peak levels observed in the rat blood samples were at least an order of magnitude greater than those found in the brain. However, the rate of disappearance from the brain was much slower than that in blood. The results from these experiments demonstrate that changes in drug levels can be measured in the individual live animal.

Conclusion

Fast atom bombardment offers the analyst a sensitive mass spectrometric ionization technique with which to analyze polar and charged molecules up to a molecular weight of about 6000. For higher molecular weight molecules, other desorption ionization techniques such as electrospray MS or matrix-assisted laser desorption MS may be applied effectively to determine the molecular mass. Continuous-flow FAB improves the capabilities of static-FAB by allowing introduction of aqueous solutions into the mass spectrometer, significantly increasing sensitivity, and decreasing the effects of ion suppression that are common with FAB. Liquid phase separation methods such as microbore or capillary LC or CE can be effectively interfaced to a mass spectrometer using CF-FAB, thus expanding the areas of investigation to which FAB can be applied. Continuous-flow FAB also provides a means by which mass specific information can be obtained online from either *in vitro* or *in vivo* experiments in which samples are obtained by microdialysis. Fast atom bombardment has been and continues to be utilized in a wide variety of applications involving biological processes and systems by providing the biochemist with a mass-specific tool to measure molecular weights of compounds isolated from biological materials, and assess the many chemical alterations that can occur in metabolic processes.

[21] Electrospray Ionization Mass Spectrometry

By J. FRED BANKS, JR. and CRAIG M. WHITEHOUSE

Introduction

Electrospray (ES) ionization interfaced with mass spectrometry (MS) has evolved into a powerful tool for the analysis of proteins, peptides, nucleic acids, carbohydrates, glycoproteins, drug metabolites, and other biologically active species.[1-8] The success of ES in this area is largely due

[1] C. M. Whitehouse, R. N. Dreyer, M. Yamashita, and J. B. Fenn, *Anal. Chem.* **57,** 675 (1985).
[2] J. B. Fenn, M. Mann, C. K. Meng, S. F. Wong, and C. M. Whitehouse, *Science* **246,** 64 (1989).

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to its ability to extract these fragile chemical species from solution intact, ionize them, and transfer them into the gas phase, where they may be subjected to mass analysis. A unique and powerful characteristic of the ES ionization source is its additional ability to generate ions carrying many charges, whereas most ionization methods generate ions with a single charge. The majority of mass spectrometers are limited to analyzing ions with mass-to-charge ratios (m/z) of only a few thousand, but by dramatically increasing the number of charges (z) through ES ionization, compounds with masses up to several million daltons can be analyzed by MS.[9] The high sensitivity and specificity of ES/MS also make it a useful technique for the analysis of smaller compounds, such as those encountered in environmental and drug metabolite studies, which generally produce singly charged ions.[10-13]

ES/MS hardware can be configured to operate with continuous sample flow introduction, discrete sample injection as in flow-injection analysis (FIA), or on-line sample separation systems such as liquid chromatography (LC) and capillary electrophoresis (CE). Also, because ES is easily configured for these on-line sample introduction schemes, the utility of electrospray mass spectrometry can be enhanced by combining it with complementary analytical techniques. In particular, enzymes can be used to cleave a protein selectively, and the resulting peptide mixture is separated by either LC or CE, which is interfaced on-line to ES/MS. Electrospray and assisted forms of ES can produce sample ions from solution flow rates ranging from 25 nl/min to more than 1 ml/min. Hence submicroliter per minute flow rates encountered with CE separations up to 1-ml/min flow rates from

[3] R. D. Smith, J. A. Loo, R. R. Ogorzalek Loo, M. Busman, and H. R. Udseth, *Mass Spectrom. Rev.* **10,** 359 (1991).

[4] J. B. Fenn, M. Mann, C. K. Meng, S. F. Wong, and C. M. Whitehouse, *Mass Spectrom. Rev.* **9,** 37 (1990).

[5] K. L. Duffin, J. K. Welply, E. Huang, and J. D. Henion, *Anal. Chem.* **64,** 1440 (1992).

[6] T. Covey, R. F. Bonner, B. I. Shushan, and J. D. Henion, *Rapid Commun. Mass Spectrom.* **2,** 249 (1988).

[7] E. Huang and J. D. Henion, *J. Am. Soc. Mass Spectrom.* **1,** 158 (1990).

[8] D. F. Hunt, R. A. Henderson, J. Shabanowitz, K. Sakaguchi, M. Hanspeter, N. Sevilir, A. L. Cox, E. Appella, and V. H. Englehard, *Science* **255,** 1261 (1992).

[9] T. Nohmi and J. B. Fenn, *J. Am. Chem. Soc.* **14,** 3241 (1992).

[10] H.-Y. Lin and R. D. Voyksner, *Anal. Chem.* **65,** 451 (1993).

[11] D. Gartiez, "Quantitative and Structural Analysis of Singly Charged Ions from Small Molecules." Presented at the ASMS Fall Workshop on Electrospray Ionization, Boston, MA, 1992.

[12] C. Dass, J. J. Kusmierz, D. M. Desiderio, S. A. Jarvis, and B. N. Green, *J. Am. Soc. Mass Spectrom.* **2,** 149 (1991).

[13] R. D. Voyksner, *Nature (London)* **356,** 86 (1992).

conventional 4.6-mm LC columns can be accommodated on-line with an electrospray ion source interfaced to a mass spectrometer.

In addition to producing intact parent molecular weight information, ES/MS may selectively provide fragment or daughter ion information from a collision-induced dissociation (CID) process, which can occur either in the ES source itself[14–16] or with the use of multiple mass spectrometer stages (MS/MS).[17,18] When in-source CID occurs and more than one parent species is present, it may be difficult to assign the CID fragment ions to individual parent species. For this reason, the CID capability of the ES source is most effectively employed when a single ion species is present. This requirement can be accomplished with complex sample mixtures if an efficient separation technique such as LC or CE is used in-line prior to mass analysis. If mixtures of ions do simultaneously enter the source, then triple quadrupole mass spectrometers can be used in the MS/MS mode, where single components can be m/z selected and exposed to CID conditions within the mass spectrometer itself. Thus, many compound mixture analysis applications requiring triple quadrupole MS/MS capability with CID can be conducted instead using the ES CID capability with a single quadrupole MS in conjunction with a separation technique that fully resolves and separates individual chemical species before they enter the ES source.

Fundamentals

An electrospray ionization source operating at atmospheric pressure was first interfaced to a mass spectrometer in Fenn's laboratory[1] at Yale University (New Haven, CT) with inspiration taken from earlier ion-stopping potential studies using electrospray ionization conducted by Mack *et al.*[19] The term *electrospray* has evolved to describe collectively a basic set of processes encompassing (1) the formation of electrically charged micron-sized liquid droplets created from a flowing liquid sample, (2) the extraction of gas phase ions from these same droplets under a high electric field, and (3) the subsequent transport of these ions into a vacuum suitable for mass spectrometric analysis.

[14] R. D. Voyksner and T. Pack, *Rapid Commun. Mass Spectrom.* **5,** 263 (1991).
[15] J. F. Banks, S. Shen, C. M. Whitehouse, and J. B. Fenn, *Anal. Chem.* **66,** 406 (1994).
[16] A. M. Starrett and G. C. Didonato, *Rapid Commun. Mass Spectrom.* **7,** 7 (1993).
[17] J. A. Loo, C. G. Edmonds, and R. D. Smith, *Anal. Chem.* **65,** 425 (1993).
[18] S. A. McLuckey, G. J. Van Berkel, and G. L. Glish, *J. Am. Soc. Mass Spectrom.* **3,** 60 (1991).
[19] L. L. Mack, P. Kralic, A. Rheude, and M. Dole, *J. Chem. Phys.* **52,** 4977 (1970).

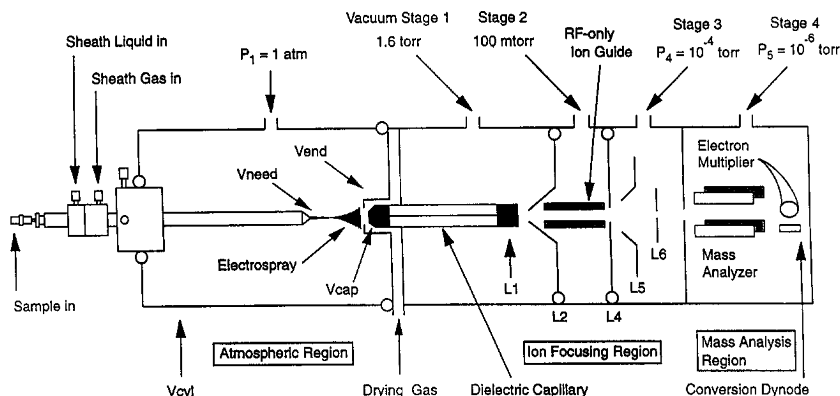


FIG. 1. ES/MS system with quadrupole mass analyzer.

Figure 1 is a schematic of an electrospray ion source as interfaced to a quadrupole mass spectrometer. Although the ES source has been successfully interfaced to magnetic sector.[20–25] Fourier transform,[26–29] ion trap,[10,30] and time-of-flight[31] mass spectrometers, the quadrupole is currently the most common mass spectrometer used with ES and is thus used for illustrative purposes in this chapter. Referring to Fig. 1, sample liquid is introduced through a tube and exits at a conductive needle tip (Vneed) which is maintained typically at 2 to 7 kV relative to the surrounding electrodes: the cylinder (Vcyl), end plate (Vend), and capillary entrance (Vcap). For the ES source illustrated, the liquid introduction needle is operated at ground potential. In conventional or unassisted electrospray, the electro-

[20] C. K. Meng, C. N. McEwen, and B. S. Larsen, *Rapid Commun. Mass Spectrom.* **4,** 151 (1990).
[21] C. Meng, C. N. McEwen, and B. S. Larsen, *Rapid Commun. Mass Spectrom.* **4,** 147 (1990).
[22] R. T. Gallagher, J. R. Chapman, and M. Mann, *Rapid Commun. Mass Spectrom.* **4,** 369 (1990).
[23] B. S. Larsen and C. N. McEwen, *J. Am. Soc. Mass Spectrom.* **2,** 205 (1991).
[24] Y. Wada, J. Tamura, B. D. Musselman, D. B. Kassel, T. Sakari, and T. Matsuo, *Rapid Commun. Mass Spectrom.* **6,** 9 (1992).
[25] R. B. Cody, J. Tamura, and B. D. Musselman, *Anal. Chem.* **64,** 1561 (1992).
[26] S. C. Beu, M. W. Senko, J. P. Quinn, F. M. Wampler III, and F. W. McLafferty, *J. Am. Soc. Mass Spectrom.* **4,** 557 (1993).
[27] K. D. Henry, E. R. Williams, B. H. Wang, F. W. McLafferty, J. Shabanowitz, and D. F. Hunt, *Proc. Natl. Acad. Sci. U.S.A.* **86,** 9075 (1989).
[28] K. D. Henry, J. P. Quinn, and F. W. McLafferty, *J. Am. Chem. Soc.* **113,** 5447 (1991).
[29] J. A. Loo, J. P. Quinn, S. I. Ryu, K. D. Henry, M. W. Senko, and F. W. McLafferty, *Proc. Natl. Acad. Sci. U.S.A.* **89,** 286 (1992).
[30] G. J. Van Berkel, G. L. Glish, and S. A. McLucky, *Anal. Chem.* **62,** 1284 (1990).
[31] J. G. Boyle and C. M. Whitehouse, *Anal. Chem.* **64,** 2084 (1992).

static field is maintained at a sufficiently high level by setting the appropriate voltages on Vcyl, Vcap, and Vend to draw out a Taylor cone from which a thin liquid filament extends. The filament then breaks up into charged droplets primarily due to mechanical Rayleigh fluid instability propagation. The electrosprayed droplets shrink in size until the Rayleigh limit is reached, at which point smaller droplet emission occurs from the primary droplet. Eventually, through this stepwise process of evaporation and droplet breakup, the diameter of the droplet is reduced to the point at which ion emission spontaneously occurs from the droplet surface. The ions produced are then driven by the electrostatic field toward Vend and Vcap, through an orifice, and thence into a vacuum. This movement opposes the drying gas flow of heated nitrogen, which helps to evaporate the droplets. The dielectric capillary in Fig. 1 serves as the orifice into vacuum and has conductive electrodes coated on each end. Positive charged droplets leaving the ground potential liquid introduction needle are driven by the electrostatic field to the Vend and Vcap counterelectrodes, which are set typically at −2 to −7 kV. The ions produced from the charged droplets are swept into the capillary orifice and pulled against the electrostatic field between the capillary entrance and exit electrodes by the gas flowing into vacuum. The ions then enter vacuum at the capillary exit potential, approximately 50–400 V for positive ions with quadrupole mass spectrometers. Because the glass capillary acts as an electrical insulator, the negative kilovolt potentials in the ion production region at atmospheric pressure can be optimized independently of the more modest potentials in the ion-focusing region under vacuum. For conductive metal capillary and nozzle orifices, the orifice potential (exit and entrance) is again typically 80–400 V for positive ions, so that the liquid introduction needle must then be operated at positive kilovolt potentials.

The ions exiting the capillary tube in Fig. 1 are transported through a free jet expansion into vacuum, where a series of dynamic and electrostatic lenses focuses the ions into the mass spectrometer for mass analysis. As illustrated in Fig. 1, highly efficient multipole ion guides operated in the radio frequency (RF)-only mode are often incorporated into the vacuum lens system. The neutral background gas, usually nitrogen, is removed through one or more vacuum-pumping stages between the orifice exit and the mass analyzer chamber. A total of four vacuum stages is shown in the ES/MS quadrupole system in Fig. 1.

The mechanism by which ions are produced from charged liquid droplets is not well understood; however, two models exist that help to describe the ion emission process. In one model initially proposed by Mack et al.,[19] the charged droplets continue their breakup and evaporation until only one molecule is left in each droplet. The charge left behind as the solvent

evaporates is then attached to the sample molecule, forming an ion. Work by Fenn[32] has indicated that this ion production mechanism may apply only to larger molecules having molecular weights over 500,000 Da, and that a second model, commonly referred to as the *ion evaporation model* and proposed by Iribarne and Thomson,[33] more accurately describes the mechanism of ion emission from charged droplets for molecules having a molecular weight below 500,000. This second model postulates that through a series of droplet breakups and evaporation, the shrinking radius of the evaporating charged droplet reaches a size and surface charge density at which the electrostatic field at the droplet surface is sufficiently strong to overcome the energy of solvation of an ion on the surface, causing it to "lift-off" into the gas phase.

Variations in the ES source design have emerged, in which electrospray chamber source components are heated and heated metal capillary tubes or nozzles are used as the vacuum orifices.[34,35] When a heated capillary or nozzle is incorporated into an ES source, the charged droplets produced in the atmospheric pressure region may only partially evaporate in the atmospheric pressure chamber and continue to evaporate as they are transported into vacuum. With this drying method, solvent from the evaporating charged droplets is swept into the vacuum along with solute ions and nitrogen carrier gas so that recondensation of the solvent on the ions must be prevented in the rapidly cooling free jet expansion. This solvent recondensation can be avoided by supplying sufficient heating to the expanding gas and setting the voltage differentials on the electrostatic lenses high enough so that ions are accelerated through the background gas in vacuum, causing the CID breakup of any remaining solvent clusters. Also, the nozzle or capillary exit in these heated systems is usually set off centerline such that the unevaporated droplets of high mass-to-charge ratio are deflected and not accelerated line-of-sight into the mass spectrometer, an event that would cause considerable signal noise to be observed. Alternatively, ES sources have been configured so that the line-of-sight trajectory between the ES needle and the orifice into vacuum is blocked by a heated element. In this case, charged droplets are evaporated as they flow around this heated element aided by concurrent gas flow directed toward the orifice into vacuum. Whatever the method employed in a particular electrospray ion source, efficient drying of charged droplets is essential to the production of a stable ion signal.

[32] J. B. Fenn, *J. Am. Soc. Mass Spectrom.* **4,** 524 (1993).
[33] J. V. Iribarne and B. A. Thomson, *J. Chem. Phys.* **64,** 2287 (1976).
[34] S. K. Chowdhury, V. Katta, and B. T. Chait, *Rapid Commun. Mass Spectrom.* **4,** 81 (1990).
[35] M. H. Allan and M. L. Vestal, *J. Am. Soc. Mass Spectrom.* **3,** 18 (1992).

Operating Range

The stability of the mass spectrometer ion signal with ES ionization is a direct reflection of the stability of the charged droplet formation and the efficiency and consistency of the droplet-drying processes. At atmospheric pressure, unassisted electrospray ionization has a window of stable operation that to a large extent is a function of the range of operating conditions within which uniform charged droplet formation can occur. For consistent unassisted ES, stable charged droplet formation and a stable Taylor cone, filament, and droplet breakoff from the filament must be maintained. Liquid flow rate, solution conductivity, solution surface tension, and operation in the negative ion mode can affect the stability of the charged droplet formation process in unassisted electrospray.

Liquid Flow Rate

If the liquid flow rate fluctuates rapidly, the Taylor cone may become unstable, resulting in an erratic ion signal. When a stable Taylor cone is formed, the diameter of the protruding filament is essentially proportional to the two-thirds power of the liquid flow rate,[36] and the size of the charged droplets produced is proportional to the filament diameter.[37] The net result is a proportional increase in droplet size with liquid flow rate. The production of larger diameter charged droplets has three negative effects on ion evaporation efficiency and hence on overall electrospray performance. First, larger droplets are more difficult to dry in the brief time between droplet formation and entrance into the vacuum system. Second, as the droplets increase in size, the net charge-to-mass ratio of the droplets decreases. Consequently, there is potentially less charge available per solvent molecule. Both effects can cause a fluctuating or reduced ion signal. Third, as the liquid flow rate increases, the filament diameter increases to a point at which the Taylor cone becomes unstable for a given liquid introduction tube tip diameter. If the Taylor cone becomes unstable, then the charge droplet formation is nonuniform, resulting in an unstable ion signal. For an ideal case in which a solution of 100% methanol is electrosprayed at a flow rate of 3 μl/min, the primary charged droplets formed have a mean diameter of 2.9 μm. The implications here for the use of ES/MS as an LC on-line detector are obvious. Because conventional ES has optimum performance at only a few microliters per minute, either some type of

[36] R. C. Willoughby and E. W. Sheenan, "Ion Production in Electrospray: A Thermodynamic Approach." Presented at the 11th Montreux Symposium, Montreux, Switzerland, 1991.
[37] K. Tang and A. Gomez, *Phys. Fluids* **6,** 2317 (1994).

flow splitting or additional technology is required to employ ES/MS as an LC detector.

Solution Conductivity

Direct observation of the electrospray process when spraying solutions with conductivities over 800 micromhos at a few microliters per minute has shown that the filament originating at the Taylor cone can extend for several tens of centimeters without breaking into droplets. The more highly conductive solution counteracts the destabilizing effect of surface tension in the liquid filament. This may be explained as a competition of surface tension and charge repulsion forces. As surface perturbations form along the liquid filament as a result of the surface tension, the diameter of the filament contracts, reducing the radius of curvature of the filament and creating a local region of higher electrostatic field. The net charge rapidly accumulating in the higher conductivity filament region damps out the wave propagation, preventing the surface tension forces from dominating and pinching off a droplet. The result is that charged droplets do not break from the liquid filament extending from an electrospray Taylor cone, as has been observed. If charged droplets are not formed, then no signal can be observed because the ion emission process has been prevented. Higher conductivity solutions can be electrosprayed using lower liquid flow rates in the submicroliter per minute range.[38] Lower liquid flow rates result in smaller filament diameters from which charged droplets can break off with solutions of higher conductivity. The higher the solution conductivity, the lower the liquid flow rate required to achieve stable electrospray. Again, there are serious implications here for the use of ES/MS as an LC or CE detector. The addition of salts, acids, and bases to LC mobile phases and CE buffers is a common and necessary practice. In general, the applied concentrations of these modifiers will increase the conductivity of the solution to a point at which stable unassisted electrospray at higher flow rates is not possible.

Higher Surface Tension Solutions

The higher surface tensions of aqueous solutions can pose a problem for maintaining a stable electrospray ion signal as well. The Taylor cone must pull out sufficiently for a liquid filament to form from its tip. The force applied by the electrostatic potential must overcome the counteracting force applied by the liquid surface tension, which is tending to hold the

[38] J. F. d. l. Mora and I. G. Loscertales, *J. Fluid Mech.* **260,** 155 (1994).

surface of the emerging liquid in a spherical shape. Unfortunately, there is a limit to the electrostatic potential that can be applied in the ES source operated at atmospheric pressure. For unassisted ES operation, if the voltage applied between the needle tip and counterelectrodes exceeds the work function of the electrodes, spontaneous electron emission, leading to a cascading gas phase breakdown and hence a sustained corona discharge, can occur. This results in a loss of or at best an unstable mass spectrometer ion signal. The onset of corona discharge loosely represents a ceiling to the relative voltages that can be applied between electrodes in an ES source operating at atmospheric pressure. Consequently, the applied electrostatic forces have an upper limit. The limited electrostatic force does not compete effectively against the higher surface tension of highly aqueous solutions and results in no Taylor cone or an unstable Taylor cone formation, which in turn results in an unstable charged droplet formation process. Adding an organic solvent to an aqueous solution effectively lowers the surface tension and can allow normal ES operation, but may not be compatible with the solution requirements for optimal LC or CE on-line separation systems. Stable unassisted electrospray can be achieved sometimes with aqueous solutions if low liquid flow rates are used in the submicroliter per minute range in combination with very sharp electrospray needle tips and electron-scavenging sheath gases.

Negative Ion Operating Mode

The sharp liquid introduction needle has a negative potential relative to the surrounding counterelectrodes when the ES source is operated in the negative ionization mode. In this configuration, the onset of corona occurs at a lower potential than when the needle has a relative positive polarity. As the voltage is increased in the negative ion mode, the work function of the metal needle tip is reached and the onset of corona discharge occurs before the field has applied enough force to counteract the liquid surface tension and form a stable Taylor cone. The onset of corona discharge occurring at the needle tip effectively lowers the local field, creating an unstable environment within which the Taylor cone forms. The addition of oxygen at the needle tip or other electron scavenger gas can suppress the onset of corona, thus allowing Taylor cone formation. Also, the use of lower surface tension solvents such as propanol decreases the voltage required for the formation of a stable Taylor cone and hence the onset of electrospray. Once the charged droplets are formed, the ion evaporation mechanisms for positive and negative ionization appear to be quite similar.

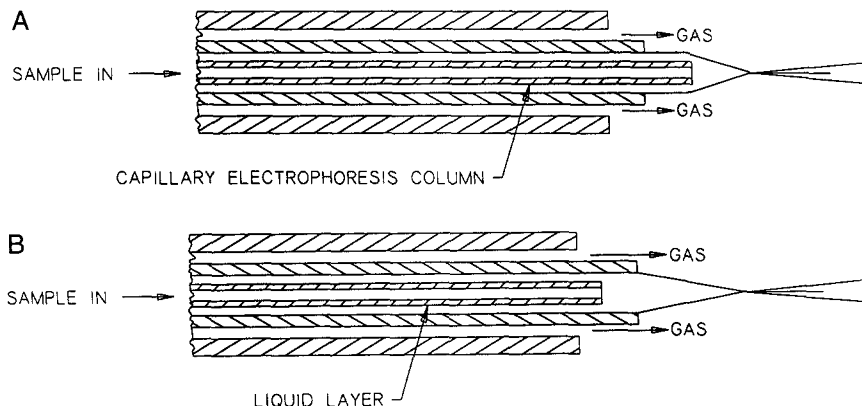


FIG. 2. Electrospray needle tip with sheath liquid flow geometry for (A) CE and (B) LC applications.

Techniques to Extend Range of Electrospray Operation

Sheath Liquid Flow

To extend the range of stable operation, techniques have been applied to electrospray to aid or assist in the charged droplet formation process. For lower flow rate operation, usually below 20 μl/min, the use of sheath liquid as well as sheath gas flows at the needle tip has proved useful in extending the range of stable unassisted electrospray operation. Figure 2 shows a typical geometry used for layering flows at the needle tip for (a) CE/ES/MS and (b) LC/ES/MS applications. The sample introduction tube can be metal or fused silica. A sheath liquid solution is introduced through the second layer tube as illustrated in Fig. 2 and gas can be introduced at the needle tip through an annulus between the second and the third layer tubes.

In this technique, the sheath solvent introduced through a surrounding tube is layered over and mixed with the sample-bearing solution emerging from the innermost tube tip. The sheath solvent can be chosen to reduce the surface tension or conductivity of the sample-bearing solution, thus making ES operation more compatible with solutions used in gradient LC or CE separations. This arrangement has been used for coupling CE to electrospray when the liquid introduction tube as illustrated in Fig. 2A is replaced by a CE column.[39,40] The flow rates in CE applications are usually

[39] R. D. Smith, C. J. Barinaga, and H. R. Udseth, *Anal. Chem.* **60,** 1948 (1988).
[40] R. D. Smith, J. A. Loo, C. G. Edmonds, C. J. Barinaga, and H. R. Udseth, *Anal. Chem.* **62,** 882 (1990).

well below 1 μl/min, and the buffers used are often aqueous and highly conductive. The addition of a solvent such as methanol or propanol through the second layer tube in this application can serve to increase the total flow rate, decrease the surface tension and conductivity, and make an electrical connection with the fluid exiting the fused silica CE column without adding dead volume. This sheath flow also tends to decouple the charged droplet formation process from the electroosmotic flow process occurring in the capillary electrophoresis column. When the sheath liquid flow technique is used with LC or CE separation systems, correct positioning of the first and second layer tip locations is important to ensure stable performance. Generally, when the first layer tube is a fused silica column, the tip is extended just beyond the second layer tube for optimal performance. When the first layer tube is metal, as may be the case with LC interfacing, the first layer tube is retracted slightly inside the second layer tube for optimal performance. The addition of layered flow to reduce the solution surface tension and conductivity can extend the solution chemistry range of operation of electrospray but may not help to extend the liquid flow rate operating range. The addition of oxygen or other electron scavenger can still be added through the third layer tube during negative ionization mode. There remains much to learn about the use of sheath liquid flow to modify solution chemistry with the goal of achieving higher ion evaporation efficiencies from charged liquid droplets.

Assisted Electrospray

From the realization that ion emission from charged droplets could proceed normally if the charged droplet formation and evaporation could be controlled, two electrospray-assisted techniques, pneumatic nebulization or IonSpray and ultrasonic nebulization or ULTRASPRAY (Analytica of Branford, Inc., Branford, CT), were developed. Both techniques greatly expand the range of solution chemistry and liquid flow rate conditions over which stable electrospray operation can be achieved by mechanically creating the fine charged droplets required for ES and thus eliminating the dependence on Taylor cone formation. In pneumatic nebulization, the addition of a gas flowing concentrically around the liquid introduction needle tip is used to shear off or pneumatically nebulize the liquid as it emerges from the needle tip in the presence of an electrostatic field (Fig. 3A). This technique first reported by Mack *et al.*[19] and later by Bruins *et al.*,[41,42] became known as *pneumatic nebulization-assisted electrospray* and

[41] A. P. Bruins, L. O. G. Weidolf, J. D. Henion, and W. L. Budde, *Anal. Chem.* **59,** 2647 (1987).
[42] A. P. Bruins, T. R. Covey, and J. D. Henion, *Anal. Chem.* **59,** 2642 (1987).

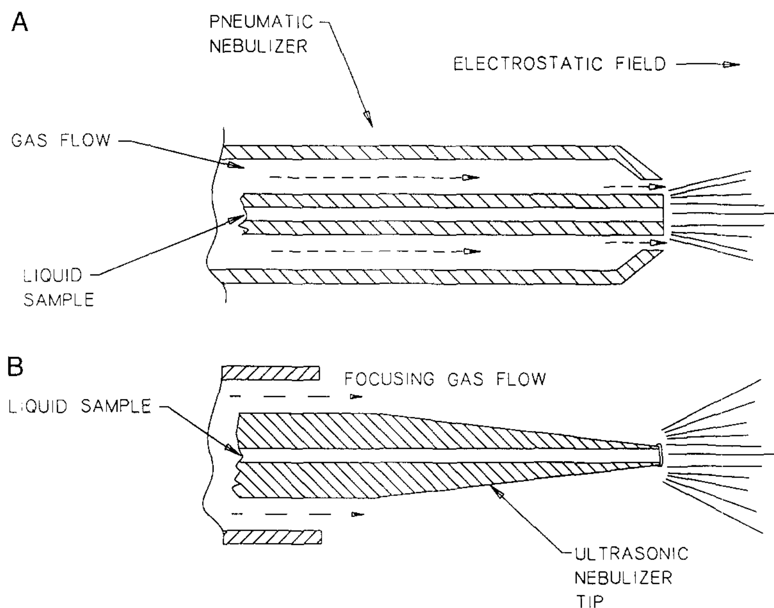


FIG. 3. Assisted electrospray needle tips configured for (A) pneumatic and (B) ultrasonic nebulization.

also as *IonSpray* (Sciex, Thornhill, Ontario, Canada). Pneumatic nebulization tends to create a broader droplet size distribution when compared with unassisted electrospray, and the droplet distribution will change with solution chemistry and liquid flow rate.[43]

The ULTRASPRAY, or ultrasonically assisted electrospray, technique has incorporated a high-frequency ultrasonic nebulizer at the electrospray needle tip to assist in the droplet formation process in the presence of an electrostatic field, as shown in Fig. 3B. This technique creates a narrow range of droplet sizes independent of solution chemistry or liquid flow rates.[44] Both ultrasonic and pneumatic nebulization techniques have been used to extend the liquid flow rate range to over 1000 μl/min. Changes in solution conductivity and surface tension have little effect on the signal stability with assisted electrospray. Both ES-assisted techniques create liquid droplets by mechanical means; however, the addition of a strong electrostatic field at the liquid introduction needle tip is still required to achieve

[43] M. A. Tarr, K. L. Goodner, B. A. Williams, G. Zhu, and R. Browner, "LC-MS Interfacing: Fundamental Studies of Aerosol Formation and Transport." Presented at the Pittsburgh Conference, New Orleans, LA, 1992.

[44] J. F. Banks, J. P. Quinn, and C. M. Whitehouse, *Anal. Chem.* **66,** 3688 (1994).

efficient charging of the droplets created. Negative ionization can be used with assisted ES techniques without the need of an electron-scavenging gas such as oxygen. Sheath flow can also be used with either nebulization-assisted electrospray technique.

Factors Affecting Sensitivity

Electrospray Ionization–Mass Spectrometry as a Concentration-Dependent Detector

The behavior of charged droplets and emitted ions during the droplet evaporation step, coupled with transport into vacuum, cause the ES/MS system to respond as a sample concentration-dependent detector. This dependence is analogous to an ultraviolet (UV) detector in LC applications, where the signal response is a function of how much absorbing sample per unit volume of liquid is present. The mass spectrometer ion signal obtained from an ES source for a given compound in solution is, to a first approximation, dependent on the concentration of the sample in a unit volume of solution and is relatively independent of liquid flow rate.

The concentration dependence in ES seems to be the result of droplet repulsion due to space charging in the ES chamber. Work by Gomez[45] has shown that the highly charged droplets produced in electrospray move rapidly away from each other owing to space charge repulsion as they evaporate and drift from the liquid introduction needle toward the counter-electrode or end plate through the atmospheric pressure gas. Because of this space charge repulsion at the droplet level, a limited number of charged droplets can occupy the gas volume in the region of the office into vacuum. The number of ions that are entrained in the gas entering the orifice into vacuum is a function of how much sample is present in each evaporating charged droplet. It is important to note that the space charge limit appears not to occur at the ion level but is established rather at the charged droplet level before the droplets enter the region of ion evaporation.

As an example of the response of an ES/MS system to the concentration of an analyte, Fig. 4 shows the relative ion signal from cytochrome *c* [1 pmol/μl in 1 : 1 (v/v) methanol–water, 0.1% acetic acid] vs concentration. For this experiment, an HP5989A (Hewlett-Packard, Palo Alto, CA) mass analyzer equipped with an Analytica of Branford ES ion source (Analytica of Branford, Branford, CT) was scanned from 600 to 1300 m/z at the rate of 1 sec/scan. The data show that the instrument response is linear over four to five orders of magnitude until the concentration reaches approxi-

[45] A. Gomez, Presented at the LC/MS Workshop of the ASMS, Washington, DC. 1992.

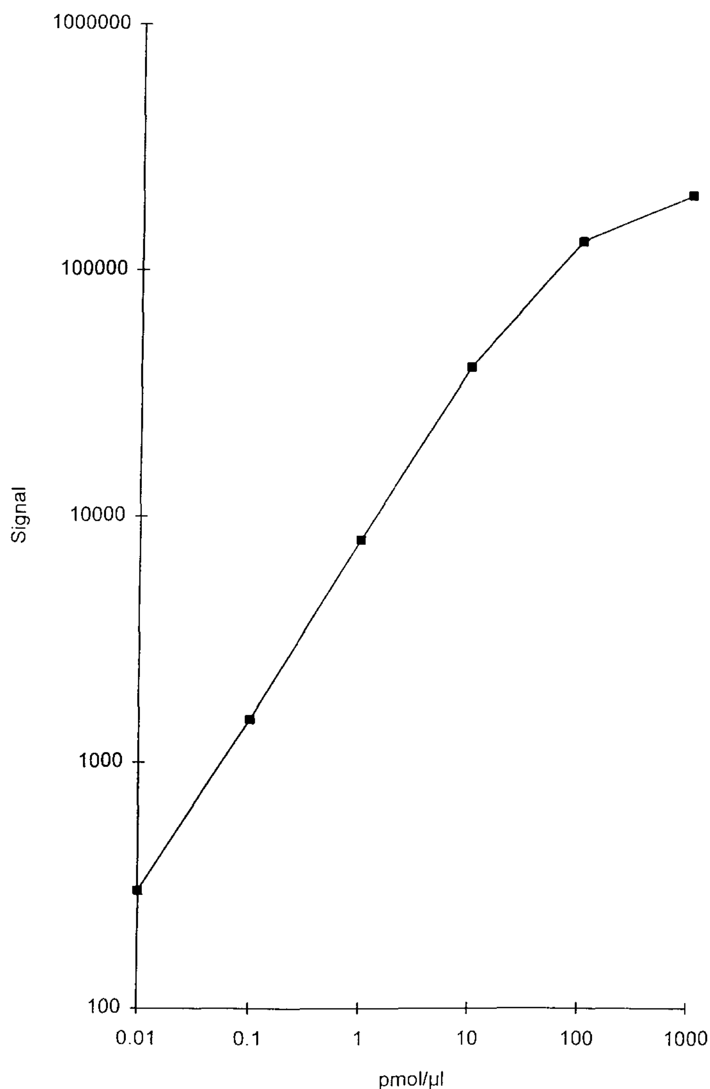


FIG. 4. Cytochrome *c* ion signal vs concentration. Amplitude values are the sum of all multiply charged peak maximums.

mately 100 pmol/μl, at which point a characteristic signal plateau begins. Details relating to this phenomena are discussed below.

A significant consequence of this concentration-dependent behavior is that for a given sample concentration, increasing the solution flow rate

increases the amount of sample per time introduced into the ES source but may not result in higher ion currents and hence signal to noise detected by the mass analyzer. In sample-limited applications, lower flow rate separation systems such as microbore LC, capillary LC, or CE will result in higher overall system sensitivity. Running the ES source at higher flow rates with flow injection or higher flow LC applications may simplify the LC coupling and operation but does not significantly increase sensitivity. In reality, for flow rates above a few microliters per minute, the ES source is effectively splitting the sample during the spraying process, sending only a small portion of the total ions produced to the mass spectrometer. Comparisons of on-line LC/ES/MS sensitivity as a function of LC column size used in the LC separation step have been reported.[46] This also suggests that a great portion of LC effluent may be split off for either fraction collection or detection by some other means without loss of ion signal.

Finally, for samples in which the analyte is very low in concentration, preconcentration of the sample on the head of an LC column using gradients or at the beginning of a CE column initially running in the isotachophoretic mode can significantly enhance ES/MS signal to noise.[47]

Sample Handling

An important point to note is that limits in sensitivity when using ES/MS are often imposed by sample-handling techniques. A sample concentration in the low femtogram per microliter range with only a few picograms of total sample present can often be detected by an ES/MS system if the sample is successfully delivered by the liquid flow system to the electrospray needle tip. Such a small quantity of sample can easily be absorbed onto the transfer tube walls or be diluted in dead volume areas of the injector valve or couplings on its way to the electrospray needle tip. Care should be taken in choosing materials that the sample solution will encounter to minimize adsorption effects. Polyetheretherketone (PEEK) tubing is often chosen over stainless steel when analyzing proteins. Capillary electrophoresis columns are in effect specialized transfer lines for which specific coatings can be chosen to minimize sample adsorption to the walls. When mixing, storing, or diluting samples for analysis, losses can occur to the sample container walls. Container materials such as glass can introduce a number of unwanted contaminants into solution, including sodium and potassium. A good grade of polypropylene is preferred, such as those found in Eppendorf-brand microcentrifuge tubes. Samples once in solution should be analyzed

[46] T. Covey, "Practicle Principles and Concepts for Analytical Applications of Ion Evaporation Mass Spectrometry" Presented at the ASMS Fall Workshop on Electrospray Ionization, Boston, MA, 1992.

[47] T. J. Thompson, F. Foret, P. Vouros, and B. L. Karger, *Anal. Chem.* **65**, 900 (1993).

as soon as possible to minimize adsorption losses or decomposition in solution.

Solution Chemistry Effects on Ion Emission from Charged Droplets

The sensitivity achievable for a specific analysis may depend heavily on how the solution chemistry chosen affects the efficiency of ion evaporation. The complexity of the ion emission process from charged liquid droplets poses a challenge to the operator to attain optimal performance for a given application but also provides the greatest opportunity for exploring many chemical processes that are difficult to probe using other techniques. A brief attempt is made here to list the solution variables that affect ion emission from droplets and consequently system performance. Positive ions in electrospray are formed by the attachment to the sample molecule of one or more positive cations. H^+ is a common cation for proteins and peptides. Carbohydrates will not show very sensitive response when the charge carrier is hydrogen but will preferentially accept sodium, ammonium, or another cation species as a charge carrier or adduct ion. For negative ions, the charge is usually formed by the removal of a cation, leaving a net negative charge behind. Presumably the most important region on the droplet for these charge transfer processes to take place is at the droplet surface. Hence the species donating or accepting a cation from the sample molecule may play an important role in the charge transfer process. The following is a list of some but certainly not all properties that may influence this charge transfer process: (1) sample concentration, (2) sample pK_a or pK_b, (3) solution conductivity, or a less general but related property, solution pH in such cases when H^+ is the relevant cation, (4) presence of other chemical species that may compete for charge or droplet surface space, (5) sample diffusion rate to the droplet surface, (6) sample molecule shape and size, (7) solution dielectric constant, and (8) droplet charge density. Some of these variables, such as solution pH, conductivity, or sample solubility, can be measured directly in the bulk solution although these values may not hold at the point of ion emission for the evaporated liquid droplet. When these variables are modified, the effect on a given signal level can be quantified.

The body of research work examining these effects has been steadily growing. For example, by examining a series of nucleosides with differing pK_a values it is found that basic analytes with higher pK_a value gave higher H^+ adduct signals in neutral solutions (pH 7.0).[15] This result is as expected after considering a rearrangement of the Henderson–Hasselbalch equation:

$$pK_a = pH + \log \frac{[BH^+]}{[B]} \tag{1}$$

where [B] is the concentration of the free base and [BH^+] is the concentration of the protonated base, respectively. From the relation above, a higher pK_a mandates that a higher concentration of the species will be present in the protonated form, or BH^+. Because it is in fact a protonated species that desorbs from the liquid droplet in ES/MS, it is reasonable that compounds with higher pK_a values and thus higher concentrations of BH^+ present in solution will give higher ion signals.

The related effect of solution pH due to the presence of dilute acids and bases has also been shown to have substantial effects on the observed ES/MS spectra of proteins and peptides. For example, Fig. 5 shows the ES/MS spectra of cytochrome *c* taken both without (Fig. 5A) and with (Fig. 5B) 0.1% acetic acid present in the sample solution [10 pmol/μl protein in 1 : 1 (v/v) methanol–water].[44] Clearly, the signal and number of charges present on each ion are much higher when the acid is present. A similar effect can be seen in the negative ion spectra of insulin, shown in Fig. 6 both without (Fig. 6A) and with (Fig. 6B) 0.01% (v/v) NH_4OH present in the sample solution.[48] Again, the signal is greatly improved by the presence of this dilute base. By the same token, the excessive amounts of acid or base can greatly reduce the ion signal observed. To illustrate this point, Fig. 7 shows the relative effect of [NH_4OH] on the 4-ion signal of insulin. While signal is greatly improved at the level of 0.01% NH_4OH, it is reduced nearly 20-fold at a composition of 1% NH_4OH.

Finally, the use of trifluoroacetic acid (TFA) in LC mobile phases calls attention to the interesting effects that solution chemistry can have on the observed ES/MS signal of proteins and peptides. Trifluoroacetic acid inhibits ion signal in ES in two ways. First, TFA is very conductive and very acidic, much more so than acetic acid, for example, and assisted ES must be used when TFA is present even in small amounts. Second, TFA associates with protein and peptide molecules to form ion pairs. This association substantially shifts the acid–base equilibria of proteins and peptides in solution so that attracting H^+ becomes unfavorable. In studying this problem, researchers[49] have found that the addition of a solution (75% propionic acid in 2-propanol) postcolumn at one-half of the sample flow rate of the TFA-containing solution prior to introduction into the ES source can greatly improve the ion signal by shifting the acid–base equilibria involved in the ion-pair formation.

[48] J. F. Banks, J. B. Fenn, and C. M. Whitehouse, "Solution Chemistry Effects in Electrospray Mass Spectrometry." Presented at the Pittsburg Conference, Chicago, IL, 1994.

[49] A. Apffel, S. Fischer, P. C. Goodley, and J. A. Sahakian, "Enhanced Sensitivity for Peptide Mapping with Electrospray LC/MS." Presented at the ASMS Conference, Chicago, IL, 1994.

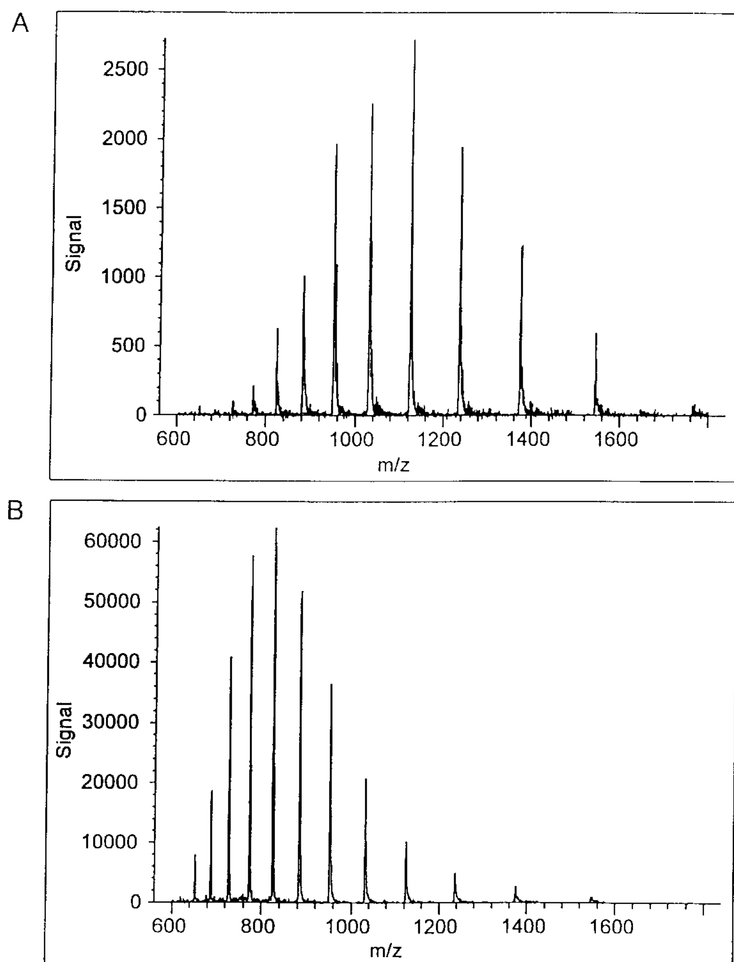


FIG. 5. ES/MS spectra of cytochrome *c*, 10 pmol/μl in 1 : 1 (v/v) methanol–water with (A) no acid present and (B) 0.1% acetic acid present.

The effect of sample concentration on ion signal has also been studied extensively. As an example, refer again to Fig. 4, in which a signal versus concentration curve for cytochrome *c* in a solution (1:1 methanol and water with 0.1% acetic acid) is shown. The signal response is linear with concentration over four to five orders of magnitude but tapers off at the higher concentrations. Solubility may be a primary factor why the signal-

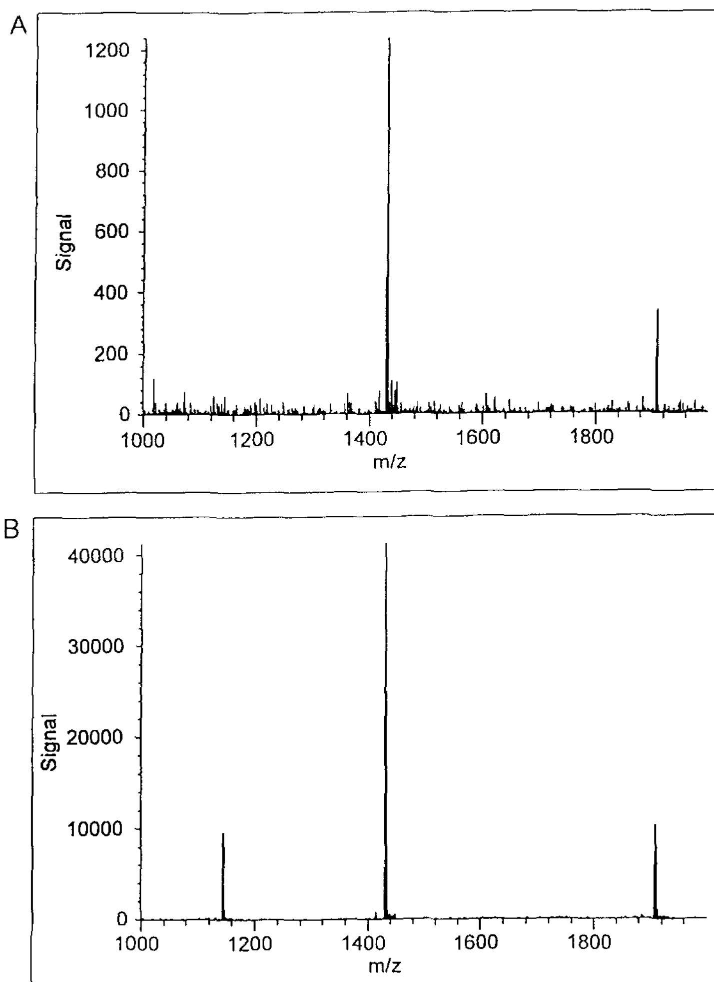


FIG. 6. ES/MS spectra of insulin, 10 pmol/μl in 1 : 1 (v/v) methanol–water, in the negative ion mode, with (A) no base present and (B) 0.01% NH_4OH present.

versus-concentration response shows a negative deviation from linearity at a higher concentration level. The signal plateau beginning at approximately 100 pmol/μl at first glance would not seem to pose a solubility limit. At the point of ion evaporation from the liquid droplet, however, much of the solvent has been evaporated and the nonvolatile cytochrome *c* sample is considerably more concentrated than it was in the original solution. As the

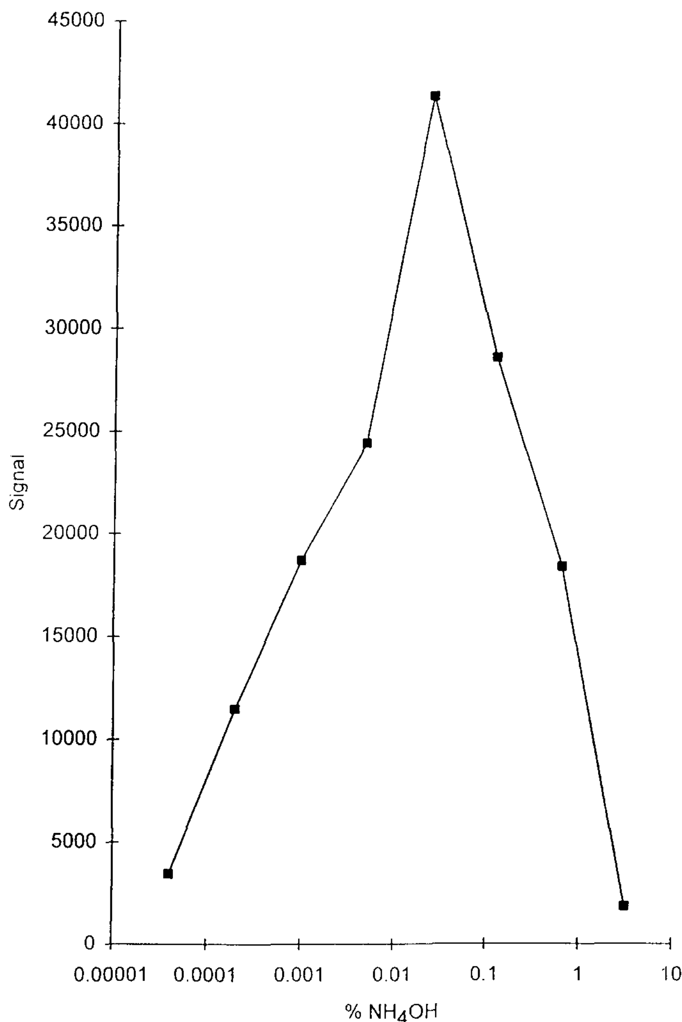


FIG. 7. Insulin −4 ion peak signal vs NH_4OH concentration in 1:1 (v/v) methanol–water.

initial sample concentration is increased beyond the signal plateau, sample clustering through precipitation is often observed in the spectra. These cluster ions appear as dimers, trimers, etc., of the sample species. Compounds with lower solubility are understandably more subject to this effect, and thus the choice of ES solvent can be important for this reason as well. Intriguing studies of system response to solution chemistry variables have

been used to study noncovalently bound complexes, including these molecular clusters and even the tertiary structure of large molecules.[15,50–53]

Mass Assignment for Electrospray-Generated Spectra

Deconvolution of Multiply Charged Ions

Electrospray produces multiply charged ions of higher molecular mass compounds that fall typically in an *m/z* window below 4000. As mentioned in the previous section, in the positive ionization mode a cation is attached to a molecule for every charge added, while in the negative ionization mode a cation is removed for every charge added. Hence for any ion appearing in the mass spectrum, the mass of the molecule can be determined from Eq. (2):

$$K_i = (M/i) + m \tag{2}$$

where K_i is the measured *m/z* value, M is the compound molecular weight, i is the charge state, and m is the mass of the charge carrier assuming the electrosprayed ion has only one species of charge carrier. Consequently, a peak appearing in a mass spectrum generated using electrospray ionization has three unknowns: the charge state (i), the charge carrier mass (m), and the compound molecular weight (M). The charge state variable can take on only integer values. There are limited possibilities for the adduct ion identity, for example H^+, Na^+, K^+, or NH_4^+, and these have precise masses associated with them. So, of the three unknowns, the compound molecular weight is the only continuum variable, whereas the charge and adduct ion mass variables have discrete values. To solve for three unknown variables, a minimum of three mass spectral peaks is required. Each related multiply charged peak in a coherent series of multiply charged peaks will satisfy the same value of M in Eq. (2). Solving for the molecular weight M is accomplished in the simplest sense by solving the set of simultaneous linear equations that describes the coherent sequence of multiply charged peaks. Whenever different data points along the *m/z* scale satisfy or solve for the same values of m and M at different integer values i, the amplitudes of these coherent data points are added and constructively contribute to the amplitude of the deconvoluted parent peak.

[50] M. Hamdan and O. Curcuruto, *Rapid Commun. Mass Spectrom.* **8,** 144 (1993).

[51] B. L. Schwartz, K. J. Light-Wahl, and R. D. Smith, *Rapid Commun. Mass Spectrom.* **5,** 201 (1993).

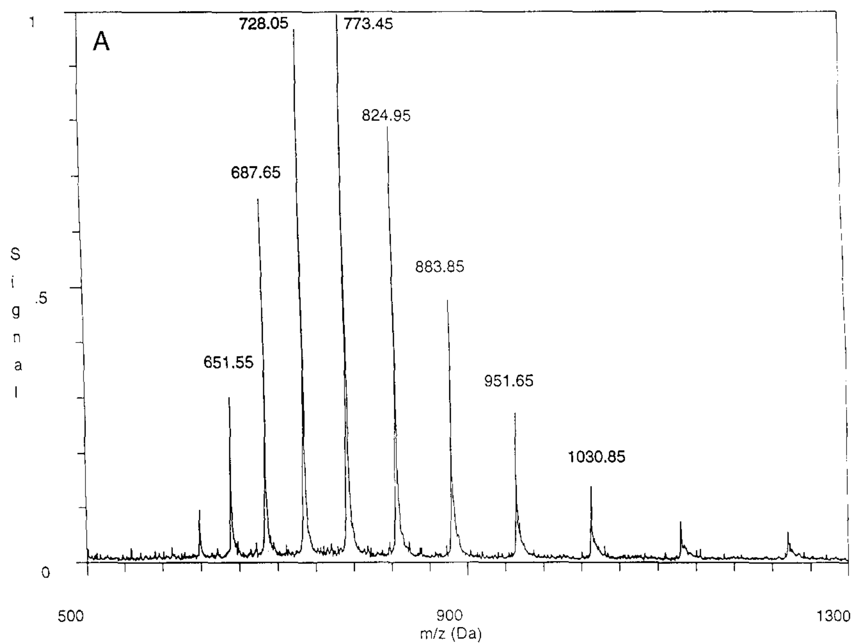
[52] C. K. Meng and J. B. Fenn, *Org. Mass Spectrom.* **26,** 542 (1991).

[53] S. K. Chowdhury, V. Katta, and B. T. Chait, *J. Am. Chem. Soc.* **112,** 9012 (1990).

Figure 8A shows a mass spectrum containing a series of multiply charged peaks of horse heart cytochrome *c*, a protein of approximately 12,360 Da in molecular mass. Each multiply charged peak contains the same molecular weight information when Eq. (2) is solved. The charge carrier or adduct ion *m* in this spectrum is H^+, as is the case with most proteins. It should be noted that the multiply charged peaks observed for cytochrome *c* in Fig. 8A are actually envelops of a mixture of unresolved isotopic peaks. If the isotopic peaks were well resolved for cytochrome *c*, as is possible with magnetic sector and Fourier transform mass spectrometry (FTMS) mass analyzers, then the isotopic spacing in m/z would give a direct measure of charge state. A related multiply charged peak with a different charge state than the first would then be required to solve for the value of *m* in Eq. (2). Computer programs are available for solving for all three variables in Eq. (2), or just *M* and *i*, assuming a value for *m*. When no assumption is made as to the value for the adduct ion mass and all three variables are solved, an indication of mass scale accuracy can be attained and hence an assessment can be made as to the precision of the parent molecular weight determination. An example of this is illustrated in Fig. 8A–C. The multiply charged spectrum of horse heart cytochrome *c* (10 pmol/μl in water with 1% acetic acid) was acquired with an HP 5989A quadrupole mass analyzer, in which the m/z scale was reasonably well calibrated. All multiply charged peaks in the mass spectrum of Fig. 8A are unresolved isotope profiles. Applying a deconvolution method[54] that simultaneously solves for the three variables in Eq. (2), a three-dimensional contour map is generated as illustrated in Fig. 8B. This contour map represents the three-variable solution that yields the deconvoluted peak maximum amplitude based on the measured m/z values and their amplitudes in the cytochrome *c* spectrum of Fig. 8A. The deconvoluted peak maximum in Fig. 8B ($M = 12{,}355.19$) falls at an adduct ion mass of 1.251 Da, not precisely the expected 1.008 for hydrogen, thus indicating an error in the m/z locations of the coherent peak maximums. A cross-section taken along the $m = 1.008$ value yields the two-dimensional deconvolution spectrum in Fig. 8C with a maximum at 12,359.13 Da, very close to the accepted value of approximately 12,360. This indicates the presence of random errors in the mass spectra peak maximum and not a mass scale error.

For lower molecular weight ions, for which fewer than three related charge state peaks occur, variables can often be eliminated by assumptions about the adduct ion mass identity and by other spectral features. Assuming H^+ is the adduct ion mass, then two variables remain: charge state and parent molecular weight. As mentioned above, the charge state can be

[54] M. Labowski, C. Whitehouse, and J. Fenn, *Rapid Commun. Mass Spectrom.* **7**, 71 (1993).



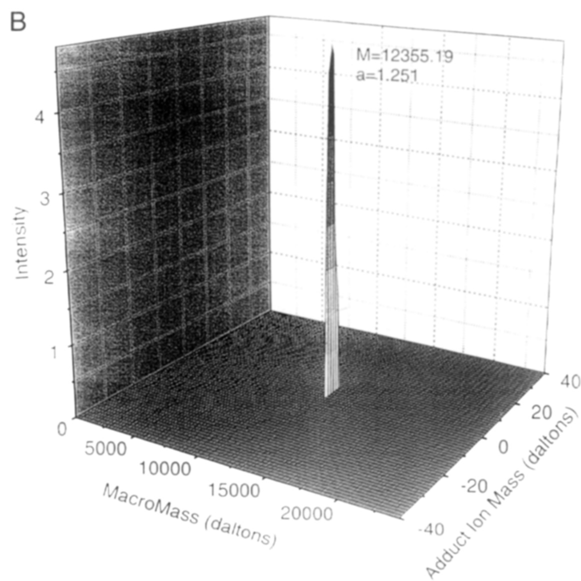


FIG. 8. (A) ES/MS spectrum of cytochrome *c* in water with 1.0% acetic acid. (B) Deconvolution of the cytochrome *c* spectrum, solving simultaneously for charge state, adduct ion mass, and molecular mass as in Eq. (2). (C) Cross-section of the deconvoluted three-dimensional surface in (B), taken along adduct ion mass 1.0008 (H^+).

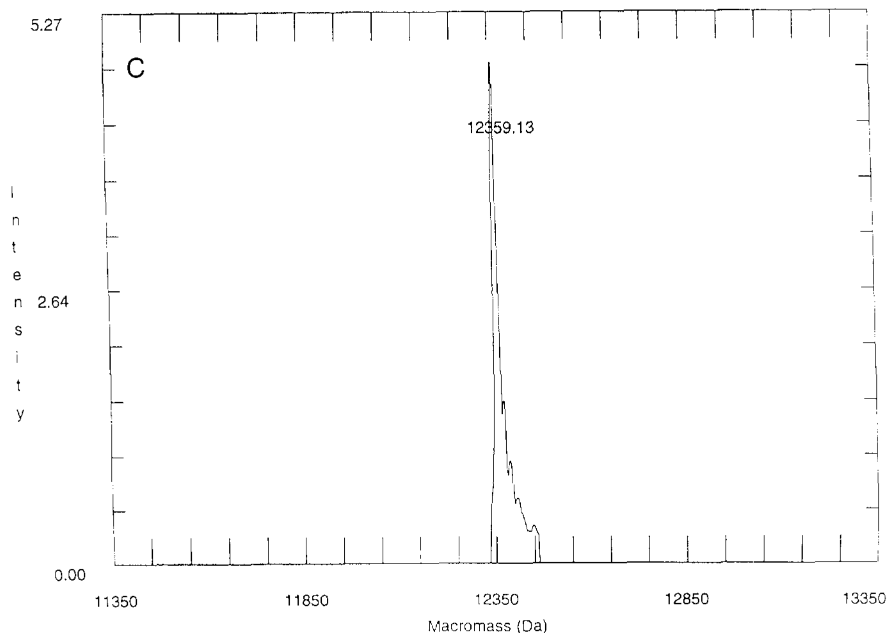


FIG. 8. (*continued*)

determined directly if the resolving power of the mass spectrometer is sufficient to resolve adjacent carbon isotope peaks. The mass-to-charge difference between consecutive carbon isotope peaks yields a direct measure of charge state as the mass difference from ^{12}C to ^{13}C is 1.003355. If the charge state can be determined, the molecular weight can be calculated directly from Eq. (2).

Mass Calibration for Multiply Charged Ions

Consider a tryptic digest from a protein being analyzed using LC/ES/MS with some CID fragmentation. When using a quadrupole mass analyzer, the doubly and triply charged peaks of tryptic fragments may not have resolved isotope profiles. Collision-induced dissociation daughter ion peaks generated from a tryptic digest parent ion may be singly or doubly charged, thus the charge state of a peak appearing in the mass spectrum must first be determined before a mass assignment can be made. Resolution defined as $M/\Delta M$ for a monoisotopic peak decreases with m/z for quadrupole mass spectrometers by the following relation:

$$\text{Resolution} = M/\Delta M = A(m/z) + B \quad (3)$$

where ΔM is the monoisotopic peak full-width at half the peak maximum amplitude (FWHM) and A and B are variables that can be set with the quadrupole mass spectrometer controls. Often A is called "resolution gain" or "high mass resolution" and B is called "resolution offset" or "low mass resolution." A typical running condition for a quadrupole mass spectrometer would be to set a constant FWHM for singly charged monoisotopic peaks along the m/z scale. For this example using a quadrupole mass spectrometer, assume that the resolution controls on the mass analyzer have been set so that $A = 2$ and $B = 0$. That is, the FWHM of monoisotopic peaks along the m/z scale is set to approximately 0.5 m/z. This resolution setting will yield approximately 12% valley separation between two equal-height, singly charged monoisotopic peaks separated by 1.0 m/z. A doubly charged peak appearing along the m/z scale would have isotopic peaks separated by 0.5 m/z and hence would not be resolved with a quadrupole resolution setting of $A = 2$ and $B = 0$.

As illustrated in Fig. 9A, the isotope profile calculated at a resolution of $M/\Delta M = 2282$ FWHM for gramicidin S + H^+ is asymmetric. Figure 9B shows the same isotope profile for gramicidin S + H^+ calculated at a resolution of 900 FWHM. Note that from the resolved to the unresolved peak profiles, the isotopic peak maximum has shifted +0.2 Da. Hence, to achieve the correct molecular weight for the gramicidin S most probable monoisotopic peak, a −0.2 Da correction should be applied to the molecular mass assignment obtained from the measured 1140.9 m/z peak, using a rearranged Eq. (2) with the correction factor added, $(K_i \times i) - im - 0.2 = M$.

In the case in which the measured peak has an unknown molecular formula, an estimate of the mass correction can often be made. If proteins or peptides are being mass analyzed, the elemental composition and relative abundance per element can be estimated.[55] Figure 10 is a plot of mass shift in peak maximum from the theoretically most probable resolved isotope (10% valley) m/z value versus resolution for a series of molecular weights. The resolution axis is linearly related to the m/z axis in quadrupoles by Eq. (3), therefore measured peaks of multiply charged ions falling at various points along the m/z scale will require different mass correction factors. The theoretical molecular formulas used for each molecular weight given in Fig. 10 were multiples of relative elemental ratios commonly found in proteins.[55] The formulas used had the elemental ratio 3.667(H) : 2(C) : 1(N) : 1(O). Note that the more symmetric the isotope profile, the less shift in peak maximum occurs for those resolutions typically

[55] T. E. Creighton, "Proteins: Structures and Molecular Principles." W. H. Freeman, New York, 1984.

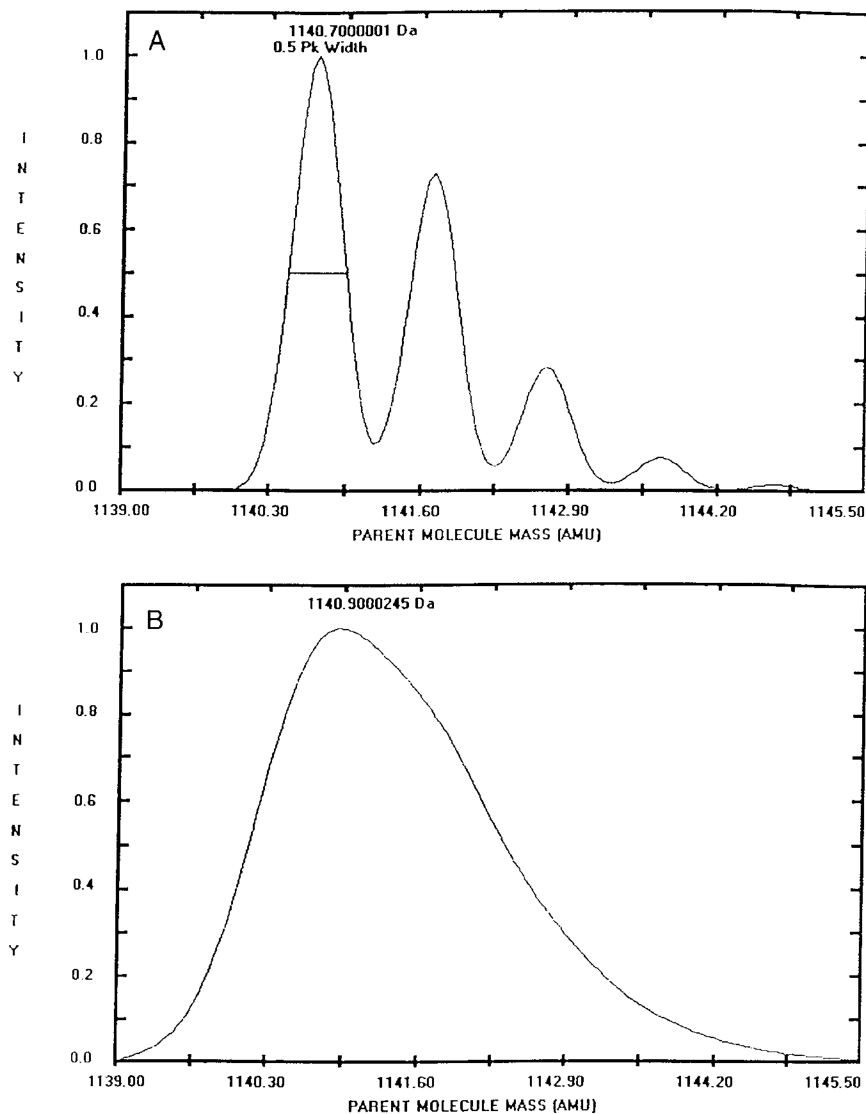


FIG. 9. (A) Calculated isotope distribution of gramicidin S + H^+ at a resolution of $M/\Delta M$ = 2282. (B) Calculated isotope distribution of gramicidin S + H^+ at a resolution of $M/\Delta M$ = 900.

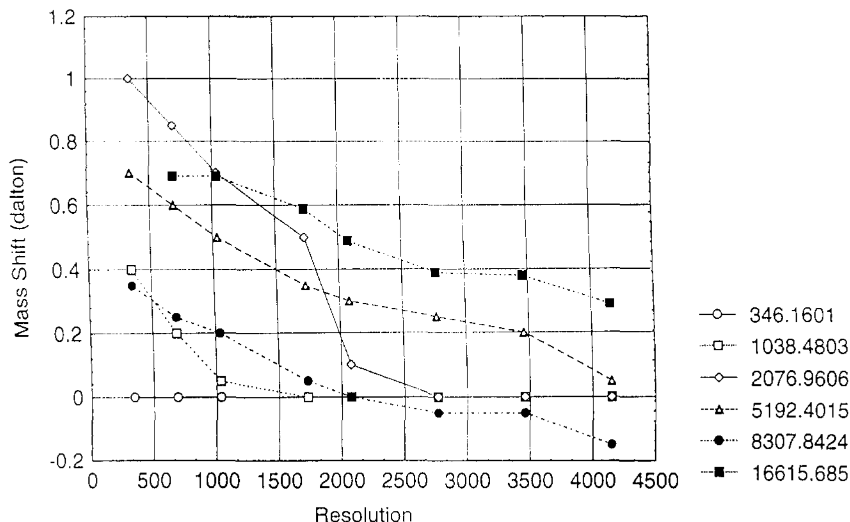


FIG. 10. Mass shift of the peak maximum vs resolution. Molecular formulas are multiples of elemental ratios: 3.667(H) : 2(C) : 1(N) : 1(O).

encountered in quadrupole mass spectrometers. Figure 10 serves as an illustration only for proteins. Specific mass correction values versus resolution can be generated for different classes of compounds being studied in a given analytical investigation. Once the charge state of a measured peak is known, the appropriate mass correction can be applied in the case when isotope peaks are not resolved. Often compounds similar to those being analyzed are used to generate calibration files to minimize or eliminate the need to apply mass correction.

Electrospray Ionization–Mass Spectrometry for Protein and Peptide Analysis

For structural elucidation, peptides and proteins are of course often chemically or enzymatically cleaved into smaller fragments that may then be analyzed by LC or CE. The combination of this procedure with MS detection via electrospray ionization results in a highly sensitive and routine method for microsequencing of peptides. As an example of this, Fig. 11 shows a total ion current (TIC) of a tryptic map of myoglobin, generated from 50 pmol of sample injected on-line into an ion trap LC/ES/MS system.[56] An Analytica of Branford ES source operated in the pneumatic nebulization mode interfaced to a Bruker ion trap (ESQUIRE) mass spec-

[56] Data were graciously provided by G. H. Kruppa, C. C. Stacey, J. H. Wronka, and F. H. Laukin of Bruker Instruments.

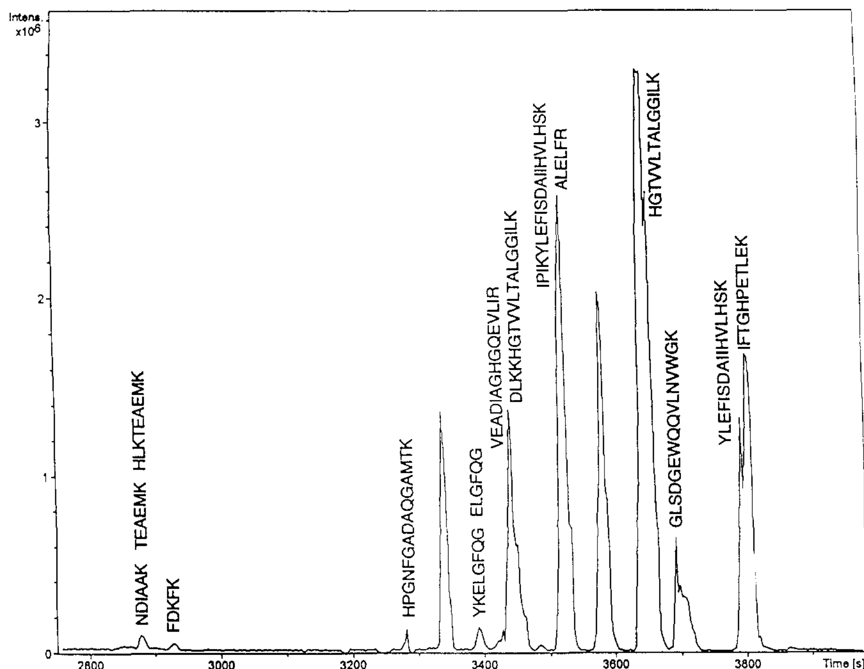


FIG. 11. LC/ES/MS TIC of a 50-pmol digest of myoglobin obtained on an ion trap MS.

trometer (Bruker, Billerica, MA) was used to acquire the data shown in Fig. 11. A 1 × 150 mm 5-μm C_{18} Michrom BioResources LC column was used with a Michrom BioResources LC (Auburn, CA). The separation was performed using a 95% A:5% B to 65% A:35% B gradient in 20 min, where solution A was 0.1% formic acid in water and solution B was 0.1% formic acid in acetonitrile. The liquid flow rate through the microbore LC column directly connected to the ES source was 50 μl/min. All detected peaks in the TIC can be assigned to the known primary sequence of a protein from the molecular weight of the individual peptides. If a mass analyzer capable of multiple stages of analysis is used, such as a triple quadrupole or in this case an ion trap, an individual parent m/z value can be selected and then subjected to MS/MS CID fragmentation. Whereas triple quadrupoles can generate only one stage of MS/MS fragmentation information, ion traps are capable of multiple [or $(MS/MS)^n$] experiments.

Future Directions

The future directions in new electrospray mass spectrometric instrumentation that can be applied to applications that complimentarily employ

chemical analysis tools are driven directly by more stringent experimental demands imposed by increasingly sophisticated analytical and biological research methods. Several improvements to ES/MS instrumentation and techniques are emerging and will continue to emerge in the near future as the full potential of this analytical tool is realized. Developments have been aimed at improving sensitivity and resolution, decreasing total analysis time, directly studying molecule-folding patterns, investigating noncovalently bound complexes and molecular folding patterns, improving ease of use, and increasing the range of compounds and solutions to which ES/MS can be applied. A few (far from inclusive) examples of new instrumentation and techniques in ES/MS and ES/MS/MS analysis are given below.

Sample-Limited Applications

On-line analysis of complex mixtures of unknown compounds when there are limited sample amounts often requires maximum sensitivity and specificity to solve problems in pharmaceutical drug development and the investigation of diseases. A particularly elegant example of a complex mixture analysis with limited sample available has been reported by Hunt *et al.* in their investigation of the class I and II major histocompatibility complexes (MHCs).[8,57] Mixtures of peptides isolated from different types of carcinoma cells were separated, detected, and sequenced to find those peptides expressed by the MHC proteins that were present in melanoma cells but not found in normal cells. On-line techniques working with individual component amounts in the low femtomolar range were designed to minimize sample loss and allow ES/MS analysis in parallel with fraction collecting. The collected fractions were used to check for biological activity and conduct additional ES/MS/MS analysis off-line. Figure 12 shows a schematic setup similar to what Hunt *et al.* employed, in which the LC analytical column used was a capillary LC column with flow splitting and fraction collecting at column flow rates of only a few microliters per minute. From this type of work, commercially available LC systems will emerge whereby small sample injection and fraction collecting in the 1-μl/min (or lower) flow rate range coupled on-line with ES/MS will become more routine.

Faster Analysis Times

Faster separation systems such as perfusion LC and CE are being employed on-line with ES/MS to decrease overall analysis times without com-

[57] A. L. Cox, J. Skipper, Y. Chen, R. A. Henderson, T. L. Darrow, V. H. Shabanowitz, V. H. Englehard, D. F. Hunt, and C. L. Slingluff, *Science* **264,** 716 (1994).

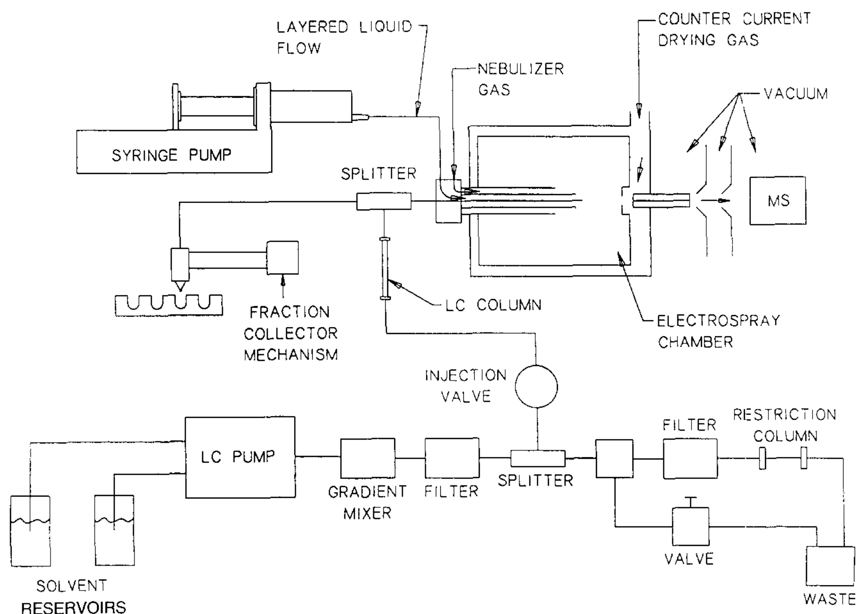


FIG. 12. Diagram of capillary LC/ES/MS system with low flow rate postcolumn fraction collecting.

promising performance. As the separation systems become faster and eluting peaks become narrower, the mass analyzer must be able to acquire data at a rate that does not degrade the chromatographic or CE separation resolution. Scanning instruments when used in scan mode and even in selected ion monitoring mode have difficulty acquiring data at a rapid-enough rate to take full advantage of the faster LC and CE separation techniques. Time-of-flight (TOF) and ion trap mass analyzers can acquire mass spectral scan data at a more rapid rate than the more conventional quadrupole and magnetic sector mass analyzers. Time-of-flight mass spectrometers can generate several mass spectra per second, which are themselves averages of hundreds of individual full mass scans. Figure 13 shows a reconstructed ion chromatogram (RIC) of a CE peptide separation in which the eluting CE peaks are approximately 5 sec wide. In acquiring these data, more than 4000 full scans per second were acquired with 500 scans averaged to produce 8 complete mass spectra per second. With this data acquisition rate, the CE peak profiles were not distorted by the ES/MS detection step. Time-of-flight mass spectrometers have the highest mass range of any mass analyzer and can acquire data at maximum instrument resolution without reducing sensitivity. The TOF analyzer used to generate

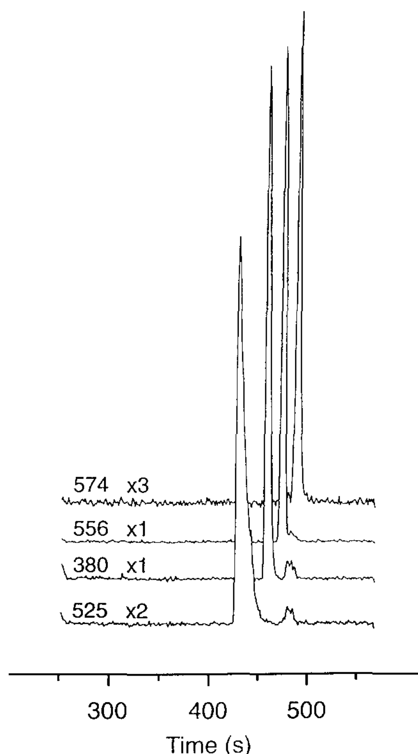


FIG. 13. Reconstructed ion currents for angiotensin (525 m/z), Val-Tyr-Val (380 m/z), Leu-enkephalin (556 m/z), and Me-enkephalin (574 m/z) analyzed by CE/ES/TOF/MS.

the TIC in Fig. 13 is capable of acquiring more than 30 mass spectra per second if necessary.[58] Ion traps, although not able to scan as rapidly as TOF, do have the capability of conducting MS/MS and $(MS/MS)^n$ analysis.

Investigation of Molecular Folding Patterns

ES/MS methods are being developed that can shed light on the folding patterns of proteins. For example, in one technique, the supporting solution of a protein sample is varied from 100% aqueous to mixtures of higher organic or acid content while the multiply charged mass spectra are acquired and compared. Relative amplitudes of the m/z peaks shift with the various protein conformation states[3,53] induced by the solution changes and can

[58] J. F. Banks, T. Dresch, P. Haren, and J. Boyle, "Fast Separations with a New ESI-TOF Mass Detector." Presented at the Pittsburgh Conference, New Orleans, LA, 1995.

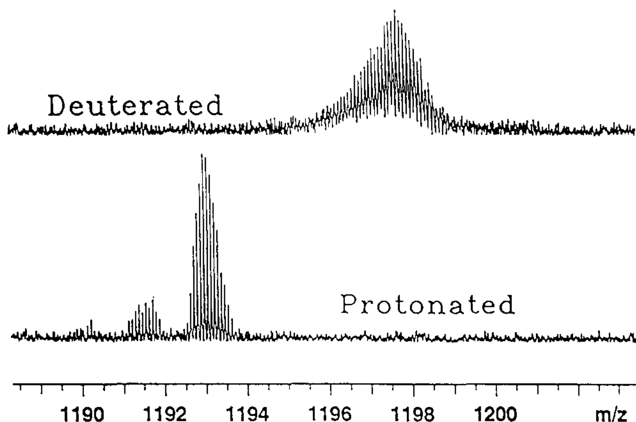


FIG. 14. ES/FTMS of lysozyme +12 charge state peak with isotope peaks resolved; comparison of deuterated and protonated forms.

then be correlated to protein structure and folding. Another technique is to exchange deuterium with exposed hydrogens of a protein and from the rate of exchange and the total percent exchange achieved, infer protein-folding patterns. Deuterium exchange has been conducted in the liquid phase[59] before electrospraying the solution and in the gas phase with electrosprayed ions trapped in FTMS cells.[60] As an example, Fig. 14 shows two mass spectra of the +12 charge state peak of lysozyme, one in its native form and the second after gas phase deuterium exchange in a Bruker ES/FTMS instrument.[61] The rate of deuterium uptake can be measured by the degree of mass shift over time, while the high-resolution capability of FTMS allows the precise measurement accuracy required for these types of experiments.

Lower Liquid Flow Rate Electrospray Ionization–Mass Spectrometry, Higher Sensitivities, Higher Resolution

Sharp electrospray needle tips with bores as small as 3 μm have been used to electrospray liquid samples at flow rates of 25 to 100 nl/min.[62,63]

[59] V. Katta and B. Chait, *Rapid Commun. Mass Spectrom.* **5,** 214 (1991).

[60] D. Suckau, Y. Shi, S. C. Beu, M. W. Senko, J. P. Quinn, F. M. Wampler, and F. W. McLafferty, *Proc. Natl. Acad. Sci. U.S.A.* **90,** 790 (1993).

[61] G. H. Kruppa, C. H. Watson, C. C. Stacey, J. Wronka, F. H. Laukien, C. V. Robinson, S. J. Eyles, R. T. Aplin, and C. M. Dobson, 1996 (submitted).

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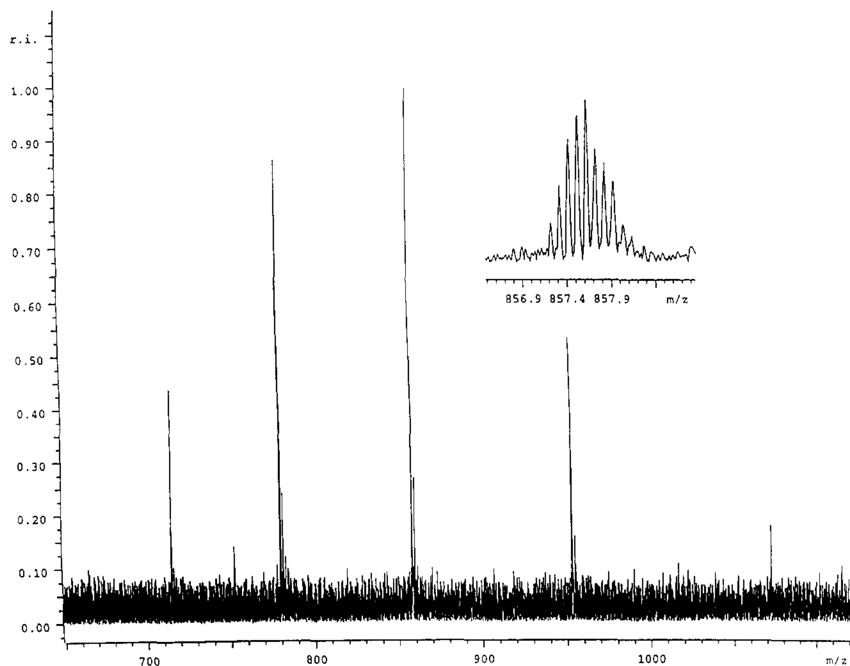

FIG. 15. ES/FTMS analysis of ubiquitin from 1 μl of a 0.1-μg/μl solution loaded into a microtip consuming only 12 amol of sample. *Insert:* +10 peak showing the resolved ^{13}C isotope distribution.

At these liquid flow rates, ES/MS or ES/MS/MS analysis times of over 30 min can be achieved while consuming less than 1 μl of sample. This translates into increased sensitivity as illustrated in Fig. 15, where a microtip was used in an Analytica of Branford ES source interfaced to a Bruker FTMS mass spectrometer. Only 15 amol of ubiquitin was consumed from a 0.1-μg/μl solution to generate the mass spectrum shown[56] with resolution separating the isotopic peaks of the 8.5-kDa protein. Ultimately the ES/MS analysis of just a few molecules may be possible, as has been reported by Bakhtiar *et al.*[64] The refinement of electrospray ion sources as interfaced to TOF, ion trap, and FTMS analyzers will enhance the capability of electrospray mass spectrometry, particularly when complemented by enzymol-

[64] R. Bakhtiar, X. Cheng, S. V. Orden, and R. D. Smith, "Charge State Shifting of Individual Multiply Charged Ions of Bovine Albumin Dimer and Molecular Weight Determination Using an Individual-Ion Approach." Presented at the ASMS Conference, Chicago, IL, 1994.

ogy and on-line separation techniques. Also, increased data analysis capability will greatly improve the range of applications to which ES/MS can be routinely applied and the speed and quantity of information that can be acquired.

[22] Matrix-Assisted Laser Desorption Ionization Mass-Spectrometry of Proteins

By RONALD C. BEAVIS and BRIAN T. CHAIT

Introduction

In this chapter, we provide a practical guide to the application of matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) for the analysis of peptides and proteins. We describe in detail the best methods that are currently available for preparing samples for MALDI-MS, because good sample preparation is the key to successful mass analysis. We consider aspects of the method that are important for obtaining high-quality data. Finally, we describe a selection of strategies for studying proteins with this powerful new technique.

Pulses of laser light have been employed since as early as 1976[1] to produce intact gas phase peptide ions from solid samples. The resulting peptide ions could then be analyzed by mass spectrometry. These early investigations and subsequent measurements over the following decade produced useful mass spectra from only a few short peptides. In addition, the probability for obtaining a useful mass spectrum depended critically on the specific physical properties of the peptide (e.g., photoabsorption spectrum, volatility) under study. This situation changed dramatically with the development by Karas and Hillenkamp.[2] MALDI-MS provides the means to volatilize proteins readily and to make the conditions for volatilization largely independent of the specific physical properties of the protein. This effect is achieved in two steps. The first step involves preparing an appropriate sample by dilutely embedding proteins in a matrix of small organic molecules that strongly absorb ultraviolet wavelength laser light. The second step involves ablation of bulk portions of this solid sample

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[2] M. Karas and F. Hillenkamp, *Anal. Chem.* **60,** 2299 (1988).

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