Journal of Chromatography, 492 (1989) 189–221
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHROMBIO 4772

REVIEW

BIOCHEMICAL APPLICATIONS OF LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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(First received November 28th, 1988, revised manuscript received January 16th, 1989)

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0378-4347/89/$03 50

LIST OF ABBREVIATIONS

5′-AMP	5′-Adenosine monophosphate
a m u	Atomic mass unit
API	Atmospheric pressure ionization
CF-FAB	Continuous-flow fast atom bombardment
CI	Chemical ionization
DES	Diethyl stilbestrol
DLI	Direct liquid introduction
EI	Electron impact
FAB	Fast atom bombardment
GABA	γ-Aminobutyric acid
GC	Gas chromatography
HETE	Hydroxyeicosatetraenoic acid
HPLC	High-performance liquid chromatography
LC	Liquid chromatography
LOD	Limit of detection
MAGIC	Monodisperse aerosol generation interface
MS	Mass spectrometry
MW	Molecular weight
OTLC	Open tubular liquid chromatography
OTMS	O-Trimethylsilane
PC	Phosphatidylcholine
PFB	Pentafluorobenzyl
RA	Relative abundance
SIM	Selected-ion monitoring
TSP	Thermospray

1 INTRODUCTION

Of all the detectors used in liquid chromatography (LC), mass spectrometry (MS) may come closest to being a universal detector Mass spectra can be obtained for numerous organic compounds and biomolecules with molecular masses less than ca 20 000–30 000 a m u [1] In addition to detecting the presence of a compound, the mass spectrometer can confirm its identity and often can even provide sufficient data to determine the structure of an unknown Although the mass spectrometer is not the most sensitive LC detector, the routine detection limits of MS [typically obtained by gas chromatography–mass spectrometry (GC–MS)], in the low femtomoles (picograms), is sufficient for most LC applications Its optimum detection limits, ca 40 amol, does

not compare too unfavorably with the most sensitive detectors such as fluorescence at 4 amol and amperometric at 2 amol For most applications, the increased selectivity of the mass spectrometer can more than compensate for any loss in sensitivity compared to other detectors.

As with most hyphenated analytical techniques there are problems associated with interfacing two disparate systems in such a way as to obtain maximum performance from both subunits, and LC–MS methodology is certainly no exception One major problem which will not be discussed further is the cost The second major difficulty is the incompatibility between the operating pressures of the mass spectrometer analyzer (ca. 10^{-6} Torr) and of the LC system (liquid at ≥ 1 atm). Since LC systems use liquid mobile phases, the pressure incompatibility is much more severe than in GC. An additional problem is introduced when magnetic sector instruments, which operate at high source potential (typically 3–8 keV), are involved since they are susceptible to high-voltage breakdowns at higher source pressures.

In the field of GC–MS numerous interface designs, such as membranes, jet separators and frits, were proposed and evaluated before the development of fused-silica capillary columns made the direct coupling of capillary columns into the MS source possible This has now become the method of choice for interfacing GC and MS LC–MS technology is presently at a stage comparable to the earlier days of GC–MS where a number of interfaces are being proposed and evaluated, but no single methodology has yet to emerge as a universal choice Thus, any discussion of LC–MS methodologies currently revolves around a discussion of the various interface types

Although every LC–MS user would probably come up with a different definition or different emphasis when defining the perfect LC–MS interface, there are several characteristics which are worthwhile to keep in mind during the discussion of interfaces.

From the mass spectrometrist's viewpoint an ideal interface should (1) require no special source, (2) be as simple as possible and, preferably, not require permanent changes to the instrument, (3) yield source pressures compatible with electron-impact (EI) data acquisition, (4) be compatible with high-voltage instruments, (5) not degrade thermally labile compounds, (6) require no splitting or make-up flows; and (7) be universally applicable to all compounds

From the chromatographer's point of view the interface should· (1) place no restrictions on the LC flow, (2) be compatible with normal- and reversed-phase chromatography, (3) be compatible with all buffers, and (4) introduce no chromatographic band broadening

2 INTERFACE DESIGNS

2 1 Transport interfaces

In transport interfaces, the LC eluent is mechanically transported from the end of the LC column to the ion source with the mobile phase being removed

prior to entry into the ion source The first successful LC–MS interface, the moving-wire interface of Scott et al [2], was of this type The first type of LC–MS interface sold commercially, the moving-belt interface designed by McFadden et al. [3], is still in use and is available from two manufacturers of mass spectrometers

In the original moving-belt interface, the eluent is deposited on a continuously moving belt The belt first passes under an infrared heat source to evaporate the mobile phase, then passes through two vacuum locks and into the mass spectrometer. Flash vaporization of the analyte is effected as the belt passes through a heated chamber adjacent to the source On the return path the belt passes under a clean-up heater and a scrubber used to remove non-volatile buffers

Modifications to improve compatibility with mobile phases containing high percentages of water and to improve sensitivity have been made Heaters placed in the vacuum locks, use of microbore columns and the development of a spray depositor with a heated nebulizer have all improved compatibility with aqueous phases Passing the belt directly into the source has also improved sensitivity and decreased the sample heating requirements [4] (Fig 1)

The moving-belt interface has several features which are advantageous. Since the mobile phase has been evaporated by the time the analyte has reached the source region, this interface is compatible with a variety of ionization modes including EI, chemical ionization (CI) and fast atom bombardment (FAB) Thus, the technique is not limited to data acquisition under CI conditions as is the case with several other interfaces Since non-volatile buffers can be removed from the belt after it has exited the source, non-volatile buffers can be used

The negative characteristics of the moving belt include (1) potentially high background from the belt and from carryover and (2) the range of analytes is typically limited to thermally stable compounds The high belt background problem has been noted at low mass [5] Since high-performance liquid chro-

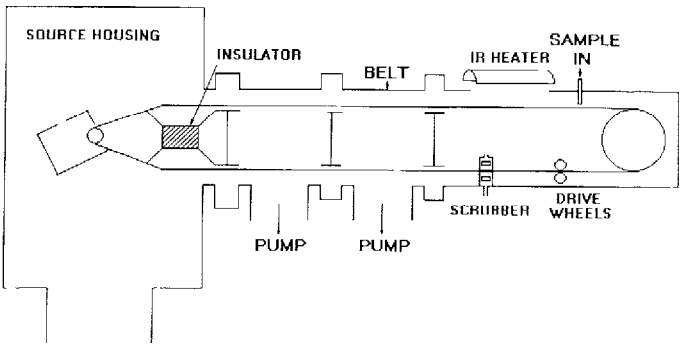

Fig 1 Schematic diagram of moving-belt interface (adapted from refs 3 and 4)

matography (HPLC) is normally used with higher-molecular-mass compounds, this often is not a severe problem Carry-over can, of course, be alleviated or removed by optimizing the scrubber process

The second problem is more fundamental Games [6] has noted that the belt interface is useful for compounds whose thermal stabilities fall in the range between those amenable to GC–MS determination and those that can only be analyzed by desorption CI. The recent combination of the moving belt with FAB ionization does remove this restriction for the more polar thermally labile compounds [7] but requires precise control of the primary ion flux to avoid degradation of analyte

2 2 *Direct liquid introduction*

As the name implies, in direct liquid introduction (DLI) LC–MS, the LC mobile phase is introduced directly into the mass spectrometer In commercial DLI LC–MS interfaces, a solvent jet is formed by passing 10–40 μl of LC effluent per min through a laser-drilled pinhole (usually 2–5 μm in diameter) in a replaceable diaphragm. To prevent premature evaporation of the solvent, the tip of the interface is water-cooled This jet then passes through a desolvation chamber where the droplets are vaporized, and the vapor enters the mass spectrometer source

Due to the limited amount of solvent which can be tolerated by the mass spectrometer's vacuum system, DLI interfaces for conventional LC columns incorporate a splitter, usually a simple needle valve and waste flow-line located downstream from the orifice. This allows all but the 20–40 μl/min of the mobile phase needed to form the jet to pass by and provides the back-pressure necessary for jet formation Unfortunately, this reduction in the amount of mobile phase entering the source leads to a comparable reduction in the amount of analyte entering the source For a 1 ml/min flow-rate, a 20-μl jet means that only 2% of the injected analyte reaches the mass spectrometer The development of microbore and minibore HPLC systems, with reduced column flow-rates, has meant that the split ratio could be improved For microbore DLI LC–MS, interfaces were designed with no split valve, and the entire column effluent forms the jet and enters the mass spectrometer [8] (Fig 2)

DLI LC–MS can be performed on a conventional CI mass spectrometer, modified by the addition of the desolvation chamber (heated or unheated depending on the manufacturer) The resulting high source pressures yield CI spectra, both positive and negative ion, with the mobile phase forming the reagent gas Ionization occurs as in conventional CI, with proton transfer in the positive-ion mode and proton abstraction and electron capture in the negative-ion mode Ionization of the analyte depends on the relative proton and electron affinities of the analyte and the mobile phase components [9]

Since the LC solvent vaporizes within the mass spectrometer, certain sol-

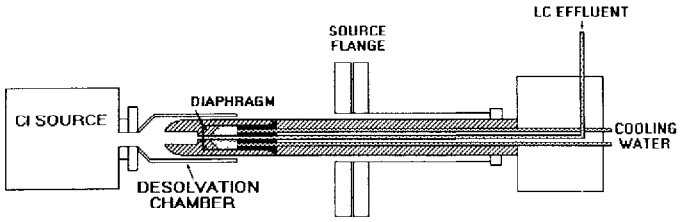

Fig 2 Schematic diagram of DLI interface (adapted from ref 8)

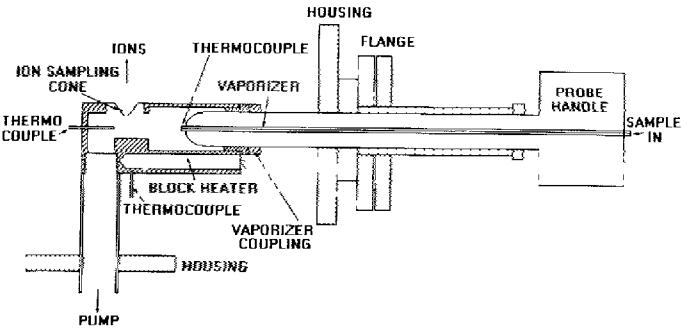

Fig 3 Schematic diagram of thermospray interface (adapted from the Vestec Interface Manual, Vestec, Houston, TX, U S A)

vent restrictions are encountered. Only volatile solvents and volatile buffers can be used (e.g., ammonium acetate and ammonium formate). The use of phosphate and sulfate buffers should be avoided. DLI LC–MS is useful for both normal- and reversed-phase systems, and high concentrations of water in the mobile phase can be used, although this may lead to reduced filament lifetimes.

Although early DLI LC–MS work was done on magnetic sector instruments [10], the high source pressures encountered in DLI LC–MS make it more amenable to interfacing with quadrupole mass spectrometers. Although arcing problems are more likely to occur with the high voltages encountered in magnetic sector mass spectrometers, DLI LC–MS is still used with some magnetic sector instruments [11]

2 3 Thermospray

Unlike DLI, thermospray (TSP) LC–MS includes a specially designed mass spectrometer source as well as an interface probe and a controller [12] In the TSP interface probe, the column effluent passes through a heated vaporizer, forming a supersonic jet Vaporizer, source block and vapor temperatures are critical for successful ionization and volatilization (Fig 3) Most sources now also include a filament, a discharge electrode and a repeller to assist in ion

production, extraction and fragmentation Usually performed with column flow-rates of approximately 1 ml/min, the entire column effluent in TSP passes into the mass spectrometer source Ions are drawn into the analyzer through a sampling cone (0 5 mm I D) while most of the solvent vapor (and certainly some of the sample) is pumped out of the source via an additional rough pump

Since the vaporization of the solvent occurs inside the mass spectrometer, the same buffer restrictions apply in TSP as in DLI Volatile buffers, such as ammonium acetate and ammonium formate, can be used, while sulfate and phosphate buffers must be avoided As originally designed, the TSP source had no ionization source other than interaction of the buffer with the analyte, and the use of a buffer was required The inclusion of the filament and discharge electrodes have eliminated this restriction and now allow the use of TSP in normal-phase systems TSP with a filament has sometimes been referred to as 'hot DLI' or 'CI mode' TSP

The actual ionization process in TSP is still under debate, although there is general agreement that no matter whether the initial ionization is gas phase or solution phase, enough collisions occur in the high-pressure source so that the ions leaving the source obey gas phase thermochemistry (i e , a chemical ionization-type process) In 'classical' TSP (i e , TSP without a filament or discharge), ions could only be produced by proton donation (usually from the NH_4^+ of the buffer) in the positive-ion mode or by proton abstraction or acetate attachment in the negative-ion mode This led to the formation of $(M+H)^+$ ions in the positive-ion mode and $(M-H)^-$ and $(M+OAc)^-$ in the negative-ion mode With the introduction of the filament or discharge, electron capture could now occur with the production of M^- ions in the negative-ion mode TSP is a fairly gentle ionization technique, and many compounds show only molecule ion species In order to get more structural information from the mass spectrum, the discharge electrode and repeller have sometimes been used to induce fragmentation in what may be a collision-induced dissociation process The discharge is thought to produce a hotter plasma which induces more thermolysis while the repeller is thought to enhance fragmentation through a collisionally activated decomposition process On suitably equipped mass spectrometers, TSP in combination with tandem MS has also proved useful

2 4 Atmospheric pressure ionization

As the name implies, in the atmospheric pressure ionization (API) instruments the source is maintained at atmospheric pressure Since the ion source operates at atmospheric pressure it does not impose any flow restraints on the LC system Ionization occurs by formation of a corona discharge in the source Ions are then electrically focussed through a 100-μm orifice into the high-vacuum analyzer region Since ionization occurs in a high-pressure region, CI spectra result (Fig 4)

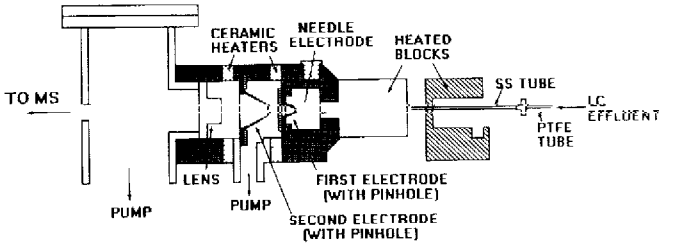

Fig 4 Schematic diagram of API source (adapted from ref 13)

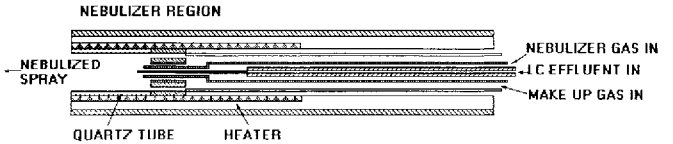

Fig 5 Schematic diagram of heated nebulizer interface for API (adapted from ref 8)

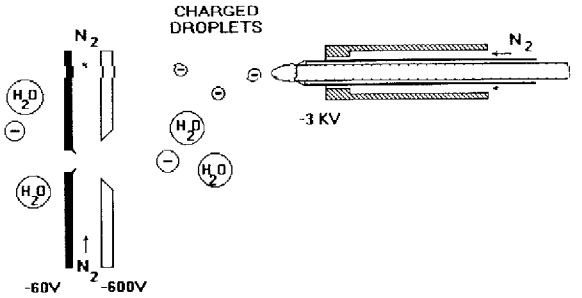

Fig 6 Schematic diagram of electrospray interface for API (adapted from ref 15)

Strictly speaking, API is not an interface design but a source design However, for the purposes of this review, it will be treated as an interface type There are three interfaces used with API MS (1) heated nebulizer [8,13]; (2) electrospray [14]; and (3) liquid ion evaporation [15]

The heated nebulizer is probably the most common of the three In this process, the LC effluent (up to 2 ml/min), make-up gas and nebulizer gas are introduced coaxially into a heated quartz tube (Fig 5) The heated nebulized spray is rapidly swept into the ion source where it is ionized by corona discharge

In the electrospray interface no heat is used The effluent passes through a metal capillary that is maintained at a high negative potential The induced charge is such that coulombic repulsion overcomes the surface tension and a fine spray results (Fig 6) The ions formed by the high potential are electrically extracted into the analyzer

The third interface, liquid ion evaporation, combines characteristics of both

nebulization and electrospray [15] The effluent is nebulized into the API source where a high-voltage electrode induces a charge on the droplets. The charged droplets then emit ions which are focused into the analyzer This technique is well suited for polar compounds

The major drawback to LC–API MS is the fact that the API mass spectrometer is highly specialized since the commercial instrument is an API-only instrument.

2 5 *Monodisperse aerosol generation interface*

In 1984 Willoughby and Browner [16] reported the design of a new type of LC–MS interface, the monodisperse aerosol generation interface (MAGIC) (Fig 7) In this interface the LC effluent passes into the desolvation chamber through a glass orifice to form a liquid jet This jet breaks up spontaneously into uniform drops A perpendicular flow of helium disperses the drops and prevents recoagulation and also aids in solvent vaporization As the drops pass through the desolvation chamber, the solvent rapidly evaporates from the drops At this point, if the analyte is a solid, the result is a high-velocity particle beam which is the basis of the alternative nomenclature for the technique, particle beam LC–MS

The analyte beam, helium and solvent vapor passes into a momentum separator The momentum separator is very similar in concept to the jet separator developed for packed column GC–MS [17,18]. The resultant source pressure is approximately 10^{-6} Torr The analyte particles strike the heated source and are then vaporized. Optimum LC flow-rates have been reported to be in the range 0.1–0 5 ml/min [16] At higher LC flow-rates the LC effluent is split before entering the interface

The low source pressure enables the analyst to obtain standard EI spectra with very little background due to the mobile phase CI spectra can be obtained by standard CI procedures The choice of the CI reagent gas is not determined by the mobile phase as it is in DLI Since flash vaporization of the analyte in the source is part of the ion formation process, MAGIC so far has been limited

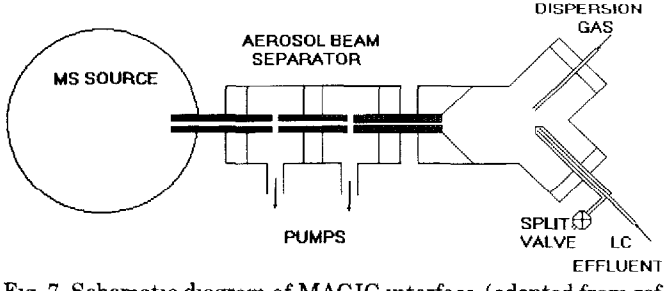

Fig 7 Schematic diagram of MAGIC interface (adapted from ref 16)

to use with volatile compounds. The more polar or thermally labile compounds such as peptides or sugars have not been observed to be compatible with the interface [19]

2 6 Open tubular liquid chromatography

Just as packed column GC–MS using jet separators has its LC–MS equivalent in MAGIC, there is an LC–MS equivalent of capillary column GC–MS in open tubular liquid chromatography (OTLC)–MS An adequate discussion of OTLC should cover two distinct areas (1) OTLC; and (2) using open tubular columns as an LC–MS interface (a subset of DLI) Although the emphasis of this review is on the coupling of LC and MS, the use of capillary columns in LC is relatively new and merits a brief introduction

OTLC columns are made from coated or chemically bonded glass or fused silica These columns typically have internal diameters of 1–20 μm and lengths of 1–10 m At an optimum internal diameter of ca 2 μm and length of 2 m, an excess of $2 \cdot 10^6$ theoretical plates should be generated within 2 h for a solute with a k' of 10 [20] Flow-rates in those columns are of the order of 5–250 nl/min OTLC columns have been used for a variety of applications including the separation of neurotransmitters found in a single neuron [21]

The mobile phase flow-rate of, e g , water, that can flow into a typically pumped MS system and still maintain a reasonable EI source pressure, ca. $5 \cdot 10^{-6}$ Torr, is of the order of 0 2 μl/min or less Thus the nl/min flow-rates encountered in OTLC should be easily compatible with the mass spectrometer. For this reason and due to the interest in OTLC within the field of separation science, interest in coupling OTLC with MS has increased

There have been two general approaches taken to coupling OTLC and MS, each of which is designed for different purposes The first is to use a piece of fused-silica capillary tubing as a transfer line from a conventional HPLC column to the mass spectrometer [22–24] In this type of interface the flow-rates are fairly high (50–100 μl/min) so that a stable liquid jet forms. The effluent is usually split before entering the fused-silica column As the flow-rate is reduced, as in packed microcapillary LC (e g , 22 mm I D) or in OTLC (20 μm I D) the tip of the column must be drawn to a fine tip to provide a stable liquid jet

In our laboratory we have used coated capillary columns as the separating columns and as integral parts of the interface (Fig 8) The OTLC column is tapered and inserted directly into the source We have observed that, if the tip is heated as well as tapered, vaporization occurs at the probe tip rather than inside the tip, as in a non-tapered interface, resulting in less plugging of the column and/or freezing of the mobile phase due to expansion [25]

Alborn and Stenhagen [26] have observed that ionization is aided by a strong electrostatic field as found in a magnetic sector instrument, and is, thus, sim-

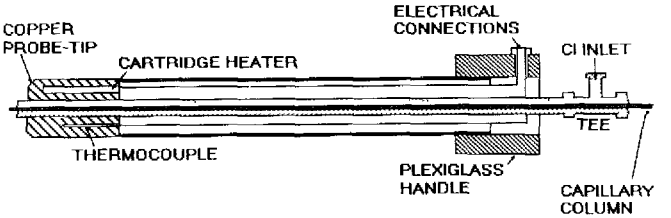

Fig 8 Schematic diagram of OTLC interface (adapted from ref 25)

ilar to electrospray ionization Problems were encountered with freezing of the effluent This problem has been alleviated by heating the probe tip which also permits use with quadrupole instruments where the electrostatic field is absent.

OTLC interfaces typically do not require instrumental modification or special sources The range of compounds amenable to analysis using this interface has not been determined, but early results indicate it lies between GC and FAB. Henion in 1985 [27] noted that OTLC permits the analysis of more complex mixtures due to higher chromatographic efficiencies and that EI data can be obtained, suggesting that more applications of OTLC–MS should appear in the future.

One limitation of OTLC–MS at present is in column preparation. Recent developments by Dluzneski and Jorgenson [28] in the preparation of coated fused-silica columns, however, indicates that this problem is being addressed successfully

2 7 Continuous-flow fast atom bombardment

The advent of FAB MS has greatly increased the range of compounds amenable to mass spectral analysis to include ionic compounds, polar compounds and thermally labile compounds such as quaternary ammonium salts, peptides and carbohydrates

The coupling of HPLC with FAB MS was soon pursued as a natural extension of the capabilities of the FAB MS [29,30] (For a review of FAB see ref 31) The major problem was to develop a means of continuously introducing analyte into the FAB source The most widely employed method is to introduce continuously the analyte in aqueous solution, typically containing glycerol The solvent flows to the probe tip through the probe shaft using a fused-silica transfer line, usually at flow-rates of 5 μl/min or greater. The probe tip is warmed sufficiently to prevent freezing (Fig. 9) Connecting an HPLC system with splitting or a microbore HPLC column without splitting was then a simple task. Similarly, we have adapted OTLC columns to continuous-flow fast atom bombardment (CF-FAB) by using a coaxial column in which the LC column

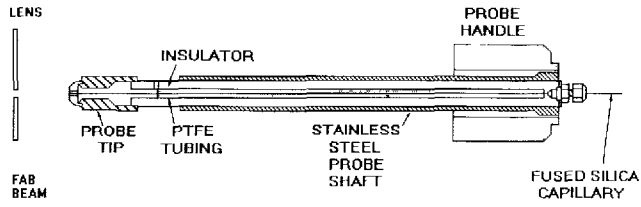

Fig 9 Schematic diagram of CF-FAB interface (adapted from VG Interface Manual, VG Analytical, Manchester, U K)

is inserted within the column carrying the FAB matrix [32].

Although CF-FAB may employ a different source and probe than does conventional FAB, the CF-FAB source and probe can be used for conventional FAB The range of compounds which may be analyzed is the same as for conventional FAB, e g , peptides, carbohydrates, complex lipids, oligonucleotides and nucleosides

2 8 Comparison

Comparing each of these LC–MS interface characteristics confirms our earlier statement that the ideal does not yet exist The DLI, TSP, API and CF-FAB are compatible with only CI (positive and negative) or FAB ionization All interfaces place limits on the LC conditions (usable buffers, maximum or minimum flows or require splitting) Some, such as TSP, require specialized MS sources and/or complex interfaces These characteristics are summarized in Table 1

TABLE 1

INTERFACE CHARACTERISTICS

Characteristic	DLI	Belt	TSP	API	CF-FAB	OTLC	MAGIC
No special source	×				×	×	×
No permanent modifications	×				×	×	
EI data		×				×	×
High-voltage compatibility	×	×	×	×	×	×	×
No additional flow requirements imposed by interface				×		×	×
Normal or reversed-phase	×	×	×[a]	×	×	×	×
Compatible with all buffers		×			×		
No band broadening	×	×	×	×	×	×	×
No thermal degradation				×	×		

[a]With the use of a filament or corona discharge assembly

3 APPLICATIONS

This section is not intended to be a complete coverage of all the papers in which LC–MS methodology has been applied (For a recent review see ref 33) Rather those techniques which have been successfully or unsuccessfully applied to a given category of compounds will be indicated Aspects which impinge on biochemical or clinical applications, such as limits of detection, will be discussed as will applications to 'real' problems To better emphasize the current state of LC–MS applications, emphasis will be on results from the last four or five years In reading these sections several caveats should be kept in mind (1) What you have is what you use Very few labs have several different LC–MS interfaces available and, thus, there is little comparative information available This also tends to emphasize applications from turnkey systems such as commercial TSP LC–MS instruments (2) Positive results are more likely to be published than negative results (3) Many industrial applications of LC–MS, e g , in drug research, are not reported in the open literature (4) A majority of papers on rapidly developing techniques are oriented to the behavior of standards, often by flow injection analysis, rather than applications in problem solving (5) With the enormous volume of scientific literature today, the reviewer is at mercy of the quirks of computerized literature searches (Some of our papers were missed so we must presume that others' were as well)

3 1 Peptides and amino acids

The qualitative and quantitative determination of amino acids and peptides by LC–MS has generated considerable interest Of the interface designs discussed above DLI, API, moving belt, TSP and CF-FAB have all been applied to these analyses MAGIC and OTLC–MS have not yet been applied successfully to the analysis of peptides

For obtaining spectra of peptides, the moving-belt interface appears to be the least sensitive and is also prone to induce thermal decomposition at low levels [34] Improved sensitivity has been achieved by derivatization (100 μg N-acetyl permethylated Leu-enkephalin) [35] Although the in-source sensitivity by DLI is quite good (ng), DLI effluents are usually split prior to analysis which decreases the on-column sensitivity by several orders of magnitude {limits of detection (LODs) of ca 10 μg injected [36]} Another problem which was observed with DLI is variable tuning Milon and Bur [37] investigated a series of polypeptides, $(Gly)_n$ with $n=2$–6, by DLI using a constant-tuning procedure for optimum sensitivity of one of the compounds of interest Under these conditions, $(M+H)^+$ ions were observed for only $n=1$–4 and no molecular ion species was seen for $n=5$ and 6 That appropriate conditions can be found for $n=5$ and 6 is evidenced by the observation of $(M+H)^+$ ions for Leu- and Met-enkephalins and α-amanites [36] When dealing with an unknown,

however, the analyst often does not have an appropriate compound to tune on and, in complex mixtures, tuning on one component may not be optimal for all components.

All three API interfaces (nebulized, electrospray and nebulized electrospray) have been successfully applied to peptide analysis Sensitivity seems to be quite compound-dependent, although it is difficult to compare LODs in different laboratories on different machines for different compounds. None the less, good full-scan spectra of peptides with molecular weight (MW) > 1000 can be obtained on less than 100 ng

The use of TSP LC–MS for the analysis of peptides has proven to be quite popular The range of peptides amenable to TSP MS analysis so far has been limited by the mass range of the mass spectrometer For example, Rudewicz [38] observed the protonated molecular ion cluster of glucagon, (M+H) = 3481 6, on a 4000 a m u quadrupole from 3 nmol of material Detection sensitivities have been reported to be of the same order of magnitude as static FAB sensitivities (100–500 pmol for full-scale spectra) As with DLI, tuning conditions can be critical and some analytes may not be observed under conditions where related compounds can be [34] This may be due to thermal decomposition which has also been noted for glutathione conjugates of xenobiotics [39]. Like FAB, the TSP spectrum sometimes includes fragment ions characteristic of the structure in sufficient intensity for structure elucidation [40] The presence of structural information is very dependent upon the compound and operating conditions

To increase the structural information obtained by TSP MS, Stachowiak et al. [41] and Kim et al [42] in Vestal's group have developed elegant on-line procedures for the determination of proteins In this technique, immobilized enzyme columns are placed in line before (and after) the LC column In this procedure a protein is subjected to endopeptidase proteolysis by passing it through a column of the immobilized enzyme, e g , trypsin The products are separated on an LC column Each separated peptide fragment is then passed through a third column containing an immobilized exopeptidase such as carboxypeptidase The resulting mixture of products arising from a single endopeptidase fragment are detected by TSP MS

The sequence of the peptide fragment can be determined by the mass differences of the product. This procedure was applied to the 58 amino acid protein, basic pancreatic trypsin inhibitor, with the result that 29 of 58 residues were identified in their proper sequence using less than 600 μg total of protein Hartman and Jardine [43] have used TSP MS for the analysis of the rabbit biliary metabolites of cyclosporine A 1-μg isolate gave good spectra of the metabolites indicating products showing addition of one to four oxygens and several N-demethylated products

In addition to structure determination of peptides, TSP MS has been successfully applied to the quantitative analysis of smaller molecules such as car-

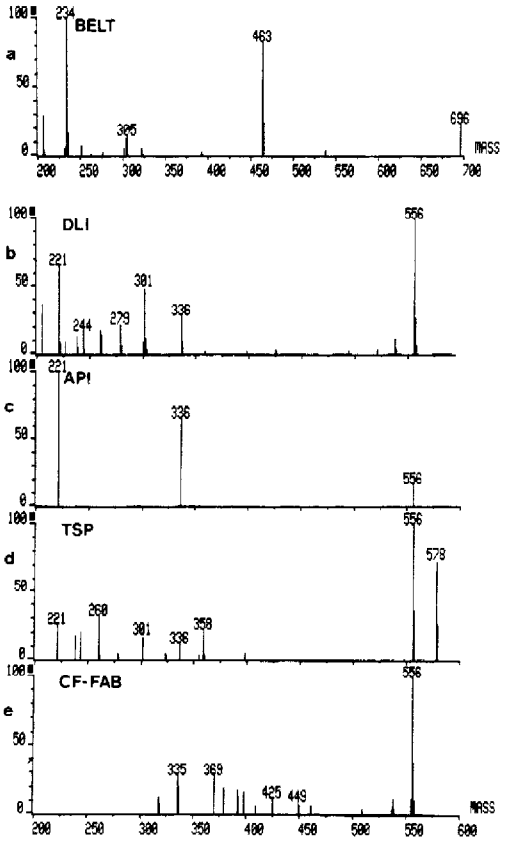

Fig 10 Spectra of Leu-enkephalin by (a) belt (as N-acetyl methyl ester derivative), (b) DLI, (c) API, (d) TSP and (e) CF-FAB Spectra were reconstructed from data in refs 35, 36, 13, 40 and 52, respectively

cinogenic tryptophan pyrolysates [44], mutagens formed in cooked food [45], indole acids in urine and serotonin, choline and acetylcholine in mouse brain homogenate [46] γ-aminobutyric acid (GABA) in brain tissue [47]. For the latter analysis, Artigas and Gelpi [47] found that the detection limit for GABA by TSP MS [10 pg, selected-ion monitoring (SIM)] was comparable or better than that obtained by GC–MS [46] In contrast, they found that catecholamines demonstrated very poor sensitivity by TSP MS (LOD $\geq$ 2 ng)

CF-FAB has been the technique most competitive to TSP MS in popularity for the analysis of peptides. Chemical noise is decreased by reducing the amount of matrix needed, and the suppression effect observed in FAB, where specific peptides not detected in a mixture are readily detected by themselves, is greatly reduced in CF-FAB [48] LODs in the full-scan mode are significantly lower for CF-FAB ($<$10 ng for peptides $<$2000 a m u), especially using microbore

columns [48,49] We have obtained attomole detection limits with 10 μm I D open tubular columns using the coaxial interface [32]

The analysis of high-molecular-mass proteins such as insulin [48] and the analysis of proteolytic digests for structure determination have been accomplished by CF-FAB [48–52] Now that CF-FAB probes and sources (basically standard FAB sources modified by the addition of a source heater) are commercially available, it is expected that the number of reported applications of this technique will increase dramatically

To compare as closely as possible the data obtained for peptides from the various interfaces, Fig 10 contains the spectra of Leu-enkephalin obtained by (a) belt (as the N-acetyl methyl ester derivative), (b) DLI, (c) API, (d) TSP and (e) CF-FAB The mass range shown was selected to keep all spectra on the same footing Since the belt contains a thermal desorption step, it is not surprising that the molecular ion species is of relatively low abundance It is surprising, however, that the API interface gives an $(M+H)^+$ of only 23% relative abundance (RA) As will be seen in later sections, for most compounds LC–API MS usually provides spectra with less fragmentation than the other interfaces

3 2 Nucleotides, nucleosides and bases

Nucleosides and related compounds have been successfully analyzed by LC–MS using moving belt [53,54], electrospray [14], API [13], DLI [55–57], TSP [58–65] and CF-FAB [66] Some of these references simply report the spectra of standards [59,66] Most work appears to be done in the positive-ion mode, using conditions analogous to ammonia CI A notable exception was the analysis of 6-thiopurine, where negative-ion TSP was ten times as sensitive as positive-ion TSP [58] With the belt technique, enhanced molecular ion information was obtained for adenosine through the use of chloride attachment negative-ion CI [53]

Varying ratios of protonated molecular ion, fragment ion corresponding to the protonated base, and the sugar moiety can be produced depending on source and interface conditions. A comparison of spectra resulting from different versions of the belt interface is given by Games et al [54], while an examination of the effect of temperature on the TSP spectrum of 5-adenosine monophosphate (5′-AMP) is given by Edmonds et al [63] This variation, as well as differences between compounds, makes comparisons between systems difficult, although sensitivities of 8 μg thymidine (on-column) by conventional column positive-ion DLI [55], 3 μg for adenosine by positive-ion electrospray [14] and 0 25 μg for thymidine by positive-ion microbore DLI [56] have been reported Better nucleoside sensitivities have been reported for API LC–MS and TSP LC–MS Reported SIM sensitivities for positive-ion API are 0 1–5 ng [13] which is comparable to those achieved by positive-ion TSP (0 1–1 ng

SIM and 10–50 ng full scan) [61]. While nucleosides and bases often yield intense protonated molecular ions, nucleotides are more difficult to analyze, often fragmenting to give the more stable protonated nucleoside, protonated base and (sugar $-H_2O$) fragments Seven of the fifteen TSP spectra reported by Edmonds et al [63] for 5′-nucleotides and methylated nucleotides showed no protonated molecular ion The other nucleotides showed $(M+H)^+$ ions ranging from 0 2 to 1.9% RA API may be better than TSP for nucleotides, since the spectrum of 5′-AMP by TSP showed a protonated molecular ion of only 0 6% RA [63] while the API spectrum of the same compound showed an 8% RA molecular ion [13]. Reported detection sensitivities of 5′-AMP for the two methods are 300 ng by TSP and 50 ng by API

Both DLI and TSP have been used for the analysis of nucleosides and bases in DNA and RNA digests Esmans et al. [56] and Alderweireldt et al. [57] have used DLI microbore LC–MS for the analysis of modified nucleosides, including pseudouridine and 5,6-dihydrouridine, in human urine extracts Naturally occurring methylated bases were detected by positive-ion TSP in calf thymus and salmon sperm DNA digests [65] and in digests of bacterial tRNA [64] For these types of studies, sensitivities of 20 ng per nucleoside in 2 5–30 μg of crude digest have been reported Although these levels are sufficient to detect some naturally occurring methylated nucleosides, they are unfortunately several orders of magnitude above the level of modified bases arising from interaction of xenobiotics with DNA

Davidson et al [62] have used TSP LC–MS for the examination of radiation damage to individual DNA bases In this study, exact mass measurements and MS–MS were used to confirm the identity of the thymidine glycol produced from radiation-treated thymidine

TSP LC–MS has also been used to identify 2′,3′-dideoxyinosine, a metabolite of 2′,3′-dideoxyadenosine (an antiviral agent) in mouse plasma [60] While no quantitation was done, the stability of the system was such that a time study could be performed over the course of 4 0 h

In another interesting application, Volk et al [58] used TSP (without separation) to identify the reaction products of a purine drug in on-line electrochemistry TSP combined with tandem MS.

3 3 Steroids

Sugnaux et al [67] have investigated DLI with microbore columns for steroid analysis. They were able to obtain reproducible full-scan spectra from 250 pg of 6-hydroxymethazone and to quantitate it in horse urine using an external standard These workers noted, however, that there was no universal recipe for optimizing sensitivity

Using the moving-belt interface, LODs of 100 pg have been reported for some steroids (SIM) [68] However, it was noted that, at low levels, thermal

decomposition occurred with some steroids and that background from the belt could create problems with identification of low-level metabolites [69].

Detection levels for steroids and steroid conjugates by LC–API MS appear to be quite good, 10 pg in many cases under SIM [13,70] This can be very compound-dependent, however For example, Weidolf et al. [70] were able to determine boldenone sulfo-conjugate and related steroid sulfates at the 10-pg level for full-scan spectra In contrast, however, a 600-ng injection of digitoxin by flow injection, not on-column, was needed to obtain a full-scan spectrum [15]. Interestingly, the dominant ion of multiply conjugated steroids, e g , a disulfate, was the $(M-2H)^{2-}$ parent [15] Although this may be suitable for targeted compound analysis the presence of multiple charges may create problems with structure identification of an unknown

Owing to the ready availability of TSP LC–MS instruments, a number of groups have examined the TSP MS behavior of steroids and conjugates In general detection limits of ca 100 pg have been reported for SIM [71–74]. Liberato et al [73] have noted, however, that tuning is critical for low-level analysis since, as with the moving-belt interface, thermal degradation of low-level analytes can occur Catlow [75] has pointed out another potential problem with the analysis of low levels of material when they exist at trace levels (0 1–0 2%) in mixtures. To obtain full-scan spectra for identification, greater than 50 μg of the mixture may need to be injected At this level precipitation of the analyte in the probe tip resulting in a plugged tip is a distinct possibility

Steroid levels in serum and urine are of interest in clinical settings. Gaskell et al [76] have rigorously compared LC–TSP MS with GC–MS for the analysis of serum cortisol levels They found that TSP sensitivity is about a factor of ten less sensitive than GC–MS as well as being less precise (standard deviation = 5%), possibly due to the lower sensitivity and signal stability observed in TSP On the other hand, the LC–TSP MS approach eliminated an HPLC step and derivatization steps, thus simplifying the procedure Esteban et al [77] have also applied LC–TSP MS analysis to the determination of daily cortisol production using isotope dilution. They observed LODs similar to those of Gaskell et al [76], with similar precision On the other hand, using isotope dilution LC–API MS with a nebulizer interface, Takatsu and Nishi [78] obtained a relative standard deviation of 0 6% which is comparable to GC–MS analysis (0 5%) The difference between the two groups' results may be due to instrumental differences (TSP versus electrospray API) or (less likely) due to inherent differences in compound behavior

Liberato et al [73] used LC–TSP MS to identify the presence of 18-hydroxycortisol in several clinical expressions of hypertension They also pointed out that LC–TSP MS may prove useful for quantitative analysis of this compound since GC–MS analysis yields two peaks on derivatization (methoxime-trimethylsilyl ether) as well as not giving any high-mass ions of significant abundance

Surprisingly, very few applications of CF-FAB for the analysis of steroids and their conjugates have been reported Full-scan spectra at 20–40 ng levels have been reported but the LODs have not been determined [79,80]. We have obtained spectra from 2 ng of corticosterone and 4 ng of disodium taurolithocholic acid 3-sulfate by coaxial open tubular CF-FAB In general, TSP MS has been the method of choice for the LC–MS analysis of steroids This is primarily due to the availability of instrumentation rather than to any inherent advantage over competitive techniques such as LC–API MS

3 4 Lipids

Lipids for the purpose of this review can be separated into two broad categories, fatty acids and derivatives and phospholipids, which will be treated separately

3 4 1 Phospholipids

The major emphasis in the analysis of phospholipids has been qualitative rather than quantitative Molecular ion species and fragment ion information have been obtained by DLI [81–87], API [13], belt [88,89] and TSP [90–93] In general, detection limits for all of these interfaces have been of the order of 1–50 ng using SIM [13,86,88,90,92]. We have been able to obtain full-scan spectra from 1 ng of dipalmitoyl phosphatidylcholine (PC) using OTLC in conjunction with CF-FAB The observed LC–TSP MS sensitivity was sufficient to profile triacylglycerols in plasma [86,87], phospholipids in egg yolk [90] and to quantify ether phosphocholines in psoriatic scale [92]

Consistent with indications from investigations of other compound classes that 'tuning' (source and interface conditions) can be very compound-dependent is a comparison of PC spectra from different groups In a study of a number of phospholipids, Kim et al [91,93] found that the TSP MS spectrum of 16 0, 18 2-PC contained an $(M+H)^+$ of ca 10% RA (Fig 11a) In contrast, by tuning specifically on the $[M+H]^+$ ion of 14 0,14 0-PC, Mallet and Rollins [92] obtained a spectrum with the $[M+H]^+$ ion as the base peak (Fig 11b) Tunability in this context refers to temperature tuning rather than conventional MS ion optics tuning In this case 'tunability' may be considered an advantage since the other interfaces gave spectra containing molecular ion species of relatively low abundance ($<10\%$) (Fig. 11a–e), except for the spectra obtained for CF-FAB using OTLC (Fig 11f) It cannot be discerned from the work by Mallet and Rollins [92] whether or not optimization on the $(M+H)^+$ ion increased or decreased overall sensitivity, however.

3 4 2 Fatty acids and derivatives

Interest in the LC–MS analysis of fatty acids and derivatives has focused more on thermospray than on the other interfaces The standards by which

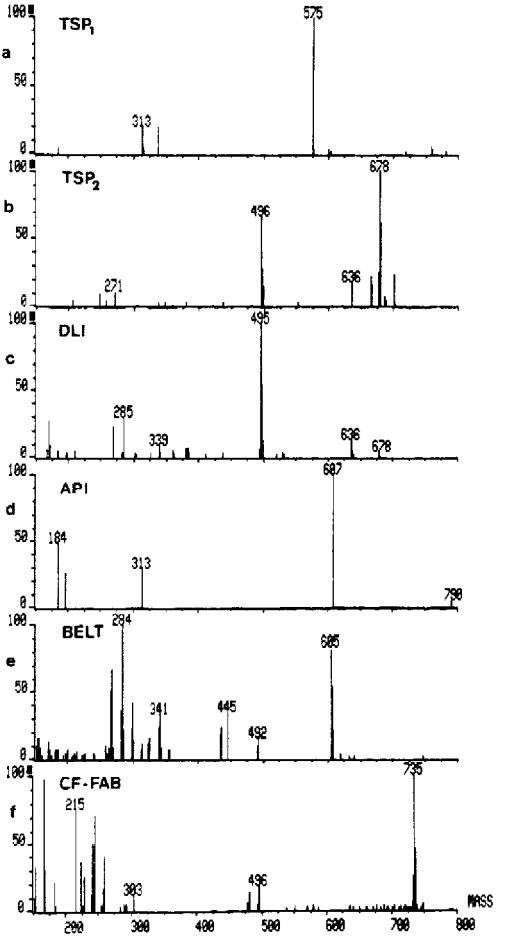

Fig 11 Spectra of phosphatidylcholine by (a) TSP, (b) TSP, (c) DLI, (d) API, (e) belt and (f) CF-FAB Spectra a–e were reconstructed from data in refs 91, 92, 81, 13 and 88, respectively

one must judge the qualitative and quantitative analysis of fatty acids and derivatives by LC–MS are set by GC–MS

The underivatized compounds, prostaglandins and hydroxyeicosatetraenoic acids (HETEs), gave positive-ion spectra dominated by water losses with little additional fragmentation [59,91,94–98] In comparison the positive-ion EI spectra of the most common derivatives [(O-trimethylsilyl (OTMS)ether methyl esters] give $(M-15)^+$ as the high-mass ion with extensive lower-mass fragments which often arise from rearrangement The TSP MS spectra of isomeric species are very similar, showing only successive H_2O losses which makes structural assignments difficult in the absence of standards Under negative-ion conditions, both the TSP MS spectra of the free acids or pentafluorobenzyl

(PFB) esters are dominated by fatty acid anion, $(M-H)^-$ or $(M-PFB)^-$, which readily provides the molecular mass It is only when analyzing the more complex leukotrienes that LC–TSP MS exhibits strengths relative to GC–MS To analyze these compounds by GC–MS, the peptide moiety must be cleaved and only the fatty acid portion analyzed In contrast, underivatized leukotrienes give spectra under negative-ion conditions dominated by the $(M-H)^-$ anion, and loss of the peptide chain is also seen The latter process dominates as the source and vaporizer become contaminated [99]

Detection limits for quantification by GC–MS and/or GC–high-resolution MS range into the mid femtograms [100] In contrast, the typical LODs of the underivatized acids analyzed by LC–MS lies in the low nanogram range (91,95,96,101–103) Voyksner et al. [101,103] have investigated several derivatives in an effort to increase sensitivity but have only reached low to mid picogram LODs (10–300 pg). Thus the major advantage of LC–TSP MS analysis for these compounds lies with its rapid analysis time and ease of molecular mass determination (negative ion) rather than for low-level quantitation

3 5 Carbohydrates

Carbohydrates may represent the class of compounds most refractive to LC–MS analysis, especially in their underivatized form DLI [96], moving-belt interfaces [53,54] and API interfaces have been applied to carbohydrate analysis but success has been limited to trisaccharides and lower. Detection limits have not yet been addressed except for the API interface where LODs by SIM ranged from 50 to 200 ng [104]

Hsu et al [105] have carried out the most extensive investigation of carbohydrate analysis by TSP MS They were able to observe $(M+NH_4)^+$ ions for seven of eight disaccharides investigated but at $<1\%$ RA for the most part The spectra of anomeric and linkage-position isomers differed, but unequivocal assignments could not be made on the basis of the spectrum of a single isomer LODs for the underivatized carbohydrates were observed to be approximately 2 ng Higher sensitivity was observed after derivatization by O-methylation Thus, the 1-O-methyl glycosides exhibited LODs of ca 20 pg Methylation also increases the range of saccharides for which a molecular ion species can be obtained. For example, the $(M+NH_4)^+$ ions of the tetrasaccharide stachyose was observed with 49% RA. Esteban et al [106] and Yergey et al [107] have applied TSP MS analysis to the study of endogenous serum glucose levels by isotope dilution LODs for these were determined to be 9 ng (by SIM)

Since FAB has been successfully applied to the analysis of carbohydrates, several groups have looked at CF-FAB as an analytical technique [66,108] Successful analyses of maltohexaose and maltoheptaose have been reported with LODs of 500 ng for full scan and 200 ng for SIM Using the OTLC–CF-

FAB MS interface, we have obtained a full-scan spectrum of maltotetrose from 5 ng of material From these reports CF-FAB appears to be the best LC–MS methodology for obtaining spectra of underivatized oligosaccharides Comparable data from CF-FAB and TSP MS for derivatized saccharides are not available. Static FAB sensitivities in the picomole range (ca 50 ng) were observed under negative-ion conditions for the ethyl *p*-aminobenzoate derivative of maltoheptaose [109] Therefore, it is probable that reasonable sensitivities for derivatized higher-molecular-mass carbohydrates might be achieved by CF-FAB and possibly by TSP MS

3 6 Xenobiotic metabolism

An area of ongoing concern in environmental and biomedical chemistry is the fate of xenobiotics in the body Both DLI and TSP have been used to characterize xenobiotic metabolites including conjugates such as glucuronides [110–112], sulfates [110] and members of the glutathione family [39,113], as well as non-conjugated metabolites [110,114,115]

Detection limits for the full-scan spectra of 2-nitrofluorene metabolites [114], benzo[*a*]pyrene metabolites [110] and trinitrotoluene metabolites [115] were observed to be within the range of products expected to be obtained from challenged or environmentally exposed animals or people, 20–500 ng In their study of benzo[*a*]pyrene by negative-ion DLI, Bieri and Greaves [110] observed that the spectra were extremely sensitive to ion source and desolvation chamber temperature Thus, at 250°C $(M-H)^-$ ions were not observed for the sulfate or glucuronide conjugates or the tetrols At lower source pressure, some tetrols gave molecular anions but at the expense of sensitivity and poor peak shape

In our application of TSP MS to xenobiotic metabolites we observed that glutathione conjugates often gave spectra dominated by thermal processes and that molecular ions of the conjugates of polynuclear aromatics were of low abundance or absent After derivatization (N-trifluoroacetyl methyl ester) molecular ions could be observed [39] Yergey et al [116] also observed indication of thermal degradation occurring during the TSP MS analysis of acylcarnitines They noted that the fragment ions by FAB MS are different than in TSP MS and that the TSP MS was sensitive to temperature of the transfer line We have also noted in an LC–TSP MS study of the metabolites of blue dyes that under tuning conditions optimized for the parent xenobiotic, molecular ions for conjugates were not observed Similar behavior has been noted by Mellon et al [117] and resulted in a comment that is applicable here: "We have frequently observed that an overall loss in TSP sensitivity occurs (compared to that for the parent molecule) when a thermally or chemically labile group is attached to an otherwise stable molecule"

3 7 Drugs

The pharmaceutical industry and related laboratories are some of the most extensive users of LC–MS methodology, LC–MS is used for drug identification, metabolite profiling and identification, screening and quantification

3 7 1 Qualitative aspects

Since the purpose of the review is not to list all compounds to which LC–MS methodology has been applied, we will mention briefly the types of compounds that have been investigated. A listing of more recent LC–MS applications for a variety of drugs is presented in Table 2 Examination indicates that within this mass of spectral data there are several opportunities for comparisons of different LC–MS methodologies

Several of the more common drugs have been analyzed by more than one technique. Comparison of the spectra obtained for erythromycin is quite informative (Figure 12). The API spectrum (Fig 12a) gave $(M+H)^+$ as the base peak [87] while $(M+H)^+ - H_2O$ was the base peak in one TSP report [96] (Fig. 12b) A more recent TSP report from the same group [118] using an improved interface designed to reduce thermal decomposition indicated that $(M+H)^+$ was now 100% and loss of water was reduced to 20% (Fig 12c) Using a moving-belt interface (erythromycin B in this case), an NH_3 CI spectrum with $(M+H)^+$ as the base peak and a 70% water loss ion was obtained [130] (Fig 12d) CF-FAB [66] and DLI [96] both gave spectra with $(M+H)^+$ as the base peak but with more extensive fragmentation than reported for API or TSP The DLI fragmentation was dominated by water loss and sugar loss (Fig 12e) The CF-FAB spectra also showed sugar loss but the lower mass fragments did not correlate with those observed in the DLI spectrum (Fig 12f) In the absence of MS–MS data, it is difficult to say which fragment ions are arising from thermal processes and which are arising from mass spectral fragmentations All interfaces yield reasonable spectra for this type of compound if appropriate conditions are used

A comparison of the spectra for ampicillin by API [13], TSP [59] and CF-FAB [66] indicates that API and FAB yield $(M+H)^+$ as the base peak while the $(M+H)^+$ ion from TSP has a 25% RA Also of note is that while API showed no fragment ions, both the CF-FAB and TSP spectra contained significant but different fragment ions Thermal fragmentation in the TSP interface may be responsible for the difference [132]

Both API [13] and TSP [59] gave $(M+H)^+$ ions as the base peak in the spectrum of chloramphenicol Water loss was noted but minor for both API (2%) and TSP (ca 20%). In comparison, DLI gave an $(M+H)^+$ ion of very low RA [96]. Under negative-ion DLI conditions, an $(M-H)^-$ ion was observed but its RA was very source-temperature-dependent (100% RA at 150°C, <10% RA at 250°C)

TABLE 2

SELECTED DRUGS ANALYZED BY LC–MS

Numbers given are the reference numbers from the Reference section

Compound	DLI	Belt	API	TSP	CF-FAB	OTLC
Acetaminophen	98					
Actinamine	96					
Amastatin			13			
Antrimycin			13			
Aureothricin			13			
Beclamethasone	123	123				
Betamethasone metabolites	28,67,96			123		
Bromocriptines	96					
Chloramphenicol	96		13	59,118		
Clobazams	123					
Cocaine	96					
Diethylstilbestrol	28					
Epinephrine, phenylephrine	123			53		
Ergotamine	96					
Erythromycin	96	129	13	59,119	66	
Fluerlapine				128		
Flunixin	98					
Fluphenazine				59		
Gentamycin			13			
Heroin				122		
Ibuprofen	98					
Kanamycin			13			
LSD				59		
Labetalol				121		
Metoclopramides				59		
Neomycin		54				
Nicardipine				129		
Nodusmicin, nargenicin	27					
Oxytetracycline			13			
Penicillins			13	59	125	
Pentazocine				59		
Phenacetin						124
Phenothiazines	27					
Phenylbutazone				59		
Pseudomonic acid					66	
Puromycin			13			
Ranitidine	8,123	131	8	59		
Reserpine	41					
Ribostamycin			13			
Rutin			13			
Salicylamide	98					
Spectinomycin	96					
Spiramycin			13			
Sulfadimethoxine			115			
Sulfadizine			120			
Sulfamethazine	97		120			
Sulfisoxazole			120			
Tamoxifen	123					
Terbutaline				127		
Tetraacetyltylosine			13			
Tetracycline			13			
Thiazides	128					
Thiolutin			13			
Tobramycin		54				
Trenbolones				126		

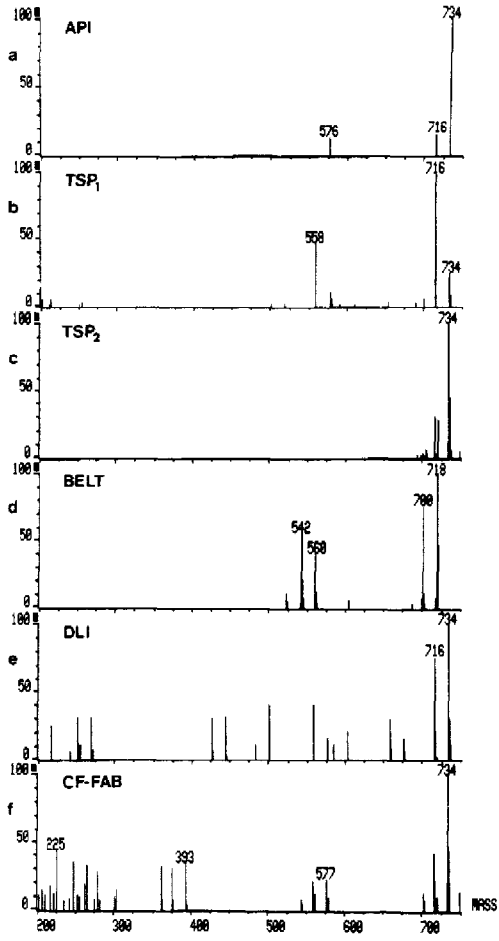

Fig 12 Spectra of erythromycin by (a) API, (b) TSP, (c) TSP, (d) belt (erythromycin B), (e) DLI and (f) CF-FAB Spectra were reconstructed from data in refs 13, 59, 119, 130, 96 and 66, respectively

Ranitidine-N-oxide, a compound prone to thermal degradation has been suggested by Carey and Martin [133] and Martin et al. [134] as a test for thermal effects in LC–MS. The spectra of ranitidine-N-oxide obtained using five different interfaces are shown in Figure 13 The moving-belt interface shows a negligible molecular ion (Fig 13a) Under DLI conditions a molecular ion is obtained but the loss of oxygen is the base peak (Fig 13b) Spectra obtained from two different TSP interfaces are significantly different (Fig 13c and d) In the earlier interface the $(M+H)^+$ has 50% RA and 100% oxygen loss while the newer design gives $(M+H)^+$ as the base peak and only 69% oxygen loss The spectrum obtained from an API interface (Fig 13e) shows

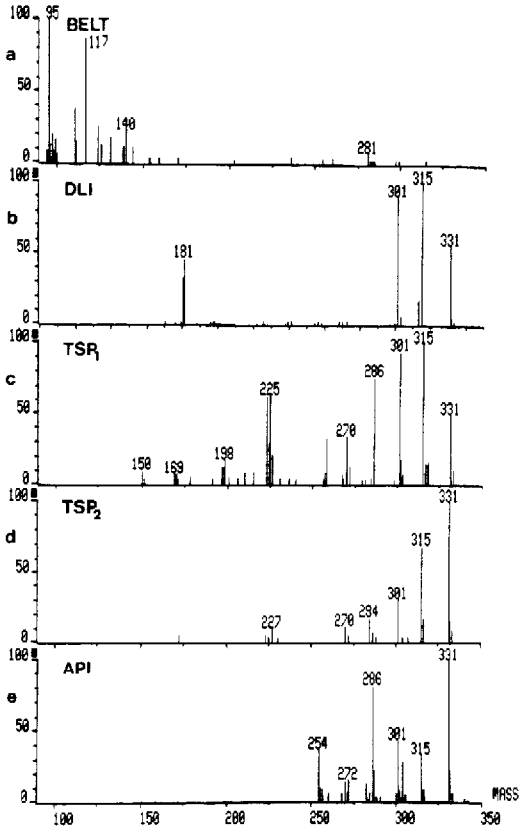

Fig 13 Spectra of ranitidine-N-oxide by (a) belt, (b) DLI, (c) TSP, (d) TSP and (e) API Spectra were reconstructed from data in refs 131, 8, 8, 59 and 8, respectively

the least amount of oxygen loss, 30% Thus, from these data, the API instrument appears to be the least prone to thermal effects Unfortunately, comparable data for the other interfaces such as CF-FAB, MAGIC or OTLC are not available

Several other compounds show evidence of thermally produced fragments These include aphidicolin glycinate [135], labetalol [121] by TSP and beclamethasone by moving belt [123] and DLI [124]

In a comparison of DLI and TSP spectra of betamethasone using a dual TSP–DLI probe, Covey and Henion [40] observed no $(M+H)^+$ ion under DLI conditions but a 98% relative abundance $(M+H)^+$ peak under TSP conditions

One of the most exciting developments in the analysis of drugs is the coupling of CF-FAB with microdialysis as described by Lin and Caprioli [125] They have used this combination for the on-line in vivo analysis of drugs in animals.

3 7 2 Quantitative aspects

Since the types of compounds and interfaces considered in this section are very different, observed detection limits are quite diverse also The detection limits in the full-scan mode (API MS) for 25 antibiotics ranged from 1 to 50 ng while thiolutin and aureothricin gave detection limits of 10 pg on the same instrument [13] Similar detection limits were observed for candidate antimalarial drugs by TSP (10–50 ng full scan) [136], LSD by TSP (10 pg, SIM) [59] and DLI (100 pg, SIM) [96], reserpine by TSP (20 pg, SIM) [40], trenbolone and epitrenbolone in bovine liver by API [126], diethylstilbestrol (DES) by API (10 ng full scan, 10 pg, SIM) [15], bromocriptines by DLI (100 pg, SIM) [96], terbutaline by TSP (4 pm, SIM) [127] and thiazides by DLI (1–10 ng full scan) [128].

These levels are adequate for most biochemical analyses and several biomedical applications have been reported

Although betamethasone did not give a molecular ion under DLI–negative-ion CI conditions, by use of a fragment ion, Lee and Henion [27] could study the disappearance of betamethazone from equine urine over a 48-h period The same group [67] also quantitated betamethasone and 6-hydroxybetamethasone by SIM in an equine urine sample, and the levels found were 6 and 0 6 ng, respectively, well above the detection limit. Full scans could be done on 250 pg, and estimated detection limits were <1 pg by SIM on the intense fragment ions The authors note evidence of thermal degradation in the lack of molecular ion and extensive fragmentation observed for these compounds Prednisolone gave a strong $(M-18)^-$ fragment ion which could be used for its determination in equine urine by microbore DLI–negative-ion CI MS at levels of 10 ng in a 20-μg biological matrix In this study on improved desolvation chamber design, the authors comment that multiple injections of tuning solution and adjusting the ratios of the solvent ions made daily tuning more reproducible

The synthetic estrogen DES has been analyzed by Bruins et al [15], using API LC–MS, in the negative-ion mode Detection limits of 10 ng full scan and 10 pg SIM were reported DES and dienestrol were also done by the same group [27] using microbore DLI LC–MS These compounds produced $(M+H)^+$ ions in the positive-ion mode Detection limits of less than 50 ppb full scan could be achieved A study on DES residues was performed in which a calf was dosed with 10 mg DES ten days prior to sacrifice DLI microbore LC–MS showed liver levels at approximately 40 ppb [$^2H_8$]DES was used as the internal standard A gas-nebulized DLI interface was used by Appfel et al. [123] to obtain spectra of tamoxifen and DES, and the authors report "excellent" spectra

Labetalol in human plasma had a detection limit of 5 ng/ml by positive-ion TSP [121] Quantitation was done with a deuterated analogue, and the calibration was linear over the range 10–103 ng/ml Lant et al [121] found that

quantitation could not be done without the internal standard because there was a 47% variation in peak height observed. This was attributed to variations in ionization efficiency The detection limit was much worse with a clean source and got better after several injections This was attributed to adsorption of plasma constituents onto the source, resulting in less adsorption of the target compound

In a comparison of DLI and TSP , Schellenberg et al [129] conclude that quantitation at trace levels with TSP is limited due to the noisy chromatograms, but that TSP is easier to operate than DLI. DLI was found to be less noisy, but more operator skill was required They also found the DLI jet to be less stable at very high or very low water concentrations "A serious drawback of the DLI is that it is still only an accessory, and has remained virtually unimproved for the past few years In contrast, dedicated ion sources have been developed for the TSP technique, and both interfaces and sources have been continuously updated by several manufacturers "

3 7 3 Drug metabolism

In the area of the identification of drug metabolites by LC–MS, N-oxides, sulfates, glucuronides, sulfoxides, decarboxylated products, taurine conjugates and dealkylation products have all been identified [27,104,129,137–142] in various biological media Molecular ions were observed in all cases As we have pointed out previously, thermal processes can contribute substantially to the TSP spectra obtained of some compounds Blake [138], in his paper on the metabolism of SKF 93944, invoked thermal processes, such as reaction with water in the interface, to rationalize the spectra of unknown metabolites and to help establish their identity.

4 CONCLUSION

As we stated in the introduction, LC–MS development is approximately at the stage of GC–MS development when a number of interfaces were being proposed as the standard From our discussion there are several recurring themes that indicate why LC–MS methodology is still at this stage

One reason is 'tuning' variability This refers to the fact that if instrumental conditions are set to optimize on one analyte, good spectra containing molecular ion species for other related compounds may not be obtained Thus, if one were analyzing, for example, a mixture of metabolites having optimized the instrument for the parent compound, one might obtain a good spectrum of a dealkylation product while no molecular ion might be observed for a conjugate such as a glucuronide No technique which requires foreknowledge of the results for minimal, let alone optimal, performance is very satisfying. (Which is not to say that it is not a lot better than nothing at all!)

Another reason is compound-dependent sensitivities, due to the production of ions by a CI process in many of these LC–MS techniques Success or failure

can result from fundamental gas phase acidities and basicities and from the comparative proton, electron or acetate affinities of the analyte and mobile phase components or from differing hydrophobic/hydrophilic natures. We have observed variations in sensitivities of several orders of magnitude for a set of closely related compounds even when each was optimized independently

Thermal degradation is another severe problem which can be viewed as a subset of tuning variability Those interfaces that use heat to volatilize the analyte and/or mobile phase are susceptible to thermal decomposition problems Of the interface techniques discussed here, ion spray, API and CF-FAB are least susceptible to these problems CF-FAB, however, has limited utility for less polar compounds

Some techniques such as the various LC–API MS methodologies, which appear to offer superior sensitivity in those cases where comparisons have been made, have not become popular for other reasons In the case of API, this might be attributed to lack of familiarity

What does the future hold? It is impossible at this time to predict whether or not one interface, let alone which one, will supercede all others Because of the wide range of compounds amenable to LC separation, it may very well be that two or more interfaces may dominate At the present, however, some directions for development can be discerned

At present a great deal of effort on the part of instrument manufacturers is going into improvements of TSP, MAGIC and CF-FAB sources and interfaces while DLI and moving-belt interfaces have been all but abandoned by the manufacturers It is not unreasonable to expect further improvements in those techniques where instrument development is supported and less improvement in other areas

The increasing use of microbore columns in LC can be traced to the increasing use of LC–MS It is likely that this trend will continue so that LC columns requiring flow-rates more compatible with MS will become more prevalent Whether or not this trend continues to the point where capillary LC or micropacked LC predominates will probably depend on how extensively the LC capabilities are developed, as well as adoption to these techniques by instrument manufacturers For the more polar biomolecules CF-FAB is becoming increasingly more popular and may reduce the demands on other interfaces to deal with ionic compounds

In the meantime, the current state-of-the-art of LC–MS is sufficient to provide a means of solving a variety of analytical problems and is now a major component of MS's arsenal of techniques

5 SUMMARY

The current state-of-the-art liquid chromatography–mass spectrometry (LC–MS) is reviewed with particular attention to biomedical applications The most

common LC–MS interface designs are described and compared These interfaces include transport, direct liquid introduction, thermospray, atmospheric pressure ionization, monodisperse aerosol generation, open-tubular LC and continuous-flow fast atom bombardment The relative sensitivities of the techniques are compared as much as possible, as well as their tendencies to induce thermal decomposition of the sample Applications of these various interface types to a variety of biomedically important compound classes, including peptides, nucleotides, steroids, lipids, carbohydrates, xenobiotic metabolites and drugs, are also reviewed

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