ELSEVIER

International Journal of Mass Spectrometry 200 (2000) 201–217

International Journal of Mass Spectrometry

Lipid mediators and mass spectrometry: an historical perspective

Joseph A. Hankin, Robert C. Murphy

Department of Pediatrics, Division of Cell Biology, National Jewish Medical and Research Center, 1400 Jackson Street, Denver, CO 80206, USA

Received 7 June 2000; accepted 14 August 2000

Keywords: Lipids; Lipid mediators; Mass spectrometry; Steroids; Fatty acids; Prostaglandins; Leukotrienes; Photoplate; Phospholipids; Oxidized phospholipids

1. Introduction

The development of mass spectrometric technology has been a didactic process from its inception to the current date. Developments in instrumentation have opened up new research possibilities, and unsolved research problems have prompted improvements in the technology. The chemical simplicity of small fatty acids, along with their relative volatility when chemically derivatized, made these frequently used samples for study when mass spectrometry was emerging into the arenas of organic and biological chemistry. Thus, many developments in mass spectrometry were closely linked to the study of lipids. One unique class of lipids, generally regarded as lipid mediators, is made up of fascinating molecules that serve an important role in intracellular communication and have potent actions within the biological environment. Most of these lipids are recognized by G-protein linked receptors present on cell surfaces that respond to their chemical structure by stimulation of biochemical reactions within the cell. In many cases, these molecules are derived from a single polyunsaturated fatty acid, namely 5,8,11,14-eicosatetraenoic acid, also called arachidonic acid, which can be enzymatically oxidized to several families of lipids. The best well-known eicosanoids are the prostaglandins whose synthesis is catalyzed by cyclooxygenase, and the leukotrienes whose synthesis is mediated by the enzyme 5-lipoxygenase. This perspective will focus on the elucidation of these biochemical pathways by emerging techniques in mass spectrometry as prototypic examples of the development of mass spectrometry in the area of lipid biochemistry.

Early mass spectrometric analyses were limited by sample volatility and quantity required to maintain a steady beam of ions while recording ion abundances for a range of mass-to-charge ratios. Modern instrumentation can generate gas phase ions from nonvolatile samples by a variety of ionization methods and this capability has opened new pathways for characterizing lipids of all sizes and structures. The development of mass spectrometric technology continues today, largely a result of efforts made by our commercial partners in mass spectrometry. In our view, the emergence of commercially available mass spectrometers and competitive improvements in technology have been a major force in developing modern mass spectrometry. The reduced cost and subsequent availability of mass spectrometers to a wider variety of laboratories continue to spawn additional growth

* Corresponding author. E-mail: murphyr@njc.org

PII S1387-3806(00)00307-9

and novel applications into specific areas of biochemical interest.

2. Lipid analysis (1911–1950)

There are several accounts of the development of the mass spectrometer as it emerged from physics research to become a powerful structural tool of organic chemistry [1,2]. The use of mass spectrometers for the study of lipids was an extension of the growing capabilities of this powerful technology. The advent of mass spectrometry is credited to the work done by J.J. Thompson (Cambridge, England) where he found that a beam of ionized neon gas was deflected by electric and magnetic fields [3]. F.W. Aston, one of Thompson's students, made subsequent improvements in the apparatus used by Thompson [4,5] and reported signals from a sample of ionized coal gas corresponding to H^+, H_2^+, C^+, CH^+, CH_2^+, CH_3^+, and CH_4^+ along with greater signal intensity and improved mass resolution [4].

The conclusive identification of stable isotopes of hydrogen at mass 2, ^{18}O, ^{13}C, and ^{15}N and the ability to resolve compounds by single mass units made mass spectrometry a powerful device that could be applied to studies of various processes [6,7]. It was now possible to study biochemical as well as chemical events by incorporating stable isotopes into the structure of a molecule of interest, chemically degrading the molecule to a gas such as CO_2, N_2 or H_2, and evaluating isotope ratios of the gas by mass spectrometry. Urey and co-worker [8] developed techniques to use mass spectrometry and a stable isotope of oxygen to study the chemical and biochemical events such as esterification [9]. Mass spectrometry had advantages over radioactive tracer studies in that stable isotopes persisted and did not cause ionizing radiation. Disadvantages were that only small gaseous molecules could be reliably detected by the instruments of the day. Biochemical studies were enhanced by improvements made to the mass spectrometer for measuring relative isotopic abundances by using two ion detectors with a null channel [10].

One of the first applications of the new mass spectrometers to lipid biochemistry were in studies by Shoenheimer and Rittenberg. In the 1930s these investigators [11] used specific gravity measurements to measure deuterium incorporation into various products of lipid metabolism [12,13]. With a mass spectrometer available, it was now possibly to measure (more directly) the incorporation of deuterium into lipid products. Various deuterium labeled substrates were first administered to human subjects, then metabolic products were isolated and converted into CO_2 and H_2O/D_2O by combustion. The H_2O/D_2O was converted to H_2/D_2 by passing the water vapor over zinc granules at 400 °C and the quantities of H_2/D_2 were measured by the isotope ratio mass spectrometry. This methodology was a vast improvement over the density measurements which required 100 mg of H_2O and a substantial amount of experimental time whereas the mass spectrometer required only 3–5 mg of sample and 40 min per analysis [14].

It is worth noting that activity in mass spectrometry during World War II which centered on the separation of uranium isotopes with a calutron, assisted in the ultimate development of commercial instruments. The major uses of the first commercial mass spectrometers were largely centered on the analysis of petroleum samples: the hydrocarbons derived from lipid products laid down in prehistoric sediments. These were the first applications of mass spectrometry for analysis of unknown lipidlike molecules.

Volatility of analytes was a critical issue with early mass spectrometers. Sample introduction required converting the analyte into a gas with a minimal vapor pressure of 10^{-2} Torr [15]. The development of the heated direct insertion probe was therefore a major advance in the 1950s [16] that further extended the type of molecule amenable to analysis.

As mass spectrometers found more use, the formation of fragment ions due to unexpected rearrangements, were now routinely observed, and further studies had to be carried out to elucidate the gas phase ion chemistry that took place during the electron ionization process. Stable isotopes incorporated into molecules remained an important strategy to help unravel the complexity of some fragmentation mech-

anisms. For example, stable ^{13}C isotopes were incorporated into the carboxylic acid group of low molecular weight fatty acids from formic to isocrotonic acids and the methyl esters of these compounds were then analyzed by electron ionization mass spectrometry [17]. These early experiments revealed that the observed loss of 28 daltons had to involve the loss of C_2H_4 and did not involve carbon monoxide from the carboxylic acid. Ultimately, such fundamental studies established sufficient foundation that led to the ability to identify and characterize structures of more complex lipids.

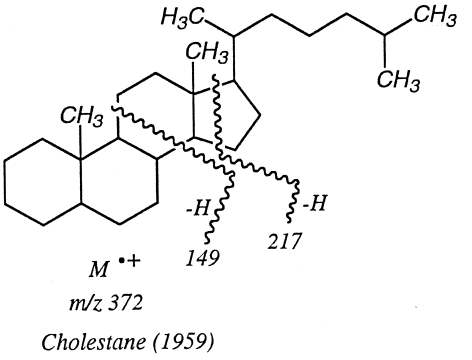
3. Volatile lipid analysis (1950–1980)

3.1. Direct inlet-electron ionization

Sample inlets for instruments of this time period typically consisted of glass reservoirs with a series of valves within an oven directly connected by a small orifice to the electron ionization ion sources of the mass spectrometer. A relatively large amount of sample (μmol) was required for these large volume reservoir inlet systems to provide sufficient gas (typically 10^{-2} torr) to generate a constant gas stream through a small orifice prior to ionization and mass analysis [15]. Carboxylic acids were typically derivatized to their methyl ester to enhance volatility to this level. Later, a direct probe was engineered to permit placement of dried samples quite near the electron beam within the ion source and with no orifice between the ion source and the gas sample [16]. This lowered significantly the sample vapor pressure required for analysis to 10^{-5} Torr or less and increased the variety of samples suitable for study.

3.1.1. Steroids

During the 1950s, the analysis of steroids by mass spectrometry emerged as one of the first applications of this analytical technique in the area of lipid biochemistry. Reed and co-worker in 1958 studied the electron ionization mass spectra of several steroid molecules including cholestane, ergostane, $\Delta^{9,11}$-lanostene, and stigmastadiene [18]. Early investigators using mass spectrometry to study steroids found that these instruments could determine molecular weight and that specific fragment ions revealed the nature of the steroid skeleton [19]. The electron ionization mass spectra of 11 different steroids were presented by Friedland and coworkers [20] by using the heated inlet system of the CEC21-103. One example of structural information derived from the electron ionization mass spectrum was the explanation of the formation of the abundant ion *m/z* 217 and 149 from cholestane [20] as suggested in Scheme 1.



Scheme 1.

Ryhage was investigating the electron ionization mass spectra of bile acids and this area was reviewed in 1960 as possibly the first review of the mass spectrometry of lipids [21]. The detailed investigation of the electron ionization mass spectrometry of steroids initiated by Budzikiewicz and Djerassi [22] was rewarding not only from the standpoint of beginning to understand the fragmentation behavior of the steroid skeleton in a wide variety of naturally occurring as well as derivatized steroid molecules, but also from the perspective of providing a wealth of information concerning fragmentation mechanisms of all organic molecules [23,24].

3.1.2. Fatty acids

By the late 1950s, a systematic study of electron ionization mass spectrometric behavior of fatty acids had been completed by Ryhage and Stenhagen in Sweden [21]. In a series of almost 20 manuscripts, a

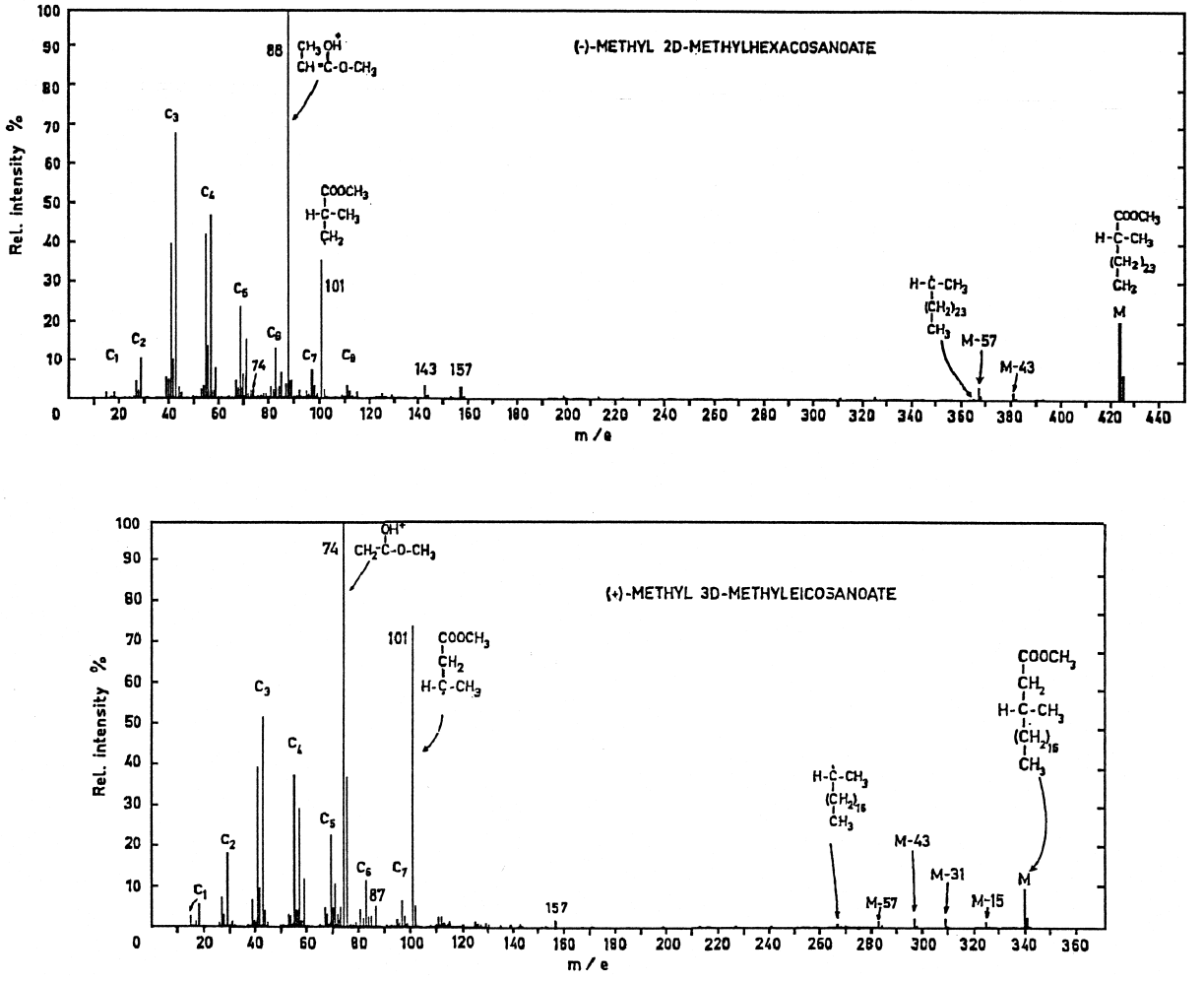
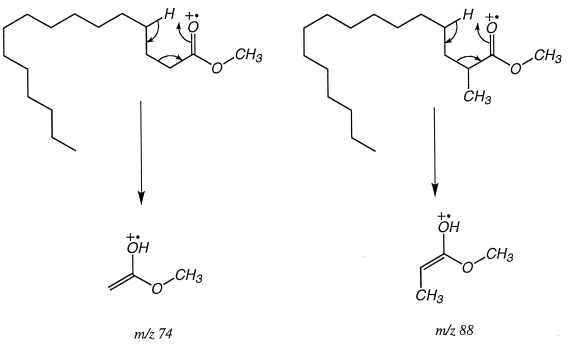


Fig. 1. Electron ionization mass spectrum of (top) methyl 2-methylhexacosanoate and (bottom) methyl 3-methyleicosanoate. With permission from [25].

large variety of fatty acids were studied in detail including straight chain, branched chain, unsaturated, polyunsaturated, as well as fatty acids that had been functionalized with various hydroxyl and keto substituents.

The systematic approach used by these investigators, can be exemplified by their studies of methyl substituted fatty acids (Fig. 1). These investigators clearly showed that the abundant fragment ion *m/z* 74, which was derived from the carbomethoxy moiety of most fatty acids was markedly affected by the presence of a methyl group close to the carbomethoxy



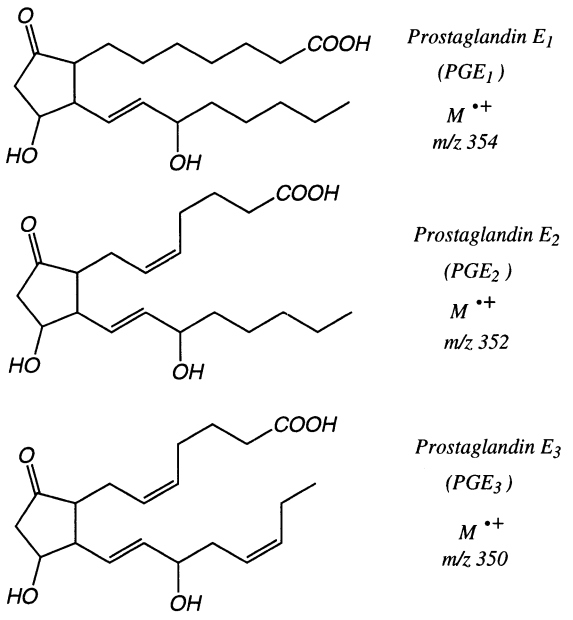
Scheme 2.

substituents [25]. As shown in Scheme 2, the major ion could be shifted from *m/z* 74 to 88 with an α-methyl substituent.

In the United States, Dr. Fred McLafferty was also studying the mass spectrometry of lipid substances and characterized the fragmentations observed in ketones and esters. He described a plausible six-membered transition state mechanism for commonly observed and abundant rearrangement ions [26]. This "McLafferty rearrangement" mechanism represented a major advance in the elucidation of fragmentation pathways induced by electron ionization mass spectrometry as well as a useful structural tool for lipid analysis.

3.1.3. Prostaglandin lipid mediators

In the late 1950s and early 1960s, one of the major successes of mass spectrometry in the area of lipid biochemistry was the structural characterization of a family of biologically active lipids that had been called prostaglandins. Prostaglandin E_1, E_2, and E_3 were isolated and purified by Professor Bergstrom and students, Bengt Samuelsson and Jan Sjovall from sheep prostrate glands in 1960–1962 [27–29]. These substances were all found to cause contraction of certain smooth muscles and reduce blood pressure in rabbits [30]. Mass spectrometry provided important molecular weight information of these purified products after formation of their methyl esters and introduction through the heated glass reservoir. The relatedness of the compounds in terms of differences of an observed 2 dalton shift for each family member and confirmation of the presence of two hydroxyl groups in each of the PGE molecules were readily established in their mass spectra (Scheme 3). Structure elucidation of other prostaglandins, PGF_1 and PGF_2 were also carried out by this research group [31]. As a culmination of these pioneering studies in lipid mediator biochemistry that involved the use of mass spectrometry, Bergstrom and Samuelsson received the Nobel Prize in medicine for 1982.



Scheme 3.

3.2. Gas chromatography/mass spectrometry and gas chromatography interface

One of the most important developments in mass spectrometric technology for the lipid biochemist was the direct coupling of a separation technique with the mass analyzer. This added the capability to separate complex mixtures of lipids from biological extracts and directly analyze individual components. Molecules that passed through a gas chromatograph (GC) inherently had the appropriate vapor pressure for mass spectrometry, making this a natural marriage of instruments. However, it was necessary to develop mass spectrometers that could scan a mass range sufficiently fast to match the elution time of compounds from the gas chromatographic column.

The development of an interface between the gas chromatograph and the mass spectrometer (MS) was critical to facilitate transfer of the analyte from a high pressure region to a low pressure region [32,33]. One of the most successful early interfaces was devised by Watson and Biemann [34] using a glass frit that

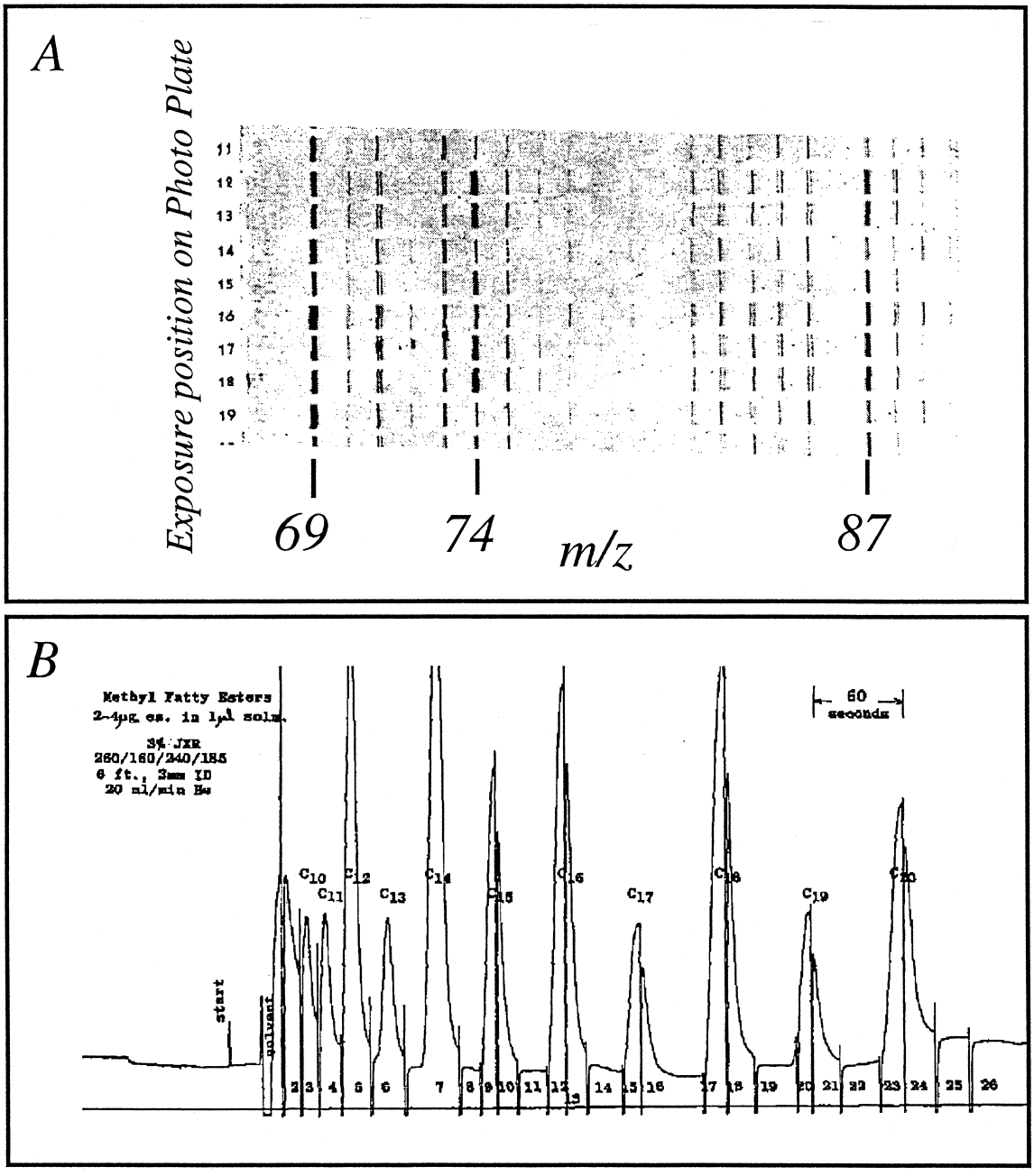


Fig. 2. Early example of GC/MS analysis of fatty acid methyl esters using a photoplate mass spectrometer (CEC 21-110). Effluent from the gas chromatograph was directed into the ion source through an effusion separator and mass spectra recorded on a photographic plate. (A) A portion of the photographic plate recording of exposure positions 11–19 from mass range *m/z* 68–90. (B) Total ion current monitoring a trace recording during exposure of the photoplate where each position of the photoplate is indicated during the elution of the fatty acid methyl esters from the chromatographic column. For example, elution of methyl stearate (C_{18}) was recorded during exposure positions 17 and 18. With permission from [35].

permitted the helium carrier gas to be preferentially pumped away due its faster effusion properties compared to the heavier analyte molecules which passed directly into the mass analyzer. The validity of this approach was demonstrated in part through the analysis of a series of fatty acid methyl esters eluting from the gas chromatograph. The mass spectra were recorded on a photoplate (Fig. 2) in an instrument with Mattauch-Herzog geometry where a new spectrum could be recorded every 10 s [35]. The time dependent intensity (exposure position number) of *m/z* 74 and 87 derived from different fatty acid methyl esters eluting from the gas chromatographic column can be seen in Fig. 2.

Analysis of arachidonic acid metabolites such as prostaglandins by GC/MS required conversion of these lipids into thermally stable as well as volatile derivatives that could pass through the gas chromatograph. Higher vapor pressure and thermal stability required chemical protection of hydroxyl substituents as well as the carboxylic acid moiety. Various derivatives were employed, but the most successful to protect the hydroxyl moiety was the formation of the trimethylsilyl ether derivative of primary and secondary alcohols [36]. This was readily accomplished using highly volatile reagents that formed the desired tetramethylsilane (TMS) ether in high, if not quantitative, yield in a short time and under microchemical conditions. As a consequence, the electron ionization mass spectra of virtually hundreds of prostaglandins and prostaglandin metabolites as TMS ethers have been collected [37].

The major drawback of this TMS derivative was the weak molecular ion and high abundance of ions at low mass-to-charge ratio. Other derivatives have also been used such as the *t*-butyldimethylsilyl ether which has more favorable electron ionization fragmentation behavior [38]. For those prostaglandins with keto functional groups, formation of a methoxime derivative was found to be highly useful to improve GC chromatographic behavior and yield highly abundant and specific ions resulting from fragmentation adjacent to the methoxime derivative [39].

3.3. Computer-based data system

It was very quickly realized that while the GC/MS instrument had incredible power in resolving complex mixtures, the analysis of individual components of the mixture generated a great deal of data in a short time interval. Ultimately, this led to the development of the gas chromatograph/mass spectrometer computer system where the computer had the requisite speed to process data coming from the mass spectrometer and generate a database corresponding to each GC/MS experiment [40]. This time-based data array was then available for off-line investigation that was also found to be of tremendous value. Representations of the data array included the total ionization plot and the mass chromatogram. This latter data processing set tool still in use today was initially developed and refined using the unique properties of fatty acid methyl esters and their formation of the McLafferty rearrangement ion at *m/z* 74 to characterize these products in a complex mixture of fatty acids isolated from Colorado Green River Oil shale (Fig. 3) [41].

3.4. Chemical ionization mass spectrometry

Ionization of samples in the gas phase by proton transfer from an intermediate with a low proton affinity was an important development that has been applied to the analysis of lipids. The gentle ionization techniques described by Munson and Field [42] resulted in reliable quantities of protonated molecules generated in the ion source of a mass spectrometer. Abundant positive ions corresponding to $[M + H]^+$ derived from low energy proton transfers from CH_5^+ were observed for a number of lipid substances [43]. Overall, this technique provided alternative structural information than that available by using electron ionization techniques, in particular molecular weight determination of the intact TMS derivatives. This technique was applied to the study of lipid mediators, initially with prostaglandins [44] and later leukotrienes [45].

One of the important developments of chemical ionization in terms of lipid applications was the discovery that abundant negative ions could be pro-

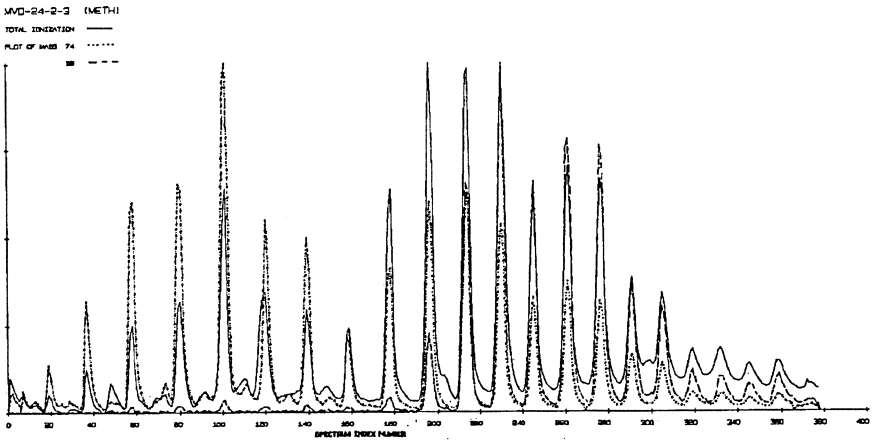


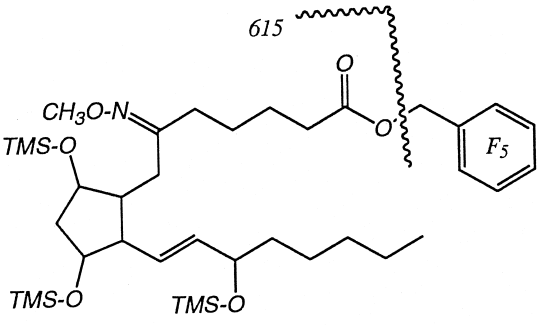
Fig. 3. An early application of a GC/MS computer system to analyze the fatty acids isolated from Green River oil shale following derivatization to their methyl esters. Components eluting from the gas chromatograph are indicated in both the total ionization plot obtained by summation of total ion current in the computer data array recorded during the entire gas chromatographic elution as well as extraction of specific ion current relevant to fatty acid methyl esters (*m/z* 74) and the methyl esters of dicarboxylic acids (*m/z* 98). The strategy of reconstructing the elution of specific ion traces was later referred to as mass chromatograms. With permission from [41].

duced under chemical ionization techniques as a result of reducing the energy of electrons present in the ion source [46]. These thermalized electrons could be efficiently captured if a molecule was derivatized with a structural moiety having a high affinity for electrons. Perhaps the most widely used derivative for lipid biochemistry was the pentafluorobenzyl ester that could be readily made from all prostaglandins since they had a common free carboxylic acid group [47]. The reaction of pentafluorobenzyl bromide under basic conditions was found to be an efficient and nearly quantitative way in which to make this derivative [48].

This development of negative ion chemical ionization GC/MS analysis is widely used even today for sensitive quantitative analysis of eicosanoids. This ionization technique proved to be exquisitely sensitive, typically requiring only 1–10 pg (3–30 fmol) of material injected onto the GC column to yield a detectable signal. This sensitivity matched a critical feature of highly potent lipid mediators, namely that they were present within tissues only at low concentrations. An important example was the use of negative ion chemical ionization mass spectrometry to measure a metabolite of prostacyclin (Fig. 4), viz. 6-keto-$PGF_{1\alpha}$ as a pentafluorobenzyl ester trimethylsilyl ether derivative (Scheme 4), in experiments that conclusively showed that prostacyclin was not a prostaglandin that circulated in human subjects [49].

3.5. Degradation of lipid mediators

Prior to 1980, lipid mass spectrometry had developed into a mature area of investigation. Various lipid mediators could be successfully analyzed both quali-



6-keto-Prostaglandin $F_{1\alpha}$
(6-Keto-$PGF_{1\alpha}$–TMS,MOX,PFB)
MW 796
$[M\text{-}PFB]^-$ = m/z 615

Scheme 4.

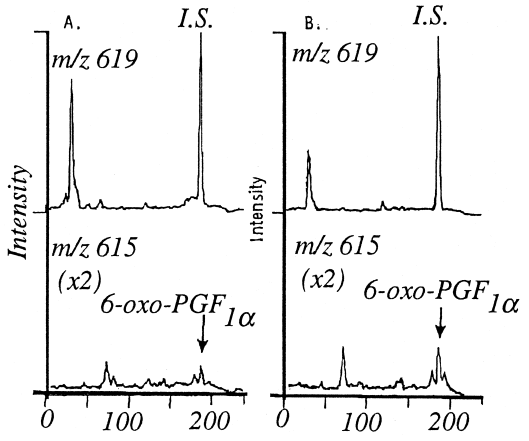


Fig. 4. Mass chromatograms for 6-keto-$PGF_{1\alpha}$ extracted from human plasma after the addition of 2 ng [$2H_4$]6-keto-PGF_{1a} to the plasma. The ion at *m/z* 615 corresponds to endogenous 6-keto-$PGF_{1\alpha}$ as the PFB, TMS, and MOX derivative and *m/z* 619 to the internal standard. (A) Endogenous 6-keto-$PGF_{1\alpha}$ present in plasma at 2.49 pg/mL. (B) Plasma to which 5 pg/mL exogenous 6-keto-$PGF_{1\alpha}$ had been added. With permission from [49].

tatively and quantitatively. The development of the gas chromatograph/mass spectrometer and later the addition of the computer for data acquisition proved an invaluable tool for the analysis of complex mixtures of lipids from biological extracts. All prostaglandins had been structurally elucidated by this time and their biosynthetic pathway delineated (Fig. 5), largely with the assistance of mass spectrometric techniques. Structures of metabolites could be determined if they had sufficient volatility to either enter into the gas chromatograph or be thermally stable and have a vapor pressure of at least 10^{-6} Torr so that it could be placed within the ion source in a direct insertion probe.

However, intact lipid mediators that were inherently nonvolatile eluded analysis by mass spectrometry. Such compounds typically decomposed by thermal processes prior to ionization in the ion source or lacked sufficient volatility even upon degradation so that no relevant structural data could be obtained.

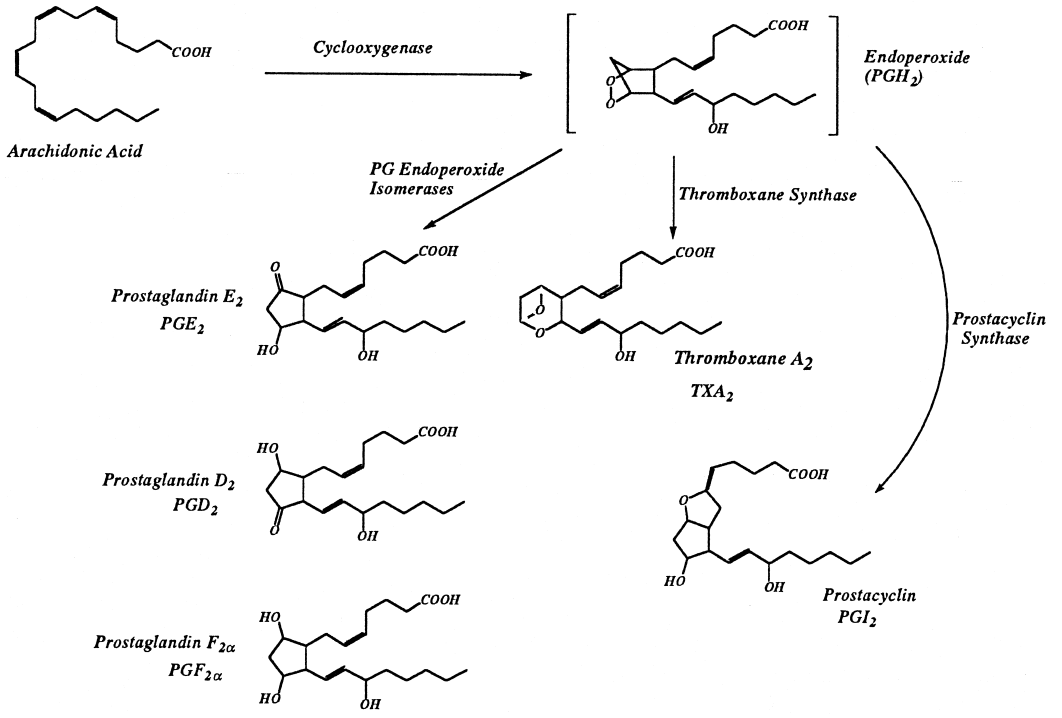


Fig. 5. Biochemical pathway for the oxidation of arachidonic acid by cyclooxygenase leading to the formation of the prostaglandins—PGE_2, PGD_2, $PGF_{1\alpha}$, TXA_2, and PGI_2 through the endoperoxide PGH_2 intermediate. All of these cyclooxygenase products are biologically active and have structures determined largely using gas chromatography/mass spectrometry.

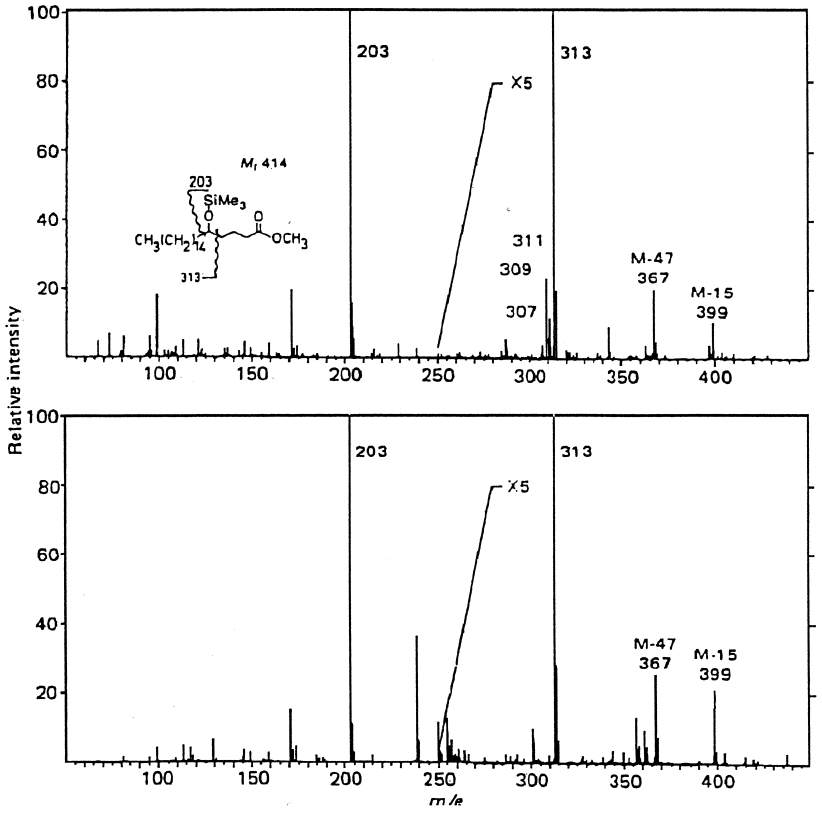


Fig. 6. Mass spectrometric analysis of the degradation product of slow reacting substance of anaphylaxis following treatment with (A) Raney nickel followed by methylation and trimethylsilylation. (B) Mass spectrometry of a trimethylsilyl methyl ester of 5-hydroxyeicosaenoic acid provided by Dr. R. Ryhage (Karolinska Institutet). With permission from [51].

While instruments were capable of rapid scanning and high mass analysis, the major impediment to further application of mass spectrometry to lipid biochemistry was the fact that mass spectrometry could not effectively deal with nonvolatile molecules. This was the case for one unique lipid mediator, slow reacting substance of anaphylaxis [50] that we structurally characterized in 1979 [51].

Slow reacting substance of anaphylaxis (SRS-A). The biosynthesis of a highly active principal, by cells within the lung, was first reported in 1938 by Kellaway and Trethewie [52] as a substance emanating from this tissue after immunological challenge or treatment with cobra venom that caused a slow and prolonged response on certain smooth muscle such as the guinea pig ileum. For the next 40 years, various attempts were made to characterize the structure of this substance without success. One of the interesting features of SRS-A was that this molecule was suggested to be an acidic lipid; however, it was not directly amenable to mass spectrometry. In part, this was due to the presence of a single sulfur atom in its molecular structure which was, in fact, was first identified by spark source mass spectrometry [53]. Attempts to derivatize and pass the molecules through a gas chromatograph or analyze the intact molecule on a solid probe of a mass spectrometer proved futile.

It was necessary for Murphy and co-workers to degrade chemically SRS-A to known products that could be characterized once relatively large quantities (1–10 μg) of biosynthetic material could be made available [51]. A key piece of the SRS-A structural puzzle came after Raney nickel desulfurization of the molecule followed by GC/MS of the resulting lipid

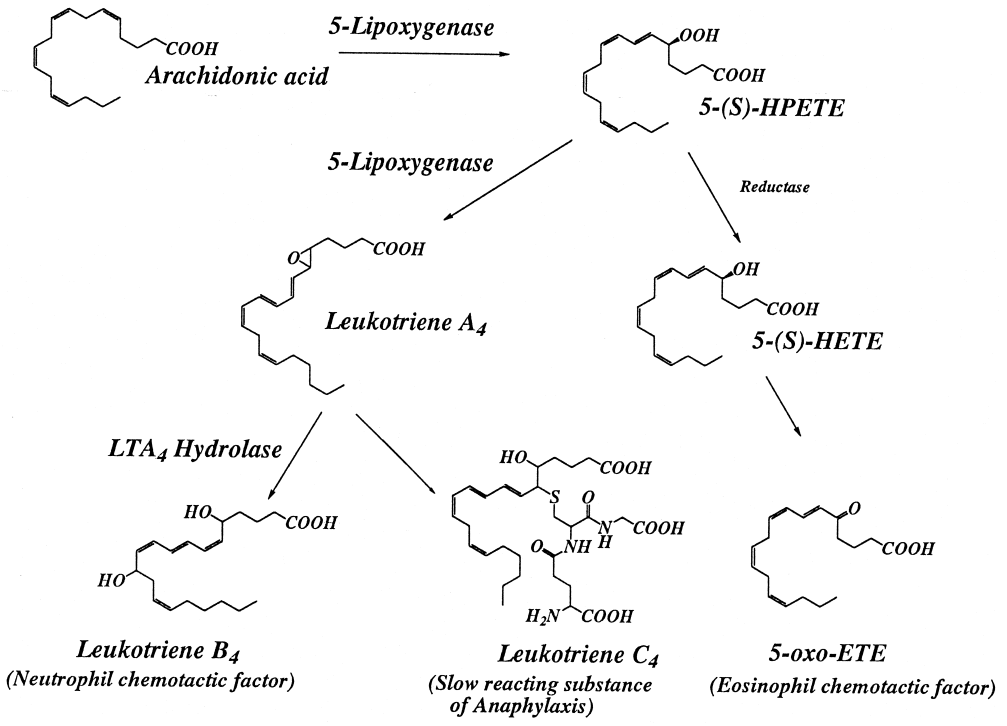
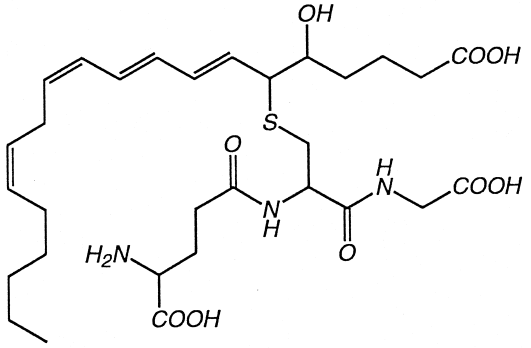


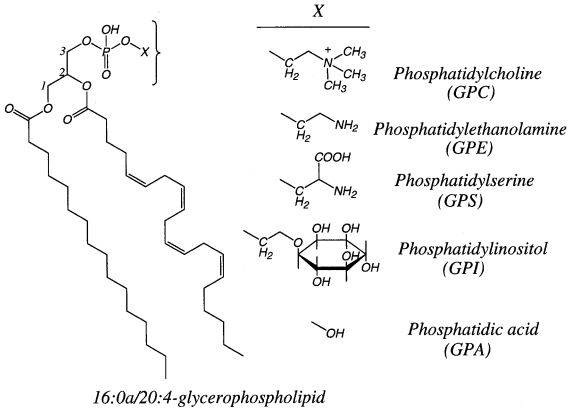
Fig. 7. Metabolism of arachidonic acid by the 5-lipoxygenase pathway leading to the formation of leukotrienes and other 5-oxygenated eicosanoids. These chemical structures were largely determined using mass spectrometric techniques.

portion of the molecule. The reduced and desulfurized lipid portion (Fig. 6) was identified as 5-hydroxyeicosanoic acid [51]. In fact, the reference material used to identify this lipid degradation product was obtained from the laboratories of Ryhage and Stenhagen, who 20 years prior to this, had studied the electron ionization mass spectrum of various hydroxy substituted fatty acids [54]. Having established arachidonate as a



Leukotriene C_4
(LTC_4)
MW 625

Scheme 5.



16:0a/20:4-glycerophospholipid

Scheme 6.

precursor of SRS-A with oxidation at carbon-5 and a unique conjugated triene absorption UV spectrum, it was possible for us to piece this molecule back together as 5-hydroxy-6-glutathionyl-6,8,10,14-eicosatetraenoic acid (Scheme 5) [51]. The name "leukotriene" was then given to this class of novel lipid mediators since the reactive intermediate was derived from a leukocyte and it had the unique chemical feature of being a conjugated triene [55]. This specific biologically active component was renamed leukotriene C_4 (LTC_4) and a new biochemical cascade was delineated (Fig. 7). After the structure of SRS-A (LTC_4) was characterized and published by Murphy and co-workers [51], other investigators were able to obtain a partial mass spectrum of the cysteinyl glycine analog (leukotriene D_4) using the solid probe and derivatization [56]. Nonetheless, it was clear that progress in the identification of this biologically reactive lipid was greatly hindered by inability to analyze the intact molecule, even though it was obtained in reasonable quantities (nanomoles) in a relatively pure state. This situation was about to change with the development of various desorption ionization techniques which would bypass this critical limitation of nonvolatility for mass spectrometric analysis.

4. Nonvolatile lipid analysis (1980–present)

4.1. Fast atom bombardment

The development of fast atom bombardment ionization mass spectrometry was a critical mile stone in the analysis of lipid substances since many of these compounds are incapable of being rendered volatile by derivatization techniques [57]. It was now possible to generate an intact molecular ion species of nonvolatile lipids without any chemical pretreatment. For example, generation of $[M + H]^+$ ions from LTC_4 was quickly demonstrated following the development of fast atom bombardment ionization [58].

This ionization method also permitted for the first time, a detailed analysis of phospholipid precursors of lipid mediators [59]. Phospholipids (Scheme 6) contain esterified arachidonic acid and are present in the bilayer of cellular membranes. Free arachidonic acid is typically found in cells in very low concentrations, yet the enzymes responsible for the production of lipid mediators require arachidonic acid as a free carboxylic acid as substrate. Therefore, activation of a phospholipase A_2 becomes a critical step in the biosynthesis of lipid mediators. Investigations into the precursor phospholipids that donate arachidonic acid and the complex movement of arachidonic acid throughout phospholipid classes was now possible by direct analysis [60]. Both positive and negative ions were abundantly generated from glycerophospholipids and the complexity of phospholipid molecular species within the plasma membrane lipid bilayer could now be directly assessed.

4.2. Collisional activation (collision induced dissociation)

Detailed structural investigation of complex lipids such as phospholipids and the sulfidopeptide leukotrienes required more information than the mass-to-charge ratio of the ion species generated by fast atom bombardment ionization. Collisional activation of ions and the resultant formation of fragment ions carrying structural information was an important experimental advancement. For example, phospholipids in the positive ion mode $[M + H]^+$ were found to generate highly relevant product ions in high yield indicative of the polar head group (Table 1). Initial studies with fast atom bombardment very rapidly progressed to the stage of being able to identify individual phospholipid classes through collisional spectra [61].

The collisional activation of phospholipid-derived negative ions was also a major advance in providing important information relative to the esterified fatty acyl chains as first described by Tomer and coworkers [62]. Collisional activation of $[M - H]^-$ derived from phosphatidylinositol [63], phosphatidylglycerol [63], phosphatidylserine [62], phosphatidylethanolamine [63,64], and phosphatidic acid [63] yielded abundant carboxylate anions from each fatty acyl group. Further, a careful look at the abundance of

Table 1
Positive and negative molecular ion species generated by fast atom bombardment of common glycerophospholipids in mammalian cells and abundant product ions following collisional activation

		Electrospray ionization	MS/MS
Positive ions	GPC	$[M + H]^+$	*m/z* 184
	GPS	$[M + H]^+$	$[M - 140]^+$
	GPE	$[M + H]^+$	$[M - 184]^+$
	GPI	$[M + H]^+$	$[M - 260]^+$
Negative ions	GPC	$[M - 15]^-$	$R1COO^-$, $R2COO^-$, $[M -15 -R'C{=}C{=}O]^-$
	GPC	$[M + OAc]^-$	$[M -15]^-$
	GPS	$[M - H]^-$	$R1COO^{\cdot}$, $R2COO^-$, $[M - 88]^-$
	GPE	$[M - H]^-$	$R1COO^{\cdot}$, $R2COO^-$
	GPI	$[M - H]^-$	$R1COO^{\cdot}$, $R2COO^-$

these specific ions made it possible to suggest the esterification position of the fatty acyl group, being from the glycerol carbon atom-1 or -2 (sn-1 or sn-2) (Fig. 8). Although initial studies showed that the abundance of the carboxylate anions carried this important information [63,65], it became clear that highly polyunsaturated fatty acids esterified at the sn-2 position or the presence of short-chain fatty acids altered this situation. Nonetheless, it was possible to assign the loss of the carboxylic acid as ketene moiety from the sn-2 position as a more abundant ion compared to that from the sn-1 position [66].

In fact, the events of charge-remote fragmentation were first discovered while working with collisional activation of fatty acid carboxylate anions [67]. The assignment of double bond position in a fatty acid has always been an important, but challenging task in lipid biochemistry. Tomer et al. [67] observed that double bond location could be readily assigned by understanding charge-remote events following activation of the carboxylate anion. Since the carboxylate anion from fatty acyl substituents of glycerophospholipids are abundant product ions, high energy activation of these ions can be used to provide double bond location [62,63].

Glycerophosphocholine (GPC) lipids were also found to generate abundant negative ions even though this phospholipid structure had a quaternary ammonium substituent within its polar head group (Scheme 7). It was found that this phospholipid readily lost a choline methyl substituent and that collisional activation of the resultant $[M - 15]^-$ ion led to the same carboxylate anions which were so useful in structural characterization of the other phospholipid classes [63,65,66].

The collisional activation of prostaglandins was also studied in some detail using FAB ionization [68]. Specific ion transitions characteristic of prostanoid ring type were observed for all the major prostaglandins as underivatized species (nonvolatile). This was subsequently found to be highly useful in the identification of isoprostanes (isomers of prostaglandins derived from free radical oxidation of arachidonic acid) [69,70]. It was also possible to study the detailed collision induced ion chemistry of the carboxylate anions derived from leukotrienes [71,72] and their metabolites [73,74]. Various novel metabolites of leukotriene B_4 (LTB_4) and LTC_4 in purified form were now readily amenable to direct analysis without the need for derivatization.

4.3. Electrospray ionization (ESI)

The next major development for lipid mass spectrometry was the discovery of the electrospray ionization technique. In many respects, the ions and ultimately gaseous ion structures formed by electrospray ionization were similar to those formed by fast atom bombardment. The critically important feature of this ionization technique was that it provided an interface system to couple directly the HPLC effluent (condensed phase) to the mass spectrometer by a mecha-

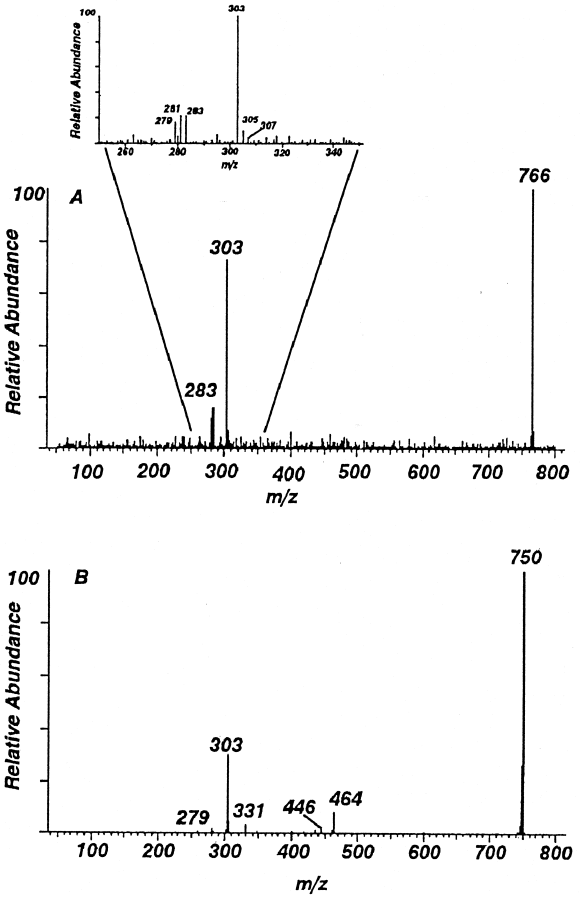
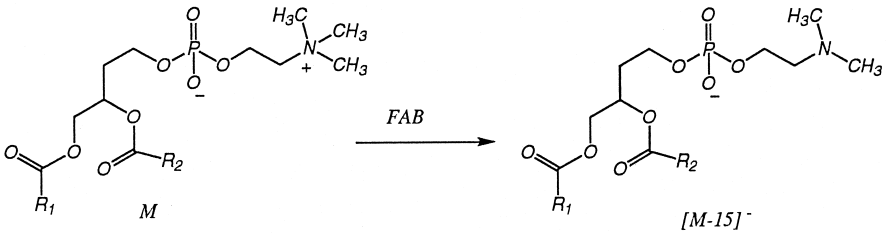


Fig. 8. Fast atom bombardment mass spectra of two abundant glycerophosphoethanolamine phospholipids isolated from human neutrophils (A) collision induced dissociation of *m/z* 766 corresponding to 1-stearoyl-2-arachidonoyl-glycerophosphoethanolamine, revealing abundant carboxylate anions at *m/z* 303 and 283. Other carboxylate anions were also present in this fraction, revealing additional isobaric molecular species (carboxylate anion region expanded in inset). (B) Collision induced dissociation of *m/z* 750 corresponding to the plasmalogen 1-O-octadec-1′-enyl-2-arachidonoyl-glycerphosphoethanolamine. The ion at *m/z* 464 corresponds to the loss of arachidonate ester at the sn-2 position as a ketene species. Reproduced with permission from [65].

nism which removed the majority of the solvent, yet maintained an efficient transmission of both positive and negative ions derived from the analyte eluting from the HPLC. Just as in the case for the major advances made by direct coupling of gas chromatography/mass spectrometry, the development of LC/MS now permitted direct separation with online mass spectrometric analysis. More importantly, many biological molecules and in particular the lipid mediators were amenable to HPLC separations with analyses which did not require derivatization prior to analysis.

The logical extension of electrospray ionization LC/MS was the development of LC/MS/MS or electrospray ionization tandem mass spectrometry. Thus, the effluent from an HPLC column could be studied without sample handling and the eluted molecule structurally characterized through collision-induced decomposition reactions. Numerous nonvolatile and thermally unstable products from metabolism studies of prostaglandins [75], leukotrienes [76], could be identified with these techniques. Lipid mediators derived from free radical reactions taking place within cell membrane phospholipids have been identified using LC/MS/MS and quantitated using stable isotope dilution and multiple reaction monitoring [77].

Advances made in lipid mediator biochemistry have emerged into the world of free radical biology with the ability to characterize the structure of biologically active compounds derived from oxidized lipids. For many decades it has been known that oxidation of low-density lipoproteins is an important event in the development of atherosclerosis [78]. However, the exact nature of the oxidized lipids that may be formed during free radical based oxidation reactions has remained somewhat of a mystery. While low molecular weight lipid degradation products such as malodialdehyde or 4-hydroxynonenal have been measured as products of lipid peroxidation, the concept that biologically active lipids were present could not be realistically addressed because of the inability to analyze trace quantities of chemically labile and biologically active lipids in highly complex mixtures derived from phospholipid bilayers. However, using strategies of LC/MS/MS, the oxidation of polyunsaturated fatty acids present within cellular membranes could now be approached. [Oxidized phospholipids were conveniently studied by collisional activation of both positive and negative ions eluting from the HPLC.] Various chain-shortened phospholipids have now been identified as biologically active components of oxidized LDL and their structures elucidated [79,80]. A rather curious isoprostane esterified to

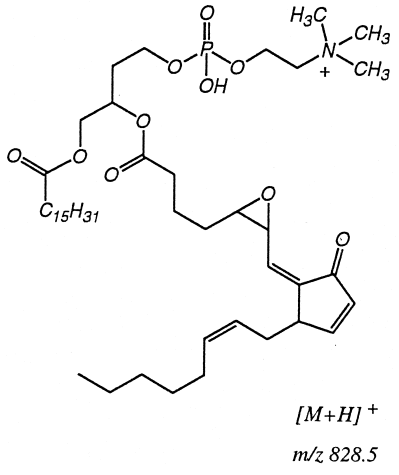


Scheme 7.

glycerophosphocholine (Scheme 8) that has the biological property of increasing the adherence of monocytes to endothelial cells has also been structurally characterized by LC/MS/MS techniques [80].

5. Conclusion

The progression of mass spectrometry from its earliest form to the instrumentation currently available at the beginning of the 21st century has been remarkable. The initial applications of mass spectrometry to lipid biochemistry necessarily involved the conversion of these complex organic substances into simple gases such as carbon dioxide and hydrogen. Even with this limitation, important information relevant to lipid biochemistry was obtained as early as the middle part of the last century. The very rapid development of gas chromatography/mass spectrometry in combination with the computer interface, permitted an unparalleled explosion of applications of the mass spectrometer to lipid biochemistry, as evident in studies of the biochemistry and pharmacology of lipid mediators. An unraveling of the biosynthetic pathways of prostaglandins and later the leukotrienes, in large part, was made possible through the structural clues of intermediates in this pathway as suggested by mass spectrometric experiments.



Scheme 8.

With the sensitivity and selectivity of ESI tandem mass spectrometric techniques, it is now possible to quantitatively measure not only the enzymatic products of polyunsaturated fatty acid oxidation, but also to probe the importance of prostaglandins, leukotrienes, lipoxins, and EETs produced in vivo. We have gained invaluable insight into the pharmacological agents that modify formation of these products through mass spectrometric measurements. Even more impressive is the ability to enter into the world of free radical biology in a meaningful way where highly complex structures are present within highly complex mixtures.

Nevertheless, many challenges remain in the area of lipid mediator biochemistry. It is anticipated that mass spectrometry will play a central role in a further unraveling of the mysteries operating in the production and metabolism of these important compounds. We are only now beginning to understand some of the complex events taking place at the level of the enzyme during the oxidation of arachidonic acid. One of the most unique reactions taking place in the biosynthesis of eicosanoids is a high incidence of suicide inactivation reactions [81]. The mechanisms involved in these suicide events are only poorly understood, yet this process likely is an important

feature regulating lipid mediator biosynthesis. It has also been known for sometime that highly reactive intermediates such as LTA_4 and the endoperoxides PGG_2 and PGH_2 can interact covalently with macromolecules [82]. A relevant example is the formation of levuglandins by chemical rearrangement of PGH_2 and the fact that levuglandins rapidly and covalently bind with lysine side-chains in proteins [83]. The free radical generation of molecules isomeric to PGH_2 (PGH_2 isoprostanes) lead to the formation of isolevuglandins [84]. The targets of these highly reactive chemical entities are virtually unknown and now it is possible through mass spectrometric-based proteomic studies to approach realistically these questions. It has also been observed that LTA_4 [85] and lipid hydroperoxide products [86] can react with nucleosides and nucleotides, raising the possibility that an interaction of this highly reactive chemical intermediate with DNA and RNA present within the nucleus may play an important role either in physiologic arenas or possibly in pathophysiology. Mass spectrometry will be challenged to carry out these studies; however, the development of more sensitive ionization techniques as well as the capacity of mass analyzers to provide relevant structural information of intact, covalently linked proteolipids will remain for our future mass spectroscopists.

Acknowledgement

This work was supported in part by grants from the National Institutes of Health (HL34303 and HL25785).

References

[1] J. Roboz, Introduction to Mass Spectrometry: Instrumentation and Techniques, Interscience, New York, 1968.
[2] G.R. Waller, Biochemical Applications of Mass Spectrometry, Wiley-Interscience, London, 1972.
[3] J.J. Thomson, Philos. Mag. 20 (1911) 752.
[4] F.W. Aston, Philos. Mag. 38 (1919) 707.
[5] F.W. Aston, Philos. Mag. 39 (1920) 611.
[6] H.C. Urey, F.G. Brickwedde, G.M. Murphy, Phys. Rev. 39 (1932) 164.
[7] H.C. Urey, F.G. Brickwedde, G.M. Murphy, Phys. Rev. 40 (1932) 1.
[8] M. Cohn, H.C. Urey, J. Am. Chem. Soc. 60 (1938) 679.
[9] I. Roberts, H.C. Urey, J. Am. Chem. Soc. 60 (1938) 2391.
[10] A.O. Nier, E.P. Ney, M.G. Inghram, Rev. Sci. Instrum. 18 (1947) 294.
[11] R. Schoenheimer, D. Rittenberg, M. Graff, J. Biol. Chem. 111 (1935) 183.
[12] R. Schoenheimer, D. Rittenberg, J. Biol. Chem. 127 (1939) 285.
[13] D. Rittenberg, A.S. Keston, F. Rosebury, R. Schoenheimer, J. Biol. Chem. 127 (1939) 291.
[14] J. Graff, D. Rittenberg, Anal. Chem. 24 (1952) 878.
[15] K. Biemann, Mass Spectrometry Organic Chemical Applications, McGraw-Hill, New York, 1962, pp. 4–19.
[16] R.I. Reed, J. Chem. Soc. (1958) 3432.
[17] G.P. Happ, D.W. Stewart, J. Am. Chem. Soc. 74 (1952) 4404.
[18] P. de Mayo, R.I. Reed, Chem. Ind. (1956) 1481.
[19] J.H. Beynon, Nature (1954) 735.
[20] S.S. Friedland, G.H. Lane, R.T. Longman, K.E. Train, M.J. O'Neal, Anal. Chem. 31 (1959) 169.
[21] R. Ryhage, E. Stenhagen, J. Lipid Res. 1 (1960) 361.
[22] H. Budzikiewicz, C. Djerassi, J. Am. Chem. Soc. 84 (1962) 1430.
[23] R.H. Shapiro, J.M. Wilson, C. Djerassi, Steroids 1 (1963) 1.
[24] H. Budzikiewicz, C. Djerassi, D.H. Williams, Interpretation of Mass Spectra of Organic Compounds, Holden-Day, San Francisco, 1964.
[25] R. Ryhage, E. Stenhagen, Arkiv Kemi 15 (1959) 291.
[26] F.W. McLafferty, Anal. Chem. 31 (1959) 82.
[27] B. Samuelsson, J. Biol. Chem. 238 (1963) 3229.
[28] S. Bergstrom, J. Sjovall, Acta Chem. Scand. 14 (1960) 1701.
[29] S. Bergstrom, F. Dressler, R. Ryhage, B. Samuelsson, J. Sjovall, Arkiv Kemi 19 (1962) 563.
[30] S. Bergstrom, R. Eliasson, U.S. von Euler, J. Sjovall, Acta Physiol. Scand. 45 (1959) 133.
[31] S. Bergstrom, R. Ryhage, B. Samuelsson, J. Sjovall, Acta Chem. Scand. 16 (1962) 2.
[32] R.S. Gohlke, Anal. Chem. 31 (1959) 535.
[33] L.P. Lindeman, J.L. Annis, Anal. Chem. 32 (1960) 1742.
[34] J.T. Watson, K. Biemann, Anal. Chem. 37 (1965) 844.
[35] J.T. Watson, K. Biemann, Anal. Chem. 36 (1964) 1135.
[36] J.A. McCloskey, R.N. Stillwell, A.M. Lawson, Anal. Chem. 40 (1968) 233.
[37] C.R. Pace-Asciak, Mass Spectra of Prostaglandins and Related Products, in Advances in Prostaglandin, Thromboxane, and Leukotriene Research, B. Samuelsson, R. Paoletti (Eds.), Raven, New York, 1989, Vol. 18.
[38] R.C. Murphy, Prostaglandins 28 (1984) 597.
[39] K. Green, M. Hamberg, B. Samuelsson, M. Smigel, J.C. Frolich, in Advances in Prostaglandin and Thromboxane Research, J.C. Frolich (Ed.), Raven, New York, 1978, Vol. 5, pp. 39–94.
[40] R.A. Hites, K. Biemann, Anal. Chem. 39 (1967) 965.
[41] R.A. Hites, S. Markey, R.C. Murphy, K. Biemann, Proc. Annu. Conf. Mass Spectrom. 16 (1968) 302.
[42] M.S.B. Munson, F.H. Field, J. Am. Chem. Soc. 88 (1966) 2621.

[43] T. Murata, S. Takahashi, Anal. Chem. 49 (1977) 728.
[44] E.O. Oswald, D. Parks, T. Eling, B.J. Corbett, J. Chromatogr. 93 (1974) 47.
[45] R.C. Murphy, T.W. Harper, in Biochemistry of Arachidonic Acid Metabolism, W.E.M. Lands (Ed.), Martius Nijhoff, Boston, 1985, pp. 417–435.
[46] D.F. Hunt, G.C. Stafford, F.W. Crow, J.W. Russell, Anal. Chem. 48 (1976) 2098.
[47] I.A. Blair, Methods Enzymol. 187 (1990) 13.
[48] R.J. Strife, R.C. Murphy, J. Chromatogr. 305 (1984) 3.
[49] I.A. Blair, S.E. Barrow, K.A. Waddell, P.J. Lewis, C.T. Dollery, Prostaglandins 23 (1982) 579.
[50] R.P. Orange, K.F. Austen, Adv. Immunol. 10 (1969) 105.
[51] R.C. Murphy, S. Hammarstrom, B. Samuelsson, Proc. Natl. Acad. Sci. USA 76 (1979) 4275.
[52] C.H. Kellaway, E.R. Trethewie, Q. J. Exp. Physiol. 30 (1940) 121.
[53] R.P. Orange, R.C. Murphy, K.D. Austen, J. Immunol. 113 (1974) 316.
[54] R. Ryhage, E. Stenhagen, Arkiv Kemi 15 (1960) 545.
[55] B. Samuelsson, P. Borgeat, S. Hammarstrom, R.C. Murphy, Prostaglandins 17 (1979) 785.
[56] H.R. Morris, G.W. Taylor, P.J. Piper, J.R. Tippins, Nature 285 (1980) 104.
[57] H.R. Morris, M. Panico, M. Barber, R.S. Bordoli, R.D. Sedgwick, A. Tyler, Biochem. Biophys. Res. Commun. 101 (1981) 623.
[58] R.C. Murphy, W.R. Mathews, J. Rokach, C. Fenselau, Prostaglandins 23 (1982) 201.
[59] R.W. Gross, Biochemistry 23 (1984) 158.
[60] F.H. Chilton, R.C. Murphy, Biomed. Mass Spectrom. 13 (1986) 71.
[61] M.J. Cole, C.G. Enke, Anal. Chem. 63 (1991) 1032.
[62] N.J. Jensen, K.B. Tomer, M.L. Gross, Lipids 21 (1986) 580.
[63] N.J. Jensen, K.B. Tomer, M.L. Gross, Lipids 22 (1987) 480.
[64] K.A. Kayganich, R.C. Murphy, Anal. Chem. 64 (1992) 2965.
[65] H. Munster, H. Budzikiewicz, Biomed. Environ. Mass Spectrom. 13 (1986) 423.
[66] Z.-H. Huang, D.A. Gage, C.C. Sweeley, J. Am. Soc. Mass. Spectrom. 3 (1992) 71.
[67] K.B. Tomer, F.W. Crow, M.L. Gross, J. Am. Chem. Soc. 105 (1983) 5487.
[68] J.A. Zirrolli, E. Davoli, L. Bettazzoli, M.L. Gross, R.C. Murphy, J. Am. Soc. Mass. Spectrom. 1 (1990) 325.
[69] R.J. Waugh, J.D. Morrow, L.J.I. Roberts, R.C. Murphy, Free Radic. Biol. Med. 23 (1997) 943.
[70] J.A. Lawson, H. Li, J. Rokach, M. Adiyaman, S.-W. Hwang, S.P. Khanapure, G.A. FitzGerald, J. Biol. Chem. 273 (1998) 29295.
[71] P. Wheelan, J.A. Zirrolli, R.C. Murphy, J. Am. Soc. Mass Spectrom. 7 (1996) 129.
[72] M.J. Raftery, G.C. Thorne, R.S. Orkiszewski, S.J. Gaskell, Biomed. Environ. Mass Spectrom. 19 (1990) 465.
[73] B. Fruteau de Laclos, J.A. Zirrolli, R.C. Murphy, Biol. Mass. Spectrom. 22 (1993) 9.
[74] A. Sala, K. Kayganich, J.A. Zirrolli, R.C. Murphy, J. Am. Soc. Mass Spectrom. 4 (1991) 314.
[75] J. Hankin, P. Wheelan, R.C. Murphy, Arc. Biochem. Biophys. 340 (1997) 317.
[76] P. Wheelan, J.A. Zirrolli, J.G. Morelli, R.C. Murphy, J. Biol. Chem. 268 (1993) 25439.
[77] Z. Mallat, T. Nakamura, J. Ohan, G. Leseche, A. Tedgui, J. Maclouf, R.C. Murphy, J. Clin. Invest. 103 (1999) 421.
[78] U.P. Steinbrecher, S. Parthasarathy, D.S. Leake, J.L. Witztum, D. Steinberg, Proc. Natl. Acad. Sci. USA 81 (1984) 3883.
[79] G.K. Marathe, S.S. Davies, K.A. Harrison, A.R. Silva, R.C. Murphy, H. Castro-Faria-Neto, S.M. Prescott, G.A. Zimmerman, T.A. McIntyre, J. Biol. Chem. 274 (1999) 28395.
[80] A.D. Watson, G. Subbanagounder, D.S. Welsbie, K.F. Faull, M. Navab, M.E. Jung, A.M. Fogelman, J.A. Berliner, J. Biol. Chem. 274 (1999) 24787.
[81] L. Orning, J. Gierse, K. Duffin, G. Bild, G. Krivi, F.A. Fitzpatrick, J. Biol. Chem. 267 (1992) 22733.
[82] M. Lecomte, R. Lecocq, J.E. Dumont, J.M. Boeynaems, J. Biol. Chem. 265 (1990) 5178.
[83] O. Boutaud, C.J. Brame, R.G. Salomon, L.J. Roberts, J.A. Oates, Biochemistry 38 (1999) 9389.
[84] C.J. Brame, R.G. Salomon, J.D. Morrow, L.J. Roberts, J. Biol. Chem. 274 (1999) 13139.
[85] D. Reiber, R.C. Murphy, Arc.Biochem.Biophys., in press.
[86] D. Rindgen, M. Nakajima, S. Wehrli, K. Xu, I.A. Blair, Chem. Res. Toxicol. 12 (1999) 1195.