QUANTITATIVE MASS SPECTROMETRY

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PREFACE

The driving force for the rapid expansion of mass spectrometry in the 1960s was the recognition by organic chemists of the vast amount of structural information which could be obtained by the technique. Workers in the area of medicine and biochemistry soon became convinced of the value of mass spectrometry as a qualitative tool, but naturally, having identified compounds of biological importance in their extracts, their attention turned to the quantitative determination of these substances. The recent growth of mass spectrometry as a quantitative technique is a direct result of the demands of these workers, and indeed the pioneering work in mass spectrometry in this area was carried out by biochemists and pharmacologists. Thus, not surprisingly, the majority of the examples in this book concern the determination of compounds of biochemical importance.

Although the increasing importance of quantitative mass spectrometry in the last few years has been obvious to anyone attending conferences on mass spectrometry, particularly where applied in medicine and biochemistry, this importance has been recognized in print only by the publication of a few reviews of specific areas. This book has been written to emphasize the fact that quantitative mass spectrometry is now a subject in its own right; many vastly different problems from a variety of disciplines can be tackled by a broadly similar approach. From this point of view, the problems of the perfume chemist are not very different from those of the pharmacologist or the quality control chemist. Each wishes to quantify a very small amount of a substance in a very complex mixture, and this book is intended to help each of them.

The book is also meant to bring to the attention of those who have not yet considered mass spectrometry to be a quantitative technique the considerable progress which has been made in harnessing the unrivalled sensitivity and specificity to quantitative determination. If such workers in other areas of research can be encouraged to apply mass spectrometry to their problems, then

their experience gained in other analytical techniques cannot but help to ensure the continuing progress of quantitative mass spectrometry.

Finally, I would like to express my sincere thanks to Professors Catherine Fenselau and Clyde Williams for devoting so much of their time to reading the typescript, and for their many helpful suggestions which have been incorporated into the final book.

London
September, 1977

Brian J. Millard

INTRODUCTION

There are still a few adherents to the school of thought that analytical instruments can be treated as 'black boxes', into which solutions are fed, and from which numerical results are obtained without worrying too much about what occurs inside the box. There are many occasions where such an approach can be reasonably satisfactory, for example when the black box is a simple, rugged instrument with few knobs to turn, and when the particular analysis is not pressing the capabilities of the instrument near to its limits of performance. By no stretch of the imagination can mass spectrometry be considered to be amenable to such an approach. Mass spectrometry is a relatively expensive technique, and more often than not analysts will have turned to it as a last resort because less expensive instrumentation is either not sensitive enough or not specific enough. In such circumstances the mass spectrometer will frequently be operating near to its limits.

If the instrument is not well maintained its sensitivity will inevitably decrease, sometimes by a factor of many hundreds, and the precision of the quantitative analyses will fall off, perhaps to intolerable levels. A fundamental understanding of how ions are produced, separated and detected is essential to the efficient utilization of a mass spectrometer, and the first two chapters of this book are devoted to this aspect, with a discussion of basic instrumentation and the more recent developments which are relevant to quantitative work.

The error involved in a measurement is a prime consideration in analytical work, and the various sources of error, from the initial sample manipulation to the final measurement of a response on the chart paper or by a computer, are covered in Chapter 3. Any discussion of errors inevitably moves into the realm of statistics, and simple statistical operations are utilized throughout the text. It was considered useful to gather together the more important equations and tables and place them in an Appendix at the end of this book for rapid reference.

The construction of calibration curves is a much larger topic than is apparent from the literature of quantitative mass spectrometry, and much more thought should be put into their determination. The various points which should be considered in connection with calibration lines are covered in Chapter 4.

Although, when quantitative mass spectrometry is mentioned, one's thoughts turn almost automatically to the use of selected ion monitoring in a combined gas chromatography mass spectrometry system, a study of the literature shows that much useful work is carried out by means of direct sample introduction. Indeed, there are many compounds that are too involatile or thermally unstable to pass through a gas chromatograph, and direct introduction is the only method of obtaining the necessary data. Without a doubt, this is a much neglected area, and one which would benefit considerably from further applications such as the determination of substances in biological tissues under high mass spectrometric resolving power.

At the moment, selected ion monitoring in a g.c.m.s. system is the fastest growing area of quantitative mass spectrometry, and therefore, not surprisingly, accounts for the largest chapter in this book. The three most important aspects of the method to consider are sensitivity, specificity, and the choice of internal standard. The first two are, to some extent, capable of being traded off against each other. For small amounts of a given component, single ion monitoring will be the most sensitive technique, but may result in a loss of specificity when compared with the monitoring of say four different ions in the spectrum. A choice between 1, 2, 3 or 4 channel monitoring will depend therefore very much on the nature of the problem, such as how little of the compound of interest is present, and how many interfering substances are present.

The use of single ion monitoring, which generally means that homologous internal standards have to be used which yield an ion in common with the compound of interest, has perhaps been emphasized rather strongly in this book. This is mainly an attempt to achieve a balance vis-à-vis the current literature on internal standards, where stable isotope labelled analogues are usually preferred. There are many instances where stable isotope labelled analogues are preferable, but it can be argued that there are many more occasions where they are chosen because they are fashionable, rather than because the relative merits of various internal standards have been tested rigorously and the labelled compounds have been shown to be superior.

It is intended that this book should stimulate a more questioning approach to the whole concept of quantitative determinations by mass spectrometry, so that the development of an assay is given the careful thought and attention to detail necessary if disappointment is to be avoided.

CONTENTS

CHAPTER 1

BASIC INSTRUMENTATION

A mass spectrometer may be defined for our purposes as an instrument that produces ions and then separates them according to their mass-to-charge (m/e) ratio. The ions can be produced in many ways; for example, by electron impact, chemical ionization, charge transfer, field ionization, field desorption and in a spark source. The ions can also be separated in many ways, by magnetic, quadrupole and time-of-flight analysers. Both positive and negative ions are formed in the mass spectrometer source, but the majority of instruments are designed for the efficient formation and examination of positive ions.

ION PRODUCTION

Electron impact

In an electron impact (e.i.) source, the sample is bombarded with a beam of electrons in the vapour phase. Usually, the energy of this beam can be adjusted from about 5 to 80 eV, but by convention operates normally at 70 eV. Unless otherwise stated, it can be assumed that published mass spectra have been obtained at this beam energy. It has been estimated that even at this high energy in the most efficient sources available, only about one molecule in a thousand is ionized. Of all the ions formed, only a small proportion are negatively charged. In the case of positive ions, most are singly charged, a few are doubly charged, and very occasionally triply charged ions may be formed. The occurrence of doubly charged ions is very dependent on the type of compound being ionized, and such ions are more prevalent in the mass spectra of aromatic compounds containing several rings.

For an organic compound M, the situation is as follows:

$$M + e \rightarrow [M]^{+} + 2e$$

$$M + e \rightarrow [M]^{2+} + 3e$$

$$M + e \rightarrow [M]^{3+} + 4e$$

The $\dot{+}$ notation for the species $[M]^{\dot{+}}$ implies that the latter is an ion radical, i.e. an ion with an odd number of electrons. The doubly charged species has an even number of electrons. Ions derived from the molecular ion by fragmentation may be even or odd electron species, depending on the identity of the precursor ion and the nature of the fragment lost.

The amount of energy necessary to remove an electron from M is called the ionization potential (IP). Ionization potentials can be measured by the mass spectrometer and by other techniques. The energy needed to form a particular fragment ion can also be measured, and this is called the appearance potential (AP) of the ion in question. When the electron beam is operating at 70 eV, an average of several electron volts of energy in excess of the ionization potential is transferred to the molecular ion. It is this excess energy which causes the molecular ion to fragment. A hypothetical energy distribution in the molecular ions formed from a compound under electron impact may look like the example shown in Fig. 1.1.

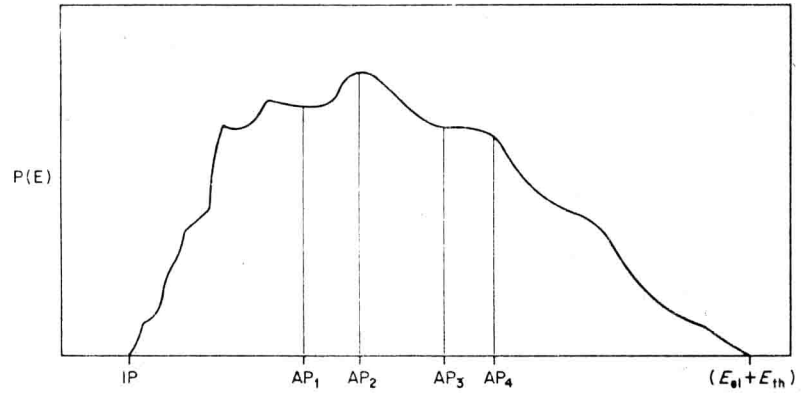
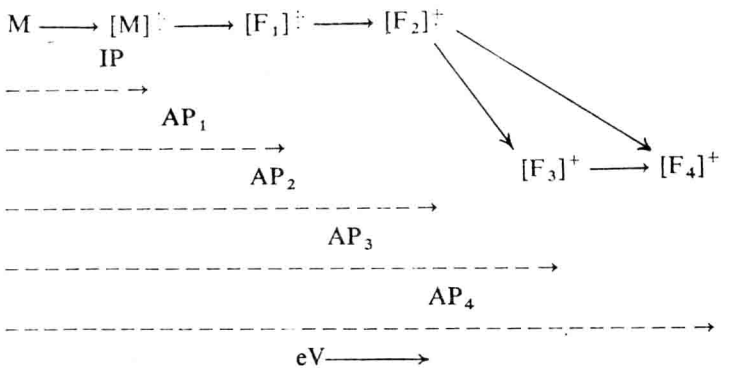


Fig. 1.1. A hypothetical energy distribution in molecular ions formed from an organic compound under electron impact. $P(E)$ represents the probability of occurrence of an ion with energy E. IP is the ionization potential of the compound. AP_1 to AP_4 are the appearance potentials of four different fragment ions in the mass spectrum. $(E_{el} + E_{th})$ is the sum of the electron beam energy and the thermal energy of the molecule.

The appearance potentials AP_1 to AP_4 represent the energy required to form fragment ions F_1 to F_4, where F_1 is the most easily formed of these ions, i.e. requires least energy. The fragment ions may be ions $[F_3]^+$ or ion radicals $[F_1]^{\dot{+}}$ and may be formed from two or more precursor ions.



Ions are considered to be more stable than ion radicals, and tend to fragment by loss of a neutral molecule to form another ion, rather than losing a radical to form an ion radical. Thus, fragmentations involving the consecutive loss of two radicals are rare.

Taking a simplified view, only those molecular ions with energies between IP and AP_1 will be observed as molecular ions in the mass spectrum. Ions with energy greater than AP_1 will have decomposed to the particular fragment ion for which they have sufficient energy. Therefore, the abundance of the molecular ion in the final mass spectrum will depend upon what proportion of the total area under the curve is represented by the area between IP and AP. The maximum energy which an ion may have, i.e. at the point represented by the high limit of the curve, will be the sum of the thermal energy of the molecule E_{th} and the energy of the electron beam E_{el}. For a molecule such as 1,2-diphenylethane, the average thermal energy has been calculated as 0.3 eV at 75 °C and 0.7 eV at 200 °C.[1] This is significant when compared with ionization potentials which are typically in the range 7–20 eV.

The simple view outlined above neglects what is called the kinetic shift. In a magnetic instrument, normal fragment ions observed in the mass spectrum are those which have been formed in the source. Since the residence time of ions in the source is about 10^{-6} s, the ions have to be formed with a unimolecular rate constant greater than 10^6 s^{-1} in order to be observable in the mass spectrum. The kinetic shift is the excess energy required above the theoretical appearance potential in order for decomposition to occur with this rate constant. Thus, the appearance potentials AP_1 to AP_4, as determined in the mass spectrometer, will be higher than the theoretical values.

A typical e.i. mass spectrum, for example that shown for chlorpromazine in Fig. 1.2, exhibits a large number of fragment ions in addition to the molecular ion.

In some cases, for example barbiturates, the molecular ion decomposes so readily that it is not observed in the spectrum. Since the most important item of information to be gleaned from a mass spectrum is the molecular weight of the

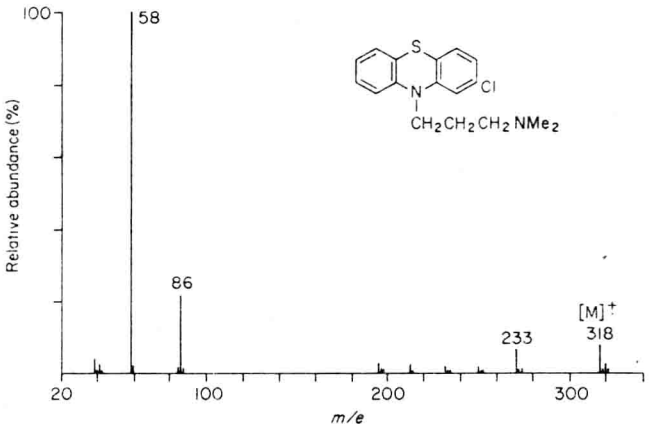


Fig. 1.2. Mass spectrum of chlorpromazine. The molecular ion is at *m/e* 318.

compound, the major disadvantage of using e.i. conditions on an unknown compound is that it is never quite clear that the highest *m/e* value encountered is that of the molecular ion. One way of surmounting this problem is to use the technique of metastable refocusing,‡ whereby it is possible to determine the precursor ions for those ions which are of interest. Thus, it can be determined whether or not the supposed molecular ion has a precursor, and if so, its *m/e* value may be ascertained. At present this method is not easily applicable to samples eluting from a gas chromatograph into the mass spectrometer source, and therefore is of limited interest to, say, workers in the biomedical field. The most useful solution to the problem is to use the softer ionization techniques of chemical ionization, field desorption or fission fragmentation. These techniques can reduce the wide energy spread of molecular ions, as shown in Fig. 1.1, to a very narrow band, so that insufficient energy is transferred to cause any appreciable fragmentation. These alternative ionization methods must always be considered to be complementary to electron impact ionization, mainly because no structural information is given simply from knowledge of the molecular weight of an unknown compound. In general, the more fragment ions there are in a mass spectrum, the greater is the amount of structural information that may be obtained.

A much larger proportion of the total ion current will be carried by the $[M + 1]^+$ ions typical of chemical ionization spectra than is carried by the molecular or fragment ion in the e.i. spectrum. If it is allowed that the total yield of ions from a given weight of sample is approximately the same, this means that there is a greater sensitivity for the particular compound. Another factor which becomes especially important when working with complex mixtures is that the

‡ See Chapter 2

total number of different ions produced from this large number of compounds is greatly reduced, thereby lessening the chances that some other compound can interfere with the analysis by producing an ion of the same m/e value as the one to be monitored.

Once a compound of interest has been identified and attention turns towards its quantitative determination, in the majority of cases it can be argued that chemical ionization in particular is of greater use than electron impact ionization.

Chemical ionization

In chemical ionization (c.i.) mass spectrometry, the compound under investigation is ionized by reaction with a set of reagent ions. These reagent ions are formed from the reactant gas by a combination of electron impact ionization and ion–molecule collisions. The proportion of compound to reactant gas is usually of the order of 1 to 1000, so that electron impact ionization of the compound does not occur. One of the most popular reactant gases is methane, and the electron impact ionization and ion–molecule reactions of methane can be summarized as follows:

$$CH_4 \rightarrow [CH_4]^{\cdot +}$$
$$[CH_4]^{\cdot +} + CH_4 \rightarrow [C_2H_5]^+ + CH_3\cdot$$
$$[CH_3]^+ + CH_4 \rightarrow [C_2H_5]^+ + H_2$$
$$[CH_3]^+ + 2CH_4 \rightarrow [C_3H_7]^+ + 2H_2$$
$$[CH_2]^{\cdot +} + 2CH_4 \rightarrow [C_3H_5]^+ + 2H_2 + H\cdot$$
$$[CH_2]^{\cdot +} + CH_4 \rightarrow [C_2H_4]^{\cdot +} + H_2$$
$$[CH_2]^{\cdot +} + CH_4 \rightarrow [C_2H_3]^+ + H_2 + H\cdot$$

At a source pressure of about 1 Torr, the ions formed from methane consist mainly of $[CH_5]^+$ ions (48%), with lesser amounts of $[C_2H_5]^+$ (40%) and

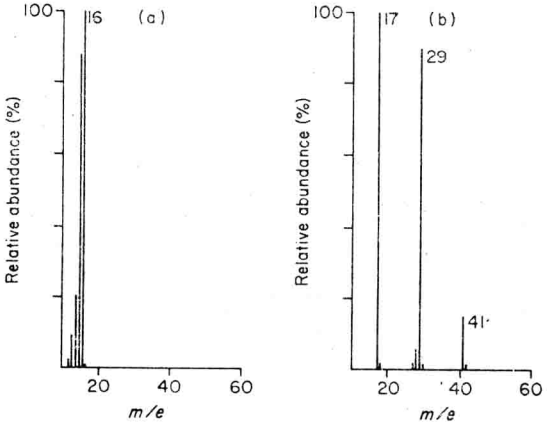


Fig. 1.3. Mass spectra of methane: (a) at 10^{-5} Torr; (b) at 1 Torr. (Reproduced with permission from Ref. 2.)

$[C_3H_5]^+$ (6%).[2] This can be seen in Fig. 1.3, where the mass spectrum of methane is shown for normal source pressures and at the high pressure of 1 Torr.

The reactant ions $[C_2H_5]^+$ and $[CH_5]^+$ react with the sample (BH) mainly by proton transfer or hydride abstraction:

$$[CH_5]^+ + BH \rightarrow [BH_2]^+ + CH_4$$
$$[C_2H_5]^+ + BH \rightarrow [BH_2]^+ + C_2H_4$$
$$[C_2H_5]^+ + BH \rightarrow [B]^+ + C_2H_6$$

To a lesser extent alkyl transfer reactions can occur to give $[M + 29]^+$ and $[M + 41]^+$ ions. Thus,

$$BH + [C_2H_5]^+ \rightarrow [BHC_2H_5]^+$$
$$BH + [C_3H_5]^+ \rightarrow [BHC_3H_5]^+$$

The ions $[M + 1]^+$ ($[BH_2]^+$) or $[M - 1]^+$ ($[B]^+$) have often been described as *quasimolecular* ions, but the term is now falling into disuse. Since these ions are even electron species, they are inherently more stable than the $[M]^+$ ion produced

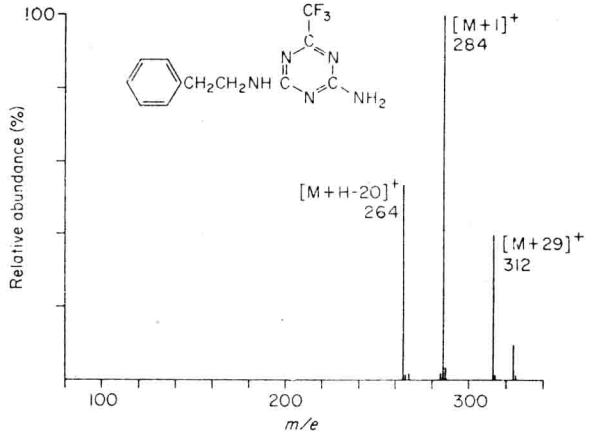


Fig. 1.4. Methane c.i. mass spectrum of a derivative of phenformin. (Reproduced with permission from S. B. Matin, J. B. Knight, J. H. Karam and P. H. Forsham, *Biomed. Mass Spectrom.* **1**, 320 (1974).)

by electron impact. Furthermore, the amount of energy transferred to the quasimolecular ion is much lower than that transferred in an e.i. source, but is highly dependent upon the reagent gas used. Because of these factors, the amount of fragmentation is usually greatly reduced, and the quasimolecular ion is normally the most intense ion in the mass spectrum. This is illustrated in Fig. 1.4, where the methane c.i. spectrum of a derivative of phenformin is given. The only significant fragmentation is the formation of an $[M + H - 20]^+$ ion. The alkyl transfer ion is intense.

There are many cases where the quasimolecular ion is not the most intense ion in the spectrum, however, for example the prostaglandins,[3] where the $[M + 1]^+$ ion loses one and two molecules of water to give more intense fragment ions.

Although earlier work on c.i. mass spectrometry was carried out using methane as reagent gas, much effort is being directed at present to the selection of reagent gases which will control the amount of fragmentation and also selectively ionize particular components in a mixture. Isobutane is a very popular reagent gas, where at high pressures the $[t\text{-}C_4H_9]^+$ ion accounts for over 90% of the ions formed. This ion is a weaker Brønsted acid than the $[CH_5]^+$ ion and transfers a proton with much less energy. Consequently, isobutane c.i. spectra are characterized by much less fragmentation, as shown in a study of a large number of drugs by Milne *et al.*[4] With the exception of aspirin, only $[MH]^+$ ions were produced. Isobutane transfers a proton only to the more basic compounds, so that strong acids are not ionized well. Frequently $[C_4H_9]^+$ is added to compounds such as alcohols.

Argon water mixtures have also been used successfully in c.i. work.[5] In a study of explosives, it was found that while methane and isobutane gave no $[MH]^+$ ions, water was successful in yielding intense $[M + 1]^+$ and $[M + 19]^+$ ions.[6] Ammonia has also been used, for example in a study of mono- and disaccharides,[7] where molecular weight information was obtained. Nitric oxide can condense with ketones, esters and carboxylic acids to give $[M + NO]^+$ ions and can also abstract hydride from aldehydes and ethers.[8]

There are some slight disadvantages to c.i. mass spectrometry which should be mentioned. In most cases the spectra are extremely temperature dependent, leading to poor reproducibility, a very important factor in quantitative work. The tight source design, combined with the fact that c.i. mass spectrometry is a high pressure technique, means that the source has to be cleaned much more frequently than is the case with e.i. sources. It is also extremely important to use high purity reagent gases, since the impurities present can lead to a very high background spectrum. Isobutane c.i. spectra frequently show cluster ions due to reaction with water present, so that very dry gases should be used. In combined gas chromatograph mass spectrometer (g.c.m.s.) systems, current practice is not to use the reagent gas as the carrier gas (the number of useful carrier gases is limited anyway), but to bleed the reagent gas into the system at a point after the molecular separator, since much better control over pressure, for example, is maintained. There are references, however, to the use of methane as a combined carrier gas and c.i. reagent gas.[8-10]

Three reviews by Field[11-13] are to be recommended as introductory reading to the topic of chemical ionization.

Charge exchange

In the above account, the reagent gases were shown to function mainly by transferring H^+ with the consequent formation of even electron ions. There is another category of c.i. reactions in which the reagent ions transfer an electron to form an odd electron species. This is called charge exchange or charge transfer, and usually involves a rare gas such as helium, argon or xenon. Since an odd molecular ion is formed, not surprisingly, charge exchange spectra look fairly similar

to e.i. spectra, although the degree of fragmentation can be controlled. It is this latter point that is important, for the molecular ion is much enhanced compared with e.i. spectra, but one still has the advantage of structurally significant fragment ions. A mixture of nitric oxide (5–10%) in helium, nitrogen or argon has proved to be especially useful for enhancing the molecular ion, for example in the case of the trimethylsilyl (TMS) ethers of bile acid methyl esters.[14,15] Figure 1.5 shows a charge exchange mass spectrum of heroin obtained through

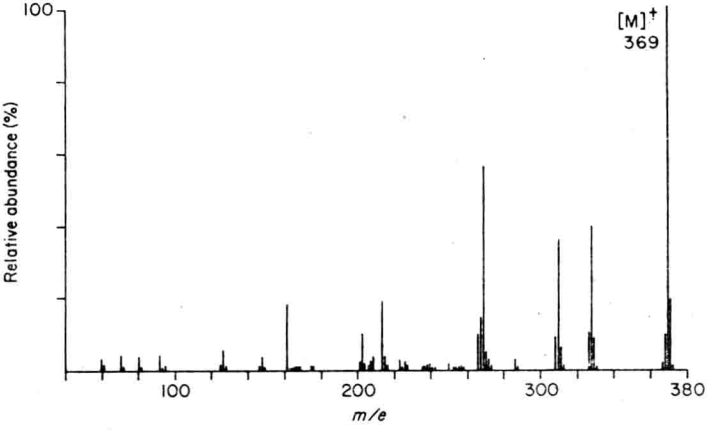


Fig. 1.5. Charge exchange spectrum of heroin via a g.c.m.s. system. Reagent gas 10% NO in N_2. (Reproduced with permission from Ref. 16.)

a g.c.m.s. system, using 10% nitric oxide in nitrogen.[16] One percent mixtures of nitric oxide in nitrogen have also been used.[17]

Field ionization

The technique of field ionization is well described in an early book by Beckey, the pioneer of the technique.[18] The anodes in early field ionization (f.i.) sources were sharp blades, wires or points. Present-day commercial sources employ wire emitters, and cathodes with slits in them. A high voltage of the order of 2 volts per Å is present between the two electrodes. Organic molecules subjected to this electric field can lose an electron by the tunnel effect to form positive ions. These are accelerated out of the source through the slit in the anode and then separated in the usual way by the mass spectrometer. Originally, f.i. sources suffered the serious drawback of very low sensitivity, but the activation of the emitter with benzonitrile has overcome this problem to some extent.[19]

Compared with the effort now being put into field desorption mass spectrometry, field ionization has received little attention of late, but the increased availability of field desorption sources which can be used readily for f.i. work may rectify matters. Although only a few references are available[20,21] to f.i.