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Natural products

A retrospective view of mass spectrometry and natural products—sixty years of progress, with a focus on contributions by R. Graham Cooks

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Abstract

Modern mass spectrometry as used by chemists has prospered for the past sixty years. During the first decade of this period, the primary compounds studied were from petroleum, although other organic compounds soon attracted interest. By the mid nineteen fifties, mass spectra were being recorded on thousands of compounds each year. Most of this activity was in industrial laboratories, although by 1960 the academic world had realized the potential of the technique. This retrospective, reviewing the past sixty years, is divided into four quarters. The first two quarters cover the thirty-year period to 1970, by the end of which R. Graham Cooks had begun to plant firm roots into mass spectrometry. The major feature of the third quarter is the development by Cooks and his students of tandem mass spectrometry using isobutane or ammonia chemical ionization mass spectrometry coupled to the mass-analyzed ion kinetic energy spectroscopy (MIKES) technique for the direct, rapid, and efficient analysis of complex biological matrices. A milestone in the work was the direct introduction of plant material into the mass spectrometry (MS) source. The fourth quarter of the review covers an explosive growth in the breadth of mass spectrometry. Work in the Cooks laboratory was focused on the application of a series of ion generation and sample introduction techniques, such as desorption chemical ionization, surface-induced dissociation, secondary ion mass spectrometry, and membrane introduction mass spectrometry, to analyze increasingly complex biological matrices. In contrast to the earlier MIKES technique on a sector spectrometer, tandem MS became more common using a triple quadrupole spectrometer, with the natural products being studied ranging in complexity from ethanol to proteins and DNA. (Int J Mass Spectrom 212 (2001) 65–79) © 2001 Elsevier Science B.V.

Keywords: Tandem mass spectrometry; Natural products; Analysis of complex biological matrices

1. The first two quarters: thirty years from 1940

Studies on mass spectrometry between 1911 and 1940 focused on the instrumental aspects and the determination of isotopes [1]. By the early nineteen forties, mass spectra of simple organic compounds, primarily hydrocarbons, had been recorded and the technique reached such a level of development that commercial instruments were sold by the Consolidated Engineering Corporation for use in the analysis of gas oil samples from petroleum refining [2,3]. These analyses were spurred on by military needs

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Dedicated to R. Graham Cooks on the occasion of his sixtieth birthday.

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arising from World War II, but were the beginning of mass spectrometry being used as an analytical tool for natural products, if it can be allowed that petroleum distillates are natural products. This retrospective will attempt to survey progress in the combination of natural products and mass spectrometry (NPMS) during the period 1940–2000 and will attempt to place into perspective the contributions to NPMS made by Graham Cooks during 33 of these 60 years.

Mass spectrometry at this time was largely confined to quantitative analysis of mixtures of known hydrocarbons in industrial laboratories. In 1954, John Beynon at Imperial Chemical Industries noted in a seminal paper in Nature [4] that mass spectrometry could be used for qualitative analysis of unknown compounds. He pointed out that spectra collected by the American Petroleum Institute [5] could be readily correlated with structure; for example, aromatic compounds give intense molecular ions and certain functional groups are associated with certain low-mass ions. Beynon proceeded to state some of the now familiar "rules" for deriving information from the *m/z* value of an ion (such as the nitrogen rule) and that knowing this value accurately can provide a unique molecular formula for the ion. He then illustrated the principles with examples using logical analyses of the fragmentation patterns. These principles laid the foundation for the use of mass spectrometry in the analysis of natural products development being rapid. After a few months, Beynon elaborated on these ideas [6] and also described the use of gas-liquid chromatography for the separation of a mixture, prior to mass spectral analysis.

The year 1954 also saw chemists at the Atlantic Refining Company in Philadelphia team up with a dermatologist at the University of Chicago to analyze fatty alcohols from human hair [7].

Beynon's papers are noteworthy for their paucity of references. By contrast, another seminal paper appeared in 1956. This paper, from Fred McLafferty at the Dow Chemical Laboratories in Midland, Michigan [8], has 37 references and provides a wealth of mass spectral information on a diverse range of boron-, oxygen-, nitrogen-, phosphorus-, sulfur-, and halogen-containing organic compounds. The paper is titled "Mass spectrometric analysis—broad applicability to chemical research" and graphically illustrated how important mass spectrometry had become to industry as early as 1955, by noting in its conclusion that the Dow Chemical Laboratory was running mass spectra on 10 000 samples per year. It is amazing to consider this number of spectra being counted and measured manually, computerized data acquisition systems being still years away!

Up to this time, instrumental development and studies on the chemistry of ions in the gas phase had made steady progress [9,10]. However, the end of the fifties saw the beginnings of mass spectrometry applied to natural products moving out of analytical applications in industrial laboratories into elucidation of structures and fragmentation patterns. Mass spectrometers became more widely available in academic and government laboratories [11–21] and development expanded rapidly. The stage was thus set for the appearance of two classic texts.

The first text was written by Beynon in 1960 [22]. Most of the work deals with instrumentation, with only one chapter in ten relating the mass spectra of organic compounds to their structures.

A milestone in NPMS came in 1962 with the publication of Biemann's monograph [23], the explicit intention of which was to alert organic chemists to the power of mass spectrometry (MS) in structure elucidation. Biemann noted that much of the earlier literature in mass spectrometry was published in analytical or physical journals and proceeded to describe mass spectra taken from a wide range of natural products. These included chapters on fatty acids and glycerides, amino acids and peptides, alkaloids, steroids, triterpenes, carbohydrates, nucleosides, and a topic of interest in the sixties, "bitter principles" from hops.

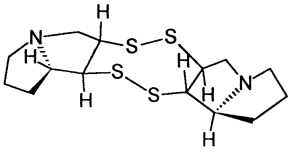
This list suggests that NPMS was already a mature field. In fact at that time electron ionization mass spectrometry (EIMS) was in reality only useful on certain derivatives from many of the previous substances. Nevertheless, gas chromatographs had already been coupled to mass spectrometers (GCMS) [24,25], including a high-resolution mass spectrometer [26], and Biemann described obtaining useful

mass spectra of underivatized nucleosides using a time-of-flight (TOF) spectrometer [27]. It is instructive to recognize that many important instrumental advances have been known for years. Many are tools that today are taken for granted, although often at first their applicability was very limited and developments were slow. TOF mass spectrometers, for example, fall into this category. The 1961 joint meeting of the A.S.T.M. E-14 Committee and the Mass Spectrometry Panel of the Hydrocarbon Research Group was the second of its kind and the precursor of the current series of International Mass Spectrometry Conferences. At this meeting, papers were read on field ionization mass spectrometry [28], popular for a limited time as a technique to circumvent some of the drawbacks of electron ionization of sensitive molecules, together with several papers describing double focusing mass spectrometers [29,30], instruments indispensable for the accurate determination of ionic masses, later giving rise to tandem mass spectrometry (MS/MS).

A very successful application of mass spectrometry to natural products chemistry at this time was in structural work on indole alkaloids, primarily the work of Biemann's own laboratory. This included development of the shift technique [23], the careful comparison of mass spectra from homologs or derivatives, which has led to understanding fragmentation pathways in the EIMS of many compounds.

Research in mass spectrometry from this time forward has progressed in leaps and bounds. Books on the subject proliferated, with two works by Budzikiewicz, Djerassi and Williams [31,32] and that of Reed [33] being especially relevant to NPMS. The application and study of mass spectrometry mushroomed dramatically within a very short period of time [34].

It was Biemann's work using MS to solve structures of indole alkaloids which caught this author's eye when he was a young graduate student attempting to determine structures of southern African *Strychnos* alkaloids and which resulted in a fruitful collaboration [35,36]. At the same time, Cooks was beginning his graduate studies in the same group, one aspect of which was the work on the novel, sulfur-containing

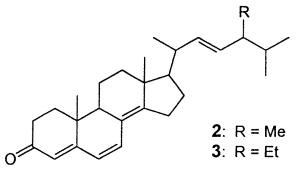


Structure **1**.

alkaloid cassipourine, **1**[37], the structure of which was later confirmed by x-ray crystallography [38] and synthesis [39]. This research group, led by the late Frank L. Warren, was active in studying alkaloids from indigenous *Senecio*, *Amaryllidaceae*, *Strychnos*, and *Rhyzophoraceae* species, in addition to triterpenes from *Euphorbia* resins. Work in this group proved to be an excellent training ground for organic chemistry in what was at that time a relatively isolated part of the world, laying the ground for the connections between this author, Graham Cooks, and mass spectrometry.

2. Years 30–45 — developing tandem mass spectrometry after 1970

Approximately thirty years had elapsed since mass spectrometry had evolved from being performed on specialty instruments in the laboratories of physicists to commercial development. It had now emerged as an essential tool in the laboratories of chemists. By this time Cooks had spent three productive years in Cambridge, U.K., mostly working on mass spectrometry but not specifically on NPMS. However, his first work after moving to Kansas State University in 1969 was appropriately relevant to the U.S. grain belt, in keeping with his initial South African training. Collaboration with colleagues led to a classic paper on the structural elucidation of fluorescent compounds from mold-damaged wheat flour [40]. Careful chromatographic separation was followed by the use of a full range of physical techniques, including the measurement of mass spectral fragmentation patterns and accurate masses. The compounds were identified as ergostatetraene-3-one, **2**, and its C-24 ethyl homolog, **3**. This approach was an efficient replacement for classical natural products chemistry, practiced for the



Structure **2**: R = Me. Structure **3**: R = Et.

preceding century. Structural problems could now be solved in months rather than years, or even lifetimes, and chemists could profitably collaborate with biologists in wide-ranging taxonomic and other studies, which used chemical skills in ways that were fast becoming almost routine. Mass spectrometry has played a large part in this revolution.

Other work at Kansas saw Cooks make some preliminary moves in the mass spectrometry of carbohydrates [41,42] and complete one of those obligations of a young faculty member, namely the writing of a review, which was the NPMS chapter [43] in the first Specialist Periodical Report of the Chemical Society entitled “Mass Spectrometry”. This series has had a limited life, but performed a valuable review role for some twenty years. As a measure of the dramatic growth of NPMS since 1960, this review [43] covered a two-year period during 1968–1970, discussed topics from oligomer sequencing to compounds giving rise to the aroma of strawberries and cited 322 references.

From Kansas, Cooks moved to Purdue University in 1971, where he has developed a thriving, multifaceted research program in the Aston Laboratory for Mass Spectrometry. Beynon had taken a position at Purdue in 1969 and remained there, although not full time, until 1975. Much of the early work in the Aston Laboratory during this time was focused on fundamental mass spectrometry. Developments from this work led to a major research theme on the use of tandem mass spectrometry, initially mass-analyzed ion kinetic energy spectroscopy (MIKES), for the direct analysis of mixtures [44,45]. The year 1977 saw the MIKES method used in the discovery of a new alkaloid in a crude plant extract [46]. Subsequently, the method was developed through other forms of tandem MS into a series of over a fifty papers. In the most common MIKES procedure, ions are mass selected in a magnetic sector, fragmented in a collision cell, and the product ions analyzed, based on their kinetic energies, using an electric sector. An eloquent personal account of the development of the technique has been recorded by Amy, Baitinger and Cooks [47]. When first introduced, the MIKES procedure was a significant advance, but its major disadvantage lies in the modest resolution inherent in electric sector analyzers. Over time, successful triple quadrupole mass spectrometers were developed [48]. These are ideal for MS/MS experiments, having typically a lower capital cost, but also better mass resolution in the selection of the product ions. Recently, the sophistication and speed of the approach has been improved in other ways by the use of an ion trap which can perform both MS^n experiments and parallel monitoring of multiple targeted compounds [49].

The crux of the early MIKES NPMS work was outlined in 1978 by Kondrat and Cooks [50] in which they described the protocol for the direct analysis of unseparated plant mixtures. They compared the technique in concept to the already established analytical methods of fluorescence spectroscopy and GCMS, as outlined in Fig. 1.

One key element in this work was the use of a soft ionization technique, such as chemical ionization (CI) by isobutane, to generate only protonated molecules from the matrix to be analyzed. By generating ions with low internal energies, ion fragmentations were minimized and the procedure resulted in good sensitivity. This is in contrast to the use of electron ionization (EI), which for most compounds deposits sufficient internal energy as to cause many ions to fragment, thus substantially reducing the ion current carried by the molecular ion. An exciting element of the procedure was the ability to identify, directly, compounds present in complex mixtures, including biological matrices. This was often accomplished by direct comparison of the analyte with a known standard, although it could in principle be used to characterize unknown compounds. Not requiring regular chemical separation and purification processes reduced the possibility of artifact formation in these

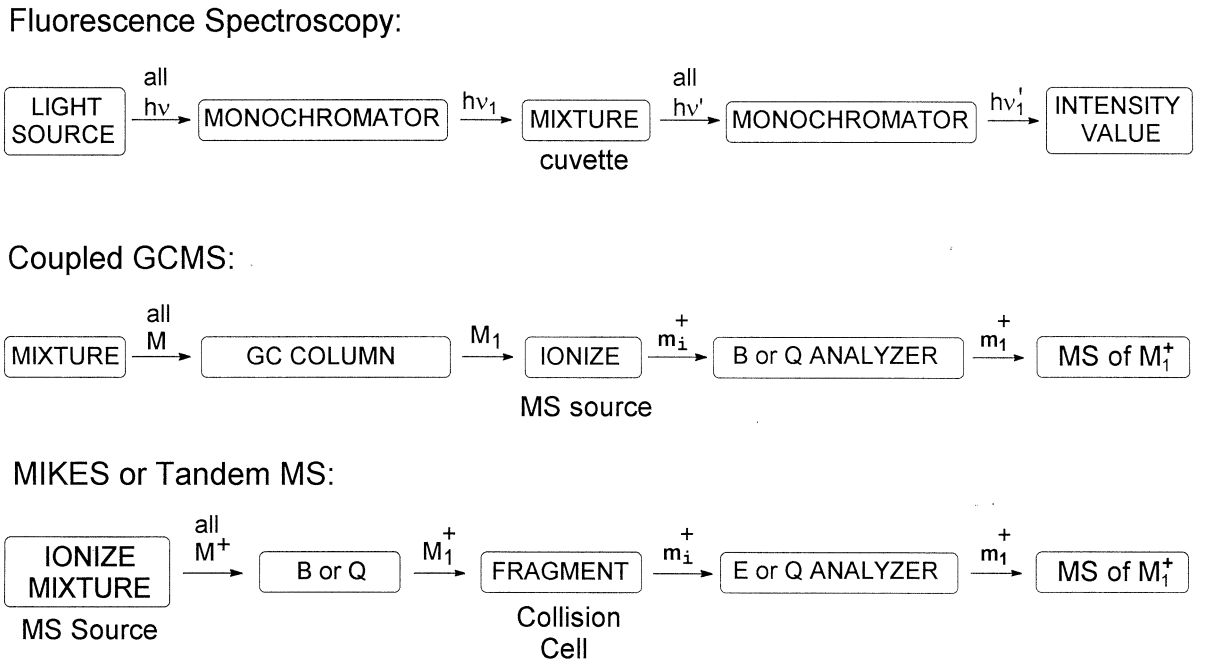


Fig. 1. A conceptual comparison of mixture analyses by fluorescence spectroscopy, coupled GC/MS and tandem mass spectrometry (adapted in part from [50]).

steps. The MIKES method was fast, showed good limits of detection, even with sample sizes in the nanogram range, and was amenable to quantification. The obvious drawback is that the method depends on only one analyte with a given molecular mass being present in the sample matrix. In some cases this problem can be circumvented if any such analytes have different proton affinities, in which cases selectivity could be achieved using different CI gases. Other combinations of mass analyzers in the form of three- or four-sector spectrometers have since been developed, allowing selection of the precursor ion at high resolution, subsequent collision-induced dissociation (CID), followed by analysis with much better mass resolution [51] than is possible with the original MIKES technique using a BE spectrometer.

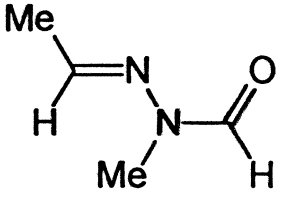
Isolation and characterization of natural products presented significant challenges only a decade prior to this work. One of the opportunities opened up by the technique was rapid screening of plant and other living materials for metabolites, thus leading to the development of phytochemistry as a discipline. Indeed, a significant number of the Cooks papers using MIKES for NPMS were written in collaboration with Jerry McLaughlin, a professor of pharmacognosy at Purdue.

The next development in the concept was remarkable, given that most mass spectrometrists trained in EIMS worry about keeping their EI source clean and are cautious about what samples they introduce into these sources. In 1978, Kondrat and coworkers published a paper in Science entitled, “Alkaloids in whole plant material: direct analysis by kinetic energy spectrometry” [52], the first of a series of papers describing the direct introduction, without chemical extraction, of biological matrices in the direct injection probe of a mass spectrometer. The paper describes experiments in which plant material was frozen in liquid nitrogen, ground, and placed into the mass spectrometer direct-introduction probe. On heating the probe, protonated molecules were formed by isobutane chemical ionization mass spectrometry

(CIMS) and mass-selected ions were subjected to the MIKES technique, leading to characteristic fragmentation patterns which could be interpreted or compared to the fragmentation patterns of known analytes. Plant materials studied included among others, poison hemlock, *Conium maculatum* L., and opium poppies, *Papaver somniferum* L. The authors estimated that they could detect the alkaloid analytes at the nanogram level.

Subsequent papers described analyses of mushroom metabolites from fresh or freeze-dried *Helvella esculenta* [53] and steroids from urine samples [54], although the latter were recognized to be complex matrices. *Helvella esculenta* had previously been shown to contain gyromitrin, **4**, a naturally occurring N-formylhydrazone.

The Cooks group placed mushroom samples into the solids probe [53] and using isobutane CIMS obtained ions corresponding to eight different protonated molecules. Use of the MIKES technique and 1 mg of dried mushroom enabled gyromitrin to be identified by a set of fragment ions consistent with the structure of a protonated, closed-shell ion, as shown in Fig. 2. In addition, it was possible to propose structures for four of the other ions found in the CIMS spectrum.

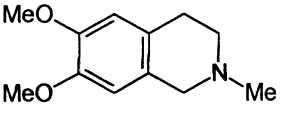


Structure **4**.

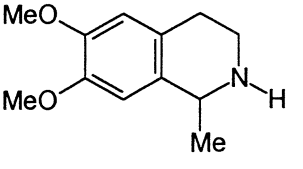
Further application of these techniques, coupled with MIKES obtained at different probe temperatures and conventional high-resolution mass spectrometry, enabled Unger and Cooks to propose structures for a range of natural products present in samples of the mushroom *Psilocybe cyanescens* [55]. The concepts were broadened to include both negative ion CIMS with charge inversion followed by MIKES [56], as well as multiple reaction monitoring in which the magnetic field was kept constant but the accelerating and electric sector voltages were selected to pass the ions of interest [57].

Arguably the seminal paper in the application of tandem mass spectrometry to plant chemistry was published in the 1979 under the title "Mapping of Cocaine and Cinnamoylcocaine in Whole Coca Plant Tissues by MIKES" [58]. This described how the techniques outlined in the preceding paragraphs could be combined to sample the alkaloid content in milligram-sized samples of plant material taken from all parts of the plant. The technique provided reproducible results in a short time frame and is thus significant for studies in chemotaxonomy and plant physiology. The work continues to be cited regularly [59]. The concepts were developed in a series of papers on cactus alkaloids [60–65]. In one study [60], it was shown that two alkaloids, one, **5**, with an N-methylisoquinoline skeleton and the other, with a C-methylisoquinoline skeleton, **6**, were both present in different cactus species. However, the N-methyl compounds were readily converted into C-methyl compounds using classical extraction and workup procedures.

Thus, the direct analysis using plant material and MIKES gave information that was unavailable by regular methods. In another study [62], an instrumental development saw the use of combined laser desorption and CIMS to liberate ions of quaternary ammonium salts from the sample matrix, for analysis by the MIKES technique. The concepts discussed in the previous paragraphs have been ably reviewed



Structure **5**.



Structure **6**.

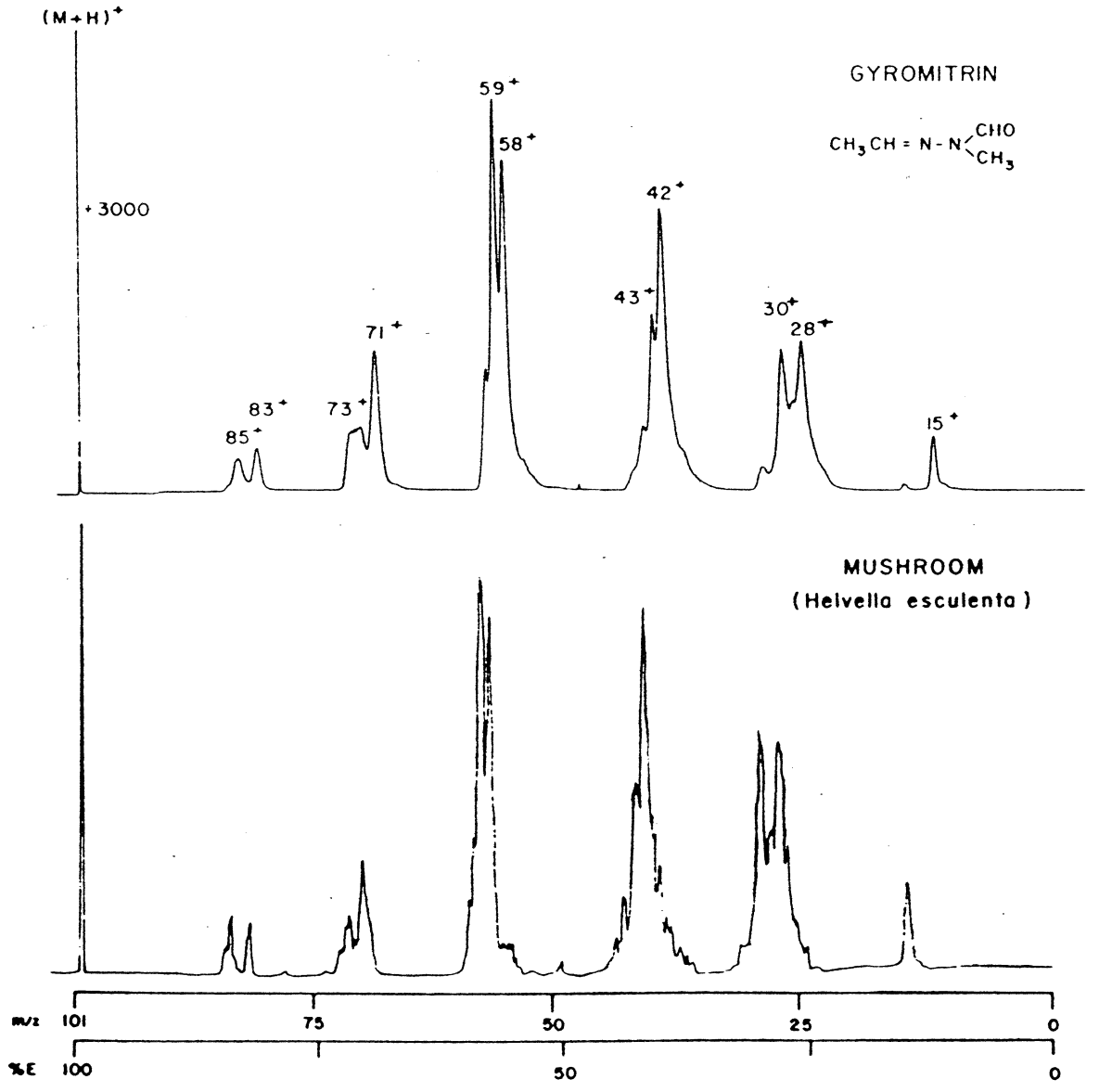


Fig. 2. A comparison of the MIKE spectra of *m/z* 101 from authentic gyromitrin, **4**, and a sample of mushroom tissue (reprinted from [53] (Fig. 1), with permission from Elsevier Science).

[66], including aspects of quantification using regular practices such as the use of standard addition or internal standards to generate calibration curves. An elegant example using a combination of temperature profiling of the solid natural material coupled with both high-energy and low-energy MS/MS showed the power of the method for the analysis of the complex mixtures present in *Myristica fragrans* (nutmeg) [67]. Similar techniques were used to study antineoplastic agents from *Psorospermum febrifugum* [68].

Coverage of this time period should include a pair of papers from 1980 on the use of a double quadrupole (QQ) mass spectrometer for the analysis of natural product mixtures [69,70]. The QQ spectrom-

eter featured a simple collision cell between two quadrupole mass analyzers, the ions of interest being selected by the first quadrupole. The resulting MS/MS spectra were comparable to spectra obtained by the MIKES technique, although the scan functions for the QQ spectrometer were simpler than those on a sector instrument. However, the triple quadrupole (QqQ) spectrometer design was found to be superior and is now widely used for such MS/MS experiments [48].

3. The fourth quarter: years 45–60—SID, SIMS, MIMS, and more

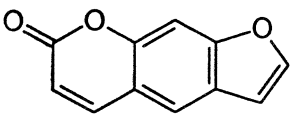
Cooks' work in mass spectrometry has always had a heavy emphasis on the fundamentals, leavened with useful applications, especially to NPMS. This section will attempt to summarize a series of developments, organized into the previous four categories, all of which have an NPMS component.

The first category deals with surface-induced dissociation (SID) of ions, as an alternative to CID for imparting sufficient internal energy to ions to cause fragmentation. The initial paper [71] describes novel processes, including the decomposition,

$$CO_2^{2+} \rightarrow CO^{+\cdot} + O^{+\cdot}$$

of the doubly charged carbon dioxide ion, arguably a natural product.

The paper concludes with the words, "this work suggests the possibility that a surface might be substituted for a collision gas as a convenient, simple, and efficient method of exciting gaseous ions". One might quibble with the "convenient and simple," but the concept has nevertheless proved to be valuable, although not widely used because of the rigorous demands placed on instrumental geometry [72]. It was suggested that the SID process deposits more internal energy in larger ions than does the conventional CID process [72,73]. SID and a range of other mass spectral techniques were used to identify isomeric structures [74] in a range of tricyclic, fused furobenzopyranones, typically structural isomers of psoralen, **7**, found in *Psoralea coryfolia* L., and a variety of other plants. These compounds are highly reactive



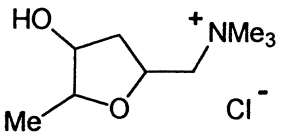
Structure **7**.

photoreagents towards DNA, have many structural isomers, and are difficult to characterize.

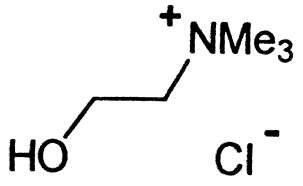
The techniques developed in the Cooks laboratory enabled five dimethyl isomers to be distinguished from each other, with SID being the most effective technique for distinguishing among the isomers. Detailed reviews of SID have been published [75,76] and the technique has now been extended for use in an ion cyclotron resonance mass spectrometer [77].

The second category deals with another technique studied by the Cooks group and applied to NPMS, namely, secondary ion mass spectrometry (SIMS) which is one of a series of desorption methods. In the SIMS method, an analyte deposited on a surface is bombarded with an ion beam having a very low current density, leading to sputtering of ions derived from the analyte into the mass spectrometer source. Benninghoven reported using this technique to obtain excellent mass spectra of either protonated or deprotonated amino acids, depending on whether the spectra were acquired in the positive or negative ion mode [78]. The technique could be applied to neutral species, which invariably become protonated, or become cationized by a complexing metal ion [79]. Alternatively, preionized analytes could simply be sputtered off the surface into the mass spectrometer. The key feature of these techniques is that they permit the direct study of thermally labile analytes, such as carbohydrates or amino acids and their derivatives. The technique has been extensively reviewed by Cooks and coworkers [80–82]. Variations in techniques studied were the use of solid ammonium chloride as a matrix for the analytes [83], deposition of the analyte directly on roughened silver foil [84], or on to a platinum or graphite support [85,86].

An interesting study on extracts from the mushroom *Inocybe napipes* showed that it was possible to obtain useful mass spectra of the muscarine, **8**, and choline, **9**, present in the extract directly by argon ion



Structure **8**.



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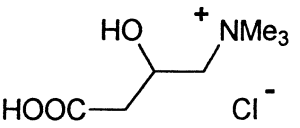


Structure **10**.

bombardment of spots on a thin-layer chromatogram (TLC) of the extract [84]. The results were compared with those from an early form of high-performance liquid chromatography coupled using a moving belt interface to a mass spectrometer with a CI source (HPLC/CIMS). The results were also compared with those from a direct probe sample combined with conventional CI and MS/MS. The direct SIMS results were more useful because of high background signals in the HPLC/CIMS attempts and the fact that the conventional CIMS spectra gave only fragment ions rather than the intact quaternary ammonium cations. A later attempt to develop a TLC scanner used laser-desorption and a heated transfer line to allow volatile samples to be carried from the TLC plate into the source of a QqQ mass spectrometer by the CI gas [87]. This was fairly successful for volatile compounds, but quite unsuitable for ionic compounds such as could be handled, albeit slowly, by the Cooks system [84].

Another intriguing facet of the SIMS technique was described in a study of carnitine, **10**, on silver and graphite surfaces with a range of different matrices [88]. The interesting finding was an intermolecular transmethylation reaction that was shown to occur in the selvedge region between the condensed and gas phases. This result and several of those described previously highlight the fact that mass spectrometrists using traditional EIMS need only be concerned with the chemistry of unimolecular decomposition processes. Other ionization processes may give unexpected chemical reactions, traps to catch the unwary.

The previous work [88] can be classed as NPMS. It could also be classed as surface science. The same applies to the final paper listed under the SIMS category, in which nucleotides and oligonucleotides were attached to a gold surface as monolayers. These were then probed using the SIMS technique [89] at both high and low collision energies. The method was intended to analyze relatively small molecules and the products of interactions between these molecules and the immobilized species. These species could be as large as nucleic acid residues.

The third category in the final quarter of the period covered by this retrospective deals with volatile organic compounds being introduced into the source of a mass spectrometer by their permeation through a semipermeable membrane, now known as membrane introduction mass spectrometry (MIMS). The method has particular potential for obtaining mass spectra of modestly sized organic compounds present in an aqueous solution. As mentioned previously, many techniques in mass spectrometry have been known for some time and MIMS is no exception, it having been first described in 1963 [90]. The first foray into this area by the Cooks group was in the form of a simple direct interface between a liquid flow and a triple quadrupole mass spectrometer [91]. Subsequent activity in the area has been regularly reviewed [92–95].

Initially, there was collaboration between the Cooks group and workers at the Dow Chemical Laboratories in Midland, Michigan, with one notable paper describing the in vivo analysis of organic contaminants in the blood of a rat [96]. A significant advance in the technology was the development of a robust sheet-membrane, direct-insertion probe for a quadrupole mass spectrometer [97]. This probe circumvented difficulties with previous probes, such as

memory effects, high detection limits, poor reproducibility, and analyte loss in heated transfer lines, leading the way to on-line monitoring of bioreactors using MIMS with flow injection analysis. Two microorganisms, *Bacillus polymyxa* and *Klebsiella oxytoca* were selected for study, primarily since their metabolism was well understood, but also because each produces, relatively cleanly, a different diastereomer of 2,3-butanediol [98]. The diastereomers were readily distinguished from each other by the MS/MS spectra of their diastereomeric phenylborate esters, prepared using a mixer reactor as part of a flow-injection analysis system [92].

Features of Cooks' MIMS work included the use of both EIMS and CIMS, the latter using methane or isobutane reagent gases. The use of EIMS permitted the quantification of gases dissolved in the fermentation broths, whereas the different CI gases allowed for control of the ionic species to be analyzed. Protonated molecules formed by isobutane CIMS were fragmented in the collision cell of the QqQ mass spectrometer that permitted characterization of the ions, including those from minor species. For example, both ethanol and formic acid have protonated molecules at *m/z* 47, but only ethanol gives a fragment ion at *m/z* 27, thus permitting identification of formic acid as a minor species present in the broth of *Klebsiella oxytoca*. All results were checked using GCMS. These experiments are an elegant example of the power of modern mass spectrometric techniques, namely a detailed, rapid analysis of a complex mixture in an aqueous matrix, using both gas phase ion chemistry and tandem mass spectrometry.

The concepts in this work have been extended to allow for online monitoring with feedback control [99]. The system studied here was a genetically engineered yeast, strain 1400, which is capable of withstanding high concentrations of ethanol. Batch fermentations under full feedback control with MIMS monitoring allowed a broth containing 50 g L^{-1} to be prepared [99]. Other applications of MIMS were described in a recent Biennial Review on Mass Spectrometry [100]. MIMS is a sophisticated, attractive technique for real-time monitoring of chemical processes, including those that generate natural products, such as microbiological fermentations. However, as with all technology, competition is relentless. It is possible to monitor fermentations with simple, cheap "electronic noses," a set of gas sensors based on semiconductor technology [101]. Such devices can be set up to monitor specific compounds, but the simplicity is at a cost of specificity and versatility. MIMS can be set to monitor a variety of compounds, or if concentration and sensitivity are less important, MIMS can allow for repetitive scanning of mass spectra. With modern computer software, these data can be manipulated to provide a total ion chromatogram from which individual mass chromatograms can be extracted. The information available from MIMS is simply much more detailed than can be obtained from one or more sensors that are sensitive to only a limited number of compounds.

The final "more" category of this section covers a miscellaneous collection of papers related to NPMS. An interesting pair of papers from the Aston Laboratory [102,103] explored the topic of gas-phase ion chemistry in the analysis of natural products. The work showed that the use of ethyl vinyl ether as a collision gas in a QqQ spectrometer led to reaction at low collision energies with protonated molecules containing proximate hydroxyl and ketone functionalities. This reaction was observed because the ions exiting the collision cell showed ions corresponding to both $M + H\rceil^+$ and $M + C_2H_2\rceil^+$. A reasonable mechanism for the formation of the latter ions was proposed. The concept of using gas-phase ion chemistry for selective analytical work is intriguing and ion-molecule reactions continue to be widely studied in finding solutions of analytical problems in natural products [104,105].

A series of papers on chemical modifications to nucleotides and nucleic acids has arisen from joint work between the Cooks group and that of Professor Ching-jer Chang. Much of the work has involved the selective analysis, at very low concentrations, of methylated species as part of carcinogenicity studies. Some effects of carcinogens have been linked to alkylation of DNA, although some alkylating agents are used clinically as anticancer drugs. Understanding the alkylation process in vivo is therefore important.

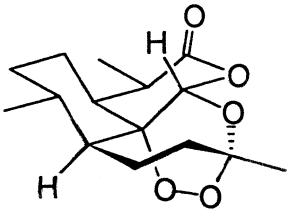
Early work made use of HPLC separation, as well as tandem mass spectrometry [106], with later work being done on a QqQ spectrometer using desorption chemical ionization (DCI) together with MS/MS [107,108]. Analysis on an alkylated dinucleotide was achieved at the subpicomole level. A detailed analytical protocol for studying the in vivo alkylation of DNA has been published [109]. The challenge is to achieve a quantitative analysis at the 10^{-14}-mole level of a series of similar, thermally labile nucleosides present in complex matrices. The alkylated species comprise only a small fraction of the overall mix of nucleosides. The nucleosides were derived from the alkylated DNA by enzymatic degradation, followed by HPLC separation [109]. The DCI technique was found to be the key to the high sensitivity of the method. Further refinements have led to a quantitative precision of at least 7% on methylated samples at the level of 10^{-13} mole in complex matrices [110].

Cooks has also made a couple of forays into the complex area of polypeptide mass spectrometry [111,112]. The latter paper is especially interesting as it provides evidence that some proteins possess different charge state envelopes as they are introduced in a QqQ mass spectrometer using electrospray ionization mass spectrometry. The different charge state envelopes could be observed readily by varying the collision gas pressure. Thus, for example, horse heart apomyoglobin (MW 16 951 Da) showed a high charge-state distribution centered about $M + 20H]^{20+}$ when no collision gas was present, whereas the envelope was centered around $M + 9H]^{9+}$ with an argon pressure of 2 mtorr [112]. Both envelopes were present at an argon collision gas pressure of 0.5 mtorr. At a pressure of 1 mtorr of argon, the lower charge state could be made to disappear leaving only the higher charge state present simply by making the potential of Q3 more negative with respect to Q2. In the presence of collision gas, the higher charge states were predominant at acidic pH values, whereas the lower charge states were predominant at a pH = 10.

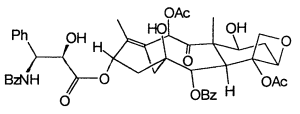
On the basis of considering the proteins as hard spheres and the collisions to be elastic or quasielastic [112], arguments were made that the two charge states represent different conformations and hence cross sectional areas, which would possess different ion mobilities. The higher charge states having larger cross sections (more open geometry) would undergo more collisions than the less charged states (more folded geometry) and hence would show sufficient energy loss that they were unable to exit Q2, unless a greater potential difference with respect to Q3 was present. Information was provided on the behavior under these conditions of eight other proteins. This heavily cited paper is an elegant example of collision processes in a mass spectrometer and the topic of protein conformations in the gas phase continues to attract much attention [113–115].

The conclusion to this section is a return to the beginning, namely the rapid screening of natural products found in complex plant materials. Artemisinin, **11**, is an antimalarial drug found in low concentrations in *Artimisia annua* L. (Compositae) and is unique among antimalarial drugs in that it does not contain a nitrogen atom [116]. Its most interesting chemical feature is an unstable endoperoxide ring and the compound also possesses other useful biological activity.

Extracts of *Artimisia annua* L. are very complex mixtures and the paper describes the development of a simple, rapid screening procedure for artemesinin and related compounds from a hexane extract of the plant leaves [116]. The techniques used were isobutane or ammonia DCIMS on a QqQ spectrometer leading to protonated molecules or $M + NH_4]^+$ ions, which could be studied by MS/MS techniques, such as constant neutral loss scans. These showed the presence of both known and new compounds. Structures could be suggested for some of the new compounds, even though no traditional isolation and



Structure **11**.



Structure **12.**

separation had been done. Artemisinin continues to attract attention [117].

Finally, the Cooks and Chang groups have produced three papers on the rapid screening and identification of taxanes, including paclitaxel, **12**, in crude plant extracts. In the first paper, electron-capture, ammonia DCI of taxanes was combined with MS/MS in a QqQ spectrometer to screen six different *Taxus* species [118]. Concentrations of active compounds were in the mg L^{-1} range and extracts could be examined at the rate of six per hour. A second paper compared other analytical procedures [119], whereas a third paper dealt with the screening of metabolites from a suspension cell culture of *Taxus brevifolia* [120]. As with the artemesinin work complex matrices presented challenges that led to the use of isotopically labeled standards for quantification. Several new taxanes were detected. Paclitaxel continues to be a compound of great pharmaceutical interest [121].

4. Conclusions

The concept of tandem mass spectrometry as an analytical tool for the analysis of mixtures has been discussed at length over the past two decades. Two reviews by McLafferty [122,123] compared the identification of a compound present in a complex matrix, such as an extract from a plant or a microorganism, to the analogy of finding “a needle in a haystack”. These two reviews were a prelude to a comprehensive monograph edited by McLafferty on “Tandem Mass Spectrometry” which included a chapter on the analytical aspects of the topic by Busch and Cooks [124] plus a chapter on NPMS by Maquestiau and Flammang [125]. More recently, the topics have been discussed in some detail by Busch and coworkers [126].

Cooks’ contributions to the analysis of natural products by tandem mass spectrometry have laid a substantial foundation for the use of the technique. His contributions are substantial, as has been described in the present work. However, the field is vast in scope as indicated by the dramatic growth in mass spectrometry since the early days documented at the beginning of this paper. McLafferty [123] actually lists some 46 topics to which tandem mass spectrometry had been applied by the end of 1980, of which 21 could be classed as NPMS. The list of 21 topics omits those related to petroleum chemistry.

The early tandem mass spectrometry using the MIKES technique must be seen as an approach to extract useful structural data from CID and it was an effective tool for its time. The concept of using sector MS/MS competitively against GCMS for routine analyses was novel but has not withstood the rapid changes in instrumentation that have occurred over the past twenty years. Early GCMS had a high capital cost and was not an easy technique to master. MIKES as an analytical tool required an expensive mass spectrometer, suffered from poor resolution of the fragment ions and needed a skilled mass spectrometrist to acquire and interpret the data. By contrast, a modern autoinjector, capillary-column GC coupled to a quadrupole or ion trap MS can be had for a modest capital cost. It is possible to search the massive data outputs of these automated machines against a library of spectra. Thus, modern analytical laboratories have a powerful machine that requires minimal intervention by technicians having a only modest level of training; even the interpretation of routine analyses can now be largely automated. This is a popular modern tool for rapid analytical screening of routine mixtures and, if the compounds are not thermally stable, similar automated HPLC/MS instruments using electrospray ionization are now available for a significantly lower cost than the old sector spectrometers.

However, this is not to suggest that tandem mass spectrometry has lost its usefulness for the analysis of natural products; rather it is a mature technique [127], alive and flourishing for more specialized work, as shown by a cursory look through current journals. Two examples of tandem mass spectrometry applied

to challenging natural product problems were selected at random. One reports on a structure determination and total analysis of complex sphingomyelin mixtures [128], the other describes a multicomponent quantification of diastereomeric amino sugars using MS/MS/MS [129] and both are logical extensions of work begun years ago by a master mass spectrometrist, R. Graham Cooks. It is reasonable to expect the need for analyses of biological materials to continue, even to increase, especially given the current popular interest in "natural medicines" and "natural foods." At present most of these are not analyzed for chemical content, but this will undoubtedly change in the future. When this happens, the need for rapid screening of complex matrices, for so long a trademark of work from the Cooks laboratory, will increase and no doubt many of the techniques used will rest on foundations laid by Graham Cooks.

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