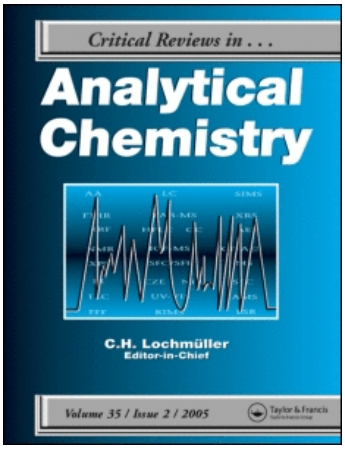

Critical Reviews in Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713400837

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To cite this Article Halpern, B. and Jellum, Egil(1981) 'Biomedical Application of Gas Chromatography-Mass Spectrometry', Critical Reviews in Analytical Chemistry, 11: 1, 49 — 78
To link to this Article: DOI: 10.1080/10408348108542706
URL: http://dx.doi.org/10.1080/10408348108542706

BIOMEDICAL APPLICATION OF GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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I. INTRODUCTION

During the past decade, integrated gas chromatography-mass spectrometry (GCMS) has become recognized as one of the most versatile and powerful tools in analytical chemistry. The ultimate strength of this technique resides with the unique capability to separate and simultaneously identify the individual components of even the most complex mixture. Sufficient sample volatility is a prerequisite of a successful GCMS analysis. Although this is a serious limitation of the method, GCMS does span an impressive volatility range. An effective identification and a reliable quantitation of a trace component within a complex mixture is largely dependent on the capability of separation. Highly efficient and inert capillary columns and direct sample injectors for these columns are now commercially available, and these have improved sensitivity and reproducibility due to narrower peak profiles and reduced column bleed. Alternatively, short capillary columns can serve extremely well in applications where lesser number of plates are sufficient or where the separation of higher boiling components is desired. A review of the current "state of the art" shows that significant progress continues to be made each year and that many of the new applications of the technique originate from improved instrumentation. The instrumental innovations include: positive and negative ion chemical ionization sources, interfaces for high-resolution glass columns, automated data handling and library searching facilities, and multiple ion detection capabilities.

The new generation of GCMS systems are designed for ease of operation and reliability. Many commercially available systems make use of quadrupole analyzers which are ideally suited to fast scanning and multiple ion detection. Some magnetic mass spectrometers have also been adapted for these applications by the use of accelerating voltage scanning. However, the key feature of most of the newer instruments is the use of microprocessors and dedicated computers to give total push-button control of all parameters needed for system operation. The benefits to the user of such a digitally controlled GCMS is in the ease of operation and the improved reliability of the system. The availability of preprogrammed options allows the operator to reproduce the exact instrumental condition of every run, and thus repetitive GCMS assays can be implemented. This facilitates the use of GC retention indexes to improve the specificity of identification which is particularly useful in cases where related (e.g., isomeric) compounds have to be distinguished. This feature also improves measurement precision where it is essential to control various instrumental parameters quite rigourously on a day-to-day basis. Most commerical instruments are suitable for biomedical applications provided they incorporate an all-glass GCMS interface. In the writer's laboratory the

commercially available equivalent to the open split-type connection of Henneberg[1,2] was shown to be superior to most other systems. The open split-type connection maintains atmospheric pressure at the end of the column; it is suitable for both packed and capillary columns and it allows for a rapid and safe column change. In addition, the incorporation of a scavenger flow into the splitting region makes it possible to dilute the GC effluent to a certain extent, so that the MS receives only a certain proportion of the sample. This peak dilution is of value when large amounts of solvents or reagents are present in the sample, which normally cause problems if admitted into the ion source.

The rate at which information can be generated by a GCMS system is so large that it cannot be utilized completely without an automatic data system. Many commercial units include mini- or microcomputers and suitable disc-oriented operating systems, graphic display and plotter units for data acquisition, analysis, and mass spectral library searching. The new top-of-the-line systems provide both CI and EI capabilities, a mass range of 10 to 1000 amu, sensitivity to picogram levels and programs for automatic calibration, real-time data acquisition, and data reduction. One manufacturer supplies a simultaneous but separate CI and EI option, which offers the advantages of two types of analysis with only one sample injection and the certainty that the two spectra originate from exactly identical sample components.[3] Many systems also offer a selected ion monitoring (SIM) option which has become a very widely used technique in all branches of analytical chemistry in the life sciences. In this mode, the mass spectrometer is used as a fixed mass filter to monitor the GC effluent and respond selectively to molecules which give rise to ions of the predetermined mass or masses. Molecules which do not generate ions at these specific masses are not detected. Because of this selectivity and the time averaging of the signal achievable in the fixed focus mode, the noise level is greatly reduced and the sensitivity is increased by a factor of up to 1000 compared to that obtained for the same ions during a mass scan. This quantitative SIM option has revolutionized the measurement of low molecular weight organics by its achievement of unsurpassed specificity and sensitivity in the picogram range.

Nowadays the major limitation on GCMS sensitivities is the presence of chemical noise in the sample. This is usually introduced during derivatization and chromatography. Interference by reagents, solvents, column bleed, and by previously adsorbed samples on the column often occur. Since there is no simple characteristic that distinguishes the sample from the chemical noise, both are recorded as ions making up the mass spectrum. While detection signals can be improved by certain signal-to-noise enhancement methods, there is still a limit to how much information is contained in the data. The analyst must not lose sight of the fact that the validity of the data is tied to the sample, the detector, and the actual measurement scheme with all the variables involved therein.

Operation in the negative ion mode at high sensitivity does require additional precautions. Deterioration of ion peak shape and dramatic sensitivity decreases in the negative ion mode precede deterioration in performance in the positive ion mode. Future improvements in chromatography, ion source design, and quadrupole configuration are necessary before high, sensitively negative chemical ion MS can become a routine operation.[4]

Some 500 citations on GCMS appear in the literature each year and a comprehensive coverage of biomedical applications is no longer possible in a review article of this size. Instead, the review is intended to illustrate some of the capabilities and limitations of the technique, the types of biomedical problems that are amenable to GCMS analysis, and the most important fields of applications and their relevance to current practice in biochemistry, medicine, pharmacology, and clinical chemistry. The applications, arranged in six sections, are

1. the detection and quantitation of underivatized volatile compounds in biological fluids
2. the selection of derivatives for gas chromatographic separation and mass spectrometric detection
3. metabolic profiling of volatilizable components of biological fluids by GCMS
4. biochemical applications of GCMS using alternative methods of ionization
5. applications of stable isotopes for studies of metabolism and biosynthesis
6. quantitative applications of GCMS

Since part of the subject matter has been reviewed previously,[6-9a,b] and some texts on MS include chapters on biomedical applications,[10-14a] most of the literature reviewed here covers the period 1973 to July 1979. Papers given at the conference on Quantitative Aspects of Mass Spectrometry Applications in Biomedical Research held in Naples, Italy in November 1978, the Second Conference on Applications of Negative Chemical Ionization Mass Spectrometry held in Chapel Hill, N.C. in March 1979, and the Eighth International Mass Spectrometry Conference held in Oslo, Norway in August 1979 have not been reviewed, as abstracts of the pertinent papers were not available.

II. THE DETECTION AND QUANTITATION OF UNDERIVATIZED VOLATILE COMPOUNDS IN BIOLOGICAL FLUIDS BY GC AND MS

Humans emit large numbers of volatile chemicals in the form of waste products produced by normal body functions. The means of exuding these chemicals may be oral (breath and saliva), skin (sweat and sebum), dermal cells, urine, and feces. Much of the earlier work to establish the nature of human effluent was performed in connection with the U.S. space submarine development programs.[15] In recent years the U.S. Naval Research Laboratory has used GCMS to analyze samples collected and desorbed from charcoal. The complexity of the work is illustrated by the fact that more than 5000 contaminants were found in nuclear submarine atmospheres. The GCMS analysis of underivatized volatile compounds in biological fluids has been reviewed recently.[16] A number of systems have been developed with which one can collect, identify, and measure total volatile effluents from humans at trace levels. These include: (a) direct head-space analysis which involves equilibration of the sample (blood, urine, etc.) with the head-space in a sealed container, followed by direct injection of a part of the head-space gas into a GC; (b) total or selective trapping head-space analysis, in which the heated sample is purged with an inert gas and the head-space volatiles are collected in a cold trap or are absorbed on a solid polymer support before transfer to a GC; (c) solvent extraction of the sample followed by a preconcentration of the extract prior to GC, and (d) direct chromatographic analysis, in which the untreated fluid sample is injected directly into a GC. All of the above methods suffer from some disadvantages. For example, direct injection of the head-space into a GC is not suitable for concentrations below 1 ppm; water may freeze in the trap and cause a blockage, or water may adhere to the adsorbent in head-space collection. Solvent removal may result in loss of some volatiles and repeated injection of blood and urine into a GC will leave an involatile residue which may pyrolyze during a subsequent analysis. Some of these problems can be overcome by modifications to the sample collection system and the GC injection port. Details for the construction of a suitable trap, solid probe inserter, and GC-precolumn system for selective head-space collection have been published[17-21] and modification of the GC inlet system for removal of residues accumulating in direct injection analysis have been described.[22] All of the above collection methods have been used for biomedical applications. Direct head-space analysis has been used for blood alcohol determinations

and for the identification of volatile drugs of abuse in blood and post-mortem tissues.[23] A number of solvent extraction techniques have been applied to the study of volatile organics in blood,[24] urine,[25] and cerebrospinal fluid.[26] Total trapping has been used for metabolic profiling of breath and urine[27] and selective trapping on Tenax-GC® has been applied to study volatile components in urine.[18] Direct injection of blood has been used to measure the concentration of alcohols, ketones,[22,28] and low molecular weight fatty acids.[29] Head-space collection on Tenax-GC® and Carbopack® B, followed by thermal desorption and analysis by capillary GCMS, is the preferred method for the analysis of volatiles from environmental samples. Some 150 compounds, which include hydrocarbons, aldehydes, ketones, alcohols, halogenated hydrocarbons, and esters have been shown to be present in ambient air samples. The presence of *N*-nitrosodimethylamine, a known carcinogen, in concentrations between 0.6ng m^{-3} to 32μg m^{-3} in air samples has been reported.[30] For a comprehensive literature survey on the GCMS analysis of organic volatiles in air samples the reader is referred to Alford's reviews.[31,32]

The quantitation of volatiles in head-space GC analyses by stripping and trapping components in a closed system has been discussed critically.[33] The internal standard method and the method of comparison with a model reference system are applicable, if all the substances to be determined are completely stripped out of the system and trapped in the column. The second method can be employed even if the components are not stripped out and collected completely, but it is necessary in this case that the composition of the matrix material of the reference system be at least approximately the same as that of the matrix material of the system under analysis.

Perhaps the most useful applications of the above techniques lie in the field of medicine. As human odors must, *a priori,* originate from biological processes the nature of the effluent emitted by man should provide signals indicative of a diseased state. Clinicians have known for years that characteristic body odors are sometimes associated with the diseased state. For example in maple syrup urine disease and phenylketonuria, the α-keto acids which accumulate in the urine are responsible for the odor associated with these inherited diseases. In recent years, the detection of unusual volatile metabolites from patients with characteristic body odors has been developed to the point where incorporation as a routine method in a laboratory is feasible. In the writer's laboratory the selective head-space collection was used in conjunction with high-resolution GC, odor assessment of the column effluent, and GCMS to study the influence of diet and antibiotics on the fish-like odor of trimethylaminuria patients[34] and to evaluate the effect of a choline load for the discrimination between normal individuals and heterozygotes. Selective-trapping head-space analysis has also been used in conjunction with SIM to study patients with diabetes mellitus before and after treatment with insulin, sulphonylurea, biguanide, and special diets.[35] GC profiles of volatile compounds in sera of normal and virus-infected patients have been interpreted by a combination of manual and computer techniques, and a two-peak ratio method was used for the classification of normal and virus-infected sera. Using the K-nearest neighbor approach, 86% of the unknown samples were classified correctly.[35a]

The clinical application of breath analysis for diagnostic purposes and for orthomolecular medicine has been reviewed.[36,37] The GC profiles of breath volatiles are quite complex and some 30 volatile organics are commonly found in the breath of human subjects. The breath of patients suffering from cirrhosis of the liver contains increased concentrations of short-chain fatty acids, as well as mercaptans and sulfides.[38,39] It has been suggested that altered bacterial ecology in the intestine and changes in hepatic blood flow are responsible for the increased acid concentrations. The sulfur-containing compounds are presumed to be derived from hepatic metabolism of methionine and cystine. The mercaptans accumulate due to the inability of the liver to further metabolize

the compounds formed by the normal thiol metabolism. A breath volatile profile may have some diagnostic value for liver function as changes in fatty acid concentration can be detected at an earlier stage than in other body fluids. It should be noted that breath-sample collection presents some unique problems. Because dead-space air (the volume of air that has not engaged in any gaseous exchange across membranes) differs from individual to individual, it is difficult to predict when expiration of alveolar air, which contains the volatiles of interest, commences.

The analysis of tissue for volatile constituents is in its infancy. The potential of the GCMS for tissue analysis has been noted by Goodman et al.[40] Politzer et al. have applied the technique of head-space trapping to examine volatile constitutents of lung, brain, and liver tissues.[41] A comparison of the volatile patterns showed marked differences between different tissues. A micro-method for the determination of volatile metabolites in biological samples, such as liver, has been published.

III. THE SELECTION OF DERIVATIVES FOR GAS CHROMATOGRAPHIC SEPARATION AND MASS SPECTROMETRIC DETECTION

There is substantial literature[42,43] on the derivatization of organic compounds for chromatography, but information on suitable derivatives for GS separation and MS detection is scattered throughout the literature. The following is an attempt to review the current state of the art.

The suitability of organic substances encountered in nature for direct GC analysis is the exception rather than the rule. For polar, nonvolatile molecules in the molecular weight range to a few hundred daltons, sufficient volatility for GCMS analysis can often be obtained by chemical derivatization. While the prime function of derivatization is to enhance volatility, the chemistry employed should also improve thermal stability and detectability by MS. The derivatization step should be simple, rapid, and quantitative, and the compounds should be stable and have good GC properties. In addition, it is important that the mass spectrum of the derivative should contain a prominent ion, preferably in the higher mass region for improved structural identification by EIMS and quantitation by SIM. Alternative derivatization methods can also be used to obtain additional structural information when the mass spectrum of an unknown cannot be fully interpreted.

For biomedical applications, contamination of the sample before GCMS analysis is a special problem. Many of the potential contaminants are introduced into the sample during work up and derivatization. Sources of contamination which are of possible concern include (1) antioxidants, (2) preservatives, (3) plasticizers, (4) background material extracted from TLC plates or ion exchange resins, (5) impurities present in solvents, and (6) artifacts introduced into the sample during derivatization. In order to make an informed choice about suitable derivatization procedures, it is important to know the likely artifacts and problems associated with alternative derivatives.

Trimethylsilyl (TMS) ethers and esters are probably the most popular derivatives for GCMS studies. TMS derivatives are easy to prepare, safe to handle, and most of them have excellent chromatographic properties. TMS derivatization often confers distinctive chromatographic behavior to diastereoisomers and in some cases these isomers show different MS fragmentation patterns. For example, the relative intensity of the rearrangement ion $^{m}/_{z}$ 147 due to [$Me_2SiOSiMe_3$] has been shown to reflect the steric disposition of trimethylsilyloxy groups on the steroid nucleus and carbohydrates.[44] The molecular ions of TMS ethers are often weak or absent with the $[M-15]^+$ ion obtained by cleavage of a methyl to silicon bond being more prominent. As for all ethers, α-cleavage

of the bond next to oxygen is favored and the ion $^{m}/_{z}$ 73 $[CH_3Si]^+$ is usually the base peak in the mass spectrum. The TMS group undergoes a large number of intramolecular migrations and rearrangements on electron impact to give prominent silicon-containing ions. In many cases TMS substituents also tend to direct the fragmentation of the molecule and are thus instrumental in the formation of diagnostic ions. For example, TMS derivatives of 6-, 11-, and 16-hydroxy steroids exhibit TMS-containing fragment ions which are characteristic of the position of the trimethylsilyloxy substitution on the steroid nucleus.[45] Low resolution EIMS fragmentation pathways can be conveniently interpreted with the aid of a duplicate experiment in which derivatization is repeated with a commercially available perdeuterated silyl reagent.[46] The observation of the shift in the molecular ion for the two spectra is a useful method of indicating the number of silyl groups introduced into the molecule which gives an indication of the number of functional groups present in the compound. Detailed information on how to prepare TMS derivatives has been published.[47] The introduction of a TMS group changes the character of the molecule from polar and active to nonpolar and inert. Normally silylation is applicable to all compounds containing active hydrogen functions. Individual functional groups, such as hydroxyl, may show different orders of reactivity towards different silyl donors. This has been exploited in the case of hydroxylated compounds where "mixed" labeled and unlabeled TMS derivatives were prepared to assist in the interpretation of mass spectral fragmentation.[48]

There is extensive literature on biomedical applications of TMS ethers and esters which includes studies on steroids, catecholamines, bile- , fatty- , and organic acids, prostaglandins, cerebrosides, and carbohydrates. For a detailed listing of references of biomedical application up to 1978 the reader should consult References 7 and 9. Some of the more recent applications of TMS ethers and esters include the identification of sugars by GC retention time and EIMS,[49] a study of anabolic steroid metabolism,[50] an analysis of urinary steroids in pregnancy,[51] the identification of abnormal deoxyribose metabolites,[52] the fingerprinting of carbohydrates of streptococcus mutans,[53] the analysis of biogenic amines,[54,55] the identification of amino sugars from bacterial lipopolysaccharides,[56] and the determination of conjugated double-bond positions in fatty acids.[57] Among the main disadvantages of TMS derivatization are the high mass increment (72 amu per functional group), the tendency for partial conversion of enolizable ketones into enol-TMS, and the ease of hydrolysis of some TMS ester and amide derivatives. The upper molecular weight range for GCMS of persilylated compounds is between 600 to 800 daltons. In the writer's laboratory the protonated molecular ion of TMS-Zeatin-riboside (mol wt = 639) was used for quantitative analysis by isotope dilution and SIM.[58] While TMS derivatives of enolizable ketones have been used for GCMS, it is best to convert the ketones to oximes,[59] or methyl-[60,61] or benzyl-oximes prior to silylation. TMS methyloximes usually show prominent molecular ions and M-31 fragmentation which are particularly suitable for SIM. Similarly, TMS derivatives of α-keto acids are suitable for metabolic profiling, but TMS oximes,[62] methyloximes, and quinoxalinols,[63] are preferred for quantitative MS. The TMS quinoxalinols in particular have very favorable properties for MS detection and quantitation due to the presence of high-abundance fragment ions at $^{m}/_{z}$ 217, 232, and 245, which are suitable for SIM. Provided TMS derivatization is carried out in screw-capped vials, containing a Teflon®-faced septum through which withdrawals can be made by syringe, TMS esters are quite stable and hydrolysis is usually not a problem. The major exceptions are TMS derivatives of dicarboxylic acids and some amides, such as acylglycines, which tend to partially hydrolyze to the mono TMS derivatives on standing. TMS derivatives are ideally suited for metabolic profiling, as many TMS reference mass spectra of interesting

compounds are included in the EPA/NIH Mass Spectral Data Base,[64] and in the Markey data file entitled Mass Spectra of Compounds of Biological Interest.[65]

In recent years various new silylating reagents have become available. Among the most useful are the t-butyldimethylsilyl ethers [t-BDMS][66] which tend to be more resistant towards solvolysis than the TMS ethers and are stable to acidic and basis conditions at room temperature. Selective cleavage back to the alcohol is possible by a treatment with tetra-*n*-butylammonium fluoride. The t-BDMS have been used for the GCMS analysis of prostaglandins,[67] steroids,[68] and nucleosides.[68] The mass spectra of t-BDMS ethers resemble those of TMS ethers with the exception that a very intense $[M-57]^+$ ion, due to the ready loss of the t-butyl radical, is present. This ion is usually the base peak and is useful for quantitative analysis by SIM. The t-BDMS derivatives have been shown to give more effective information for elucidating the structure of phospholipids than the corresponding TMS compounds.[69] For sterically hindered substrates the formation of the less bulky dimethylethylsilyl [DMES] and dimethylpropylsily [DMPS] ethers are preferred. These compounds yield characteristic ion clusters at $[M]^+$, $[M-15]^+$, and $[M-29]^+$ or $[M-43]^+$, which are useful for molecular weight determinations and for structural elucidation of complex molecules such as hydroxylated steroids[70,71] and bile acids.[72] In recent years the more volatile dimethylsilyl [DMS] ethers have been favored for high molecular weight polyol derivatization.[73,74] The relative retention times of the DMS derivatives of carbohydrates may be half that of the TMS ethers. The general features of the mass spectra of DMS ethers resemble those of the TMS ethers with the exception that they may show prominent molecular ions and that they may exhibit a pronounced rupture of the carbon-to-carbon bond adjacent to the oxygen atom within the alkyl moiety, in marked preference to cleavage within the silyl substituent. DMS ethers have also been used as a substitute for the more expensive deuterated TMS derivatives for the elucidation of mass spectral rearrangements.

For dicarboxylic acids and acylglycines a derivatization with diazomethane to methyl esters is recommended. A convenient technique for esterification of microgram quantities of organic acids with diazomethane has been reported.[75] About 500 acidic components of urine have been separated by GC via their methyl ester derivatives and some 200 of these were identified from their mass spectra. Retention data and key fragments of the mass spectra of the methyl esters have been tabulated.[75a] The EI mass spectra of 21 metabolically important *N*-acylglycines methyl esters have been published,[76] and *N*-dicarboxylmonoglycines have been characterized by GCMS.[77] The compounds are easily identified by the presence of diagnostic fragments at $[M-32]^+$, $[M-59]^+$, $[M-88]^+$, and $[M-116]^+$.[76] The advantages of methyl ester derivatization for acylglycines was demonstrated recently by the identification of suberylglycine as one of the major metabolites of dicarboxylic aciduria.[78] No evidence for the presence of this metabolite could be found in the TMS organic acid profile of the same urine. There is still considerable interest in the GCMS analysis of fatty acids via their methyl ester derivatives. Some of the more recent work has been concerned with the identification of monomethyl branched saturated fatty acids, which occur in trace amounts in the liver lipids of the baboon[79] and of midchain ethyl-substituted fatty acids which are present in the subcutaneous adipose tissue triacyglycerols of lambs.[80] The mass spectra of the monoethyl-substituted fatty acid methyl displayed several diagnostic features, which included a base peak at $^m/_z$ 74 and two sets of ketenes, one corresponding to the loss of methanol and methanol plus water from the ion $[CH_3(CH_2)_m CH(CH_2)_n COOCH_3]^+$ and the other, more intense one corresponding to the loss of methanol and methanol plus water from the ion $[CH_3 CH_2CH(CH_2)_n COOCH_3]^+$. In addition, ions corresponding to $[(CH_2)_n COOC_3]^+$ and a doublet due to proton transfer corresponding to $[(CH_2)_n$

$COOCH_3+H]^+$ and $[(CH_2)COOCH_3+2H]^+$ were found to be diagnostic for these compounds.

The interaction of alkyl- or arylboric acids with polyhydroxylated compounds offers an attractive alternative to TMS derivatization.[81] The MS fragmentation of the resulting cyclic boronates is frequently suppressed and as a result the ions containing the boron usually retain the intact ring structure. Stable methyl and butyl boronates have been prepared from corticosteroids[82] and cannabinolic acids[83] and the derivatives have been used to characterize isomeric acids. Cyclic boronates have also been used for the GCMS analysis of sphingosines and prostaglandin Fα.[84] A further extension of the method involves the oxidation of alkenes to diols and subsequent conversion to phenylboronates for GCMS analysis.[85]

Acylation is also an attractive alternative to silylation for compounds containing amino, hydroxyl, and thiol groups, giving more stable derivatives with amines, for example. Practical procedures for acylation have been reviewed by Blau and King.[86] Most of the methods are straightforward and can be readily adapted to both qualitative and quantitative GCMS analysis. The main factors in the choice of derivatives are sensitivity of detection, ease of preparation, and volatility of the resulting product. The acylation reaction is also suitable for the introduction of halogen-containing groupings into molecules to give electron-capturing derivatives suitable for negative ion chemical ionization MS.[4] In favorable cases this may enhance the sensitivity of detection by 10- to 100-fold. A number of suitable derivatives for this purpose have been listed in Section V. Although there are a large number of acyl groups available for derivatization, most of the GCMS work has been done with the trifluoroacetyl[TFA], pentafluoropropionyl[PFP] and heptafluorobutyryl [HFB] protecting groups. The TFA, PFP, and HFB derivatives tend to give more useful and identifiable ions in the high mass region of the mass spectrum than ordinary acetates. The TFA derivatives of phenols show molecular ions and major fragment ions corresponding to the loss of CO, CF_3·, and $COCF_3$· from the molecular ion, while the TFA derivatives of carbohydrates show less intense $[M]^+$ and fragment ions corresponding to the loss of TFA followed by losses of trifluoracetic acid.[86] Biomedical work on amines is dominated by investigations relating to biogenic amines. Perfluoracetylation is a convenient process for derivatizing low concentrations of amines for both positive and negative ion MS.[4,87,88] Perfluoracyl derivatives of amines give abundant ions corresponding to $[C_n F_{2n+1} CONHCH_2]^+$ and $[C_n F_{2n+1} CON(CH_3)CH_2]^+$ which are suitable for SIM. Such ions $^m/_z$ 240 $[C_3F_7 CONHCHCH_3]^+$ and $^m/_z$ 306 $[M\text{-}C_2F_5 CONHCH_2]^+$ were used for the quantification of picomole levels of plasma amphetamine[89] and brain 5-methoxy-tryptamine, respectively.[90] For GCMS analysis of amino acids the *N*-TFA *n*-butyl esters are the derivatives of choice. A large amount of mass spectral data is available for these derivatives.[91] The compounds fragment, by a loss of either of the terminal carbon substituent, with the dominant fragment being $[M\text{-}COOC_4H_9]^+$, or by a cleavage of the Cα-Cβ bond and subsequent fragmentation into nonspecific fragments. It is possible to select a significant ion for each amino acid for quantitative isotope ratio GCMS analysis.[92] *N*-perfluoroacyl peptide methyl esters are also suitable for the sequence analysis of polypeptides by GCMS. The method involves an initial partial hydrolysis of the polypeptide to yield a complex mixture of oligopeptides, which is derivatized to the TFA methyl esters to enhance volatility prior to GCMS analysis. A review on sequence analysis of polypeptides by GCMS has been published.[93]

Both acylation and silylation reactions can also be used to increase the volatility of pyrimidine bases, nucleosides, and nucleotides for GCMS analysis. The molecular ions as well as the $[M\text{-}CH_3]^+$ ion fragments of TMS uracil and thymine are suitable for SIM analysis.[94] It was also shown that the ion $[M\text{-base}]^+$ at $^m/_z$ 501 is significantly more

abundant for 3'- than for 5'-nucleoside monophosphoric acid per-TMS derivatives, permitting differentiation between the 3'- and 5'- nucleoside monophosphoric acids.[95] Because of the large mass increase upon acylation and silylation, permethyl derivatives are preferred for GCMS analysis of nucleosides. The permethylation reaction can be done with methyl iodide, silver oxide, or methylsulphinyl carbanion,[96] or with trimethylanilinium hydroxide [TMAH].[97] These derivatives, i.e., pentamethylurydilic acid [mol wt = 394], give EI mass spectra with prominent molecular ions and fragment ions which can be used to determine the composition and structure of both the base and sugar component of the nucleoside.[97] The pyrolysis methylation with TMAH is ideally suited to GCMS analysis, as the derivatization step can be done "on-column". The "on-column" TMAH methylation reaction has also been applied to the derivatization of drugs,[98] amino acids,[99] and peptides.[100,101] A number of other derivatives, such as the condensates of amines and amino acids with aldehydes,[102] ketones,[103] and carbon disulphide,[104] are also suitable for GCMS investigations. In particular, the isothiocyanates[104] and pentafluorbenzylidene[105] derivatives of amines are useful for the quantitative analysis of biogenic amines. Suitable ions for the SIM analysis of the isothiocyanates are the base ions $[M\text{-}CH_2NCS]^+$ and $[M\text{-}CH(CH_3)NCS]^+$. For a comprehensive listing of derivatives which have been used in GCMS investigations, the reader is referred to the Cumulative Subject Index in the *Chemical Society Specialist Report on Mass Spectrometry*, Vol. 5, 1979.

IV. METABOLIC PROFILING OF VOLATILIZABLE COMPONENTS OF BIOLOGICAL FLUIDS BY GCMS

The concept that individuals might have a "metabolic pattern" that would be reflected in the constituents of their biological fluids was first developed and tested by Williams in the late 1940s.[106] Utilizing data from a large number of paper chromatograms he was able to show that excretion patterns for a variety of substances varied greatly from individual to individual but that the patterns were relatively constant for a given individual. The utility of this type of analysis lay dormant until the 1960s when advances in GC and GCMS enabled the studies to be repeated. The term "metabolic profile" was introduced by the Hornings in 1971 to cover "multicomponent" GC analyses that define or describe metabolic patterns for a group of metabolically or analytically related metabolites.[107] Commenting on the potential of this type of analysis the Hornings suggested that ". . . profiles may prove to be useful to characterizing both normal and pathological states, for studies of drug metabolism and for human developmental studies." This definition of profiling still holds today although the present emphasis is more directed to the quantitative nature of the analysis. Most of the metabolic profiling work done to date make use of a computer-based repetitive scanning technique, first developed by Biemann, in which complete mass spectra are taken at frequent intervals and the spectra are stored in the computer.[108] By plotting intensity vs. time for key ions for each scan one obtains traces which are essentially equivalent to SIM data. Since sampling frequency for each ion is lower than for SIM, sensitivity and quantitative precision are lower, but this can be partially overcome by shortening the mass scan. Sample preparation and derivatization techniques of varying complexity have been published for the metabolic profiling of amino acids,[109] prostaglandins,[110] organic acids,[111,112] steroids,[113,114] and volatiles.[115] The metabolic profiling of volatiles is reviewed in Section II. It should be noted that during the process of sample preparation a variety of artifacts may be introduced into the profile. Traces of water may cause hydrolysis, particularly of TMS derivatives, and drugs and diet may be responsible for significant changes in the appearance of the profile. The particular isolation technique used will influence the

number, kind, and yield of the metabolites extracted. For example, an organic acid profile of urine will be artificially enriched in aromatic acids if solvent extraction is used. There has been considerable interest in the profiling of urinary organic acids because the identification of abnormal metabolites or the presence of acids in abnormal amounts has already led to the diagnosis of some thirty metabolic disorders.[111,116-118] An excellent summary of the potential values of GCMS profiling to the diagnosis and study of metabolic diseases is given in the proceedings of the Montreal Children's Hospital workshop held in 1973[119] and the Tübingen Symposium held in 1977.[119a]

One of the major impediments to quantitation of profiling data by GCMS has been the limited resolution of packed GC columns, hence, most of the more recent profiling work has been concentrated on the application of high-resolution column technology and/or automated data processing techniques to achieve deconvolution of unresolved GC peaks. With capillary columns, resolution is significantly better than with packed GC columns and thus the introduction of highly efficient, stable small-diameter columns and direct sample injection systems has led to more reproducible metabolic profiles. The alternative approach, which has been developed by Sweeley[120,121] and the Stanford group[122] uses "clean up" programs to remove background contributions of overlapping components based on the profiles of well-resolved MS peaks. The processed spectra are then compared to a library of spectra obtained from previous analyses of similar spectra. The traditional method of identifying certain components in a metabolic profile has been to compare their mass spectra with a library of reference spectra to find the best match.[123] This forward library searching requires large data bases and a number of such reference collections are now commercially available. Forward library searching is quite time consuming, but a presorting of the library file according to compound classes, functional groups,[124] or elemental composition, such as the presence of silicon if TMS derivatization is used, reduce search time.

An alternative approach has been termed the "inverted file" method. A prior analysis of the data base is carried out to establish two reference indexes. The first is a table of the frequency of occurrence in the data base of each integer mass value. The second consists of a set of tables, one for each integer mass value, listing the location within the data base of each mass spectrum having that mass value as a prominent peak. In attempting to identify an unknown compound, the first reference index is used to select the most diagnostic prominent peaks in its mass spectrum, i.e., those having the lowest frequency of occurrence in the data base. These are then checked in the record index for the existence of one or more mass spectra having those diagnostic peaks in common. Finally, the full mass spectra of those compounds which emerge from the search are compared with the mass spectrum of the unknown compound. Thus a search of even a very large data base reduces to the comparison of a mere handful of mass spectra. Although the necessary analysis of the data base to produce the reference indexes requires a large amount of computational effort, it needs to be done only once for any given data base. One principal advantage of the method then is that this prior analysis can be carried out on a large fast computer but the actual search of the indexed files can be left to a small mini- or even microcomputer. Some of these features are included in commercially available library search systems. For example, the Finnigan Incos forward and reverse search takes an unknown spectrum and attempts to find whether that spectrum occurs in the library (a purity search), whether any library spectrum after renormalization is present in the unknown (a fit search), or whether there are any fragmentation similarities between any part of the unknown and any library spectrum (a reverse fit search).

GC retention indexes can also be used in combination with mass spectral correlations to further identify compounds by both forward or reverse library searches.[125-129] One of the problems in constructing a successful library matching system is the selection of a

suitable coding scheme which will compress the data sufficiently to reduce search time. Abbreviation of the spectra often improves search performance by eliminating or minimizing the variability of recorded spectra. For example, the use of a minimum intensity threshold ensures that low intensity peaks of doubtful reproducibility are not used in the matching procedure. The most popular coding systems use the intensity of a limited number of peaks, usually the 10 most intense ions. This method is simple and direct both in encoding and subsequent matching and allows a surprisingly unique description of each component. A natural development of this approach is the encoding of N peaks every M mass units, intervals of 14 amu being most popular for this purpose. Other methods of selecting the peaks to be stored in the library include structurally relevent peaks or those chosen on the basis of statistical tests for the most discriminatory peaks in a mass spectrum.[130] This procedure, which is now available in time-share systems, uses "probability based matching of mass spectra". It involves examination of the following factors: the uniqueness of a particular ion relative to all of the $^{m}/_{z}$ values in several thousand reference spectra, the abundance of the ion in the reference spectrum, the degree of "dilution" of the spectrum by other spectra, and the "window tolerance" or degree of variability permitted as compared to the reference spectrum. These criteria are used to compute a "confidence index" which must be above a certain value for a spectrum to be declared a match against a reference spectrum. Peysna et al. have also published a study of the uniqueness of various masses[131] and Altwater et al. a procedure for matching of mixture mass spectra by substraction of reference spectra.[132] A comprehensive discussion of methods for comparing a spectrum with a library of reference spectra is given in Chapman's book.[133]

Quantitation is usually based on comparing the summed ion intensities for each peak to the area of an internal standards.[121,122,134] In this way quantitative data for 134 acidic substances in human urine have been determined. Recently capillary columns have been used in conjunction with high-resolution MS for urine[135] and serum[136] organic acid analysis. The detection limits of the system were in the range of 5 to 10 ng per component, and several compounds previously labeled as "unknowns" or tentatively identified by GC and low-resolution MS were assigned structures.[135] Accurate MS was also shown to be useful for distinguishing components which yield fragments of the same nominal mass but which different elemental compositions.[135]

A complete profile of all the constituents of a biological fluid is at present impossible with available analytical techniques. Most workers aim at the development of a complete profile of a selected group of substances or of compounds with similar physical properties. A comparison is then made between the profile obtained with normal samples and pathological samples to establish quantitative differences that might be of value for diagnostic purposes. In the case of metabolic disorders, where a few compounds are present in large amounts, the clinical significance is usually readily determined. In this field GCMS is well established, as some 60 metabolic disorders can be diagnosed by multicomponent analysis of endogenous metabolites in body fluids. A number of hospitals now use the method on a routine basis and some 100 papers with clinical relevance have been published in this field during the last few years. However, in the more common case, where the disturbance of metabolism is more subtle, the resulting chromatograms are so complex that the resulting information can no longer be handled by empirical means and the use of pattern recognition techniques and computer sorting are obligatory. Only a few, usually rather preliminary, studies have been completed in this field; these include Witten et al.,[137] Markey et al.,[123] and Chalmers et al.,[138,139] who examined the effect of diet on the urinary organic acid profile; Blau et al., who quantitated urinary aromatic acid excretion for the diagnosis of phenylketonuria heterozygotes,[140] and Thompson et al.,[141] who quantitated urinary organic acids by age

group in a healthy pediatric population. Because only a few clinical metabolic profiling studies have been done, little has been published that deals with statistical analysis of this type of data. Johnson[142] has related the concept of the metabolic profile to multivariate statistics. Robinson and Pauling have examined urine "volatiles",[143] and Dirreu et al. amino acids,[144] and applied pattern-recognition procedures to identify peaks and compared the collected data from several runs using a statistical ranking procedure. The most recent statistical techniques that have been applied to profiling are computerized pattern recognition, which may find data patterns that are not apparent from traditional statistical analysis,[144a] and SIMCA, which analyzes data in terms of similarity and analogy.[144b,c]

Most profiling work reported to date has been on constituents in urine and metabolic profiling of other physiological fluids such as amniotic fluid and cerebrospinal fluid has been somewhat neglected. It is known that a number of organic acidurias give rise to increased organic acid concentrations in amniotic fluid taken at 14 weeks of pregnancy. Since quantitative metabolic profiling of organic acids is technically easier than the determination of enzyme activity in cultured amniotic cells,[145,146] in utero diagnosis of some pregnancies at risk by GCMS will be used routinely for the antenatal diagnosis of a number of organic acidurias. Amniotic fluid profiling will also be of value in following the effect of any prenatal treatment such as the use of vitamin B_{12} for methylmalonic aciduria.[147] Cerebrospinal fluid also contains substances representing intermediates and end products of cerebral metabolism. The establishment of quantitative metabolic profiles for various classes of compounds, such as biogenic amine metabolites, as well as the determination of selected constituents in pathological samples, has potential in neurodiagnosis and in the study of drug-induced changes in cerebral metabolism.[148]

Analytical methodology developed for the study of endogenous constituents such as organic acids, steroids, and volatiles, can be easily adapted to studies of drugs and drug metabolites. Methylene screening and a specialized library containing the mass spectra of some 400 drugs and their metabolites permits fast identification.[149] GCMS has been used routinely to identify drugs in body fluids of drug overdose victims.[150]

In conclusion, GSMC metabolic profiling has become a valuable analytical tool in clinical biochemistry. The technique is very suitable for the diagnosis and study of inborn errors of metabolism as these defects often result in abnormal excretion or accumulation of metabolites in the cells and in the body fluids. The value of metabolic patterns associated with other diseased states, such as cancer, has not been established.[151] Further study and documentation of normal metabolic profiles is required before metabolic pattern changes can be associated with the diseased state.

V. BIOCHEMICAL APPLICATIONS OF GCMS USING ALTERNATIVE METHODS OF IONIZATION

The mode of ionization of a molecule has a strong influence on its behavior in MS.[152] In chemical ionization (CI) developed by Munson and Field, a reagent gas is first ionized by electron impact and these ions then react "chemically" with the substrate in fast acid-base type reactions to form ions which subsequently fragment to various extents. In this process the degree of ionization of the substrate is determined by the structure of the substrate and the nature of the reagent gas. The fundamental condition for CI is fulfilled for proton transfer if the proton affinity of the substrate exceeds that of the conjugate base of the reagent ion. Chemical ionization can also be effected by charge exchange between ions of the reagent gas and molecules of the substrate. Here too, the reactivity of the initially formed ion can be controlled by a suitable choice of the ionizing agent. In this case the energy content of the molecular ion is determined by the difference

between the recombination energy of the reagent ion and the ionization potential of the substrate. Much of the power of CI stems from the finding that the characteristics of the spectrum produced is dependent on both the nature of the reagent gas and the type of ion-molecule reaction used to ionize the sample.[153] Different structural information can be obtained with different reagent gases. As a consequence, results can range between extremes of high ionization selectivity and reduced fragmentation on the one hand, and extensive fragmentation and low selectivity on the other. For example, isobutane can be used for the analysis of nitrogen isotopes in ammonia as the $[t-C_4H_9]^+$ ion will protonate ammonia but not water so $[H_3O]^+$ cannot interfere in the analysis of $[^{15}NH_4]^+$. Ammonia is a useful gas for the analysis of compounds such as sugars, which do not give a stable protonated molecular ion with methane or isobutane. The $[NO]^+$ ion from nitric oxide is a very selective hydride abstractor, but it can also condense with ketones and carboxylic acids to form $[M+NO]^+$ ions. Nitric oxide diluted with nitrogen acts principally as a charge-exchange reagent giving abundant $[M]^+$ ions. Helium-water and argon-water mixtures have also been used for CIMS, here the $[He]^+$ ions cause dissociative charge exchange and the $[H_3O]^+$ ions protonate the substrate molecules.

Technically, combination of GC with CIMS is simpler than with its EI counterpart.[154,155] The reagent gas (for example, methane) can be passed through the GC and thus serve at the same time as a carrier gas. Because of the higher pressure required in the ion source, a direct connection between the GC and MS is possible and this reduces sample loss. Independent optimization of separation and reagent gas properties are possible if different carrier and reagent gases are employed. In this arrangement the reagent gas must be added to the carrier gas flow after the GC separation is complete. In capillary GC-CIMS where carrier gas flow is relatively low (1 mℓ/min), such optimization of CI conditions is easily achieved. For packed columns which require high carrier gas flows of the order of 20 to 30 mℓ/min, it is necessary to remove most of the carrier gas with a molecular separator before the reagent gas is added. This ensures complete flexibility but at the expense of some loss of sensitivity owing to unavoidable losses of material on pressure reduction. Proper control of CI conditions is important for the analysis of multicomponent systems and for structural studies. In the analysis of complex mixtures by GC-CIMS, a sequential reaction with different reagent gases, one at a time, may allow elimination of ion current contributions from interfering components. For analytical purposes CIMS offers reliable molecular weight information and the possibility of controlling fragmentation processes.[156] Optimization of $[MH]^+$ ions results in high detection sensitivity and reduces the risk of interferences from chemical noise, especially at the higher $^m/_z$ values. The control of ionization also gives some flexibility as to the selectivity desired and in some cases the complete exclusion of co-eluting contaminants is possible. Although identification, proof of structure, and elucidation of fragmentation processes of organic molecules by EIMS is well established, CIMS has not been extensively utilized for structural examination of complex organics. Some interesting applications of CI for this purpose are the use of on line hydrogen-deuterium exchange in capillary GC-CIMS for structure analyses of complex mixtures,[157] the use of hydrogen and isobutane CIMS to characterize carotenoids,[158] the differentiation between 1-2 and 1-4 linked dissacharides,[159] the characterization of the stereochemistry of glycoside linkages,[159] and the identification of bile acid methyl esters by means of GC retention volumes and methane CIMS.[160] Another important application of CIMS is the use of ammonia and trideuteroammonia as reagents for the determination of active hydrogen in organic compounds.[161] The method is based on an exchange of active hydrogen with deuterium during CIMS with trideuteroammonia. A comparison of the $^m/_z$ ion for the ammonium adduct $[M+NH_4]^+$ with the trideutero-ammonium adduct $[M\text{-}nH+nD+ND_4]^+$ yields the number of active hydrogens. Both the

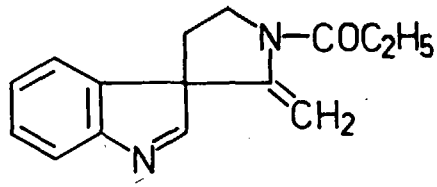

FIGURE 1.

number of active hydrogens and the nature of the functional group can be identified concurrently in favorable cases. The procedure has already been applied to the identification of functionality in C_{27} steroids.[162] The GC-CIMS instrumentation has also been used to determine the steric purity of amines and amino acids by reacting the compound to be analyzed with a mixture of N-TFA-R-prolylchloride and N-TFA-S-[1-^{2}H]-prolylchloride, separating the diastereoisomers by GC and quantifying the amount of each diastereoisomer present by SIM.[163] Many of the biochemical applications of GC-CIMS have been concerned with the identification and detection of known substances. This is reflected in the inclusion of CI spectra in large general collections of mass spectra and the availability of specific collection of CI reference spectra for drug and drug metabolite identification.[164] For example, the Batelle Institute GC-CIMS drug identification program depends on a match of CI fragment ions to distinguish between isomeric drugs.[165] For quantitative determinations of very low concentrations in multicomponent mixtures, GC-CIMS in conjunction with SIM is very effective. For these applications the use of standards multiply labeled with stable isotopes of high isotopic purity is recommended as this precludes overlap in the recording of the selected ion pair even in the presence of $[MH]^+$, $[M]^+$, and $[M-H]^+$ ions. The versatility of chemical ionization in the biomedical area is amply demonstrated by numerous papers on the qualitative and quantitative determination of drugs,[166] metabolites, and other endogenous substances.[167,168]

The utility of electron capture negative ion technique for quantitating organic compounds by conventional GC-CIMS and SIM methodology has been demonstrated.[169-171] The method depends on some sample molecules capturing near thermal energy electrons which converts them to either stable molecular anions M^- or high molecular fragment ions. When this process proceeds with high efficiency it becomes ideally suited for organic trace analysis. While many organic molecules afford negative ion currents comparable to that observed in the positive ion mode, very few organic structures capture thermal electrons and form stable molecular ions or high mass fragments efficiently. Since most biomedically important molecules have to be chemically modified to make them sufficiently volatile or GCMS, it is possible to enhance high efficiency electron capture by suitable derivatization. Selection of a suitable electron capturing derivatizing agent is a critical element, as it is possible to efficiently capture electrons and then fragment to an anion which derives only from the reagent. According to Markey and Levy,[4] cyclic derivatives or polycyclic compounds will generally yield structurally specific anions. For the quantitative analysis of melatonin in human plasma by negative CIMS (methane) the spirocyclic pentafluoropropionyl derivative (Figure 1) formed by reaction of melatonin with pentafluorpropionyl anhydride, could be determined at or below the picogram level.[4] Other derivatives which have been used successfully for negative CIMS with methane reagent gas are the silylated Schiff base formed from pentafluorobenzaldehyde and dopamine (Figure 2), the

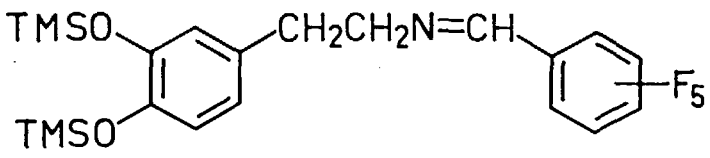

FIGURE 2.

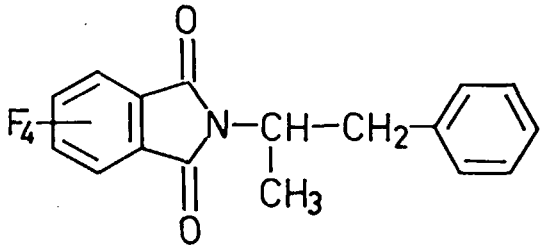

FIGURE 3.

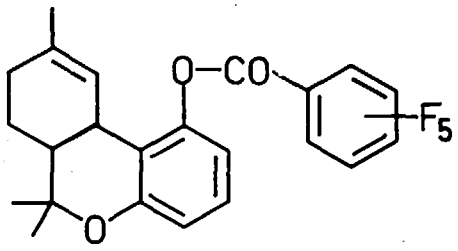

FIGURE 4.

tetrafluorophthaloyl derivative of amphetamine (Figure 3), and the pentafluorobenzoyl derivative of Δ^9-tetrahydrocannabinol (Figure 4).[169,171]

While considerable effort has been devoted to exploring the potential of various reagent gases for positive ion CIMS, very little has been published on the analytical potential of negative ion CIMS reagents. The high pressure of methane carrier gas primarily moderates the energy of the electrons to thermal energies so that resonance capture mechanisms can operate to form an "enhanced" negative ion mass spectrum.[172] There have been several reports on the use of oxygen as the reagent gas either alone or in a carrier gas, such as methane or isobutane. The superoxide anion $O_2\cdot$ has been shown to be an excellent reagent for CIMS analysis as it will react by proton transfer with a wide range of organic compounds. The [OH] ion has also been used as a reagent for negative ion CIMS of a number of drugs.

Atmospheric pressure ionization (API)[173,174] is a variant of CI in which the ion source is at atmospheric pressure. Ionization occurs by the production of primary ions in a gas by a radioactive source or by an electric discharge which in turn form ions from the sample molecules by a series of secondary ion-molecule reactions. Both positive and negative ions are produced which pass into the analyzer section. The significant difference between API and CIMS is that the product ions are in thermal

equilibrium within the source when the usual API conditions are used, while this is not true for conventional MS procedures. Very short-lived as well as very long-lived ions have been detected, and subpicogram sensitivity of detection has been achieved for both positive and negative ions. Current studies are concentrating on negative ion MS and on conditions for obtaining high sensitivity of detection. Examples of bioanalytical applications of API are the determination of diphenylhydantoin[175] in plasma and the detection of nicotine in urine.[176] The use of Cl^- and O_2^- in an API source has been described.[177]

Recently new methodology, pulsed positive ion — negative ion CIMS [PPNICI], which facilitates recording of positive and negative ion CI spectra on a mass spectrometer, has been described.[171] With such an instrument, which is already commercially available,[178] the GCMS user can detect both positive and negative ions in the CI mode with equal ease, as the MS may be operated to detect positive ions only, negative ions only, or collect positive ions on one scan and negative ions on the next. A mixture of hydrogen-oxygen has been used for some PPNICI experiments. Electron bombardment of oxygen at 1 torr affords O_2^+ and O^+ in the positive ion mode and O_2^- and O^- in the negative ion mode. The additional of hydrogen (10%) to the reagent gas eliminates O^- from the reagent spectrum by the reaction $O^- + H_2 \rightarrow H_2O + e$ and greatly simplifies the CI spectra from aromatic compounds. Under these conditions aromatics afford $[M]^{\ddagger}$ in the positive ion mode and $[M + 15]^-$ in the negative ion mode. Methane PPNICI mass spectrometry can also afford sample ion currents under electron capture conditions in the negative ion mode that exceed ion currents in the positive ion mode by two or three orders of magnitudes. The user thus has the benefit of an increase in the sensitivity of detection and any additional structural information which may be available by operating in the negative ion CI mode.

VI. APPLICATIONS OF STABLE ISOTOPES FOR STUDIES OF METABOLISM AND BIOSYNTHESIS

Many diagnostic methods for the assessment of the normal and diseased state are not recommended for some patients such as pediatric or pregnancy cases, or for clinically oriented studies, because the tests involve oral or i.v. administration of radioactive isotopes. Many countries are now reluctant to permit the administration of radioactive isotopes to humans. The increasing commercial availability and the more realistic cost of stable isotope-labeled compounds makes it possible to convert many existing methods to a protocol utilizing nonradioactive probes. For most applications stable isotope tracers offer the same sensitivity for detection and yield the same diagnostic information as radioactive isotopes without the accompanying radiation hazard.[179] No significant health hazard from stable isotopes has been demonstrated with the quantities required for clinical and human studies as the normal body content of naturally occurring stable isotopes such as ^2H, ^{13}C, ^{15}N, and ^{18}O, is usually much larger than the quantity which would be used for tracer purposes. Great care must be taken in deciding upon where the isotopic label is to be placed in the molecule, as it must be retained in the compound and its metabolites throughout the pathway to be studied. It is also undesirable to introduce just one atom of ^2H or ^{13}C into a molecule, because of the natural abundance of ^{13}C and the generation of $[M+1]^+$ ions in the EI source through ion molecule reactions. It is best to aim for a mass difference of at least 3 amu between the labeled and unlabeled species. In addition, the label should not produce a significant isotope effect in distribution or metabolism and it must be retained during MS in one or more abundant ions. One of the advantages offered by stable isotopes over radioisotopic tracers is the capacity to expand the number of tracer variants which can be distinguished from one another by MS

analysis, thus multiple labeling experiments are possible. Such labeled variants have been used for kinetic analysis of events from a single analysis, for estimation of extraction efficiences, and for quantitative work.[180] The instrumental requirements for stable isotope ratio measurements are those needed for SIM, except that both high precision and sensitivity are necessary for circumstances in which ratios may be as low as $1:10^2$ or $1:10^3$. In the final analysis, the attractiveness of using stable isotopes in biomedical applications depends on how well the method competes with other methods.[181] While in general, stable isotope-labeled compounds cost more than radioisotopes, no real cost comparison is meaningful without considering such parameters as the sensitivity of the method,[182] the enrichment required, and the time required for any needed chemical modifications. As might be expected, the number of references to the use of ^{13}C are fewer than to the use of ^{2}H, because of the greater cost involved to produce enriched ^{13}CO as opposed to $^{2}H_2O$. Similarly, ^{17}O is very much more difficult to produce and more expensive than ^{18}O and hence ^{17}O is very seldom used. Both ^{18}O and ^{15}N have found wide application as no convenient radioisotopes of oxygen and nitrogen are available.

Biomedical and clinical[183,184] applications of stable isotopes have been reviewed recently and many examples may be found in the proceedings of four international conferences on stable isotopes in chemistry, biology and medicine.[185-188] Compilation of selected references to the use of ^{2}H, ^{13}C, ^{15}N, ^{17}O, ^{18}O, and ^{34}S, in biochemical, pharmacological, clinical, and environmental application for the period 1971 to 1976 have also been published.[189-192] Some typical applications include estimation of pool size, turnover rate of endogenous compounds,[194-196] determination of total body water,[197] bile acid kinetics,[198] and procedures for measuring nitrogen retention in growth hormone deficiency.[199] Some of the most valuable clinical uses of stable isotope-labeled substrates are in the study of biosynthesis and metabolic pathways.[200-202] Examples of such applications are investigations of glucose metabolism in children,[203] a study of phenylalanine metabolism in normal and phenylketonuric patients,[204] and studies of bile acids,[195] steroid,[205-207] fatty acid,[208] biogenic amines,[209,210] and prostaglandin[211,212] metabolism. It is now becoming increasingly apparent that one cannot glibly assume that a metabolic disturbance which would be predicted from the enzyme assay in vitro will necessarily be the most important biochemical effect in vivo. This point has been demonstrated by stable isotope load studies in one patient with tyrosinemia in whom an enzyme secondarily inhibited by accumulating substrate became the most important functional block in metabolism.[213] There are reasons to believe that this general pattern is true in many other inborn errors of metabolism and thus there is a need to combine enzymic assays performed in vitro with labeled metabolic load studies performed in vivo.

In all of these applications stable isotope tracers and subsequent MS analysis provide information regarding both the presence of a label and its position in the molecule. Similar information can also be derived from radioactive tracer experiments, but in many cases the molecule has to be extensively degraded before the exact position of the label can be established.

Pharmacological applications of stable isotopes and the role of stable isotopes in drug metabolism have been reviewed recently[214,215] and only a few applications are highlighted here. Stable isotopes are particularly suitable to establish the importance of stereochemistry for pharmacological activity. When a drug molecule possesses one or more chiral centers, biological activity usually resides largely in one enantiomer. The relative proportions of enantiomers of a drug and its metabolites recovered, following metabolism of the racemate, may be determined by MS when one enantiomer is isotopically labeled. Racemates containing one deuterium-labeled enantiomer have been used to study the differential metabolism of the enantiomers of propanolol[216] and

cyclophosphamide.[217] Species variations in the metabolism of drugs have been observed and deuterium-labeled drugs have been used to optimize the specificity in tracing minor metabolites of unknown structure by mass spectrometry.[218] The need for careful examination of bioavailability during development of oral medication has been established. Stable isotope-labeled drugs have been used for bioavailability studies in man and animals.[219-221] The preferred technique utilizes comparison of either the plasma concentration or urine levels, resulting from administration of a labeled drug solution, coadministered with a test drug formulation. Drug concentrations are then determined by GC and the ratio of labeled to unlabeled drug is obtained by GCMS. Stable isotope tracers also have considerable potential in the analysis of compartmentation problems in steady-state pharmacokinetics.[222,223] The preferred procedure is to establish a steady-state by repeatedly administering the unlabeled drug to the patient. One dose of the labeled variant is then given after which the unlabeled compound is readministered. Serial analysis of plasma by SIM permits measurement of the time course of a single dose which can be compared with the time required to metabolize a single dose given to another patient.

A novel method to facilitate the recognition of metabolites has been described. This involves partially labeling a drug with stable or radioisotopes in such proportions that the $[M]^+$ and $[M+1]^+$ ions are of equal abundance, thus metabolites are formed with a similar ratio of molecular ion intensities, while fragment ions containing the labeled center similarly appear as characteristic "twin ions".[224]

The use of labeled tracers for biomedical studies is associated with a number of difficulties. One is the possibility of kinetic isotope effects, which are changes in rate that result from substitution of a normal abundant isotope with another. These are undesirable and tend to obscure the information sought. They occur if C-H bond cleavage is the rate-determining step and this hydrogen has been replaced with deuterium during the labeling process. Such deuterium-isotope effects are quite common in enzyme reactions and they can be determined by deliberately exchanging such a hydrogen with deuterium. So far, large isotope effects in in vivo metabolism have only been observed with deuterium, thus stable isotopes of carbon, oxygen, and nitrogen, are to be preferred for tracer studies. Another problem is associated with the presence of ions, such as [M+1] and [M-1] in CIMS or other abundant ions in EIMS. This complicates the ion current ratio determination and is further complicated by variation in temperature, pressure, and background contribution in the MS, which may change the ratio during the daily operation of the machine. A refined system for quantitative analysis of unlabeled and polydeuterated compounds has been developed and is based on computer evaluation of spectra obtained by repetitive scanning.[225] It has been applied in studies of steroid metabolism and biosynthesis. A number of calculation procedures for isotope dilution determination for GCMS experiments have also been compared.[226] A model has been developed for the simultaneous quantitative determination of several isotopically labeled substances of the same chemical structure in the picogram range with SIM.[227]

Finally, it should be noted that an efficient GC column frequently shows significant separation of isotopic variants and this can cause inaccuracies if only peak height measurements are recorded. This problem is most noticeable if several hydrogen atoms in an organic compound are replaced by deuterium; it is less likely to occur with the heavier elements or if only one hydrogen atom is replaced with deuterium.

VII. QUANTITATIVE APPLICATIONS OF GCMS

During the initial development period of bioanalytical GCMS, the most important objective was to identify the components of a biological sample. While this is still an

important function, the major emphasis of current work is upon the development of quantitative procedures for trace organic analysis. Quantitative analysis with MS sets particular demands, since the signal intensity depends not only on the amount of the sample but also on a number of other experimental parameters such as the ionization yield, focusing of the ion beam, and the amplification factor of the detector. Since it is difficult to keep these parameters constant over a long period, nearly all quantitative applications of MS are based on a comparison of ion current obtained from the compound of interest in the sample with the ion current obtained from a standard. Two methods for quantitation are in general use, both based on the integrated ion current technique and SIM. The first method requires the continuous admission of a reference sample at a constant rate concurrently with the sample under investigation. An elution profile is obtained by repetitive scanning alternatively across a reference peak and a suitable analyte peak. The scan yields an envelope of the analyte peak, the area under which is proportional to the integrated ion current and thus to the concentration of the analyte. The reference compound serves as an internal standard to monitor any variations in instrument sensitivity. The second method is based on the use of an internal standard which is added to the sample prior to MS analysis. It is now generally accepted that quantitative analysis for compounds of biological interest are best carried out using stable isotope-labeled analogs as internal standards. Isotope dilution assays of this type have been described for several hundred different compounds and are capable of great precision and sensitivity. Although the label is usually a stable isotope, radioisotopes have also been used for reasons of availability.[228,229] However, the use of radioisotopes for this purpose can lead to contamination of the instrument if a large number of analyses are to be done. If labeled standards are not available, structurally similar compounds, such as homologs can be used.[230,231] The chemical and physical properties of standards should permit them to be isolated, derivatized, and purified, with efficiencies and degrees of sample loss comparable to those of the compound of interest. An alternative approach is to use enantiomer labeling which involves the use of the unnatural enantiomer as an internal standard. With chirasil-val, a chiral stationary phase, the enantiomers of amino acids and a variety of compounds can be separated and quantitated. The accuracy of amino acid analysis by enantiomer labeling is equal to that of hitherto known methods.[231] An analysis of variance errors associated with SIM led Clacey et al.[232] to conclude that stable isotope-labeled internal standards, as opposed to the use of homologs, produce the lowest variance factors due to instrumental stability and sample manipulation errors. A common argument for the use of a stable isotope analog is that it can be added in excess (100:1 to 1000:1) so that it can act as a carrier to minimize adsorptive and other losses of the endogenous compound during the work-up of the sample. Such a carrier technique was used successfully in the determination of picogram amounts of prostaglandins.[233] Haskins et al.[234] also confirmed the existence of a carrier effect, as he found a sevenfold increase in sensitivity in the diphenoxylate assay when the tetradeuterated analog was used as carrier and internal standard. This might not always hold as was shown by the analysis of octopamine by Millard and co-workers[235] in which serial injections of nanogram quantities of octopamine prior to analysis of picogram amounts resulted in improved responses. However, when nanogram quantities of the deuterium-labeled internal standard were co-injected with picograms of unlabeled compound, the signal from the unlabeled compound was not significantly increased. In a recent letter to the editor of *Biomedical Mass Spectrometry*, Self[236] discusses these contradictory results. He poses the following questions. Does the carrier effect only apply under certain conditions? Is it due to the presence of unlabeled impurity in the standard? Is the use of a strongly deactivated column essential?

Although the work-up procedure for each determination must be adapted to the

particular compound to be analyzed, most published methods have certain features in common. For example, the internal standards should be added to the samples immediately after they have been collected and they must be uniformly distributed in the sample. Since drugs and metabolites are often excreted as highly polar conjugates it is usually necessary to use a hydrolytic procedure or to release the aglycone enzymatically. Unless the substances to be analyzed are present in very high concentration and are sufficiently volatile for GCMS analyses, a preconcentration step such as a solvent extraction, chromatography, or a combination of both, will be required. In practice, it is not unusual to find that a number of purification steps are necessary. Finally, the compounds must be chemically modified in order to increase volatility or thermal stability for GCMS. If the analysis is to be done by EIMS it is necessary to search for a suitable derivative, which has both good vapor phase properties and characteristic fragment ions at high mass values, so that the compound can be monitored with a minimum of background interference. The preferred method of recording the ion current is by single ion monitoring [SIM] or repetitive scanning over a narrow mass range. The choice of which recording method is chosen will depend on whether specificity can be relaxed. For example, when testing for a particular compound with known mass spectral properties, the sensitivity of the assay can be increased by monitoring only a limited number of ions or in the extreme case only one ion [SIM]. If various fragment ions associated with a single compound have to be monitored to increase the specificity of detection, then there will be an offsetting loss in sensitivity. Since a compound and its labeled analog may be partially resolved during the GC analysis, an adjustment has to be made for this when peak height measurements are used to calculate the ratio of the two ion currents. It is more reliable to rapidly scan the ion intensity of the two ions over the entire GC peak and sum both signals. In some cases the complexity of the mixture concerned is such that identification of the component of interest is uncertain and sensitivity is reduced by an unacceptably high contribution to the signal from components co-eluting from the GC. One approach to this problem is to use high-resolution MS to eliminate interference from ions of the same nominal mass but different elemental composition. An alternative approach is to monitor metastable peaks corresponding to fragmentations occurring in the first free region of a double-focusing MS. Gaskel and Millington[237] have used the combined electrostatic analyzer - magnet scan facility of a double-focusing MS to monitor the daughter ion peak for the fragmentation, $[M\text{-}57] \rightarrow [M\text{-}57\text{-}76]^+$, of a 5α-dihydrotestosterone-t-butyldimethylsilyl ether. The detection limit was shown to be approximately 20 pg. The experimental simplicity of the technique combined with the high degree of specificity suggests considerable potential in the field of biochemical analysis.

The requirement for accurate methods of analysis is of growing importance in routine clinical chemistry. This is essential if published values are to be meaningful. Although clinical judgment is possible within a hospital where the laboratory results are precise but inaccurate, the situation is much improved by quantitative knowledge of the inaccuracy. In principle, this can be achieved by calibration of routine methods of assay against corresponding "reference methods" that are available to the laboratory and that can, in turn, be standardized against a "definitive method", i.e., a method of highest proven accuracy. Isotope dilution-GCMS methods for the determination of metabolites such as creatinine, cortisol, glucose, progesterone, triglycerides, testosterone, urea, and uric acid, have been shown to be suitable for "reference methods" in clinical chemistry.[238] For example, the determination of total serum cholesterol[239-241] via the GC of the TMS derivative and using cholesterol-$^2H_7$ as an internal standard was carried out by focusing alternatively on the molecular ions at $^m/_z$ 458 and 465 and summing both signals. An overall relative standard deviation of 0.33% was obtained for the measurement of total

cholesterol in five different serum pools that were analyzed on four different occasions.[241] The most characteristic property of the GCMS techniques is their high specificity. To interfere with the assay a compound must have the same GC behavior and contain the same specific ion in its MS as the compound to be determined. The presence of an interfering ion can easily be tested by using other ions in the EIMS for quantitation, which should give the same result. The GCMS methods must be considered only as "reference" and not "definitive methods", as it is not possible to exactly assess the divergence of the results obtained with GCMS from the "true" or "absolute" values. It should be possible to develop the procedures into "definitive methods" if each individual step in the methods is subjected to a detailed analysis with respect to possible error. In this connection, Eldjarn et al.[241a] have shown that interference from the matrix can be completely corrected for by removing the constituent to be analyzed. For example, in the quantitative assay of glucose in serum, one aliquot of the serum can be treated with glucose oxidase to remove glucose completely and in a highly specific manner. The glucose-free serum can then be used as a blank. In a similar manner one can remove urea completely by treatment with urease, and uric acid by treatment with uricase, and so on. The accuracy of these isotope dilution-MS analyses depends in part on the wet-chemical processing steps and in the preparation of the standard solutions and the pipetting of these solutions. In many instances, the "wet chemistry" factors are best determined with radioactive tracers. While knowledge of the exact purity of the labeled internal standard is not required, it is essential to know the exact purity of a reference sample of the unlabeled material. By bracketing the sample with standards of closely similar composition prepared from the reference and the labeled compounds, sources of error in the calibration can be minimized. For a more detailed treatment the reader is referred to an excellent review[242] and two books[243,244] on quantitative MS which cover instrumentation, sources of errors, treatment of calibration data, and quantification in GCMS.

SIM and isotope dilution methods of quantitation are becoming more common, mainly because of the increasing availability and range of stable isotope-labeled compounds. In the U.S., the National Stable Isotope Resource Center to help meet the need for stable isotope-labeled compounds has been established at the Los Alamos Scientific Laboratories and funds have been made available for the synthesis of many labeled compounds in sufficient purity for isotope dilution assays. In Europe, a committee has been set up to establish definitive MS methods for steroid hormone analysis.

Because of the cost of the equipment and the expertise required for its maintenance and use, GCMS is not normally used for routine quantitative analysis. This situation might change as one manufacturer[245] already offers a GCMS system which has been specially designed for routine quantitative analysis. This system offers a high sample through-put of 100 or more samples per 24 hr, an automatic sample injection system, a capillary GC with programmable temperature and pressure options, and a low-pressure CI source in which the primary ionizing particles are produced by a gas discharge. Autosampler GC and MS are controlled by microprocessors and the data system allows for controlled 24-hr operation. Easy access to all systems elements, visual and acoustic warning signals for uncontrolled changes of state, a "no filament" ion source, and an efficient vacuum system suggest that "down time" could be kept to minimum. At present, the main application of quantitative MS is for research purposes where sensitivity and specificity are of paramount importance. Some typical recent applications are the analysis of melatonin[4] and biogenic amine metabolites in cerebrospinal fluid[246] and brain tissue,[247] the analysis of steroids,[248-251] bile acids,[252,253] polyamines,[254] and prostaglandins[225,256] in serum and urine and the determination of ^{13}C-labeled compounds in urine by GC followed by pyrolysis to CO_2 and $^{13}C/^{12}C$ ratio detection.[257] A review[258] on the detection of hepatic

enzyme activities involved in corticosteroid and testosterone metabolism by GC and mass fragmentometry has been published. A survey has also been made of about 120 papers appearing in the food literature in which mass spectrometry has been employed to measure toxic contaminant levels.[259]

GCMS with stable isotope labeled internal standards have been used extensively for quantitative drug analysis. Horning et al.[260] have reviewed the use of GCMS in drug quantification, discussing the problems of extraction methods, choice of derivatives, and internal standards. Since a book on the analysis of drugs and metabolites by GCMS has been published recently,[261] only a few references are cited here.[262-264] The increased precision and accuracy of quantitative GCMS analyses has led to a growing recognition that variability in the collection and handling of biological specimens, such as blood, may lead to changes in test values. As a consequence, the interpretation of analytical results may sometimes be difficult. The National Committee for Clinical Laboratory Standards in the U.S. has published standard procedures PSH-3 and PSH-4 which should be followed by anyone obtaining blood by venipuncture or skin punctures, respectively. Variability attributable to collection factors may be minimized by reducing the number of individuals who are allowed to collect blood. At present, there is no mechanical device that can ensure reproducibility of specimen collection. Commercial blood-separating devices should not be used prior to GCMS analyses as they may be responsible for the presence of a multitude of "unknowns" in the gas chromatograms.[265] Extensive hemolysis of a blood specimen may result in the observation of significantly lower drug levels. For example, some drugs are extensively bound to plasma proteins resulting in a grossly unequal distribution between plasma and the erythrocytes.[266] An unresolved issue is whether the collection of plasma or of serum is the appropriate specimen for drug assays. Differences between plasma and serum, which are of possible clinical significance, have been noted for some routine chemical tests. Dudley[267] has recently reported that phenytoin and phenobarbital levels in serum are 10% greater than in plasma. The quantitative analysis for metals in biological systems has less direct clinical application than organic analysis. There is, however, considerable interest in trace element analysis as copper, zinc, selenium, and iodine are known to be essential to human life, and a number of other elements found in trace amounts are suspected to be merely environmental contaminants. This interest has been stimulated by the rising concern in industrialized nations of man's impact on his environment and its biological effect on him. Most of the elements in question occur at the $\mu g/g$ level, and while other techniques such as atomic absorption spectroscopy, neutron activation analysis, and polarography have been used with considerable success, MS is probably the only technique that can be used to determine parts per million levels of several metals simultaneously. There are two MS techniques applicable for the detection and quantification of trace elements, namely, spark source MS and GCMS. Spark source MS has already been widely used for the analysis of trace metal impurities in both inorganic and biological materials. Morrison[268] has published a critical review on "Elemental Trace Analysis of Biological Materials" which includes a section on the application of spark-source MS. Very little has been published on the application of GCMS for this purpose. Mushak[269] has reviewed the GC of metal ions via chelation and nonchelation techniques. At the present time, β-diketones and β-ketoamines derived from both mono- and bifunctional amines are the preferred chelating agents. Metal halides are the best derivatives for the GC analysis of metal ions using nonchelation techniques. The potential of GCMS in the quantitative analysis of trace elements in biological materials has been demonstrated by Wolf et al.,[270] who determined beryllium and chromium levels in blood and serum using the volatile trifluoracetylacetone chelates. Concentrations below the ng/g level were determined with an accuracy of the order of 20%. A

unique use of GCMS in metal analysis is the assessment of isotope distribution ratios for a chromatographic species.[271] This was found to be of particular value in extraterrestrial sample analysis. Few other methods are intrinsically capable of furnishing these types of data.

VIII. CONCLUSIONS

During the last decade, the rapid development and acceptance of GCMS systems, especially those automated with dedicated on-line computers, has caused an exponential growth in the volume of mass spectral data derived from biomedical sources. A modern GCMS system can give an interpretable mass spectra from 10^{-9}-g samples, a quantity much too small for other techniques for molecular information. Although rapid progress is being made in computer systems for interpretation and reference file retrieval of mass spectra, there appears to be little chance that these will ever supplant the need for a skilled mass spectrometrist.

A significant limitation of GCMS is that useful mass spectra can only be obtained from volatile GC effluent and many biomedically interesting compounds are not directly amenable to GC analysis. Two other mass spectrometric methods, combined liquid chromatography (LC)-MS and the use of mass separation with metastable and collision activation spectra are at the stage where they can be applied to many problems not soluble by GCMS.

A number of approaches to the direct coupling of LC to MS have been published. They include the introduction of a small portion of the LC effluent directly into the MS through a glass capillary column,[272] the use of a micro LC with a jet separator interface, and the use of a continuously moving wire transport LCMS interface.[273,274] Undoubtedly, combined LCMS is already a viable technique and the next few years should see a rapid expansion in the use of such instruments in biomedical applications.

One shortcoming of GCMS and LCMS in trace analysis is the fact that they subject the sample to a contact phase in which both the amount of foreign material and the possibility of retention of sample or a transformation product is high. Column bleed in GC is perhaps the major limitation on GCMS sensitivities in advanced instruments.

An alternative approach to mixture analysis uses the mass spectrometer in both its mass transport and its compound identification roles. Here the mixture to be analyzed is converted into ions immediately upon introduction into the MS and the ions are subject to physical probes which affect separation and identification on the fly. This technique is currently being practiced using the mass analyzed ion kinetic energy spectrometer (MIKES), a form of double-focusing MS in which the usual order of analyzing fields is reversed.[275] The direct analysis of mixtures by MIKES is already established as a rapid, specific, and extremely sensitive method which can be applied to samples of a variety of types. A number of applications of the CI/MIKES technique to the analysis of metabolites and drugs have been published. The near future will probably see a further broadening in the range of applications, specifically into more studies on biological systems.

LCMS and MIKES in conjunction with desorption techniques such as field-, plasma-, and laser-desorption can push the molecular weight limit of molecules amenable to MS analysis well beyond mol wt 1000. Despite this, however, a vast group of high molecular weight substances still remain inaccessible to most types of direct chromatographic or spectrometric analysis and thus one must invoke degradation techniques to reduce these molecules to smaller, less complex, but nevertheless definitive, fragments. Among the degradative methods, pyrolysis is particularly suitable for many biochemical applications. Pyrolysis in combination with GCMS has been found to be

applicable to a number of biomedical problems, such as the differentiation of normal human cells,[276] and pyrolysis-MS is a rapidly expanding field. In particular, it is worth mentioning that the MIKES methodology is uniquely compatible with pyrolysis techniques. Pyrolysis delivers complex mixtures of gaseous organic compounds in small amounts directly into the ion source, which is the form in which samples are required for MIKES. The power of this combination has been demonstrated in studies on intact DNA.

Recently Yost and Enke[277] introduced the concept of a triple quadrupole mass spectrometer as a particularly simple and efficient approach to selected ion fragmentation. The instruments consist of, in series, a dual CI/EI sample ionization source, a quadrupole mass filter, an RF-only quadrupole that can be pressurized with a collision gas, a second quadrupole mass filter, and an electron multiplier. The ion fragmentation process is performed by collision-induced dissociation in which the ion acquires internal energy by collision with a neutral molecule. The RF-only quadrupole collision chamber provides focusing of scattered ions and is highly efficient. For analysis of mixtures, the molecular ions for each component can be produced by soft ionization, such as CI, and then separated by the first mass analyzer. Thus, the molecular ion species can be selected for one component at a time, the selected ion fragmented, and its fragmentation mass spectrum obtained by scanning the second analyzer. The system can be used to detect species present in a mixture including isomers without prior separation at a 10^{-15} mol level. The capability to select a desired initial mass and specific collision product mass reduces chemical noise dramatically. Among the many promising applications of the selected ion fragmentation techniques is the analysis of isotopically labeled samples, in which the first mass filter can eliminate interferences from molecules that are not completely labeled.

The use of mass spectral separation of the components of a mixture in MS/MS eliminates time delays associated with the chromatographic separation used in GCMS and LCMS. Because all the components are available at any time and in any order, only those components of particular interest need be analyzed. Continuous analysis of a sample is possible and direct insertion probes and field or chemical desorption can be used to introduce involatile samples.

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