SPECIAL FEATURE:
TUTORIAL

Electrospray: Principles and Practice

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The basic principles underlying the electrospray process are reviewed without recourse to detailed discussion of mechanisms. The essential features of the practical implementation of electrospray (at various solution flow rates) are described and the nature of the resultant gas-phase ion population is discussed. The generation by electrospray of multiply charged ions creates complications in that spectral complexity is increased and the determination of charge number must precede the measurement of mass. Multiple charging is beneficial, however, in extending the mass range and improving fragmentation yield in tandem mass spectrometry. The current breadth of application of the technique (including the analysis of non-covalently bound species) and future developments are discussed. © 1997 by John Wiley & Sons, Ltd.

INTRODUCTION

There are good reasons to wish to sample ions for mass spectrometric analysis directly from solution. In the first place, many important analytes (notably, but not exclusively, those of biological interest) are insufficiently volatile or thermally stable to permit volatilization prior to ionization. Furthermore, the direct sampling of solutions is important in a variety of circumstances ranging from the monitoring of the progress of chemical reactions to the determination of trace components of biological fluids. The development by Barber and co-workers[1,2] of the fast atom bombardment (FAB) method significantly extended the range of compounds amenable to mass spectrometric (MS) analysis. The subsequent elaboration of the technique to allow desorption from a surface continuously replenished by a flowing stream of analyte solution[3–5] introduced new capabilities for direct analyses of solutions. The 'continuous-flow'[3] or 'frit-FAB'[4] techniques have, however, been restricted in their application, partly because of experimental difficulties and partly because (by current standards) the range of molecular masses accessible to FAB (or liquid secondary ion MS) remains limited. The thermospray technique[6] was developed expressly for the coupling of MS and condensed-phase separation methods (such as high-performance liquid chromatography, HPLC); it addressed the need for sampling directly from solution but was restricted with respect to the volatility and polarity of the analytes.

The significance of the development of the electrospray technique and the reason for its enormous contribution to modern MS methodology are that it is unique in providing simultaneously a logical coupling of solution introduction of compounds for analysis and the facility for ionization of highly polar and involatile compounds. Much of the current importance of the electrospray technique derives from the pioneering work of Fenn,[7] but Dole *et al.*[8] first recognized the possibility of generating gas-phase ions of macromolecules by spraying a solution from the tip of an electrically charged capillary. This early work, however, was hampered by the use of an ion-drift spectrometer, rather than a mass spectrometer, for ion analysis. Building on Dole *et al.*'s ideas, Fenn and co-workers[9] developed electrospray as a true interface for mass spectrometry.

The breadth of application of the electrospray method is now such that no modern practitioner of mass spectrometry can afford to be ignorant of the fundamental principles and practices of the technique. This Tutorial seeks to provide such a basic coverage. It is not intended to present a detailed critique of the now extensive literature on the mechanisms involved in the production of isolated ions by electrospray, details of which remain imperfectly understood. The coverage in this area is, by design, brief and basic, although leading references are provided for the reader to explore this aspect in more detail. The practical implementation of electrospray is discussed, with due coverage of the (sometimes arcane) vocabulary that has developed. Substantial attention is paid to the nature of the ions produced by electrospray, with consideration of the analytical implications of these properties.

THE ELECTROSPRAY PROCESS

In this discussion, the term 'electrospray ionization' will be avoided for the reason that (except in a small minority of examples[10,11]) electrospray is not an ionization

process in the sense that applies, for example, to electron ionization where the process explicitly concerns the conversion of neutral molecules into ions. The principal outcome of the electrospray process is the transfer of analyte species, generally ionized in the condensed phase, into the gas phase as isolated entities. That is not to say that the experimental implementation of electrospray does not have the potential to effect, principally through ion–molecule collisions, changes in the nature and charges of the gas-phase ions produced; this is discussed in more detail below.

Experimental implementation

The essence of the electrospray process can be described with (perhaps deceptive) simplicity. A solution of the analyte is passed through a capillary which is held at high potential. The effect of the high electric field as the solution emerges is to generate a mist of highly charged droplets which pass down a potential and pressure gradient towards the analyser portion of the mass spectrometer. During that transition, the droplets reduce in size by evaporation of the solvent or by 'Coulomb explosion' (droplet subdivision resulting from the high charge density). Ultimately, fully desolvated ions result from complete evaporation of the solvent or by field desorption from the charged droplets (of which more below). Nebulization of the solution emerging from the capillary may be facilitated by a sheath flow of nebulizer gas, a technique for which the term 'ionspray' was originally coined by its developers.[12] In practice, the facility to use a nebulizer gas is commonly incorporated on commercial instruments; the need for its use or not is determined by the flow rate employed, the composition of the solvent and the sign of the potential applied to the capillary tip (since a high negative potential, in particular, may lead to a corona discharge unless suppressed by use of an appropriate sheath gas). In addition, a flow of bath gas is usually applied to the interface[9] to promote droplet evaporation; controlled heating of the interface provides an alternative approach.[13] Sampling of the fully or partially desolvated ions is made using a capillary or a skimmer device. The essential features of the experimental arrangement are shown in Fig. 1; numerous elaborations have been reported.

The majority of the electrospray literature involves implementation on quadrupole mass spectrometers, but this position is rapidly changing with the increasing use of Paul ion traps[14] and (to a numerically lesser extent) Fourier transform ion cyclotron resonance instruments,[15] the latter providing significantly enhanced resolution. Implementation of electrospray on magnetic sector instruments[16–18] is complicated by the need to avoid collisional activation during ion acceleration; enhanced resolution is achieved, however, in comparison with quadurpole instruments. Installation of an electrospray source on a time-of-flight instrument[19] also presents some advantages, which are discussed in a later section.

Brief comments on mechanism

Several excellent reviews[7,20,21] have appeared which have summarized current thinking on the mechanisms of the several stages of the electrospray process. It is not proposed to reproduce those discussions here but it is appropriate (bearing analytical implications in mind) to cover the essential points. It is convenient to divide the process into three stages: droplet formation, droplet shrinkage and gaseous ion formation.[21]

The solution delivered to the tip of the electrospray capillary experiences the electric field associated with the maintenance of the tip at high potential. Assuming a positive potential, positive ions in solution will accumu-
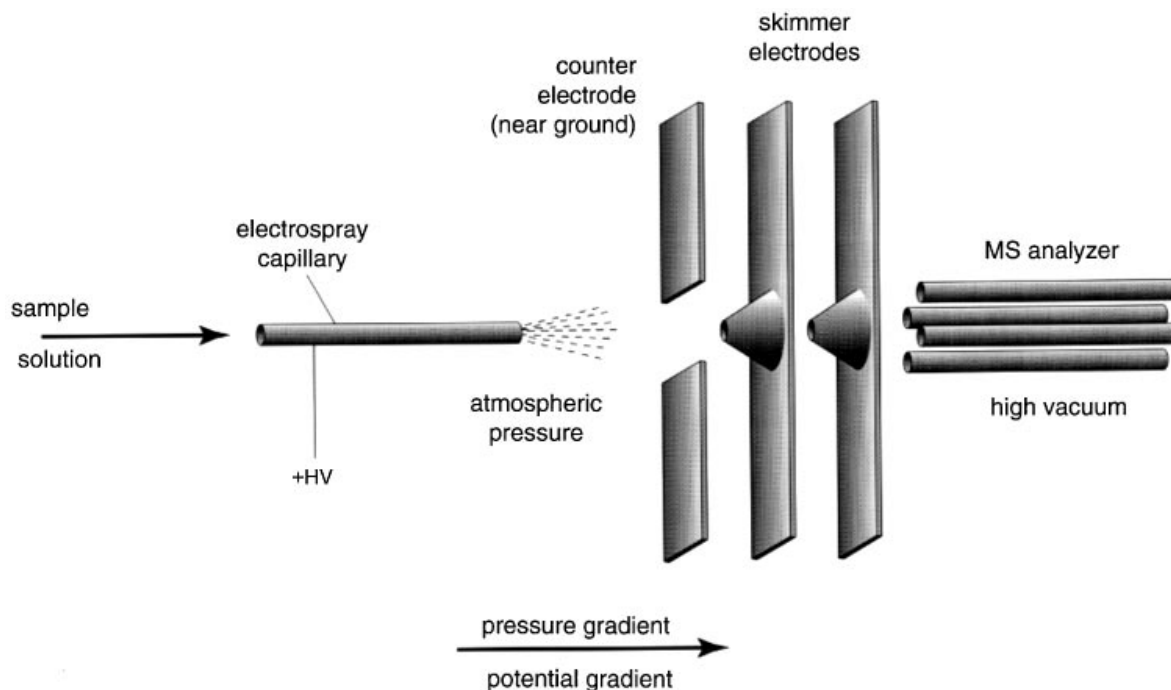

Figure 1. Essential features of the electrospray interface.

late at the surface, which is thus drawn out in a down-field direction to establish a 'Taylor cone' (Fig. 2). At a high enough imposed field, the cone is drawn to a filament which produces positively charged droplets via a 'budding' process when the surface tension is exceeded by the applied electrostatic force. The diameter of the droplets formed is influenced by a number of parameters, including the applied potential, the solution flow rate and solvent properties.[22]

Evaporation of solvent from the initially formed droplets, as they traverse a pressure gradient towards the analyser of the mass spectrometer, leads to a reduction in diameter, with collisional warming preventing freezing. Fission ('Coulomb explosion') will occur at the point (the 'Rayleigh limit') at which the magnitude of the charge is sufficient to overcome the surface tension holding the droplet together. Continuous and continual depletion of the droplet size (by solvent evaporation and fission, respectively) may be envisaged to result eventually in the formation of droplets containing a single ion, from which the non-solvated analogue may be derived by further evaporation of solvent (aided by activating collisions in the interface). A second mechanism of gas-phase ion production has been proposed based on the earlier ideas of Iribarne and Thompson[23,24]. Ion evaporation (emission) is envisaged to occur from small, highly charged droplets; the driving force is the repulsion between the charged ion and the other charges of the droplet. An extension of this model accommodates the formation of multiply charged gas-phase ions.[7] The relative importance of the single ion droplet and the ion evaporation mechanisms remains the topic of discussion and research.

Kebarle and Tang[21] noted that the ion separation in the electrospray capillary tip is essentially electrophoretic and that the interface may be considered as an electrolytic cell in which part of the charge transport occurs in the gas phase. The predicted electrochemical process at the liquid/metal interface of the electrospray capillary has been demonstrated experimentally by Blades et al.[25] Electrochemical oxidation occurring in the electrospray capillary has analytical implications in that radical cations may be generated from neutral analytes, thereby permitting, in some selected instances, the use of electrospray as a true ionization method rather than a procedure for phase transfer of pre-formed ions.[11,26,27]

Electrospray at reduced flow rates

The sample solution flow rate in an electrospray interface is most commonly in the range 3–20 μl min^{-1}. In the interests of direct compatibility with conventional scale 'analytical' HPLC, some effort has been devoted to the accommodation of much higher flow rates. Henion and co-workers,[28] for example, devised an interface for nebulizer-assisted electrospray to permit interfacing with chromatographic columns delivering up to 2 ml min^{-1} of solvent; this tolerance was achieved by incorporating a grounded metal 'shield' between the
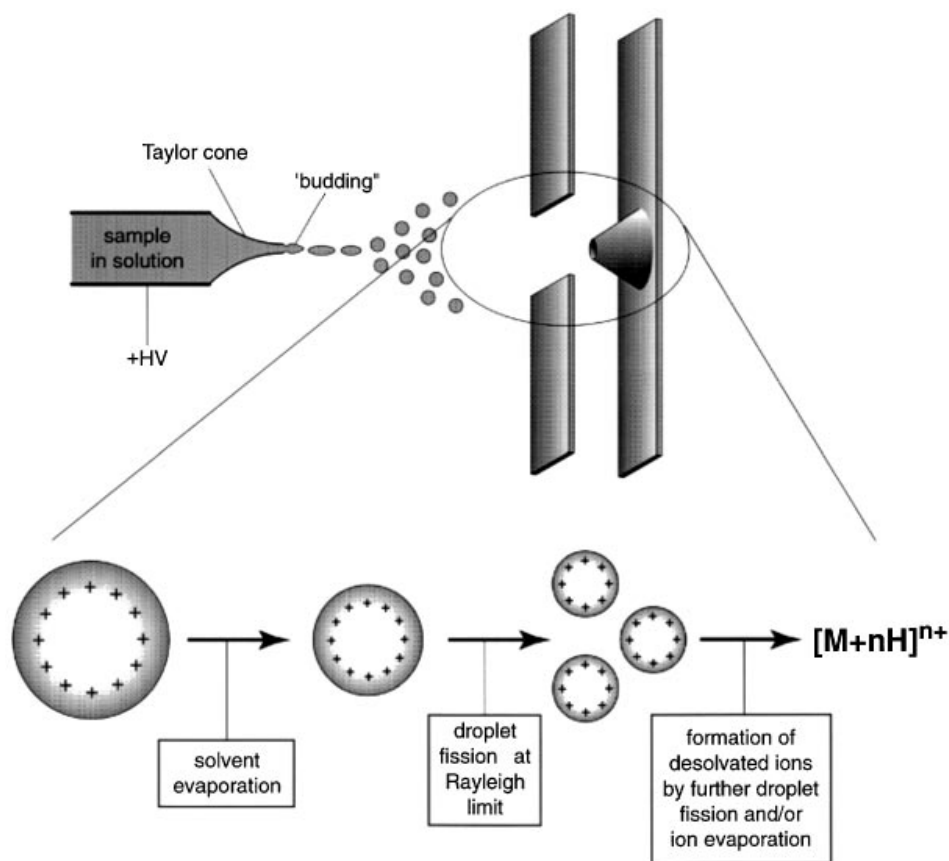

Figure 2. Droplet production in the electrospray interface.

electrospray needle and an ion sampling capillary. The device permitted the recording of full mass spectra at the nanogram level.[28] Nevertheless, it is clear that optimal sensitivities of detection using electrospray will be achieved by delivering reduced flow rates and a number of laboratories have devoted efforts in that direction. Early work on pumped low flow rate (<50 nl min^{-1}) electrospray was reported by Gale and Smith,[29] who demonstrated the achievement of high signal stability and sensitivity, together with a facility (not readily achieved using conventional-scale electrospray) to spray aqueous solutions. The fused-silica electrospray capillary (5–20 µm i.d.) was etched with hydrofluoric acid and electrical contact was established via a silver conductive coating. A sheath flow of SF_6 was used to suppress corona discharge, which can lead to signal instability, elevated background and increased ion fragmenation. This design was used in Smith's laboratory for the implementation of combined capillary electrophoresis (CE)/MS where the electroosmotic flow was estimated at 10 nl min^{-1}.[30] The fabrication of robust gold-coated fused-silica capillaries for low-flow electrospray has been reported by Kriger et al.[31] A recent report from Smith and co-workers[32] describes a refined CE/electrospray MS interface in which electrical contact is made via a microdialysis tubing connection.

Caprioli and co-workers[33] have pursued the implementation of pumped delivery of low flow rates to the electrospray needle. An electrospray source was optimized for flow rates in the range 300–800 nl min^{-1}; a fused-silica capillary was used with an i.d. of 50 µm and a wall thickness at the electrospray tip reduced by etching to 10–20 µm. For some applications, the capillary incorporated a short length of chromatographic packing material to effect sample preconcentration or desalting. The interface incorporated a coaxial flow of gas to entrain the ions en route to the analyser via a skimmer arrangement; significant reductions in background signal were achieved using ultra-pure nitrogen in preference to air. Application of this interface, albeit to the analysis of a well behaved analyte under optimal conditions, indicated the detectability of a peptide at infused solution concentrations of 320 zmol (10^{-21} mol) µl^{-1}.[33] The principal advantage of adopting a pumped flow design for low-flow rate electrospray is the compatibility with micro-HPLC, with CE and with on-line direct sampling methods. An example of the last combination is the report from Andrén and Caprioli[34] of the study of the *in vivo* metabolism of the undecapeptide substance P in rat brain using on-line microdialysis and electrospray MS. The probe was used both to introduce the peptide substrate and to sample the metabolic products.

The designation by Caprioli and co-workers of their technique as 'micro-electrospray' was intended to denote miniaturization of the conventional-scale technique rather than imply a particular range of flow rates. The interface designed by Wilm and Mann,[22] introduced independently of Caprioli and co-workers, was subsequently denoted 'nanospray.' This interface does indeed operate at low nl min^{-1} flow rates and involves the formation of droplets with diameters in the nm rather than µm range. A critical distinction between the two approaches, however, concerns the use by Wilm and Mann of sample capillaries which are installed with a backing gas pressure to aid flow stability but without pumping to impose a specific solvent flow; essentially the flow is determined by the electrospray process itself. Effectively, the capillary acts as the sample reservoir with the signal lifetime determined by the ratio of the total sample volume (typically of the order of 1 µl) to the flow rate. The efficiency of conversion of condensed-phase analyte to gas-phase ions that is achieved by this electrospray source was estimated[22] to be approximately two orders of magnitude greater than conventional-scale sources. A contributory factor to this efficiency gain is evident from a detailed theoretical treatment of the spray process.[22] Use of an electrospray capillary with an orifice diameter of 1–2 µm is estimated to yield droplet diameters of <200 nm, corresponding at typical solution concentrations to an average of one analyte molecule per droplet. Charge to volume ratios are accordingly high and the formation of analyte cluster ions is minimized. The model also predicts improved analyses of solutions with high salt concentrations and this is indeed observed in practice.[35] This feature is likely to prove of particular value in view of the generally poor tolerance of the conventional electrospray experiment for buffers and salts. The Wilm–Mann interface has been used recently in the electrospray/tandem MS characterization at the sub-picomole level of proteins recovered from polyacrylamide gels.[36]

McLafferty and co-workers[37] adopted an essentially similar approach to Wilm and Mann but further reduced the flow rate to below 1 nl min^{-1}; the combination with Fourier transform ion cyclotron resonance allows the recording of high-resolution spectra with attomole consumptions of analyte.

THE PROPERTIES OF ELECTROSPRAYED IONS

The mechanisms underlying the electrospray process and the manner of its implementation have significant implications for the properties of the gas-phase ions produced. Three issues are important:

(i) The charge states of the gaseous ions reflect the charge states in the condensed phase, although somewhat modified following ion–molecule collisions in the interface; thus, multiply charged species are commonly observed.

(ii) The transfer of ions to the gas phase is not an energetic process; indeed, the desolvation process effectively cools the ions. Under appropriate conditions, therefore, low internal energy ions are introduced into the mass spectrometer for conventional analysis or for selection as precursor ions in a tandem MS analysis. The work of Wysocki and co-workers,[38] relating the onset of precursor ion fragmentation to the collision energy in surface-induced decomposition experiments, has demonstrated that electrosprayed ions are considerably 'cooler' than their counterparts produced by fast atom bombardment. Nevertheless, conditions can be established in the electrospray interface to achieve effective collisional activation. Application of a suitable potential difference between focusing components in a

region of intermediate pressure allows ions to achieve kinetic energies consistent with the applied potential and therefore undergo activating collisions.

(iii) The electrospray process involves the stepwise disruption of non-covalent interactions (principally the removal of molecules of solvation); interception of this process may allow the preservation of relatively strong non-covalent interactions of analytical significance.

Each of these aspects of the properties of electrosprayed ions will be considered in this section.

Determination of the charge states of electrosprayed ions

The comfortable assumption of a charge number of one that is applicable to most earlier methods of generating gas-phase ions is, as noted above, not appropriate to electrospray, reminding the analyst that 'mass' spectrometry fundamentally determines mass/charge ratio. Furthermore, a single molecular species may be represented in the gas-phase ion population (as in solution) by a number of charge states, providing an apparent complexity to the mass/charge spectrum. In simple cases, the values of mass and charge are readily inferred. Figure 3, for example, shows the electrospray spectrum of a 16-residue peptide with the sequence Tyr-Leu-Glu-Phe-Ile-Ser-Asp-Ala-Ile-Ile-His-Val-Leu-His-Ser-Lys. A series of signals are observed corresponding to the isotopic envelopes of ions of differing protonation state; the resolution of the quadrupole analyser is insufficient (as is commonly the case in the analysis of multiply charged ions) to separate individual isotopic variants. The relationship between the m/z ratios of the ion series

allows independent determination of the average molecular mass (1885.2). Thus, the three principal signals occur at m/z 472.3 ([M + 4H/4), m/z 629.4 ([M + 3H/3) and m/z 943.6 ([M + 2H/2). The highest and most abundant protonation state represented in the spectrum is 4 and corresponds to the number of sites with significant basicity in solution (two histidine and one lysine residues, together with the N-terminal primary amine group).

Independent determination of mass and charge may be less obvious, even for low molecular mass analytes, if a single charge state predominates to the exclusion of others or if interpretation is complicated by the presence of multiple components. If the mass spectrometric resolution is sufficiently high, the separation of the components of the isotopic cluster for each charge state allows determination of the charge number; thus, the separation in m/z units is $1/n$, where n is the charge state. (Cooks and Rockwood[39] have suggested the introduction of the thomson (Th) as the unit of mass-to-charge number ratio. This suggestion will be followed for the remainder of this review.) Alternatively, the observation of different cationized species of the same charge number and derived from the same neutral analyte may allow direct determination of charge number. Thus, for example, sodium cations may replace protons in the formation of positive ions during electrospray, yielding ions of the general form $[M + Na + (n - 1)H]^{n+}$, which are separated from the fully protonated analogue, $[M + nH]^{n+}$, by $22/n$ Th. Samples isolated from biological sources commonly contain significant concentrations of sodium salts, so that sodium-cationized and fully protonated analogues
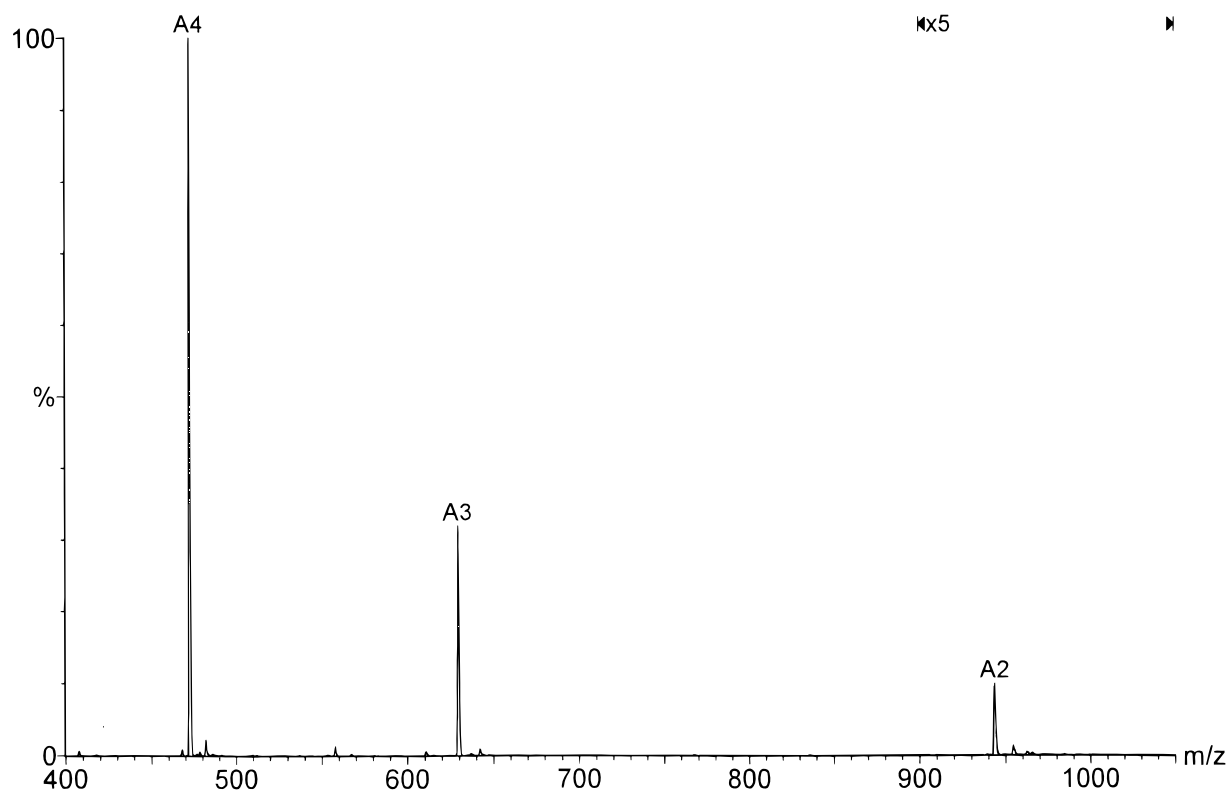

Figure 3. Electrospray spectrum of a 16-residue peptide with the sequence, Tyr-Leu-Glu-Phe-Ile-Ser-Asp-Ala-Ile-Ile-His-Val-Leu-His-Ser-Lys. The annotations of the major peaks indicate that a single series is recognized, with the protonation states shown.

are observed in the electrospray spectrum; in other instances, addition of a sodium or other metal salt may be made to the electrospray solvent[40,41] (although excessive concentrations may distribute the analyte signal between multiple additional peaks and may lead to suppression of the total signal).

An interesting elaboration of this general approach has been reported by Cunniff and Vouros,[42] who detected protonated analogues of analytes and their 1:1 adducts formed with 18-crown-6 (M_r 264) added to the electrospray solvent. The separation on the m/z ratio scale between a pair of ions of the same charge state (n) derived from the same analyte is $264/n$ Th. Moreover, low energy collisionally activated decomposition (CAD) of the adduct ions in a tandem quadrupole instrument effected ready dissociation of the non-covalent complex so that linked scanning of the two analysers with an offset of $264/n$ Th revealed those precursor ions of charge state n.

During the analysis of higher molecular mass compounds, such as proteins, oligonucleotides and oligosaccharides, a broad range of charge states is commonly observed, yielding a substantial redundancy of data for the independent determination of mass and charge. Figure 4(a), for example, shows the electrospray spectrum of rho protein; the molecular mass is $\sim$47 000, so that the observed range of m/z values indicates charge states of approximately 40 to +70. Conveniently, the multiplicity of protonation observed under normal electrospray conditions is such that the electrospray spectra of proteins may be recorded (with suitable choice of solution pH) in the m/z range below 2000. Indeed, this observation, permitting the use of MS analysers of modest m/z range, accounts for much of the explosive growth of the use of electrospray in biological MS. The multiplicity of protonation is certainly related to the number of basic amino acid residues in the protein structure; it should be noted, however, that the maximum charge state may exceed or be less than the number of sites that are protonated in solution,[43,44] reflecting, *inter alia*, the influence of gas-phase processes.[45,46] (The occasional desirability of using condensed-phase conditions which result in the formation of gas-phase ions of relatively low charge state, is discussed below in the context of the analysis of non-covalent adducts.)

If a positive ion series is assumed to represent different protonation states, then the mass/charge ratios, x_1 and x_2, of adjacent members of the ion series are given by

$$x_1 = (M + n)/n$$

and

$$x_2 = (M + n + 1)/(n + 1)$$

where M is the molecular mass. Solving these equations gives

$$n = (x_2 - 1)/(x_1 - x_2)$$

and allows the estimation of M. In practice, such conversion of m/z data to a 'true' mass spectrum is carried out by the mass spectrometer data system; the redundancy of data allows a concomitant estimate of the precision of determination of molecular mass.[47] Figure 4(b) shows the spectrum obtained in this way for
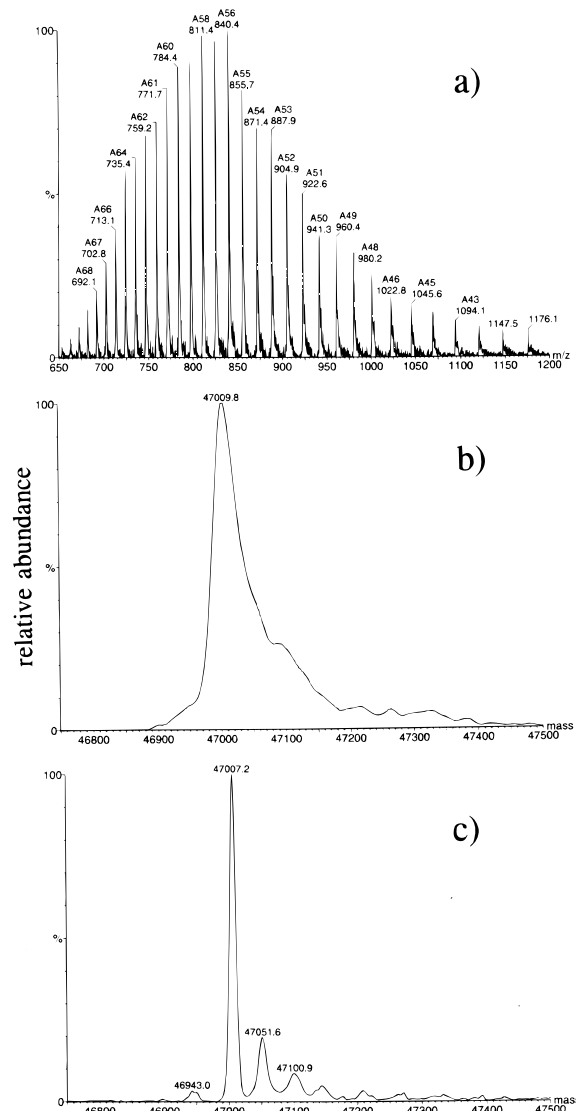

Figure 4. Electrospray spectrum of rho protein (M_r 47005). (a) Acquired m/z spectrum; the charge states of the members of the major ion series are indicated; (b) spectrum transformed to a mass scale; (c) spectrum obtained after maximum entropy processing of the data.[48]

rho protein. A number of refinements of this deconvolution approach have been presented;[48–50] a detailed discussion is beyond the scope of this Tutorial but potential benefits are evident from Fig. 4(c), which shows the mass spectrum of rho protein after maximum entropy processing[48] of the recorded electrospray data. Enhanced resolution of components closely separated in mass is achieved by application of a probabilistic method which functions by repeatedly processing trial spectra of different mass and charge and comparing with the experimental data.

For the majority of electrospray analyses of compounds with molecular masses in excess of (at most) a few thousand Da, the resolutions achieved by the common analysers are insufficient to permit determination of the monoisotopic molecular mass: the experimentally determined value is the average molecular mass. In principle, the assignment of charge state for multiply charged ions derived from high molecular mass analytes can be made (as for low-mass
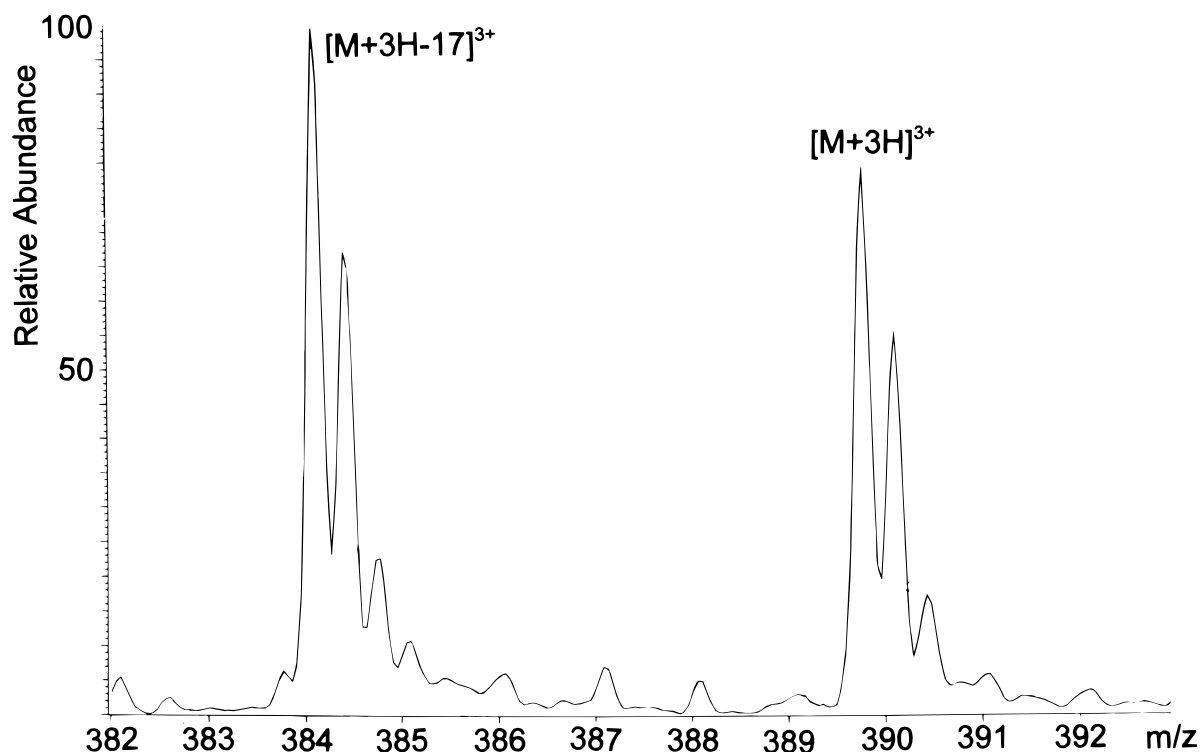
Figure 5. Portion of the product ion spectrum obtained following collisional activation of $[M + 3H]^{3+}$ ions derived from the nonapeptide Lys-Gln-Tyr-Gln-Lys-Ser-Thr-Glu-Arg. The spectrum was recorded using a hybrid tandem instrument comprising a quadrupole mass filter, hexapole collision cell and reflectron time-of-flight analyser.

compounds) by the separation of isotopic variants, yielding estimates of molecular mass for specific iso-topomers. Perversely, the implementation of such refined analyses using Fourier transform (FT) ion cyclo-tron resonance (ICR)[15] or quadrupole ion trap[51] instru-ments introduces the new problem of identifying the component of the isotopic cluster corresponding to an all-light isotopic composition. McLafferty and co-workers[52] suggested one approach to this problem, involving statistical tests to compare the observed iso-topic distribution with that calculated for a model mol-ecule of the same average molecular mass. This technique does not depend on the detection of the monoisotopic species (which may be of minor abun-dance relative to higher mass isotopomers).

Smith *et al.* [53] noted that the mass and charge of a *single* ion trapped in an ion cyclotron resonance cell may be independently determined. Detection of a single ion is made possible by repeated remeasurement and by the high charge state of macromolecular species. In an illustrative experiment,[53] stepwise changes in the observed m/z ratio of a polyethylene glycol ion incorp-orating multiple sodium cation attachment were attrib-uted to sequential losses of Na^+ following collision with added neutrals (such as crown ethers) or residual back-ground gases. At present, the elegance of the experiment and the importance of the concept exceed the practical applicability of the technique.

Fragmentations of multiply charged ions

Numerous studies have investigated the fragmentations of multiply charged ions following collisional activation,

either in the electrospray interface, the collision region of a 'tandem-in-space' instrument (such as a triple quadrupole), or in an ion trap (Paul trap or ion cyclo-tron resonance instrument). Collisions are an integral component of the process of generation of desolvated gas-phase ions in the electrospray interface. Interface conditions may usually be established to achieve col-lisional activation sufficient to accomplish complete desolvation of electrosprayed ions without promoting fragmentation. Under these conditions, the ions produc-ed have been demonstrated to be internally colder than those typically produced in a liquid secondary ion MS experiment.[38] If ions are subjected to more energetic collisions in the interface, then useful fragmentation can be induced, equivalent to that promoted by collisional activation in the decomposition region of a tandem quadrupole instrument (although obviously no connec-tivity is established between precursor and product ions). The technique is variously (and loosely) termed 'cone voltage fragmentation,' 'nozzle–skimmer disso-ciation' and others.

Clearly, multiply charged precursor ions can yield products of the same charge state or any lower charge state; interpretation of fragmentation pathways is facili-tated if the charge states of the product ions are appar-ent. This may be obvious (as for product ions of higher mass/charge than a doubly charged precursor) or may require determination. The problem may be exacer-bated by the absence of ion series corresponding to ions of similar mass but different charge state, so that appli-cation of the deconvolution approaches developed for conventional spectra may be precluded. Selection of a precursor ion population that includes more than one isotopic variant affords isotopic clusters for each

product ion species with a separation of $1/n$ Th (where n is the charge state of the product ion). Figure 5 shows an example of this approach in the CAD of an $[M + 3H]^{3+}$ ion obtained by electrospray of a non-apeptide; the portion of the product ion spectrum shown includes triply charged precursor and product ions. In this instance, the requisite product ion resolution was readily achieved by using a tandem quadrupole/time-of-flight instrument incorporating orthogonal acceleration of product ions into the second analyser. Application of this approach exploiting the powerful resolution capabilities of Fourier transform ion cyclotron resonance instruments has allowed charge assignment to fragment ions derived from the proteins myoglobin (M_r 17 kDa) and ubiquitin (M_r 8.5 kDa).[54]

McLuckey et al.[55] introduced a procedure for the charge state determination of product ions in a quadrupole ion trap via observation of the increase in mass/charge ratio upon collision with a basic neutral molecule. This approach is conceptually the same as that adopted by Smith et al.[53] and discussed in the previous section, in which the charge state of a single ion trapped in an ICR cell was determined by observation of sequential discrete changes in m/z ratio.

Fragmentation of multiply charged ions by losses of a common neutral fragment (N) may be determined by constant neutral loss scanning, as for singly charged precursors but with the experimental variation that the loss in terms of mass/charge ratio (Th) is given by N/n, where n is the charge state of both precursor and product. The exploitation of this scan mode in charge state determination[42] was discussed in the previous section. The added selectivity afforded by specification of the charge state as well as the difference in mass/charge ratio of precursor and product ions can also be used to advantage in screening complex mixtures, such as biological extracts, for targeted compound types. This approach may be exemplified by work in this laboratory[56] on the concomitant detection of conjugates of xenobiotics with glutathione and the analogous thiol, trypanothione (Fig. 6). Selective detection of glutathione conjugates was demonstrated some years ago to be achieved by scanning for the constant neutral loss of 129 Da (corresponding to the γ-glutamyl moiety less a hydrogen) from the singly protonated precursor derived by fast atom bombardment.[57,58] Electrospray of glutathione conjugates yields predominantly singly protonated species and the same tandem MS strategy may therefore be applied to their selective detection.[59,60] Electrospray analysis of trypanothione conjugates gives predominantly doubly protonated ions so that loss of a neutral fragment of 129 Da is measured experimentally as a decrement of 64.5 Th. Figure 7 shows several modes of electrospray and tandem MS analysis for the detection of glutathione and trypanothione conjugates of 1-chloro-2,4-dinitrobenzene. Scanning of precursors of m/z 130, corresponding to the γ-glutamyl moiety (Fig. 8), achieves detection of both classes of conjugate [Fig. 7(a)]. Constant neutral loss scanning, with specification of a 129 Th loss favors detection of the glutathione conjugate [Fig. 7(c)], whereas the equivalent scan, specifying a 64.5 Th loss, is specific for the trypanothione conjugate [Fig. 7(b)]. A further commonality of fragmentation of doubly
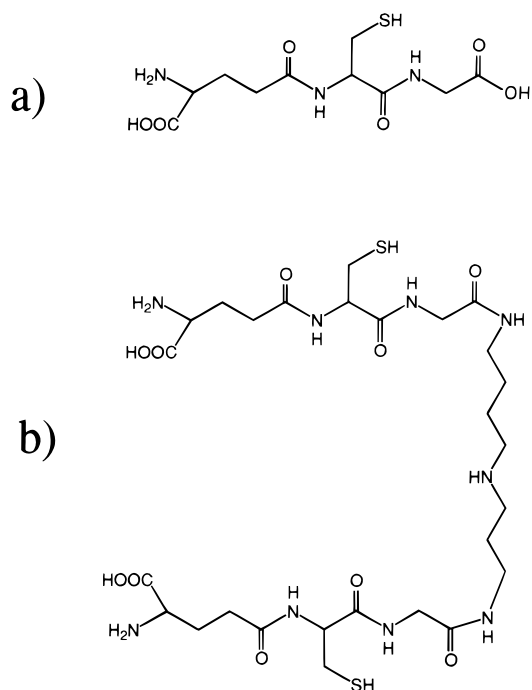

Figure 6. Structures of glutathione and trypanothione.

charged trypanothione conjugates is the same cleavage to yield complementary singly charged ions of m/z 130 and m/z M $-$ 130. In principle, therefore, selective detection might also be achieved using a constant ion loss scan, but this non-linear scan mode has yet to be implemented.

Substantial differences in the extent of collision-induced fragmentation are frequently observed for multiply protonated ions and their singly protonated counterparts. In making such a comparison, of course, account must be taken of the increased collision energy, for a given collision potential, associated with the higher charge state. Thus, for example, a collision
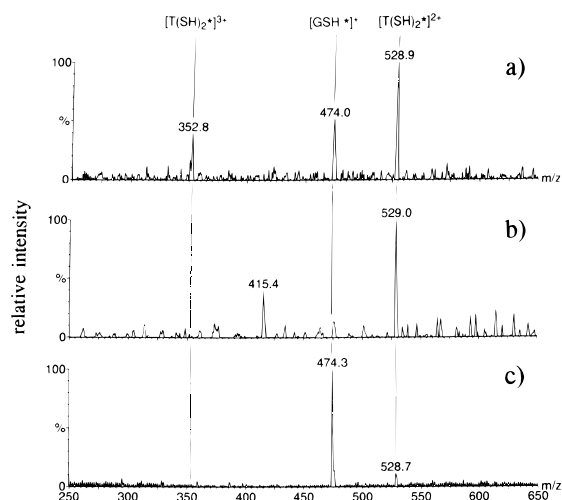

Figure 7. Electrospray tandem MS analyses (after desalting) of an extract of Trypanosoma brucei brucei spiked with 25 pmol of the glutathione and trypanothione conjugates of 1-chloro-2,4-dinitrobenzene. (a) Scan of precursors of m/z 130; (b) scan detecting the constant neutral loss of 129 Da from doubly charged ions (corresponding to a decrement of 64.5 Th); (c) scan detecting the constant neutral loss of 129 Da from singly charged ions.
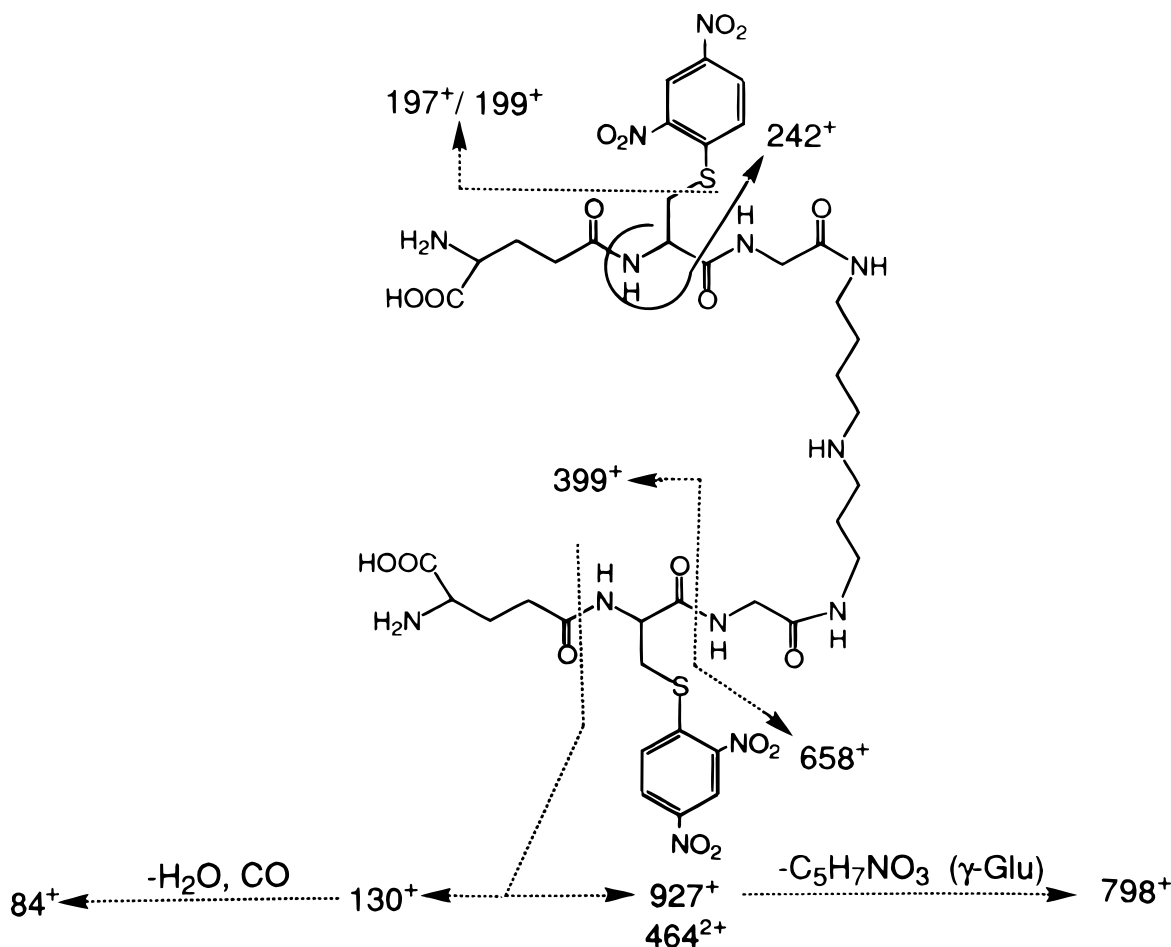
Figure 8. Principal fragmentations of $[M + 2H]^{2+}$ ions derived from electrospray of the trypanothione conjugate of 1-chloro-2,4-dinitrobenzene.

potential set to 20 V corresponds to a collision energy, in the laboratory frame-of-reference, of 20 eV for singly charged ions and 40 eV for doubly charged ions. Several early reports[61-63] described high CAD efficiencies for doubly protonated peptides derived from trypsin digestion of proteins, yielding peptides with basic arginine or lysine residues at the C-terminus. It is now appreciated that this observation (and, by implication, equivalent observations for other classes of analyte) can be explained by simple extensions[64,65] of the mechanistic ideas developed for charge-directed, low-energy cleavages of singly charged ions. Specifically, peptide ion fragmentation is promoted by a 'mobile' proton[66], or, equivalently, by a precursor ion population which is heterogeneous with respect to site of charge.[67-69] A basic amino acid side-chain (particularly the guanidino group of arginine, which is strongly basic) can sequester an ionizing proton, with proton transfer to the peptide backbone disfavoured by a significant energy barrier. In doubly charged tryptic peptides the initial sites of protonation, following electrospray transfer of the doubly charged ions to the gas phase, are the basic side-chain of the C-terminal residue and the primary amine group at the N-terminus. The energy barrier to transfer of the latter proton to the peptide backbone (and to transfer between different sites on the backbone) is low so that a variety of charge-directed fragmentation channels are promoted. Much of the

attention with respect to electrosprayed peptide ions has focused on the doubly protonated analogues because of their general prominence, but generalizations based on the desirability of a multiplicity of possible charge sites in the precursor ion population apply equally to other charge states. Thus, for example, low-energy CAD of the $[M + 2H]^{2+}$ ion derived from the nonapeptide Arg-Leu-Cys-Ile-Phe-Ser-Cys-Phe-Arg gives a very low yield of product ions. CAD of the triply protonated analogue, under identical collision conditions, yields products with an efficiency two orders of magnitude higher.[70] The difference is clearly attributable to the sequestration of two protons by the highly basic guanidino functional groups in the two arginine side-chains; a third proton in the $[M + 3H]^{3+}$ analogue is 'mobile' and therefore available to promote diverse fragmentations.

Fragmentation of precursor ions to products of lower charge state clearly implies the formation of complementary ions. For the example of multiply deprotonated oligonucleotide ions fragmented in an ion trap instrument, complementary products of simple fragmentation are indeed observed provided the collision conditions are sufficiently gentle to avoid further fragmentation of one of the first generation products.[71,72] The observation of complementary pairs facilitates interpretation of the product ion spectrum. Tang and Boyd[64] noted that the fragmentations of

tryptic peptide $[M + 2H]^{2+}$ ions yielded product ion spectra in which the b-type ions (with charge retention on the N-terminal fragment) appeared at considerably higher relative abundance than their y-ion complements. After consideration of several possible explanations, Tang and Boyd[64] concluded that the observation was attributable to a higher stability of the b-ions. This suggestion is consistent (bearing in mind the comments made above about proton mobility and the consequent efficiency of fragmentation) with location of the site of charge primarily on the C-terminal amino acid residue side-chain in the b-ions whereas the y-ions have no strongly favoured site of charge.[69]

Remarkable data, obtained using either tandem quadrupole or ion cyclotron resonance instruments,[73] have been reported for the CAD of multiply charged ions of proteins, analytes of much higher mass than hitherto considered amenable to tandem MS analysis. Presumably the success derives partly from the elevated collision energy associated with high charge states, partly from Coulombic interactions[74] and partly from the multiple protonation sites providing the driving force for fragmentation. Witkowska *et al.*,[75] for example, have described the confirmation of the structure of a variant haemoglobin using electrospray tandem MS performed on a tandem quadrupole. The product ion spectrum obtained by CAD of $[M + 18H]^{18+}$ ions derived from the normal α-globin chain showed a high decomposition efficiency and was interpreted with knowledge of the sequence. A comparison of the major fragment ions with the equivalent data for the variant α-chain allowed the location of the mutation site to the sequence incorporating the 28 C-terminal residues; consideration of minor product ions pinpointed the mutation to a specific residue. Interpretation of the product ion spectrum derived from a multiply charged protein is greatly facilitated if the resolution is sufficient to separate isotopic variants, allowing inference of charge state. Impressive examples have been published illustrating the value of FT-ICR analyses for this purpose.[76]

Analyses of non-covalently bound adducts

It has been noted above that a principal function of the electrospray interface is the disruption of the non-covalent interactions between analyte and solvent molecules. The frequent observation in electrospray mass spectra, therefore, of ions attributable to non-covalently bound species suggests the presence of associations more avid than between analyte and solvent.[77] Several useful reviews of this area have been published,[77,78] including a recent authoritative coverage by Przybylski and Glocker;[79] this Tutorial seeks only to highlight the major issues and to provide illustrative examples.

Non-covalent interactions of interest may be usefully categorized as follows:

(i) intra-ionic interactions influencing the three-dimensional ion structure;

(ii) multimeric species of identical or sturcturally similar components;

(iii) specific interactions between structurally dissimilar species which are related by biological function.

Chait and co-workers[80] noted the presence of three discrete charge state distributions in the electrospray spectra of the protein cytochrome c recorded with electrospray solvents of differing pH. The observations were interpreted in terms of solution populations with differing conformations, resulting in differing availabilities of protonation sites. Thus, the properties of the gas-phase ions are a reflection of the condensed-phase structure; no assumption is necessary, however, concerning the retention in the gas phase of the condensed-phase conformation (and on this evidence alone no such conclusion is warranted). Similarly, conclusions concerning condensed-phase structure of peptides and proteins may be drawn from the electrospray MS determination of the incorporation of deuterium by exchange in solution prior to analysis;[81-83] the complementarity of this approach to analysis by NMR spectroscopy has been convincingly illustrated.[84] In contrast to such examples of the use of electrospray MS to probe intramolecular interactions in the condensed phase, McLafferty and co-workers[85] have performed hydrogen-deuterium exchange experiments *in the gas phase* using protein ions trapped in an ICR instrument. The existence of three distinct gaseous conformers was indicated by different extents of isotope exchange; the three populations corresponded to the three charge-state distributions noted previously, implying that, at least for this example, there *was* a correspondence between condensed-phase and gas-phase structures. Interconversion between gas-phase conformers was not spontaneous but could be induced by infrared laser heating or by charge stripping.[86]

Ions corresponding to multimeric species are commonly observed in electrospray spectra and may be assumed to represent forms present in the condensed phase (although possibly only in the electrosprayed droplets rather than the bulk solution). While cautioning against over-facile interpretation of such data as evidence of *selective* interactions, Ding and Anderegg[87] confirmed by electrospray MS the preferential formation of oligonucleotide dimers which maximized Watson-Crick base pairing. Smith and co-workers have observed ions corresponding to oligonucleotide duplexes[88] and quadruplexes,[89] the latter only in the presence of metal cations. The formation of non-covalently bound complexes may be favoured by solution conditions which do not maximize the charge state of the electrosprayed ions, thereby increasing the required m/z range of the mass spectrometer. Smith and co-workers[88] used an extended range quadrupole but a more generally applicable approach involves the installation of electrospray ionization on a time-of-flight instrument.[19]

The study of non-covalent associations of structurally dissimilar species which are related by biological function represents a particularly fertile area for research using electrospray MS. Early examples include the detection of ions corresponding to receptor-ligand complexes[90] and a ternary complex between a dimeric enzyme and a substrate-based inhibitor.[91] In an interesting extension of these ideas, Smith and co-workers[92,93] have proposed the use of electrospray and FT-ICR for the screening of complex mixtures (such as combinatorial libraries) of ligands for binding to target

macromolecules in solution. Gas-phase ions corresponding to non-covalent ligand–target complexes that survive the electrospray process are selectively accumulated, detected and subjected to collisional activation to release the bound ligand. Subsequent trapping and collisional activation of the ligand ion yield product ions indicative of structure.

OUTLOOK

The development of electrospray has had a major impact on the mass spectrometric analyses of a broad range of analytes, notably those of biological interest. There is no doubt that the technique will continue to be of central importance to analytical mass spectrometry for some years to come. This Tutorial has briefly surveyed some aspects of the current state of the art in electrospray; a number of important areas of current and future development can be identified.

(i) The recent emphasis on the use of low flow rate electrospray is likely to be reinforced in future work, with the associated benefits of high sensitivity and efficient coupling to low-flow separation techniques.

(ii) The sensitivity and selectivity of analyte detection using electrospray MS (and tandem MS) is now such that the limiting factors in trace detection frequently relate to sample work-up and presentation to the mass spectrometer. Great scope exists for the integration of several (in some cases, all) stages of analysis from sample extraction to analyte detection. The extraordinary potential of the combination of microscale sample handling, low-flow separation techniques and high-sensitivity/high-resolution MS is elegantly illustrated by the recent demonstration of the analysis of haemoglobins in single human erythrocytes using capillary electrophoresis/electrospray Fourier transform ion cyclotron resonance.[94]

(iii) Further emphasis is needed on the study of fragmentations of multiply charged ions, bearing in mind the capability (already demonstrated) of inducing structurally informative fragmentations of macromolecular ions.

(iv) Continuing work can be expected on the detection of non-covalent complexes in the population of electrosprayed ions. Secure conclusions concerning the properties of condensed-phase analytes will, however, be predicated on the performance of well controlled studies (acknowledging, for example, the potential contributions of non-specific interactions) and on improved understanding of all stages of the electrospray process. The desirability of maximum flexibility in condensed-phase composition for these studies will ensure greater emphasis on the use of electrospray in conjunction with time-of-flight analysers (with less severe restrictions on the requisite charge state of the electrosprayed ions).

(v) The facility (provided by Paul traps and ion cyclotron resonance instruments) to trap electrosprayed ions and examine their gas-phase chemistry is likely to prove of great benefit in advanced analytical applications. Key features of this approach are the generation of internally cold ions by electrospray, the possible retention of condensed-phase properties (such as conformation and non-covalent interactions), the control of the time-scale of multi-stage (MS^n) experiments and the potential for selective ion–molecule reactions.

Acknowledgements

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