing a hydrogen atom; (2) the hydrogen atom, because of its polarizability, is recaptured at a peptide bond, triggering primarily C^{α}–N cleavage. The process is relatively random in cleaving between the amino acids, although proline is resistant (two C^{α}–N bonds must be cut), tryptophan is slightly favored, and disulfide is strongly favored. Overall, this leads to extensive sequence information. For example, ubiquitin was cleaved at 71 of its 75 amino acid pairs by electron-capture dissociation, while even a combination of CAD and infrared multiphoton dissociation only cleaved at 25 sites [55]. The combination of these three techniques fully sequenced ubiquitin, and it is likely that electron-capture dissociation will be used as a companion method to conventional dissociation techniques for peptide sequencing. Unfortunately, as peptide size increases, the fraction of cleavage sites decreases: 75/103 for cytochrome *c*, 30/152 for apomyoglobin, and none for carbonic anhydrase.

3.6. Particle beam electron-capture-MS

In particle beam (PB) MS, a liquid sample, usually as an HPLC peak, is converted by spraying at atmospheric pressure into droplets that condense into solid particles. Because of their momentum, the solid particles follow a straight path through skimmers while residual gases are pumped away. Finally the particles impact on a hot metal surface, where the analyte is volatilized for ionization usually by electron impact, but EC also can be done. The detection limits are usually $\geq$10-fold higher (poorer) than by GC–EC-MS. For example, a vitamin D analog [56], explosives [57] and carotenoids [5] were analyzed in this way, with detection limits of 5–25 pg, 60–200 pg, and 20–20 000 pg, respectively, depending on the analyte in each case. In a study comparing PB- and GC–EC-MS techniques for the same compounds (pentafluorobenzyl derivatives of 8 tryptophan metabolites), Naritsin et al. [20], made the following observations: (1) two of the compounds not detectable by PB-EC-MS (apparently because of high volatility) were detected by GC–EC-MS; (2) one compound not detectable by GC–EC-MS (apparently too polar) was detected by PB-EC-MS; (3) of the others, GC–EC-MS gave 3.6- to 66-fold lower (better) detection limits than PB-EC-MS; and (4) PB-EC-MS allowed larger injection volumes and could be more tolerant to dirty samples. The extrapolated detection limits for PB-EC-MS, aside from the compounds not detected, ranged from 500 to 3600 fg. Thus, PB-EC-MS indeed can be a useful form of EC-MS.

3.7. Laser-induced electron-capture MS

In laser-induced EC-MS (LI-EC-MS), the electron for electron-capture is liberated from an electron donor by a laser. To date this donor has been a metal surface or an organic matrix. Mostly LI-EC-MS has been performed on a time-of-flight (TOF) mass spectrometer, relying on a laser pulse to accomplish desorption with associated EC of the sample. For example, various desorption conditions were employed to detect chloromethanes [58,59], octafluoronaphthalene and dichlorobenzene [60], and nitrated polycyclic aromatic hydrocarbons [61] by LI-EC-TOF-MS. Because special analytes or equipment were involved, and only moderate detection limits or resolution were achieved, this work did not stimulate general interest in the technique.

Recently it was reported that LI-EC-TOF-MS can be accomplished with promise of high sensitivity using routine TOF-MS equipment, at least for pentafluorobenzylated compounds that readily undergo dissociative electron-capture [62,63]. This was achieved in two ways, each of which rely on a routine nitrogen laser: (1) deposit the sample on a silver target, or (2) deposit the sample on a gold target in a matrix comprising a polyaromatic hydro-

carbon with a low ionization potential. A silver target had been used before, but specialized equipment was employed (target temperature near 100 K in order to retain the chloromethane analytes), and the resolution was low [58]. As an example of the new technique for LI-EC-TOF-MS, in Fig. 8 is shown the detection of a pentafluorobenzyl derivative of estradiol, in which 10 pmol was deposited on a silver surface, the laser spot size is about 0.25% of the sample spot, and the S/N is 50. This result, along with other observations, indicate that LI-EC-TOF-MS will extend the range of analytes in terms of size and polarity that can be detected by EC-MS.

3.8. HPLC–atmospheric pressure chemical ionization MS

Electrospray and atmospheric pressure chemical ionization (APCI) currently are the two most popular techniques for directly coupling HPLC and capillary electrophoresis to mass spectrometry. In the latter technique, the flowing liquid is vaporized by nebulization and then ionized by passing through a zone where an electrical discharge takes place. Usually one relies on the ions (e.g. H^+ or OH^-) generated from the solvent to secondarily ionize the sample by proton adduction or removal. Singh et al. [64] recently reported that the discharge region can provide efficient electron-capture of strong, dissociative electrophores such as pentafluorobenzyl derivatives of carboxylic acids, and the nucleobase form of DNA adducts. They relied on MS–MS for high sensitivity, and were able, for example, to detect 200 fg of a pentafluorobenzylated derivative of 11-dehydrothromboxane. The structure of this derivative and its parent and daughter APCI electron-capture mass spectra are shown in Fig. 9. The unmodified OH groups of this derivative make it an unlikely analyte

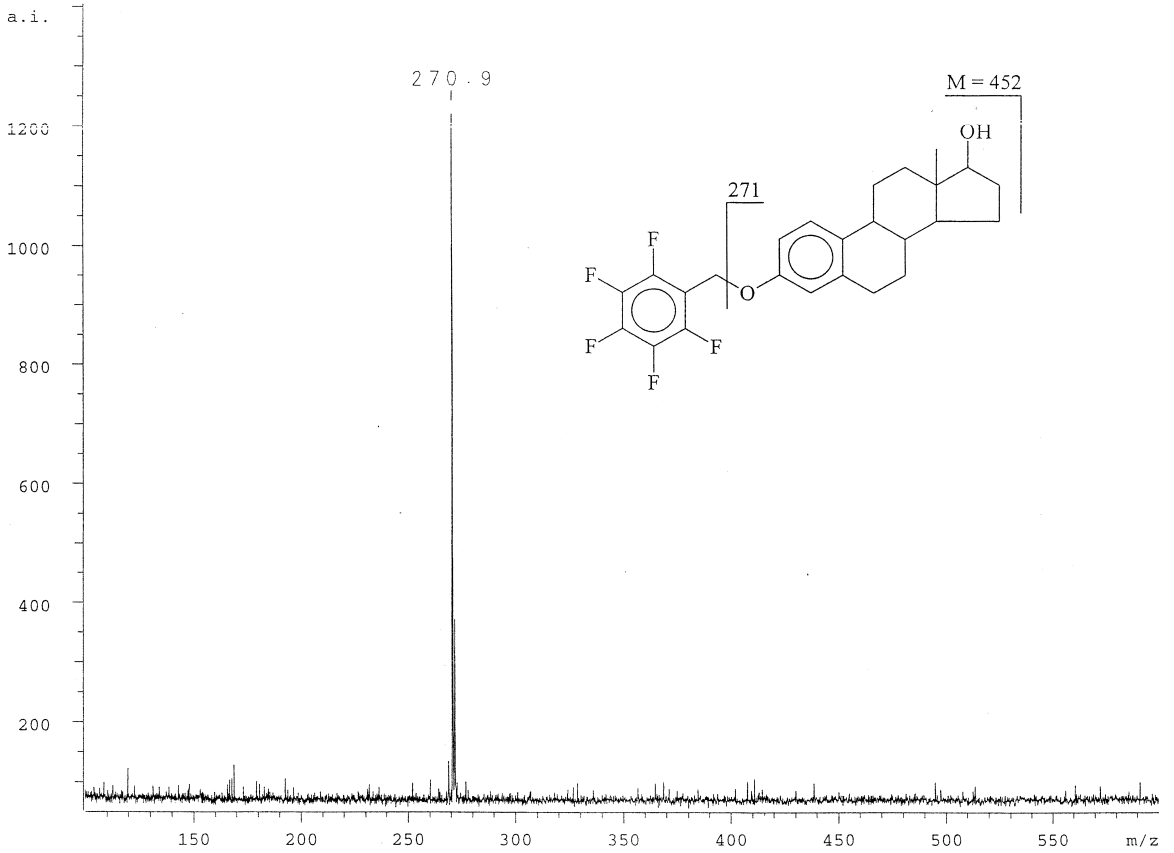


Fig. 8. Detection of pentafluorobenzylated estradiol by LI-EC(Ag)-TOF-MS. Sample deposition: 10 pmol in 1 μl of ethyl acetate giving a 2 mm spot. Laser spot size: 100 μm (0.25% of sample). Number of laser shots: 50. $S/N = 50$. Reprinted with permission from [63].

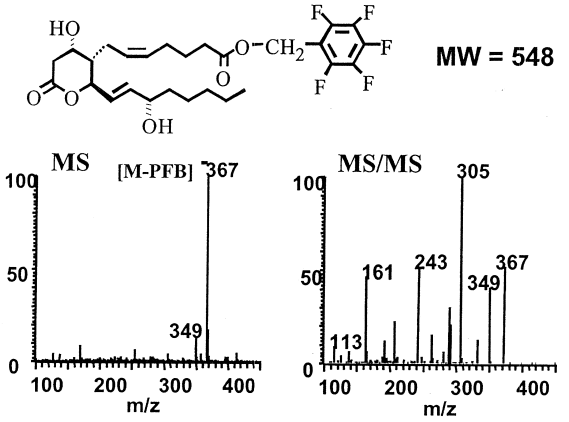


Fig. 9. Liquid chromatography atmospheric pressure electron capture mass spectrum and tandem mass spectrum of a pentafluorobenzyl ester derivative of 11-dehydrothromboxane. Reprinted from [64].

for detection by GC–EC-MS, especially with high sensitivity. This general feature, along with the routine availability of APCI equipment, promise a bright future for this new technique.

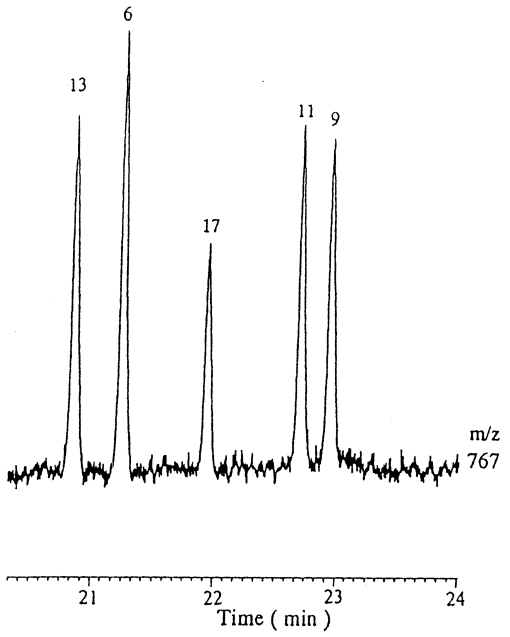


Fig. 11. Detection limit of bile acids (1 fg each) as pentafluorobenzyl, dimethylethylsilyl derivatives. 6=CA-1β-ol, 9=CA-2β-ol, 11+CA-4β-ol, 13=CA-6α-ol, 17=3β,4β,7α,12α-tetrol. Reprinted with permission from [25].

4. Applications

While a number of applications for EC-MS already have been cited in this review, here we will discuss two of them in more detail to further illustrate EC-MS methodology.

4.1. Bile acids

Murai et al. [25] reported the detection of bile acids, based on GC–EC-MS, in several biological samples: serum, dried blood, urine and meconium. The liquid samples were treated with 1 ng of internal standard, subjected to acid and base hydrolysis to deconjugate the analytes, extracted on C_{18}–Si, extracted on piperidinohydroxypropyl Sephadex LH-20, derivatized with pentafluorobenzyl bromide followed by dimethylethylsilylimidazole, and analyzed

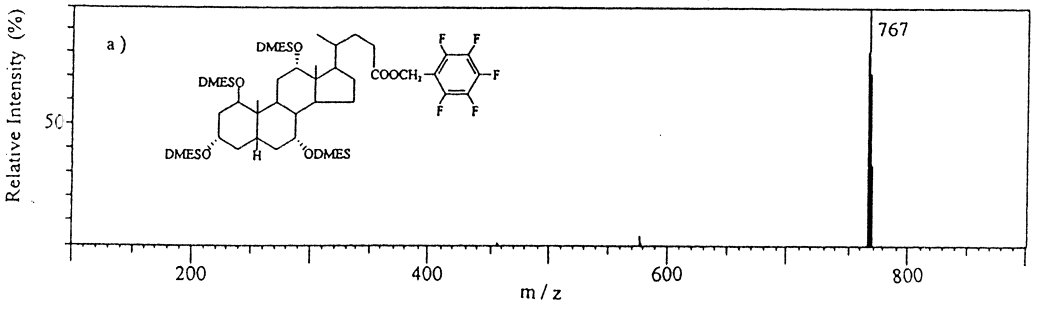


Fig. 10. Electron-capture mass spectrum of a pentafluorobenzyl, dimethylethylsilyl derivative of 1β,3α,7α,12α-tetrahydroxy-5β-cholanoic acid. Reprinted with permission from [25].

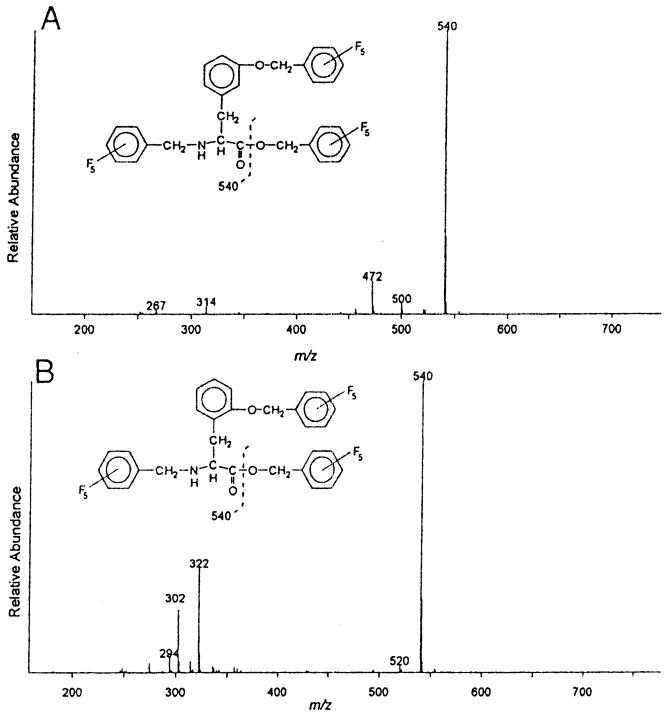


Fig. 12. Electron capture mass spectra of the mono-*N*,di-*O*-pentafluorobenzyl derivative of (A) *o*-tyrosine and (B) *m*-tyrosine. Reprinted with permission from [25].

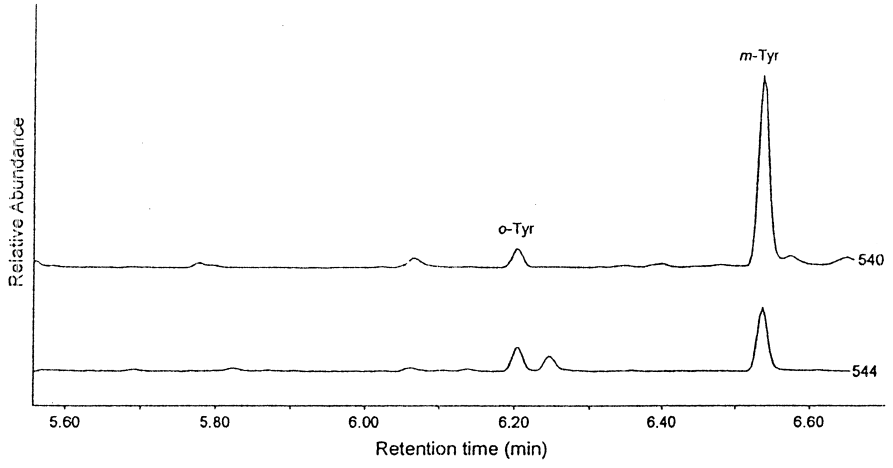


Fig. 13. Representative GC–ES-MS data (selected ion monitoring mode) for the quantification of *o*- and *m*-tyrosine in protein hydrolysates, in this case from 50 μg of oxidized bovine serum albumin. The relative responses of *o*-and *m*-tyrosine to internal standards were compared with external calibration curves to permit calculation of their levels (1.58 and 3.74 ng, respectively) in the sample. Reprinted with permission from [23].

by GC–EC-MS. While this is a multi-step procedure, multiple analytes are being detected simultaneously. When the initial sample was a dried blood disc, it was sonicated in methanol before these steps were begun. An EC mass spectrum and structure of one of the derivatives is shown in Fig. 10. Detection at the ng level in real samples was adequate for the samples tested, due to the relatively high level of analytes present. Standards, in spite of their complexity, could be detected at the low femtogram level as shown in Fig. 11. One can speculate that the round shape, sequestered polar sites, relatively high mass (which moves the GC peak and monitored ion away from background), excellent electron-capture properties of a pentafluorobenzyl ester, and perhaps other factors, all contribute to the intense response of these compounds by GC–EC-MS.

4.2. Oxidized phenylalanine

There is increasing interest in monitoring oxidative damage at the molecular level in people, since such damage apparently contributes to several disease processes such as arthritis and cancer. Examples of reactive oxygen species (ROSs) that produce such damage are superoxide, hydrogen peroxide and hydroxyl radical. One target of ROSs are the phenylalanine residues in proteins, which are converted into *ortho*- and *meta*-tyrosine (by replacement of an H with an OH).

Blount and Duncan [23] have reported a method, based on GC–EC-MS, for the detection of these two, unusual tyrosines (normal tyrosine contains an OH at the *para* position) in proteins. In their method, the protein is combined with stable isotope internal standards for both of these analytes and also of phenylalanine, and digested to amino acids with pronase E (a mixture of bacterial proteases). Lyophilization followed by derivatization with pentafluorobenzyl bromide was done according to Naritsin et al.'s procedure [20] already discussed above. The derivatives were extracted into decane prior to injection into a GC–EC-MS. Fig. 12 shows the structures of the derivatives and EC mass spectra, and Fig. 13 shows a representative GC–EC-MS chromatogram when a protein that was oxidized in vitro with hydrogen peroxide and ferrous ammonium sulfate was tested. Significant amounts of the two analytes, attributed to their presence in pronase E, were found in the blank samples (no target protein). When the method was applied to protein precipitated from plasma by ethanol, the amounts of *ortho*- and *meta*-tyrosine were about 4 and 0.4 residues, respectively, per 10^4 phenylalanines, after correction for the amounts of these analytes found in the blank sample (about 7 and 0.5 in 10^4 phenylalanines, respectively). The detection limits for derivatized standards of ortho- and meta-tyrosine were 30 and 10 fg, respectively, in spite of the residual NH in these compounds. Obviously this NH is sequestered in these derivatives.

5. Conclusion and future

This is an interesting time for EC-MS. Advances in chemical as well as instrumental techniques are taking place that are broadening both the range of its analytes, its ability to provide qualitative analysis, and its overall performance. Ultrasensitive, multi-analyte detection with high specificity is an important capability of EC-MS that has made it the method of choice for detecting some inherent electrophores such as halogenated, volatile pesticides. Advances in sample preparation including derivatization are bringing these same advantages to the detection of other classes of trace analytes. Structural discrimination of compounds both qualitatively and quantitatively is advancing based on ionization with an electron monochromator. Via release tags, electron-capture dissociation, laser-induced electron-capture, and electron-capture coincident with atmospheric pressure chemical ionization, applications to larger, more polar molecules are emerging. Both the present and future are bright for EC-MS.

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