

PRACTICAL IMPLICATIONS OF SOME RECENT STUDIES IN ELECTROSPRAY IONIZATION FUNDAMENTALS

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In accomplishing successful electrospray ionization analyses, it is imperative to have an understanding of the effects of variables such as analyte structure, instrumental parameters, and solution composition. Here, we review some fundamental studies of the ESI process that are relevant to these issues. We discuss how analyte chargeability and surface activity are related to ESI response, and how accessible parameters such as nonpolar surface area and reversed phase HPLC retention time can be used to predict relative ESI response. Also presented is a description of how derivitizing agents can be used to maximize or enable ESI response by improving the chargeability or hydrophobicity of ESI analytes. Limiting factors in the ESI calibration curve are discussed. At high concentrations, these factors include droplet surface area and excess charge concentration, whereas at low concentrations ion transmission becomes an issue, and chemical interference can also be limiting. Stable and reproducible non-pneumatic ESI operation depends on the ability to balance a number of parameters, including applied voltage and solution surface tension, flow rate, and conductivity. We discuss how changing these parameters can shift the mode of ESI operation from stable to unstable, and how current–voltage curves can be used to characterize the mode of ESI operation. Finally, the characteristics of the ideal ESI solvent, including surface tension and conductivity requirements, are discussed. Analysis in the positive ion mode can be accomplished with acidified methanol/water solutions, but negative ion mode analysis necessitates special constituents that suppress corona discharge and facilitate the production of stable negative ions. © 2002 Wiley Periodicals, Inc., Mass Spec Rev 20:362–387, 2001; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/mas.10008

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I. INTRODUCTION

Electrospray ionization mass spectrometry (ESI-MS) is a choice method to accomplish mass determination of biomolecules. It is ideally suited to biochemical analysis because it allows for large, non-volatile molecules to be analyzed directly from the liquid phase, and thus can be coupled to separation techniques such as high performance liquid chromatography (HPLC) and capillary electrophoresis (CE) (Gelpi, 1995; Niessen & Tinke, 1995; Slobodnik, van Barr, & Brinkman, 1995). ESI-MS is significantly more rapid, sensitive, and accurate than traditional techniques of mass determination for biological molecules such as gel electrophoresis and equilibrium sedimentation. As a result, the technique has become indispensable in the analysis and sequencing of proteins (Fenn et al., 1989; Mehlis & Kertscher, 1997; Cao & Moini, 1998; Owens et al., 1998; Troxler et al., 1999; Alomirah, Alli, & Konishi, 2000; Le Bihan, Pinto, & Figeys, 2001; Whitelegge, Gunderson, & Faull, 2001) and oligonucleotides (Miketova & Schram, 1997; Muddiman & Smith,

1998; Owens et al., 1998), and is also used extensively in analyzing drugs, natural products (Herderich et al., 1997; Strege, 1999), pesticides (Jeannot & Sauvard, 1999; Steen et al., 1999), carbohydrates (Shen & Perreault, 1998; Yeung, Porter, & Vath, 1997; Apffel et al., 1998), and other small molecules.

Despite the widespread adoption of ESI-MS as a technique for biochemical analysis, many users still tend to view the technique as a “black box.” Sources of instability, background, interference, competition, and suppression are not always understood. As a result, the application of ESI can be fraught with frustration. We are confident that some recent work in ESI fundamentals can help guide applications development, and have undertaken this review with the goal of presenting information that is relevant and interesting to ESI users at large.

II. THE MECHANICS OF ESI-MS

In ESI-MS, a dilute solution of analyte is pumped through a capillary at a very low flow rate (0.1–10 $\mu\text{L}/\text{min}$). A high voltage (2–5 kV) is applied to the capillary. This voltage can be either negative or positive, depending on the analytes chosen. The applied voltage provides the electric-field gradient required to produce charge separation at the surface of the liquid. As a result, the liquid protrudes from the capillary tip in what is known as a “Taylor cone” (Fig. 1). When the solution that comprises the Taylor cone reaches the Rayleigh limit (Taffin, Ward, & Davis, 1989) (the point at which Coulombic repulsion of the surface charge is equal to the surface tension of the solution), droplets that contain an excess of positive or negative charge detach from its tip. These droplets move through the atmosphere towards the entrance to the mass spectrometer, and generate charged analyte molecules (ions) by one of several proposed mechanisms (Kearle & Peschke, 1999). The coulomb fission mechanism assumes that the increased charge density due to solvent evaporation causes large droplets to divide into smaller and smaller droplets, which eventually consist only of single ions (Dole et al., 1968). A second mechanism, known as ion evaporation, assumes that the increased charge density that results from solvent evaporation eventually causes coulombic repulsion to overcome the liquid’s surface tension, resulting in a release of ions from droplet surfaces (Iribarne & Thomson, 1976). Regardless of the mechanism by which they are produced, the ESI process generates vapor phase ions that can be analyzed for mass-to-charge ratio within the mass spectrometer. The various ways in which these ions may be produced are discussed in detail later on.

The charging of new surface as the droplets form and leave the tip requires a flow of charge from the power supply. The electrical circuit that sustains this current can

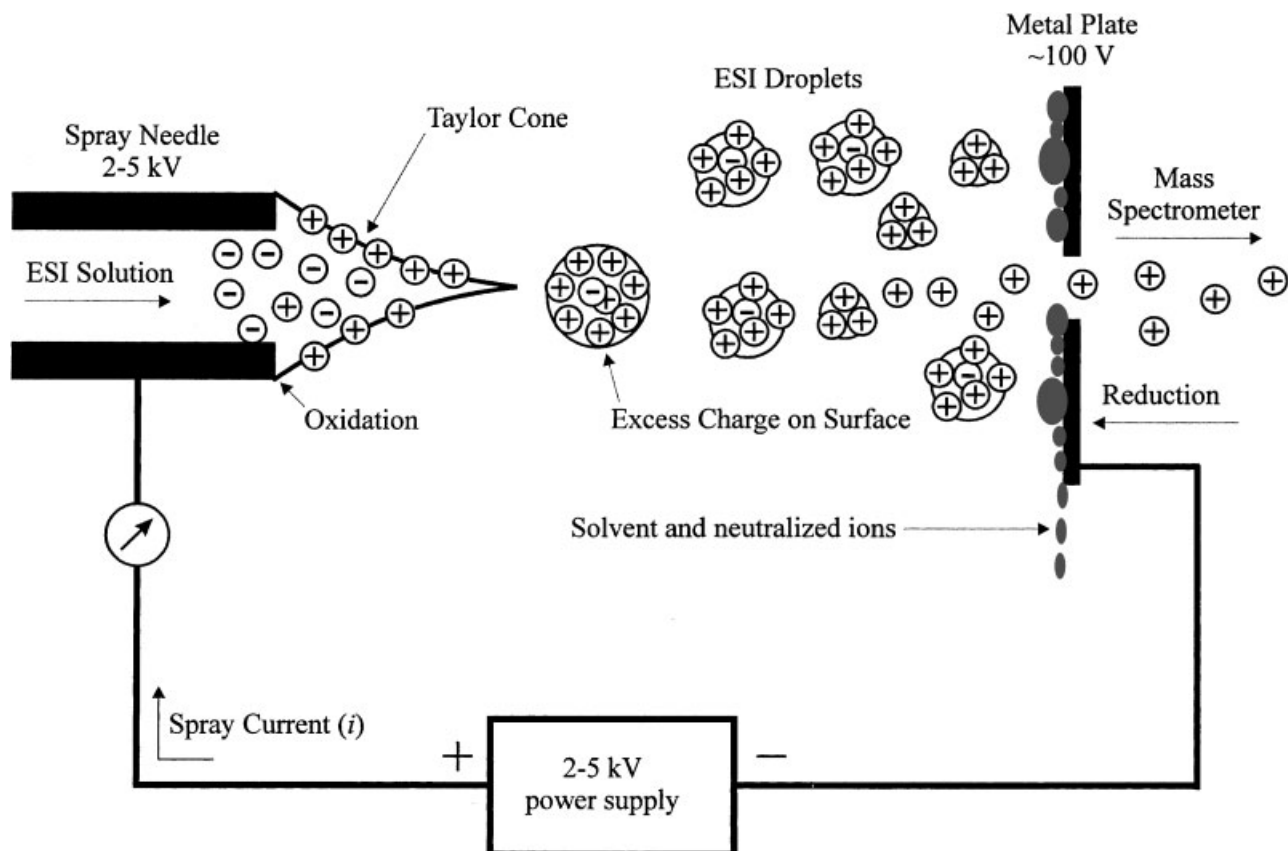


FIGURE 1. Schematic of the electrospray ionization process. The analyte solution is pumped through a needle to which a high voltage is applied. A Taylor cone with an excess of positive charge on its surface forms as a result of the electric field gradient between the ESI needle and the counter electrode. Charged droplets are formed from the tip of the Taylor cone, and these droplets evaporate as they move towards the entrance to the mass spectrometer to produce free, charged analyte molecules that can be analyzed for their mass-to-charge ratio.

be traced from the positive terminal of the power supply, to the solution via a metallic contact, through the gap that separates the capillary tip and the counter electrode, to ion neutralization at the counter electrode, and back to the negative power supply terminal (For negative ion ESI, the power supply is reversed). The current through each element of the series circuit must be the same. Thus, the current measured by the current meter shown in Figure 1 is a measure of the rate of the charge separation produced in the solution and leaving the capillary tip. The amount of charge on the droplets is, therefore, equal to the amount of charge separation. This charge is sometimes called the excess charge (Enke, 1997) to differentiate it from the cations and anions in the droplet that are neutralized by counter-ions. It is important to distinguish between these excess and neutralized charges because the neutralized charges are not likely to result in gas-phase ions. The formation of gas-phase ions from neutralized charge would require desolvation energies in excess of the

Coulombic forces between the ions and their counterions. Consequently, the maximum rate of production of vapor phase ions is equal to the rate of charge separation, and the amount of vapor phase ions produced cannot exceed the amount of excess charge introduced into the droplets.

The continuous flow of charge from the metallic contact to the sample solution must occur via an electrochemical reaction at that contact (Van Berkel, 1997). The dominant reaction in positive ion ESI is oxidation, whereas in negative ion ESI it is reduction. The equivalents of electrochemical reactant consumed and electrochemical product produced by the electrochemical reaction are exactly equal to the amount of charge separation that occurs at the capillary tip. The amount of analyte charged in ESI cannot exceed the amount by which the solution's chemical composition has been changed, because the amount of charge going to the ESI and the electrochemical process is the same.

The ESI process takes place at atmospheric pressure. Because mass analysis can only be accomplished at low pressure, it is necessary to transfer ions from the atmospheric pressure region to a low-pressure region before they can be analyzed by the mass analyzer. To accomplish this transfer of ions from high pressure to low pressure, differential pumping is generally employed, such that the mass spectrometer consists of separate chambers at decreasing pressures. These chambers are separated by orifices through which the ions must pass before reaching the mass analyzer.

Because the ESI process produces neutral species, ions, and clusters of ions with neutrals, the first challenge that must be overcome in introducing ions into the vacuum region is to separate the ions from the neutrals and to accomplish complete desolvation. Before ions even enter into the mass spectrometer, droplet formation can be aided by using a co-axial flow of neutral gas (known as sheath gas) around the needle tip. This sheath gas also aids in droplet desolvation. Electrospray mass spectrometry using a sheath gas is known as pneumatically assisted ESI (Bruins, Henion, & Covey, 1987). The separation of ions from neutrals can be aided by positioning the spray capillary slightly off-axis from the entrance to the mass spectrometer (Bruins, 1997). Off-axis positioning is helpful because the outer region of the spray generally consists of smaller, lighter, more desolvated droplets (Taffin, Ward, & Davis, 1989), and thus off-axis positioning maximizes the amount of desolvated analyte that enters the mass spectrometer while selecting against the unevaporated droplets.

There are several ways to accomplish introduction of ions from atmospheric pressure into the first stage of the mass spectrometer (Bruins, 1997). SCIEX (Ontario, Canada) uses a dry nitrogen "curtain" gas, which effectively drives neutral species away from the mass spectrometer orifice. Charged species penetrate the curtain because they are electrostatically attracted toward the orifice by virtue of an electric field gradient. A similar design, manufactured by Analytica of Branford, uses a glass tube coated on each end with a conductor in place of the orifice. Finally, Thermo Finnigan (San Jose, CA) uses a heated metal capillary interface for droplet desolvation and introduction into the vacuum. High temperatures and collisions within this capillary aid in the desolvation process and help to accomplish the declustering of ions from neutrals.

III. ANALYTE CHARACTERISTICS AND SELECTIVITY

One significant area of research in the fundamentals of ESI has been the issue of how analyte characteristics

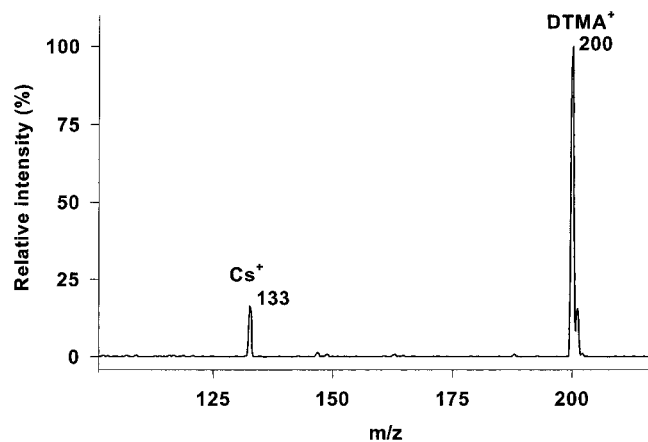


FIGURE 2. ESI mass spectrum of an equimolar (10^{-6} M each) mixture of cesium bromide (CsBr) and Decyltrimethylammonium bromide (DTMA Br) in 50% methanol, 50% water solution with 1.0 kV capillary voltage. The mass spectral response of the DTMA⁺ is significantly higher than that of the Cs⁺ due to its higher droplet surface affinity [Reprinted from Zhou & Cook, *J Am Soc Mass Spectrom*, 2001, 12:206–214 with the permission of Elsevier].

are important in determining ESI response. This issue is relevant because ESI response can vary significantly among different analytes that have identical solution concentrations (Cheng et al., 1992; Tang & Kebarle, 1993; Cech & Enke, 2000; Zhou & Cook, 2001; Cech, Krone, & Enke, 2001a) (Fig. 2). For example, Figure 2 shows the mass spectrum of an equimolar mixture of cesium bromide and decyltrimethylammonium (DTMA) bromide (Zhou & Cook, 2001). Although the solution concentrations of these two analytes are identical, the ESI responses of the two differ by approximately an order of magnitude. This difference arises because DTMA is more surface-active than Cs⁺, as discussed later on (see Analyte Surface Activity and its Effect on ESI response).

An understanding of how analyte characteristics determine ESI response is of key importance in the successful application of ESI. This understanding is necessary in accessing the suitability of ESI for analysis of a particular analyte. It is also important in troubleshooting. If the ESI analysis is not working, or if detection limits are poor, then the analyst must determine whether the problem lies in sample preparation or separation, or whether the analyte is inherently unsuitable for analysis by ESI-MS. In this latter case, it may be possible to derivatize the analyte in such a way as to improve its responsiveness. In this section, we discuss what analyte characteristics determine ESI response. We also review some of the methods of derivatization that can be employed to improve the ESI response of poorly responsive analytes.

A. Charging the Analyte

1. Ionization Through Charge Separation

As discussed in the introduction, analytes that already exist as ions in solution may become part of the excess charge when charge separation occurs within the Taylor cone. Gas-phase ions will be created when the droplets formed from the Taylor cone evaporate and the ions that carry the excess charge are released into the gas phase. Charge separation is the primary method by which ions are formed for inorganic species such as sodium, cesium, chloride, and nitrate, for organic and biological molecules with acidic or basic functional groups, and for species that contain ammonium, phosphonium, or oxonium moieties. Proteins, with their multiple basic amino acid residues, readily form positively charged ions through protonation, whereas oligonucleotides and fatty acids can be negatively charged through deprotonation of acidic groups. Positively charged ions such as inorganic cations and protonated organic bases are analyzed in the positive ion mode, whereas negatively charged ions such as inorganic anions and deprotonated organic acids are analyzed in the negative ion mode.

2. Adduct Formation

Polar analytes that do not have acidic or basic groups can be charged with ESI through the formation of adducts with various ions. Adduct formation is actually a special case of ionization by charge separation; the adduct formation occurs in solution before the charge separation process takes place.

Adduct formation is used to create ions in negative and positive ion ESI. In negative ion ESI, Cole, and Zhu have shown that adducts are formed with chloride ions when chlorinated solvents such as chloroform are used (Cole & Zhu, 1999) (Fig. 3). The formation of adducts with chloride anions allows for the successful ESI analysis of species such as polar, neutral molecules, or very weakly acidic molecules that do not generate stable negative ions through deprotonation. In positive ion ESI, analyte adducts with sodium, lithium, ammonium, or other cationic species are often observed. The addition of salts to yield these cations to samples of weakly basic or polar, neutral analytes can facilitate the formation of positive ions (Saf, Mirtl, & Hummel, 1994; Ackloo et al., 2000).

Although small concentrations of salt can facilitate ionization through adduct formation, high salt concentrations cause background that interferes with detection of the analyte. Background in the form of sodium clusters with analytes and solvents can be so severe that it completely masks the analyte signal. Some background concentration of salt, usually sodium, is almost always present in ESI samples. Low concentrations of sodium

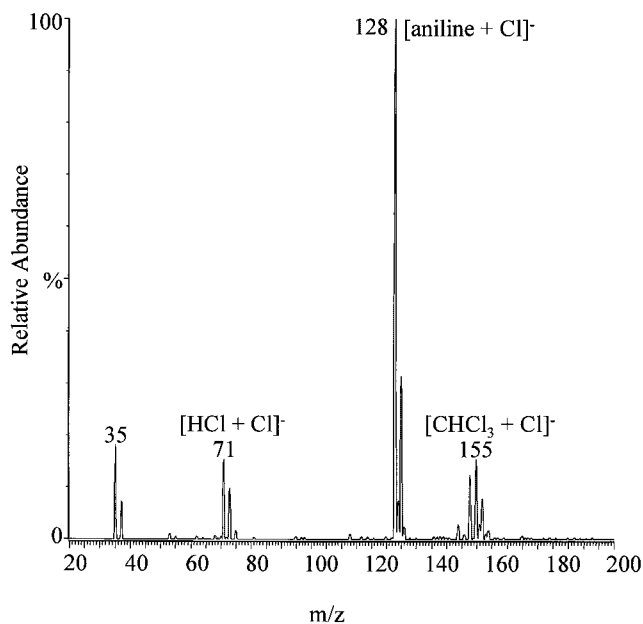


FIGURE 3. Negative ion ESI mass spectrum of aniline in a 10:1 carbon tetrachloride:chloroform solution. Aniline, which does not readily form negative ions through deprotonation, is clearly observed as an adduct with chloride ion from the solvent [Reprinted from Cole & Zhu, *Rapid Commun Mass Spectrom*, 1999, 13:607–611 with permission of John Wiley & Sons, Ltd.].

(on the order of 10^{-6} M) can derive from glassware and storage bottles, or can be present as impurities—even in analytical grade solvents. Higher concentrations of various salts can derive from biological sources, or be added as LC buffers to facilitate separations. The deleterious effects of cation adduct formation can be quite pronounced with oligonucleotides of high mass and charge state (Liu et al., 1996) due to the clustering of nucleotides with cations (Fig. 4a). Liu et al. demonstrated that interference due to salt clusters can be prevented through the application of on-line microdialysis techniques, the results of which are shown in Figure 4, parts (b) and (c) (Liu et al., 1996). Microdialysis can also be performed off-line, thus permitting the analysis to be accomplished at lower flow rates and consuming less analyte (Liu et al., 1997). Salt removal can also be accomplished on-line by packing the spray capillary with HPLC beads (Emmett & Caprioli, 1994).

3. Ionization Through Gas-Phase Reactions

Because the ESI process occurs at atmospheric pressure, and because large quantities of charged solvent molecules are generated in addition to the charged analyte molecules, the ESI-MS response can be affected by gas-phase interactions (Ogorzalek Loo & Smith, 1994; Stephenson &

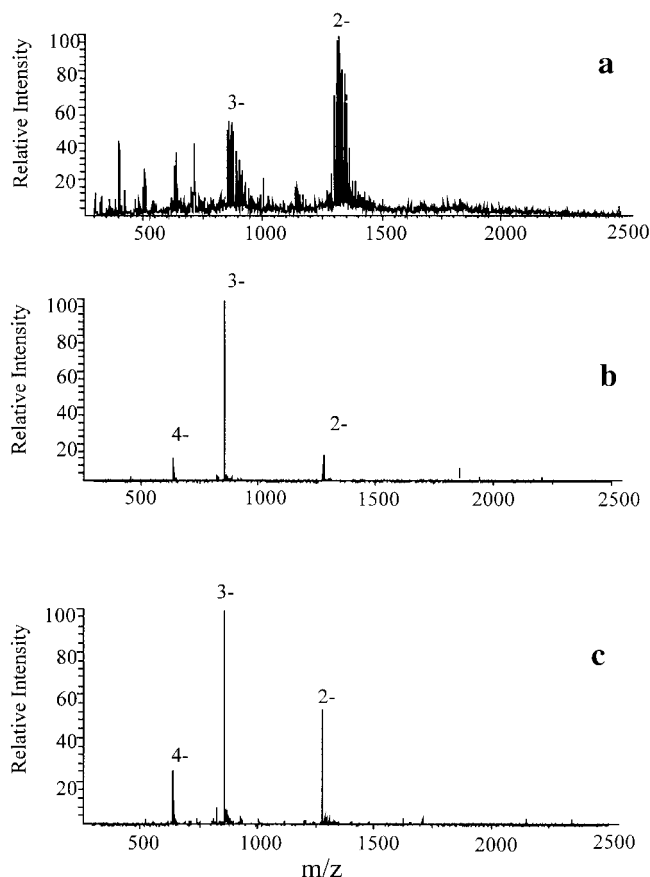


FIGURE 4. Three negative ion ESI mass spectra of the same oligonucleotide: (a) in 10 mM NH_4OAc from direct infusion, (b) in 10 mM NH_4OAc after on-line microdialysis, and (c) in 10 mM NH_4OAc and 250 mM NaCl after on-line microdialysis. By removing salt ions from the solution, the microdialysis eliminates the salt-clustering that complicates the mass spectrum [Reprinted from Liu et al., *Anal Chem*, 1996, 68:3295–3299 with permission of American Chemical Society].

McLuckey, 1996; Kebarle & Peschke, 1999; Amad et al., 2000). These gas-phase interactions occur after the analytes have been released from solution; thus, gas-phase effects are essentially the “last word” in the electrospray process.

In ESI, analyte charging in the gas phase generally occurs through gas-phase proton-transfer reactions (Zhou & Hamburger, 1995; Yen, Charles, & Voyksner, 1996; Kebarle & Peschke, 1999; Amad et al., 2000). Upon entering the gas phase, molecules that were protonated in solution yield their protons to solvents or analytes with higher gas-phase basicity. In this way, analytes that evaporate from the droplets as neutrals can become charged through gas-phase interactions. Gas-phase proton transfer reactions occur when there is an inversion in the order of basicity among a series of molecules proceeding from the solution phase to the gas phase (Kebarle &

Peschke, 1999). It is important to note that the inversion of order of basicity occurs because solution-phase basicity and gas-phase proton affinity are not necessarily related. Species with high gas-phase proton affinities may not be highly basic in solution.

Gas-phase basicity can be expressed in terms of gas-phase proton affinity (PA), which is defined as the negative change in enthalpy for the hypothetical protonation reaction at 298 K (Bowers, 1973). Note that PA is not necessarily related to $\text{p}K_a$, which is a measure of solution-phase basicity. In the gas phase, a molecule with a high gas-phase proton affinity will abstract a proton from the protonated form of a molecule that has a lower gas-phase proton affinity. Amad et al. showed that, in cases where ESI is performed on a mixture of several solvents, protonated clusters of the solvent with the highest gas-phase proton affinity will dominate the ESI mass spectrum. From Figure 5, it is apparent that the solvent with the highest gas-phase proton affinity dominates—even if the mole fraction of that solvent is very low (Amad et al., 2000). These results indicate that the solvent molecules with higher PAs scavenge protons from other solvent molecules, thus virtually eliminating the ESI response for the solvent with lower PA.

The results shown in Figure 5 suggest that solvent proton affinity could have an effect on analyte response. Indeed, in situations where the electrospray solvent has a higher gas-phase proton affinity than the analyte, analyte response may be completely suppressed (Amad et al., 2000). Consequently, the proton affinity of the solvent provides a cut-off point in determining which analytes

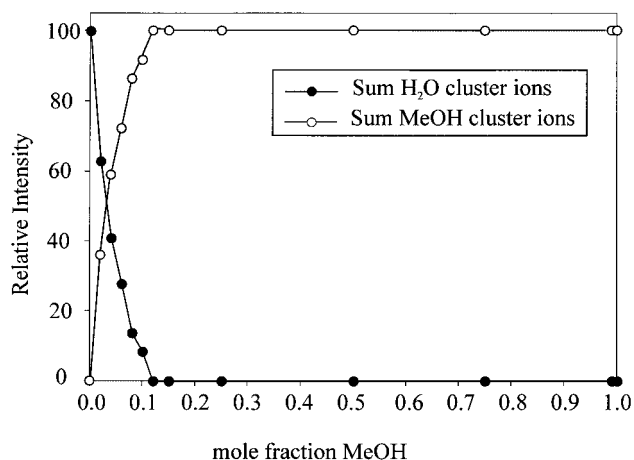


FIGURE 5. Intensity of solvent cluster response as a function of mole fraction methanol for various methanol/water mixtures. Even at very low mole fractions of methanol, the ESI response is predominantly that of methanol clusters, which have higher gas-phase PA than do water clusters [Reprinted from Amad et al., *J Mass Spectrom*, 2000, 35:784–789 with permission of John Wiley & Sons, Ltd.].

will be analyzable with ESI. Therefore, it is important to choose a solvent with a lower gas-phase proton affinity than the analyte of interest when performing analyses with ESI-MS. Low proton affinity is not so much a problem in the ESI analysis of proteins and peptides, because they tend to have very high gas-phase basicities. However, it could be a consideration in the analysis of organic compounds that are more weakly basic in the gas phase.

4. Ionization Through Electrochemical Oxidation or Reduction

As explained in the introduction, electrochemical oxidation or reduction must occur in ESI (Van Berkel, McLuckey, & Glish, 1992) to create a concentration of oxidized or reduced species equivalent to the concentration of excess charge created by charge separation. Electrochemical reactions can convert an uncharged analyte to an ionic form that is amenable to ESI, and are thus utilized as a form of ionization in some cases (Van Berkel et al., 1998). However, electrochemical reactions can be deleterious if they consume analyte in a destructive way, produce ions that add to the mass spectral background, or out-compete the analyte for the excess charge. In most cases, because only a fraction of the excess charge is converted to ionized analyte, the chemical change in the sample due to this process can be much greater than the analyte concentration.

Electrochemical reactions can change the oxidation state of the analyte, oxidize the metal contact, and/or oxidize or reduce the solvent. Electrochemical products are often not observed in positive ion analysis when their products are metallic cations such as Fe^{2+} (from oxidation of the stainless steel contact) (Van Berkel, 1998). These metal cations prefer to be solvated and are thus not effective competitors for the excess charge (see Analyte Characteristics section). In cases where deleterious electrochemical reactions are a problem in ESI, replacing the stainless steel capillary or contact with one made of a different metal can significantly alter the electrochemistry that occurs, thus changing the nature of the electrochemical products (Kertesz & Van Berkel, 2001). Changes in electrode geometry can also effect the nature of the electrochemical products by altering mass transport and current density characteristics (Van Berkel, 2000).

B. Analyte Surface Activity and Its Effect on ESI Response

In the previous section, we discussed how selectivity occurs in ESI-MS because only analytes that can be charged can be analyzed mass spectrometrically. However, there is significant diversity in ESI response even among charged species. Referring back to Figure 2, the Cs^+ and the

DTMA^+ are both positively charged species, yet their ESI responses are considerably different. This variation in ESI response among charged species arises due to differences in analyte structure.

It is generally true that analytes with high affinity for the surface of ESI droplets (*surface-active* analytes) have higher ESI response (Iribarne, Dziedzic, & Thomson, 1983; Fenn, 1993; Tang & Kebarle, 1993a,b; Karplus, 1996; Cech & Enke, 2000; Tang & Smith, 2001; Zhou & Cook, 2001; Cech, Krone, & Enke, 2001a). This effect was initially observed by Iribarne, Dziedzic, and Thomson, the pioneers of the “ion evaporation theory” (Iribarne, Dziedzic, & Thomson, 1983), who observed an increased response with atmospheric-pressure ion-evaporation mass spectrometry (API-MS) for nonpolar analytes. Iribarne et al. suggested that this enhanced response was a result of the fact that the nonpolar ions would prefer the droplet air interface, and thus would reside at the droplet surface. Consequently, these ions would enter the gas phase more readily than those in the droplet interior and have higher response.

Kebarle and Tang expanded on the ion-evaporation theory, and developed a comprehensive theory that describes ESI response in terms of the evaporation rate from ESI droplets (Tang & Kebarle, 1991, 1993a,b; Apffel et al., 1995). They studied a variety of metal cations (such as sodium, lithium, cesium, and potassium) and alkyl ammonium cations (such as tetraethyl ammonium, tetrapentylammonium, etc.) and observed a higher ESI response for those cations that were less solvated (Tang & Kebarle, 1993). This higher response was rationalized based on the fact that less highly solvated ions were expected to have higher evaporation rates and thus to be more responsive in ESI analysis. When studying more complex molecules like the drugs cocaine and heroine, Kebarle and Tang found that the ESI response could not be described in terms of evaporation rates alone, and that the additional factor of surface activity had to be considered (Tang & Kebarle, 1993).

The equilibrium-partitioning model (Enke, 1997; Constantopoulos, Jackson, & Enke, 1999; Cech & Enke, 2000; Constantopoulos, Jackson, & Enke, 2000; Cech & Enke, 2001; Sjoberg et al., 2001), originally put forth by Enke (1997), rationalized the effect of nonpolar character on ESI response without invoking ion evaporation rates. This model considered the fact that a fixed amount of excess charge is produced in the ESI process, and that this charge must necessarily reside on the surface of ESI droplets. The interior of the droplets must be electrically neutral, and will consist of cations and anions that balance each other in charge. Because neutralized analytes or ion-paired analytes would evaporate to form neutral salts, they would not be observed mass spectrometrically. The equilibrium-partitioning model, therefore, assumed that

only analytes that could become part of this excess charge phase on the surface of the ESI droplet would be responsive to ESI. Thus, the more likely an analyte is to exist as part of the excess charge, the higher its ESI response will be.

1. Surface Activity and the Fissioning Process

The process of generating ions from electrospray involves multiple steps of fissioning and evaporation. This fissioning process has been documented photographically, and it is well known that when the parent droplet reaches the Rayleigh limit and fissions, multiple, small, highly charged droplets are formed (Fig. 6) (Gomez & Tang, 1994). What is not known is exactly how many times the parent droplet fissions, and whether the ions produced by the offspring droplets result from successive fissioning events or from direct ion-evaporation. However, it is generally accepted that the offspring droplets produced in the ESI process are the major source of ions observed in ESI mass spectra (Gomez & Tang, 1994; Tang & Smith, 1999, 2001).

It has been shown that when ESI droplets fission, the parent droplet retains a smaller fraction of its charge than its mass (Taflin, Ward, & Davis, 1989). Because it is the surface layer of the parent droplet that extends into a cone and breaks up into offspring droplets, it is reasonable to assume that surface active analytes will follow the charge during fissioning and that analytes with low surface activity will not. Thus, the offspring droplets, which are enriched in the excess charge, will also be enriched in the surface-active analytes. This phenomenon was demonstrated experimentally by Tang and Smith

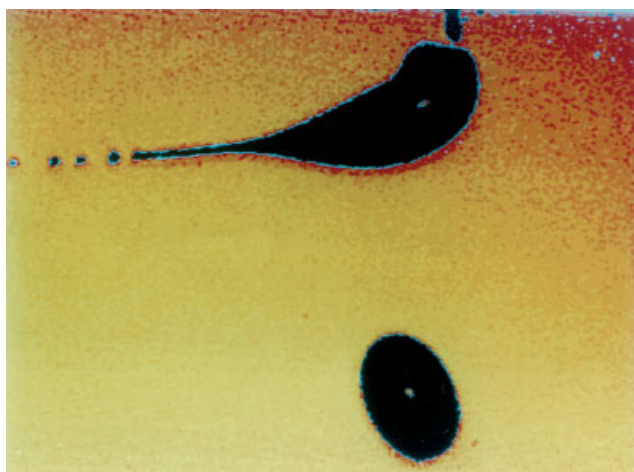


FIGURE 6. Photograph of a charged droplet in the act of fissioning. The droplet distorts, creating a miniature Taylor cone, and a string of offspring droplets are ejected [Reprinted from Gomez & Tang, *Phys Fluids*, 1994, 6:404–414 with permission of American Institute of Physics].

(2001), who photographed fluorescent species deposited on a metal counter electrode by an ESI spray. They showed that the outer ring of the ESI spray, which consists of the lighter offspring droplets, is enriched in surfactant, whereas analytes that are not surface-active are equally distributed throughout the ESI spray.

The net result of this uneven fissioning of ESI droplets is that, if analytes that are not surface-active do not end up in these offspring droplets, then they may not be charged in the ESI process. This failure to charge analytes in the droplet interior can occur even if there is plenty of excess charge in the initial parent droplets. Experimental evidence for this assertion comes from comparing the electrospray ionization response of solvophillic species and surface-active species, using conventional ESI and nanospray. Oligosaccharides and glycosides, which have poor response with conventional ESI due to their low surface activity, can be quite responsive in nanospray analysis (Bahr et al., 1997; Karas, Bahr, & Dulcks, 2000). According to Karas et al., the better response observed with nanospray analysis can be explained by the fact that the droplets generated with nanospray are already small enough to produce gas-phase ions directly, without relying on fissioning (Karas, Bahr, & Dulcks, 2000). Thus, analytes with poor surface activity are not lost in the fissioning process, but become charged in the initial nanospray droplets, which have very high surface-to-volume ratios. Ultimately, the uneven fissioning of mass and charge is another explanation for the enhancement in response of surface-active analytes as compared to those that are less surface-active (Kearle & Peschke, 1999; Cech & Enke, 2001; Zhou & Cook, 2001). This effect may compound other effects such as the partitioning within the ESI droplet and make surface activity even more important in determining ESI response (Cech & Enke, 2001).

2. Predicting ESI Response from Other Parameters

The fact that surface-activity affects ESI response means that various parameters related to analyte structure can be used to predict suitability for analysis with ESI. Consider Figure 7, which is a mass spectrum of an equimolar mixture of six tripeptides with different C-terminal residues (Cech & Enke, 2000). It is obvious that the peaks in the mass spectrum are most intense for the peptides that have the most nonpolar (hydrophobic) side-chains. The electrospray response increases as the side chains progress from glycine to phenylalanine, but the response for the peptide with tyrosine as its C-terminal residue is lower than that for the phenylalanine-containing peptide, due to the polar OH group on the phenyl ring in tyrosine. These data are in agreement with the results of Zhou and Cook, who showed that response is significantly higher for the

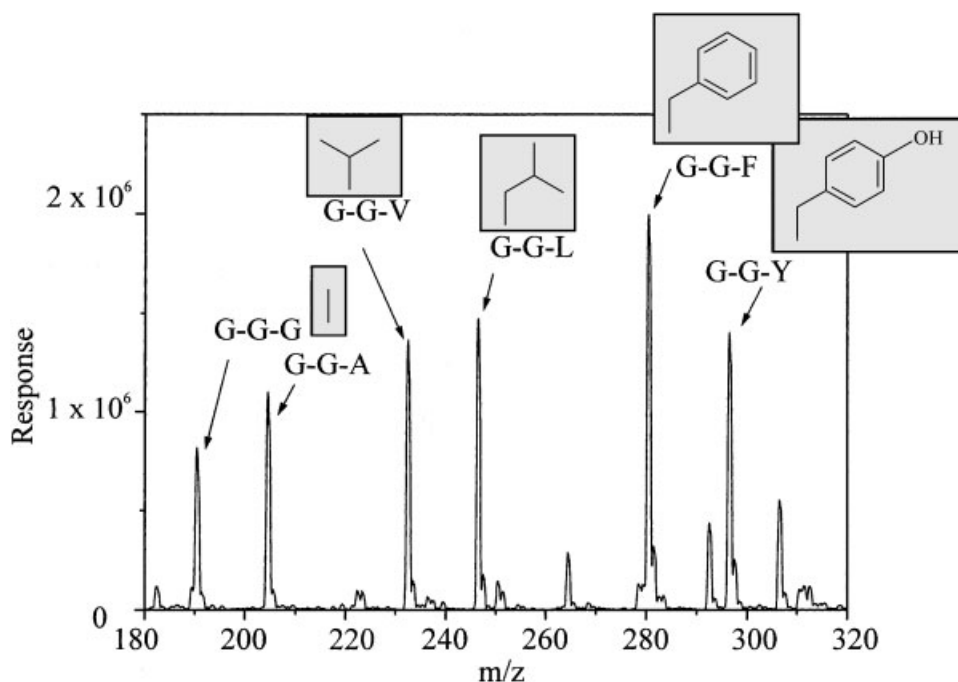


FIGURE 7. Mass spectrum of an equimolar mixture of six tripeptides that have different C-terminal residues. ESI response increases in proceeding from G-G-G to G-G-F as the side chain on the C-terminal residue becomes increasingly nonpolar (see structures in boxes). The ESI response of the peptide G-G-Y is less than that of G-G-F due to the addition of the polar OH group on the phenyl ring [Reprinted from Cech & Enke, *Anal Chem*, 2000, 72:2717–2723 with permission of American Chemical Society].

nonpolar amino acid phenylalanine than for the polar amino acid serine (Zhou & Cook, 2001). Raffaelli & Bruins (1991) also documented lower ESI response to be associated the presence of nonpolar groups as part of the analyte structure.

There are several different accessible variables related to analyte polarity that can be correlated with ESI response (Cech & Enke, 2000; Cech, Krone, & Enke, 2001a). There is a relatively linear relationship between ESI response and of nonpolar surface area (calculated from literature values (Karplus, 1996)) of the peptide (Cech & Enke, 2000). ESI response can also be correlated with Gibbs free energy of transfer from nonpolar to polar solutions. Analytes with higher Gibbs free energy of transfer (more nonpolar analytes) tend to have higher ESI response (Cech & Enke, 2000). Furthermore, it has been shown that for simple, singly charged analytes, a relationship exists between HPLC retention time and ESI response (Cech, Krone, & Enke, 2001a). The nonpolar analytes, which have a higher ESI response, are also retained longer in reversed phase HPLC (Cech, Krone, & Enke, 2001a) (Fig. 8). The relationship between ESI response and HPLC retention time for larger, multiply charged peptides and proteins has yet to be determined. However, it has been shown that the ESI response is

higher for larger peptides that have a significant number of nonpolar residues than for those that are more polar (Cech, Krone, & Enke, 2001b).

C. The Role of Analyte pK_a and Solvent pH

Under circumstances where ionization in ESI involves protonation or deprotonation, one might expect the pK_a 's of analyte functional groups to play an important role in an ESI response. However, this is not always the case. Certainly, in order for an analyte to become protonated with ESI, it must be basic in solution or in the gas phase. The same is true for acidic analytes and deprotonation. Furthermore, ESI analysis of basic analytes generally works best at low pH's, whereas the analysis of acidic analytes is best accomplished at higher pH's (Wang & Cole, 1997). However, it is well known that protonated ions of basic analytes can be observed, when ESI-MS analysis is performed with basic solutions (where the pH is higher than the analyte pK_a) (Kelly et al., 1992; Wang & Cole, 1994; Zhou & Cook, 2000) and deprotonated ions can be observed in the ESI analysis of acidic solutions (Wang & Cole, 1994; Zhou & Cook, 2000). For example, Figure 9 shows a mass spectrum of a polypeptide in a basic (pH = 10) solution (Kelly et al., 1992). Positive (Fig. 9a)

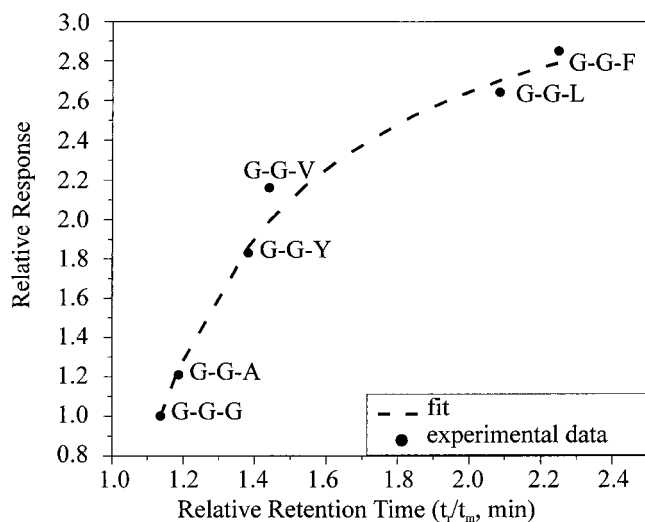


FIGURE 8. Response as a function of experimental retention time for the same tripeptides shown in Figure 7. Isocratic analyses were performed in a solvent of 65:35 water:methanol, 0.5% acetic acid. Retention time and response were both calculated relative to G-G-G (responses of all other peptides were divided by that of G-G-G and the retention time of G-G-G was subtracted from the other retention times). The general trend in the data is that, as retention time increases, so does ESI response [Reprinted from Cech, Krone & Enke, *Anal Chem*, 2001, 73:208–213 with permission of American Chemical Society].

and negative (Fig. 9b) ions were both detected, even though one would not expect protonation of the basic amino acids at such a high pH.

Many explanations have been put forth for this phenomenon. Quite simply, a fixed amount of excess charge dictated by the solution flow rate, and the applied voltage is produced in the ESI process (Enke, 1997). This excess charge must be carried by some species on the droplet surface. In the case of positive ion ESI in protic solvents, the excess charge is in the form of protons, the concentration of which depends not on the solution pH but on the flow rate and applied voltage. These protons must reside either on the analyte or the solvent. Provided that the analyte partitions to the droplet surface and has a pK_a lower than that of the solvent, it will become protonated even if its pK_a is below the solution pH. Whatever charge does not go to the analyte will go to the formation of protonated solvent molecules or clusters.

The possibility of analyte molecules being protonated once they have left the solution is also important. Gas-phase proton-transfer reactions with strong gas-phase acids such as ammonia or with protonated solvent molecules may lead to the protonation of species that are released from the droplets as neutrals (Wang & Cole, 1997; Zhou & Cook, 2000). It is also possible for positive

ions to be formed through corona discharge in neutral or basic solutions of high ionic strength (Zhou & Cook, 2000).

Further complications in attempting to establish a correlation between solution pH and analyte pK_a result because the pH of the electrospray droplets may be considerably different from that of the bulk solution. In some situations, the generation of protons through the electrolytic oxidation of water in positive ion ESI can decrease the droplet pH by as much as four pH units (Van Berkel, Zhou, & Aronson, 1997). Furthermore, the aforementioned uneven fissioning of mass and charge from parent droplets to offspring droplets may cause the offspring droplets (the major source of ions) to be significantly enriched in protons as compared to the parents (Kearle & Ho, 1997).

D. Improving ESI Response Through Derivatization

In the previous sections, we have outlined the characteristics that make an analyte amenable to analysis by ESI-MS. The analyte must already be in its ionic form in the solution phase, or be chargeable through adduct formation, electrochemical reactions, or gas-phase reactions. Furthermore, analytes most responsive to ESI analysis will have significant nonpolar regions, which give them a high affinity for the surface of the ESI droplet. Derivatization reactions enhance the ESI response of an analyte, either by making it more easily charged (Colton, Traeger, & Harvey, 1992; Hiraoka et al., 1992; Wilson & Wu, 1993; Van Berkel & Asano, 1994; Matsuura & Takashina, 1998; Van Berkel et al., 1998; Van Berkel, Quirke, & Adams, 2000) or by increasing its surface activity (Che et al., 1999; Waterval et al., 2000; Cech, Krone, & Enke, 2001b).

Most derivatization reactions for ESI introduce chargeable groups onto neutral molecules. For example, Van Berkel and coworkers have shown that neutral organic molecules such as alkenes, alkynes (Van Berkel, Quirke, & Adams, 2000), aromatics (Van Berkel & Asano, 1994), alkyl halides, alcohols, phenols, thiols, and amines (Quirke, Adams, & Van Berkel, 1994) can be derivatized by the addition of various functional groups. These functional groups facilitate ion formation through sodium adduct formation or protonation. An alternative method of derivatization that facilitates charging involves introducing electrochemically reactive functional groups into the molecular structure (Van Berkel et al., 1998). Ionization is accomplished for the derivatives through electrochemical oxidation or reduction. An example of this approach is shown in Figure 10, where a non-responsive analyte (cholesterol) has been converted to its ferrocenecarbamate ester. The molecular ion of the derivitized cholesterol can be clearly seen at an m/z value of 613 (Van Berkel et al., 1998).

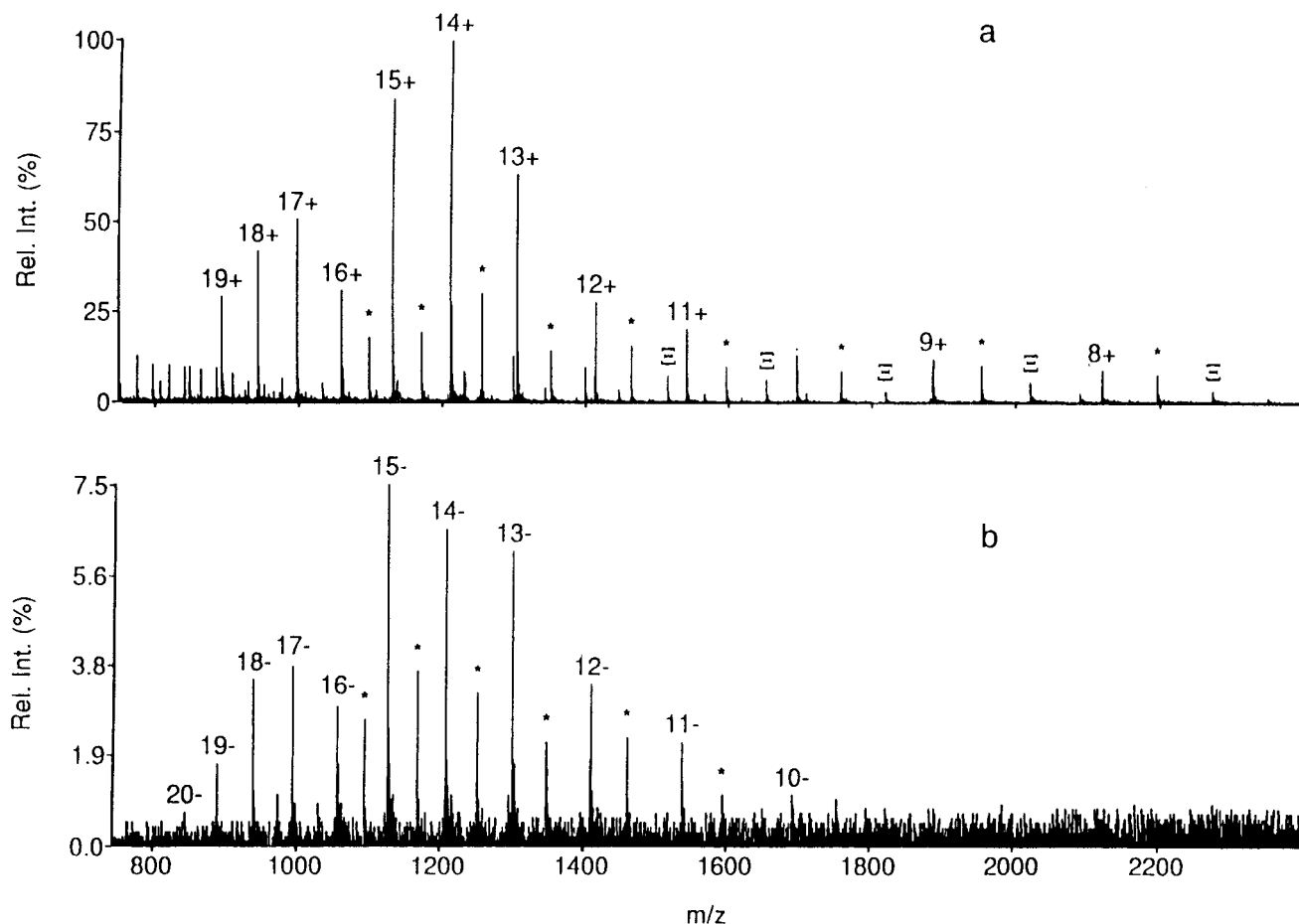


FIGURE 9. ESI mass spectra of equine myoglobin in 50% methanol, 50% water solution at pH 10. Spectrum (a) was collected in the positive ion mode and spectrum (b) in the negative ion mode. Despite the fact that the solution pH is well above the pK_a 's of the basic amino acids, protonated protein ions are clearly observed [Reprinted from Kelly et al., *Org Mass Spectrom*, 1992, 27:1143–1147 with permission of John Wiley & Sons, Ltd.].

Nonpolar groups can be added to non-responsive analytes to increase their droplet surface affinity and enhance their ESI response. For example, oligosaccharides, which are quite hydrophilic in their free form, are not particularly well suited for conventional ESI analysis (Burlingame, 1996). However, when they are modified by reductive amination with hydrophobic species prior to analysis by ESI, significant improvements in response can be accomplished (Okamoto, Takahashi, & Doi, 1995; Yoshino et al., 1995). Okamoto et al. demonstrated a 5,000-fold improvement in the ESI response of the oligosaccharide maltopentaose by reacting it with the derivatizing agent trimethyl-(*p*-aminophenyl)ammonium (TMPA). It is worth noting that this derivatizing agent increases the proton affinity and the hydrophobicity of the oligosaccharide; thus, the improvement in ESI responsiveness is due to a combination of these effects. The

use of derivatizing agents that only increased the hydrophobicity and not the proton affinity of the oligosaccharide only resulted in an increase in ESI response of approximately 500-fold (Okamoto, Takahashi, & Doi, 1995).

Most of the literature in ESI has focused on making small modifications in analyte structure to improve ESI response. An alternative approach is to bind a small organic analyte with a much larger molecule such as a peptide (Cech, Krone, & Enke, 2001b). This approach significantly improves the responsiveness of small organic molecules because peptides, which can be multiply charged and can contain many nonpolar amino acid side chains, are highly responsive to analysis by ESI. Furthermore, the signal-to-noise ratio for the analyte can be improved because the mass of the adduct is shifted out of the low-mass region of the mass spectrum—a region that

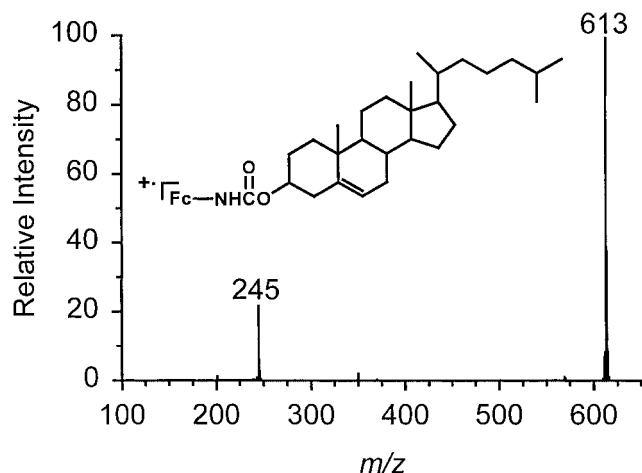


FIGURE 10. ESI mass spectrum of a ferrocenecarbamate ester of cholesterol. Cholesterol alone is non-responsive to ESI analysis, but this derivative is ionized electrochemically by ESI, and the derivatized molecular ion at m/z 613 (corresponding to the mass of the cholesterol + 277) can clearly be seen. The smaller peak at m/z 245 corresponds to a fragment (ferrocenecarbamic acid) of the parent ion, which is formed in the interface region of the ion trap mass spectrometer. [Reprinted from Van Berkel et al., *Anal Chem*, 1998, 70:1544 with permission of American Chemical Society].

is typically complicated by the presence of solvent clusters and other contaminants. An example of that approach is demonstrated in Figure 11, which shows the ESI mass spectrum of 1,3-butadiene diepoxide bound to the peptide

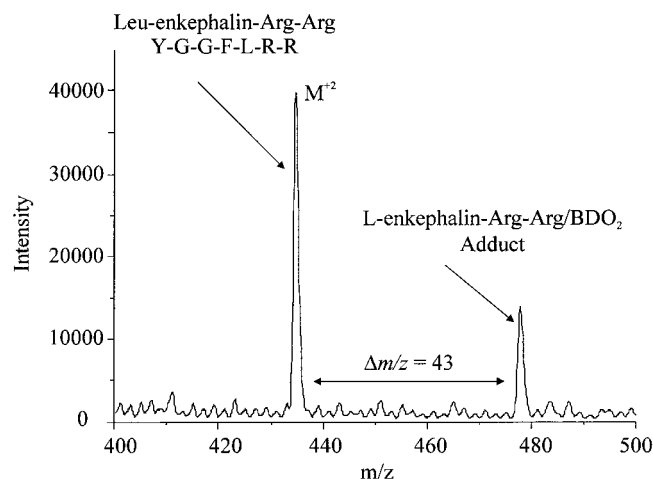


FIGURE 11. Mass spectrum of Leu-Enkephalin-Arg-Arg and the adduct of this peptide with 1,3-butadiene diepoxide (BDO_2). The adduct appears at a mass shift of 43, half the molecular weight of BDO_2 , because the peptide it reacts with is doubly charged [Reprinted from Cech, Krone and Enke, *Rapid Commun Mass Spectrom*, 2001, 15:1040–1044 with permission of John Wiley & Sons, Ltd.].

Leu-enkephalin-Arg-Arg (Cech, Krone, & Enke, 2001b). The epoxide is completely non-responsive to ESI analysis alone, but the peptide-bound adduct can be clearly observed at the characteristic shift of 43 in m/z .

IV. THE WORKING CURVE AND DYNAMIC RANGE

Ideally, the working curve obtained when quantitative analysis is performed with ESI is linear over three to four orders of magnitude. Such a curve is demonstrated in Figure 12. Deviations from linearity occurs at high concentrations (usually around 10^{-5} M (Tang & Kebarle, 1993; Zook & Bruins, 1997; Cech & Enke, 2000)) as response becomes saturated, and at low concentrations due to background interference. In the next section, we discuss the potential sources of limitation at the high and low ends of the ESI working curve, and present some potential methods to improve the linear dynamic range of ESI analysis.

A. Detection Limits With ESI

1. Background Interferences

Chemical noise is a significant source of background interference in ESI mass spectra (Ramsey, Goeringer, &

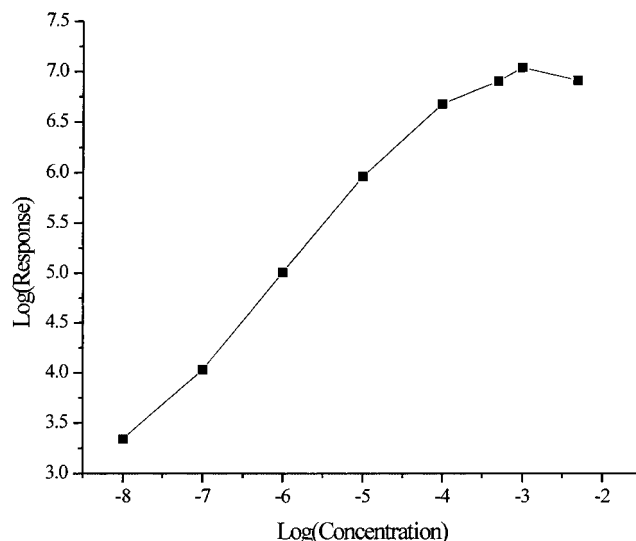


FIGURE 12. Typical ESI calibration curve generated on a TSQ 7000 triple quadrupole mass spectrometer. The analyte is the surface-active trimethyldecylammonium cation, dissolved in a solution of 50% water, 50% methanol, 0.5% acetic acid. Deviations in linearity are observed at the low end due to background interference, and at the high end due to saturation in ESI response.

McLuckey, 1993; Purves, Gabryelske, & Li, 1998; Guevremont et al., 2000). This chemical noise is fixed-pattern noise, manifested at specific m/z ratios and resulting from the mass analysis of charged species other than the analyte. Just like the analyte, the interfering species must become charged by one of the mechanisms discussed earlier. Thus, interferences are either ions or salt adducts in the electrospray solution, species generated electrochemically, or neutral species present in the atmosphere around the ESI spray that are charged in the gas phase by proton transfer.

Protonated, sodiated, or ammoniated solvent clusters are often observed in ESI mass spectra, and tend to be a particularly prevalent source of interference at low masses. Because the solvent is more volatile than the ESI analyte, it is usually possible to reduce the mass spectral response of solvent clusters by heating the electrospray droplets before they enter into the high vacuum region of the mass spectrometer. Heating can be accomplished with a heated capillary interface and/or a heated nitrogen bath gas (Bruins, 1997). It has the additional benefit of decreasing the abundance of solvent-analyte clusters, and thus further simplifying the mass spectrum.

2. Random Noise

An observation of a mass spectrum obtained with ESI-MS reveals some sort of background noise at virtually every m/z value. This ubiquity of noise throughout the mass spectrum suggests that there must be a source of random noise in ESI-MS; that is, noise for which there is an equal probability of occurrence at every m/z value. The sources of random noise in ESI-MS have yet to be determined. We speculate that one possible source of random noise is that a fraction of the ions produced in the ESI process may reach the detector without going through the mass analysis process. For these ions, mass spectral response would not be a function of m/z values, because they would arrive at the detector at times that are not correlated to the m/z setting of the mass analyzer. However, the mechanism by which ions would reach the detector without undergoing mass selection is not clear, and may differ, depending on the type of mass analyzer used (i.e., ion trap versus triple quadrupole, etc.).

Another potential source of random noise is the passage of large, highly charged clusters of analyte and solvent through the mass analyzer. Under conventional ESI conditions, some ESI droplets may not desolvate completely, and thus highly charged solvent/analyte/counterion clusters may be formed (Mann, Fenn, & Wong, 1990). These clusters might impact the deflector plate on the detector and create a shower of fragments with various m/z values (Voyksner, 2001).

3. Ion Transmission and Sensitivity

Although background noise is often the limiting factor in determining the lower limit of the linear dynamic range with ESI, sensitivity, which is defined as the slope of the working curve, is also important. Sensitivity in ESI may be determined by one of two factors, the efficiency by which molecules are converted into gas-phase ions and the efficiency by which these gas-phase ions are transferred through the various stages of the mass spectrometer and detected. The fraction of the ions produced that are actually analyzed will depend not only on the quality of the ion transfer optics (Shaffer et al., 1999) in the mass spectrometer, but also on the type of mass analyzer and the way in which it is used. For example, ion trap instruments provide full-spectrum analysis with higher sensitivity than quadrupole instruments (Voyksner, 1996) because they employ batch processing, but a quadrupole instrument operated in selected ion monitoring (SIM) mode may have the highest sensitivity for a single analyte. In general, higher sensitivity in an ESI analysis is better. Higher sensitivity analyses require fewer scans to be averaged in order to obtain a clean spectrum. Thus, less time is needed in order to accomplish the analysis, and less sample is consumed. In situations where chemical noise is not limiting, improvements in sensitivity will also result in improvements in detection limit.

The issue of just how efficient the ESI process is at creating gas-phase ions is still somewhat under debate. It is generally accepted throughout the literature that the limiting factor in sensitivity with ESI is not the ionization process itself but the transfer of the ions through the mass spectrometer (Mann, Fenn, & Wong, 1990; Zook & Bruins, 1997; Shaffer et al., 1999). This ion transfer process can result in an ion loss on the order of four to five orders of magnitude (Mann, Fenn, & Wong, 1990; Zook & Bruins, 1997). However, this conclusion is inherently difficult to support, because ions cannot be measured directly as they leave ESI droplets.

Smith et al. agree that ion transmission limits the sensitivity of ESI, and have sought to improve the efficiency of ion transfer from the source to the mass analyzer of triple quadrupole mass spectrometers. Their innovations for improved ion transmission include an "ion funnel", which consists of multiple ring electrodes of decreasing diameter (Shaffer et al., 1999; Belov et al., 2000; Kim et al., 2000a; Kim, Udseth, & Smith, 2000b), and a multi-capillary inlet (Kim, Udseth, & Smith, 2000b), a device similar to Thermo Finnigan's heated capillary, but with multiple inlets rather than only one. With the combination of the multi-capillary inlet and the ion funnel, Kim et al. have been able to accomplish ion transmission 23-times higher than that with conventional ESI ion optics (Kim, Udseth, & Smith, 2000b). Using this

configuration, Kim et al. report the ability to detect 3–4% of the ions originally produced from the ESI solution; that detection efficiency is comparable to that reported for nanospray analysis (Wilm & Mann, 1996; Geremanos, Freckleton, & Tempst, 2000).

Another route to improve the sensitivity of ESI-MS may be to improve the efficiency of charging ESI analytes. One method to improve efficiency is to implement multiple ESI sprayers rather than just one. In their initial experiments using multiple sprayers to accomplish ESI, Kostiainen and Bruins showed that the upper limit of the dynamic range in ESI is limited not by the mass of analyte (the amount) but by its concentration. They observed that the upper limit in linearity of ESI calibration curves is reached at the same analyte concentration, even if two ESI spray capillaries are used simultaneously to produce charged droplets for mass analysis (Kostiainen & Bruins, 1994). Making use of this fact, Tang et al. have developed a microarray of electrospray emitters that enables multiple stable Taylor cones, all emitting droplets, to be generated simultaneously (Fig. 13). The use of this microspray emitter facilitates operation at higher flow rates and higher spray currents, and a 2–3 fold increase in sensitivity as compared to single spray electrospray has been demonstrated (Tang et al., 2001).

B. Sources of Signal Saturation at High Concentrations

1. Limited Amount of Excess Charge

Several theories have been proposed for why ESI response is limited at high concentrations. Kebarle and Tang originally suggested a limitation in ESI response at high concentrations to be a result of an upper limit in the amount of analyte that can be charged in the ESI process (Tang & Kebarle, 1993a,b). Enke expanded on this assumption, pointing out that, because a fixed amount of excess charge is available on ESI droplets at the point at which the analyte concentration exceeds the excess charge concentration, its ESI response should level off (Enke, 1997). However, only in situations where the charging of analyte is completely efficient will the upper limit in the calibration curve correspond exactly with the point at which the analyte concentration exceeds the concentration of excess charge. If all of the analyte is not being charged, then presumably the ESI response can increase even after the analytical concentration of analyte exceeds the concentration of excess charge. Nonetheless, if no other factors were involved, then one would expect to see the saturation in the ESI response due to charge limitation occur at some point after the analyte concentration becomes greater than the excess charge concentration.



FIGURE 13. Photograph of nine electrospray Taylor cones on a microfabricated emitter array. Charging of the ESI solution is more efficient with multiple Taylor cones than with just one [Reprinted from Tang et al., *Anal Chem*, 2001, 73:1568–1663 with permission of American Chemical Society].

Bruins et al. performed experiments that led them to conclude that saturation in the ESI response is not a result of charge limitation (Kostiainen & Bruins, 1994, 1996; Bruins, 1997; Zook & Bruins, 1997). They observed that this saturation occurred at approximately the same analyte concentration regardless of the electrolyte concentration and applied voltage. We have made similar observations that, despite changes in flow rate or applied voltage, which should affect the concentration of excess charge on droplet surfaces, approximately the same saturation point is observed in ESI calibration curves generated for surface-active organic molecules. However, it is worth mentioning that, because all of the experimental parameters affect the stability of ESI operation, it is difficult to change one (i.e., applied voltage) without changing others (i.e., flow rate). Furthermore, changes in applied voltage that are significant enough to profoundly affect the available amount of excess charge are difficult to accomplish while still maintaining stable ESI operation.

2. Limited Space on Droplet Surfaces

An alternative reason for response limitation at high concentrations was proposed by Bruins and coworkers (Kostiainen & Bruins, 1994, 1996; Bruins, 1997; Zook & Bruins, 1997). They suggested that, at high analyte concentrations, the ESI droplets become so saturated with analyte that their surfaces are completely filled, and that ion ejection becomes inhibited because ions are trapped within the droplet interiors. In support of this hypothesis, Bruins presents calculations that compare the surface area of ESI

droplets with the area taken up by small organic molecules that might reside on their surfaces. These calculations show that, in theory, when 10^{-5} M solutions are used, the droplet surface could be completely covered after several fissioning steps (Bruins, 1997). Experimental evidence does indeed show that proton-bound dimers (clusters of ions with neutral ion/counterion pairs) are observed in ESI at high analyte concentrations but not at lower concentrations (Zook & Bruins, 1997). Because these proton-bound dimers could be caused by increased molecule-molecule interactions on the droplet surface, the fact that they are observed primarily at high concentrations may indeed indicate that saturation of the droplet surface is taking place.

3. Suppression and Competition at High Concentrations

When operating in the saturation region of the ESI calibration curve, the responses of some analytes will be suppressed. This suppression effect was demonstrated by Tang & Kebarle (1993), who showed that, at high concentrations of ammonia, the response of the cesium cation (Cs^+) was suppressed ten-times more than that of the surface-active tetrabutyl ammonium cation (TBA^+). They explained this observation based upon the fact that Cs^+ , being more solvated, is expected to evaporate more slowly from the ESI droplet than TBA^+ . Experiments performed in our laboratory have shown that the response of solvophilic analytes (analytes that prefer to be solvated) can be suppressed drastically in the presence of very high concentrations of surface-active analytes, but that the response of the surface-active analytes is not affected by high concentrations of solvophilic analytes (Cech & Enke, 2000). Similarly, in studying factors that affect suppression in the ESI response, Bonfiglio et al. (1999) concluded that analyte characteristics were of key importance, citing the fact that they observed their most polar analyte to undergo the most suppression in its ESI response.

Our results, as well as those of Bonfiglio and Tang, could be explained based upon a competition for a limited resource, either space or charge. Surface-active analytes would be expected to out-compete polar analytes for the limited excess charge and/or space on the droplet surface. Thus, in the suppression region of the ESI calibration curve, surface-active analytes should suppress the response of more polar analytes (Cech & Enke, 2000). Regardless of the mechanism for suppression, it is important to keep in mind that in the high concentration range, competition and suppression will occur with solutions that contain multiple analytes. For this reason, diluting the sample, performing chromatographic separations, or eliminating any solvent additives responsible for suppression may be necessary to accomplish successful analyses.

C. Improving the Detection Limit and Linear Dynamic Range

1. Extending to Higher Concentrations

Extension in the high end of the linear dynamic range could be highly advantageous for improved quantitative analysis with ESI-MS. The difficulty here lies in the fact that the reason for a limitation in the ESI response at high concentrations is still not fully understood. In actuality, this limitation in response is most likely due to a combination between instrumental factors and perhaps some fundamental limitation in the ability to produce a charged analyte in the ESI process. Thus, possible ways to extend the linear dynamic range to higher concentrations might include a more efficient charging of gas-phase analyte molecules, evaporating ESI droplets more effectively, or improving ion transmission through the mass spectrometer. However, instrument companies and researchers have explored many avenues to improve the efficiency of ESI and, as yet, have had little effect on the upper limit of the linear dynamic range. The creation of smaller-diameter droplets through nanospray techniques (Wilm & Mann, 1996; Karas, Bahr, & Dulcks, 2000) and the use of various pneumatic (Hopfgartner et al., 1993; Kostianen & Bruins, 1996) or piezoelectric (Hager et al., 1994) nebulization techniques and heated bath gases have all failed to extend the limits in the linear dynamic range much above analyte concentrations of approximately 10^{-5} M for surface-active analytes.

2. Extending to Lower Concentrations

There is keen interest within the ESI community to extend the capabilities of ESI-MS to allow for the analysis of lower analyte concentrations. In many types of biological analysis, sample size can be severely limited, and thus better detection limits with ESI-MS would be highly advantageous. We have already discussed methods that have been employed to improve the sensitivity of ESI-MS. However, when a detection limit is limited by chemical interference, improvements in sensitivity will have little effect on the lowest analyte concentration that can be detected. In order to improve the detection limit (in terms of analyte concentration), it is necessary to eliminate the chemical noise by improving the *selectivity* of the analysis; i.e., eliminating the chemical noise. When noise is no longer limiting, increases in sensitivity will improve the detection limit.

When a triple-quadrupole mass spectrometer is used, selected-reaction monitoring can be employed to improve the selectivity of the analysis. Selected-reaction monitoring is accomplished through MS-MS analysis, such that only ions that produce a particular fragment ion are selected. In this way, interfering ions that have the same

m/z ratio as the precursor ion, but do form the same fragment ions, are eliminated. Most quantitative analysis experiments with ESI-MS that require very low detection limits are accomplished with selected reaction monitoring. However, this technique only works for targeted analysis, in which the m/z ratio of the precursor ion is known. To identify an unknown species, the entire mass spectrum must be scanned, and the ability to eliminate interferences and thus facilitate detection of species of interest in this process is key.

In attempting to improve the capabilities for analysis with ESI-MS in the low-concentration range of the calibration curve, we confront the same issues as in the high-concentration range of the calibration curve. The sources of the limitation, in this case random and fixed pattern noise, are still not completely understood. Ultimately, a better understanding of the factors that contribute to noise in ESI mass spectra may be the key to improving detection limits. For this reason, continued studies into the sources of noise in ESI are highly relevant.

V. INSTRUMENTAL PARAMETERS AND STABILITY

Microscopic inspection indicates that the ESI spray is stable when the Taylor cone has a constant shape, and when there is a steady stream of droplets issuing from it (Rollgen & Juraschek, 1998) (the cone-jet mode of operation) (Cloupeau & Prunet-Foch, 1989). Unstable operation is characterized by the formation of blobs of liquid at the tip or by multiple and gyrating jets of liquid emanating from it (Rollgen & Juraschek, 1998). In general, ESI operators prefer to operate in the stable cone jet mode, where the ESI current is the most reproducible. Several variables that affect the stability of ESI operation and the ability to achieve stable and effective spray conditions include the applied voltage (V_{app}), the nebulizing gas flow rate, the distance between the spray capillary and the counter electrode, and the solution flow rate (Γ), viscosity, and dielectric constant (De La Mora & Loscertales, 1994; Chen & Pui, 1997). The results described below show that the spray is stable when a steady cone can be observed, when there are no spikes or pulses of current in the external circuit, and when operating in the constant current section of the ESI gap's current–voltage curve. Measurements of the current–voltage curve have been useful in demonstrating why stable sprays are difficult or impossible to obtain with certain solvents and operating conditions.

A. Current–Voltage Curves

The electrical characteristics of any device (excluding capacitance and inductance) can be defined by its current–voltage curve, which plots the current through the device

(Y axis) as a function of the voltage across it (X axis). The electrospray gap has been characterized as a constant-current device (Van Berkel & Zhou, 1995). The current–voltage curve for a constant-current device is a horizontal line, which indicates a current that is independent of the voltage across the device. This constant current model has been tested by the experimental determination of the current–voltage curve (Jackson & Enke, 1999).

A typical current–voltage curve for a 100 μm stainless steel capillary with a solution of 0.5% acetic acid in methanol is shown in Figure 14 (solid line with square data points). The voltage plotted is the voltage across the gap (V_{gap}). The approximate shape of this curve is that of a step with a relatively steep rise in current at the lower voltage, a relatively constant current in the middle, and a steep rise in current at the higher voltages. The three regions of the current–voltage curve correspond to three modes of ESI operation. Only in the relatively constant current region do we observe a stable Taylor cone and an orderly generation of charged droplets. In the higher current region, multiple cones and jets appear and the droplet ejection is in rapidly changing off-axis directions. In the low voltage, low current region, the Taylor cone forms briefly, ejects a droplet stream, and collapses again in relatively reproducible cycles (Fig. 15).

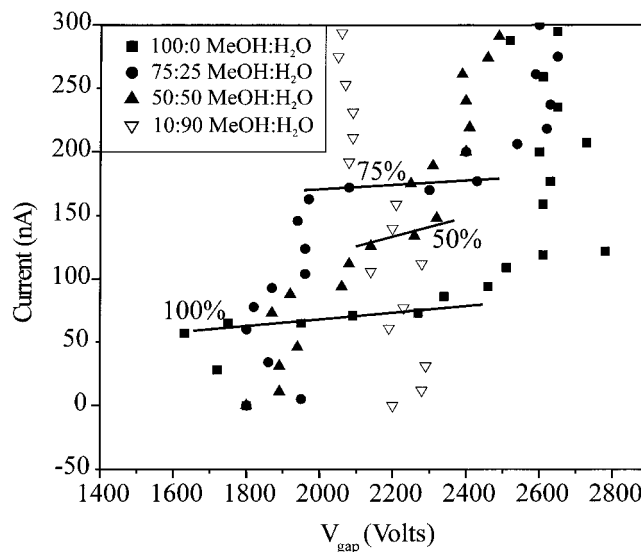


FIGURE 14. Current–voltage curves generated for acidified solutions that contain various percentages of methanol. The lines are drawn through the constant-current regions of the current–voltage diagrams for 100, 75, and 50% methanol solutions. The constant-current region for the 100% methanol solution spans approximately 800 V, whereas for the 90% water solution it is non-existent. This difference demonstrates the fact that no applied voltage will allow access to the stable cone-jet mode of operation in highly aqueous solutions.

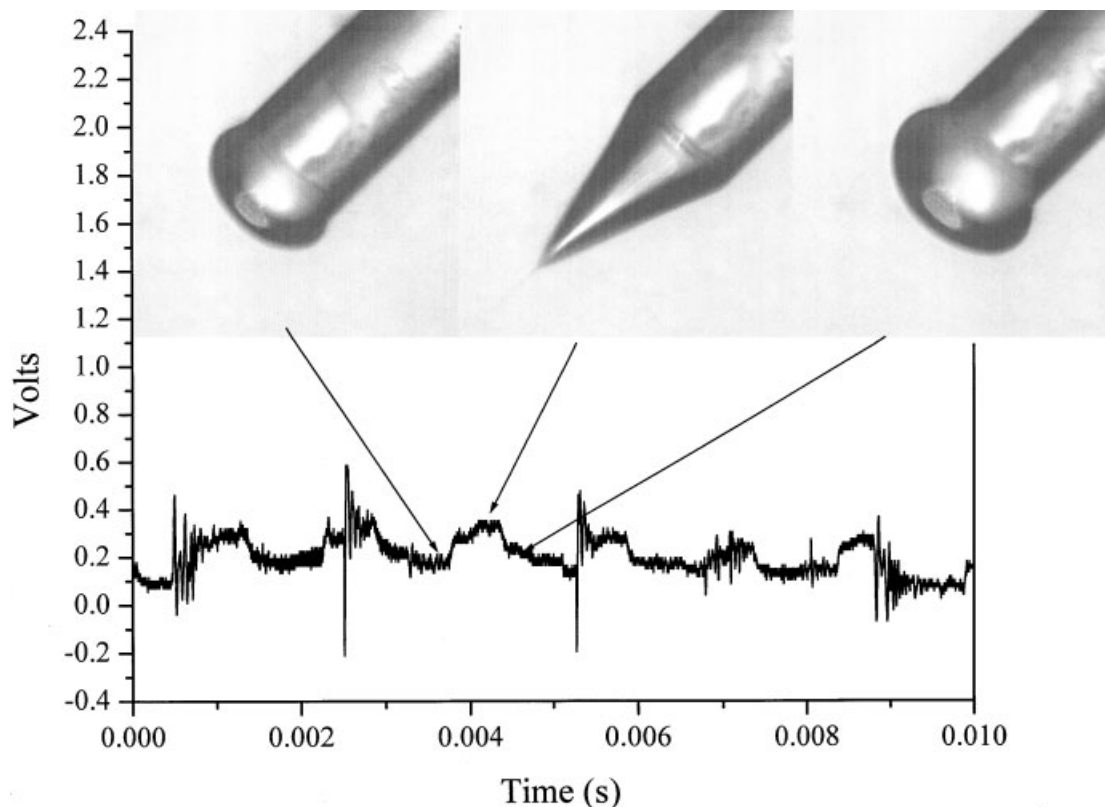


FIGURE 15. Oscillogram and photographs obtained when operating in the pulsed cone jet mode with a solution of 50% water, 50% methanol, 0.5% acetic acid. Periodic pulsations in the current give rise to peaks in the voltage–time curve. The current is observed to increase as the volume of the meniscus increases, and to maximize when the surface tension of the liquid is overcome and a jet of liquid is ejected. The current then decreases as the liquid relaxes back into a meniscus. This process repeats itself at regular intervals (on the Hz time scale).

Another aspect of stable operation is the maintenance of a steady current in the external circuit. Charge must flow in the external circuit when new solution surface is being charged at the capillary tip. If there is a steady stream of newly charged droplets emitted from the tip, then the current in the external circuit will be steady. In the low-voltage, low-current region, the Taylor cone forms, emits droplets, and collapses. This process gives rise to pulsations in the current, which occur during the elongation of the solution at the capillary tip (Rollgen & Juraschek, 1998; Amad, Cech, & Enke, 2001), as demonstrated with the oscillogram that accompanies the photographs in Figure 15. Current spikes are also associated with the high-voltage, high-current mode of operation. It has been suggested that a pulse detector in the external circuit could be used to determine when the ESI was operating in the stable mode (Amad, Cech, & Enke, 2000).

B. Effect of Instrumental Parameters on the Current–Voltage Curve

It is well known that it is difficult or impossible to obtain a stable spray in an aqueous solution without the addition of methanol or the use of pneumatic assistance. Observation of the current–voltage curve of the spray gap explains why this stable aqueous spray is so difficult to obtain. Figure 14 shows the current–voltage curve for four solvent mixtures that range from pure methanol to 90% water. As the volume fraction of water increases, the constant current region where the stable spray is observed becomes increasingly narrow and shifts to higher voltages. This shift to higher voltages makes sense, when one considers that it is necessary to balance the applied voltage with the solution surface tension in order to maintain a stable cone-jet operation. As the aqueous content (and thus the surface tension) of the ESI solution increases, it is necessary to apply a higher

voltage in order to access the cone jet mode. Unfortunately, the necessity of using higher applied voltages with solutions that have a higher aqueous content increases the likelihood of electrochemical discharge.

With a solution of 90% water, the stable region in the current–voltage curve disappears entirely (Fig. 14). The stable region of the current curve also disappears when very high solution flow rates are used. This disappearance of the stable region demonstrates the fact that, under certain experimental conditions, it is impossible to achieve stable cone jet operation at *any* applied voltage (Amad, Cech, & Enke, 2000). It is for this reason that pneumatic nebulization, which decreases the reliance on cone-jet operation for droplet formation, is necessary when operating at high flow rates or with solutions of high surface tension.

C. Self-Stabilizing Operation

Given the fact that stable ESI operation depends on the ability to balance so many variables, maintaining stable operation when solution conditions are changing (as with gradient elution liquid chromatographic introduction) can be difficult. Several solutions to the difficulty of maintaining a stable spray have been developed. If the analyte solution composition is changing or if the chromatographic effluent is not suitable for ESI, then a sheath solution is provided concentrically at, or just before, the capillary tip (Huber & Krajete, 2000; Matz et al., 2001). Alternately, the ESI process can be allowed some additional degrees of freedom so that self-adjustment is possible. For example, if the ESI process is allowed to set the solution flow rate, then it will automatically be the correct value. This self-adjustment of flow rate can be accomplished by filling (with the sample solution) a length of tubing that leads to the capillary. The back end of the tube is left open and no force is applied to the solution. The ESI process pulls the solution to the tip at the appropriate rate. This process is the typical method by which nanospray is accomplished (Wilm & Mann, 1996).

Another way to facilitate self-adjustment is to let the ESI process have some control over the voltage of the gap. A large series resistor ($\sim 10\text{ G}\Omega$) placed between the power supply and the contact to the ESI solution accomplishes this goal. With this resistor, the voltage across the ESI gap is the difference between the applied voltage and the iR drop through the resistance. If the charge generation rate decreases, then the iR drop across the series resistor decreases, and the gap voltage increases to correct the change. We have found significantly increased stability and a decreased tendency for arcing through the inclusion of this resistor in the circuit (Jackson & Enke, 1999; Amad, Cech, & Enke, 2000).

D. Non-Conductive vs. Conductive Spray Capillaries

The ESI capillary tip may be made of conducting or non-conducting material. Examples of conducting capillaries are the stainless steel hypodermic needles and metallized glass capillaries. Non-conducting capillaries are generally made of microbore fused silica tubing, which can be ground, etched, or pulled into a tip of the desired outer and inner diameter. A much smaller inner diameter is practical with glass capillary than with stainless steel. As already discussed, the smaller the inner diameter, the lower the flow rate that can be used. Because the rate of excess charge production is not significantly reduced, the concentration of excess charge is larger. In theory, this increase in excess charge concentration could increase dynamic range and reduce interferences among analytes.

Our microscopic examination of the electrospray process indicates that the liquid protruding from the electrospray capillary expands to form a Taylor cone with its base supported by the outer walls of the spray capillary. Consequently, it is the outer diameter of the capillary rather than its inner diameter that determines the diameter of the Taylor cone. Therefore, for the production of the smallest droplets, it is desirable to have a very small bore and a thin wall at the capillary tip. This combination is accomplished with microspray and nanospray sources, especially when pulled glass capillaries are used. The disadvantage to using these very small capillaries is their fragile nature and tendency to clog easily (Karas, Bahr, & Dulcks, 2000).

Another difference between conducting and non-conducting capillaries is the location of the power supply connection relative to the capillary tip. When a conductive capillary is used, the electrochemical reaction occurs very close to the tip end of the capillary. For non-conducting capillaries, a metallic union or inserted wire some distance from the spray capillary tip provides the contact between the power supply and the solution. The portion of this contact closest to the tip is the site of the electrochemical reaction. As explained in the introduction, the extent of oxidation (for positive ion formation) or reduction (for negative ion formation) is equal to the total amount of excess charge. The electrochemical reaction at this contact necessarily produces excess charge at the exact rate at which the charge separation occurs at the capillary tip. Consequently, the solution volume between the emitter contact and the capillary tip must be charged. This charged volume is very small in the case of conductive capillary. However, with a remote contact, the charged solution volume and length can be significant.

One can now consider how the excess charge will be conducted from the site of its creation (the contact) to the site of its separation (the tip). One possible mechanism is

the flow of the solution itself. If the solution contains the charge such that the velocity of charge flow is the same as the velocity of solution flow, then the conduction mechanism is completely satisfied. With such a scenario, the analytical solution need not be conducting because the conductivity of the excess charge is provided entirely by the solution flow.

Some experiments suggest that there are conditions under which solution conductivity is necessary (Jackson & Enke, 1999). The excess charge in the solution volume between the contact and the tip will be forced to the outside walls of its container. These outside walls are the walls of the capillary. For laminar flow, the velocity of a solution along the walls of a capillary through which it flows is less than the average solution velocity because of the friction between the capillary walls and the solution. This difference in velocities would cause the velocity of the charge to be less than the solution velocity. This result would require that the difference between the charge flow rate and the solution flow rate be made up by ionic conduction through the solution or by electroosmotic flow along the capillary walls. In either case, an electrical field gradient between the contact and the capillary tip would be required to sustain this process, and an electrolytic solution would be required.

In summary, the use of conductive capillaries provides the least complicated contact to the solution and the shortest time for the electrolytic products to react with the analyte solution. On the other hand, the use of disposable, pulled-glass capillary tips is very attractive because of the potential for small inner diameters and the decreased tendency of arcing and electrical discharge. Remote contact may increase the requirement for electrolyte in the analytical solution and increase the applied voltage required because of the voltage drop through the solution between the contact and the tip.

VI. SOLUTION CHARACTERISTICS

The ideal solvent composition for ESI analysis varies, depending on the application. Analysis in the positive ion mode requires different solvent characteristics than analysis in the negative ion mode, and the response of a given analyte can be enhanced or suppressed in different solvent systems. Here, we discuss which ESI solvents are ideal for analysis in the positive and negative ion modes. It is important to keep in mind that it may be necessary to deviate from this ideal in order to maintain non-covalent interactions and protein conformation, or to interface with LC gradients. However, deviations from the ideal solvent composition may result in significant compromises in ESI performance.

A. The Ideal ESI Solvent

As mentioned previously, the flow rate, applied voltage, conductivity, and liquid surface tension must be properly balanced in order to accomplish the formation of a stable ESI spray. Thus, an important characteristic of the ideal ESI solvent is that its surface tension is within the range that facilitates the generation of a stable spray. It is generally easy to create a stable spray in the positive ion mode with conductive solutions that have at least 50% of a moderately polar organic solvent such as methanol or acetonitrile, and with the rest of the solvent being aqueous. As the solution becomes more aqueous, its surface tension increases, and it becomes increasingly more difficult to adjust the ESI parameters such that a stable spray can be achieved (see the section on Current–Voltage Curves). On the other hand, it is also difficult to achieve stable electrospray operation with nonpolar liquids (Drozin, 1955) such as hexane or trichloromethane, due to their very low surface tension, high volatility, and low dielectric constant. These problems can be circumvented to some extent with the use of pneumatically assisted ESI. As mentioned earlier, the use of nebulization as an aid in droplet formation reduces the reliance on the stable Taylor cone in forming charged droplets, and thus facilitates the use of solvents whose surface tension and conductivity are not ideal. By allowing operation in more aqueous solvents and at higher flow rates, pneumatic assistance can be highly beneficial when interfacing ESI with HPLC. However, sensitivity in terms of response per mass of analyte may decrease due to a lower fraction of the analyte becoming vaporized.

The solvent composition in ESI analysis has important effects on the ESI mass spectrum. Firstly, background in the form of solvent clusters can be a significant source of chemical noise. The ideal ESI solvent wouldn't cluster to the analyte nor would it create significant amounts of background in the form of solvent clusters with added electrolyte. Analyte mass spectral response can also be dependent on solution composition. In many cases, the ESI response of typical organic analytes is higher in solutions with higher percentages of organic solvents (Zhou & Hamburger, 1995; Lu et al., 1996; Temesi & Law, 1999; Zook, Forsmo-Bruce, & Briem, 2000). This higher response may be a result of more efficient desolvation of the analyte when a more volatile solvent is used and of improved spray stability due to decreased surface tension. In some cases, however, organic content above about 80% can actually result in a decreased ESI response (Zhou & Hamburger, 1995). This decrease in response is likely due to the liquid surface tension being decreased below the ideal for maximum spray stability. Because spray dynamics are highly dependent on instrumental parameters, the percentage of organic content that maximizes ESI response will vary, depending on the parameters chosen.

As discussed in the Instrumental Parameters and Stability section, the conductivity of the ESI solution is important. ESI is generally performed with solutions that contain a significant concentration of some ionic species. The presence of ionic species is necessary for the process of charge separation at the tip. These species may be ionic analyte, added electrolyte (such as acetic acid or ammonium acetate), and/or charged products of the electrochemical reaction. Without a charge carrier to facilitate the charge-separation process, stable spray dynamics are not achieved, and the ion current obtained at the detector tends to fluctuate drastically.

B. Solvent Choice for Analysis in the Positive Ion Mode

The charge carrier in positive ion ESI can be a cation such as sodium, lithium, or ammonium, which can be added to the solution in the form of a neutral salt (such as ammonium acetate). Neutral salts are usually added to the solution to facilitate the analysis of polar, neutral analytes through adduct formation. However, positive ion ESI is most often performed with protonated solutions of acetonitrile/water or methanol/water. In this case, the charge is carried by protonated solvent clusters, which are created by reaction of the solvent with a weak acid (such as acetic, formic, or propionic acid) that is added to the solution. The low pH of acidic solutions has the distinct advantage of facilitating protonation of the analyte; thus, acidic solvents are ideal for the analysis of analytes such as proteins and organic molecules that have basic functional groups.

C. Solvent Choice for Analysis in the Negative Ion Mode

Intuitively, one might think that negative ion mode analysis could be accomplished by using the same solvents as those used in positive ion mode, but by making the solution basic instead of acidic. Early studies of ESI-MS in the negative ion mode did just that. Ammonium hydroxide was added to aqueous solutions to facilitate deprotonation of acidic analytes (Loo et al., 1992). Unfortunately, negative ion mode analysis with basic methanol or water solutions tends to be quite unstable, because stable anions are not formed to any appreciable extent, and thus no predominant species is created to carry the negative charge at low analyte concentrations. As a result, the use of methanolic or aqueous solutions that contain ammonium hydroxide for negative ion mode analysis generally yields poorer detection limits and less stability than can be accomplished with acidic solutions in the positive ion mode.

In order to accomplish more stable and successful analysis in the negative ion mode, it is necessary to use a solvent that creates stable anions. When halogenated solvents are used as electrospray solvents, they can form negative ions through electrochemical reduction. For example, chloride ions are created from chloroform when it is reduced at the electrical contact. Analyte anions are formed from this solution by proton abstraction or chloride attachment (Cole & Zhu, 1999; Zhu & Cole, 2000). Recently, a mixture of hexafluoroisopropanol and methanol, neutralized to pH 7, gave excellent results for the analysis of oligonucleotides in the negative ion mode (Apffel et al., 1997; Griffey et al., 1997; Huber & Krajete, 2000). The fluorinated solvent creates a very stable deprotonated anion that carries the negative charge at low analyte concentrations, and at neutral pH's, the negatively charged oligonucleotides are favored in solution and are thus observed in ESI mass spectra. Zhang et al. recommend the use of 10% 2,2,2-trifluoroethanol/90% methanol for negative ion ESI (Zhang, Cech, & Enke, 2002). This solvent produces the $\text{CF}_3\text{CH}_2\text{O}^-$ ethoxide ion and H_2 gas as a result of the inherent electrochemical reduction that occurs in the ESI process. The solvent spectrum for this system is exceptionally clean, and facilitates detection limits of around 10^{-8} M for fatty acids and 10^{-7} M for peptides. The 2,2,2-trifluoroethanol solvent system is also effective for the analysis of oligonucleotides without requiring neutralization to pH 7. The oligonucleotide charge states achieved with the 2,2,2-trifluoroethanol solvent are significantly higher than those achieved with hexafluoroisopropanol, likely due to the higher gas-phase proton affinity of 2,2,2-trifluoroethanol (Zhang, Cech, & Enke, 2002).

The tendency for the occurrence of corona discharge is another factor that makes negative ion ESI more challenging than positive ion ESI. Corona discharge (electrical discharge from the capillary tip (Kearle & Ho, 1997)) results in the chemical ionization of the gas-phase analyte and solvent molecules that are present in the space around the ESI spray capillary. An ESI mass spectrum obtained during corona discharge is often characterized by significant amounts of background (due to ionized solvent molecules) and poor stability. Furthermore, arcing tends to occur in the corona discharge mode. This arcing not only causes a loss in ESI current, but can also damage electronic components of the mass spectrometer. For these reasons, negative ion mode analysis is generally performed with electron-scavenging gases (Cole & Harrata, 1993; Straub & Voyksner, 1993) or with halogenated solvents (Cole & Harrata, 1992; Hiraoka et al., 1992; Cole & Harrata, 1993); both suppress corona discharge. The large series resistor discussed earlier also suppresses corona discharge to some extent, and eliminates arcing.

D. Compatibility Between ESI and Liquid Separation Techniques

The presence of non-volatile, ionic species such as phosphate and sulfate buffers in the ESI spray is deleterious. These non-volatile species cause salt deposits to form on the metal surfaces within the instrument and can result in a complete loss of ion transmission. Furthermore, the issue of ion-pairing has been presented as a potential problem with the use of inorganic buffers (Cole & Harrata, 1992, 1993; Hiraoka et al., 1992). If the anion or cation pairs too strongly with the analyte, then the analyte ions may be prevented from carrying the excess charge on the droplet surfaces; as a result, the ESI response for the analyte may be very low. For these reasons, when ESI is interfaced to HPLC or CE, volatile buffers composed of weak acids and bases are generally used in place of non-volatile buffers. Common choices of buffers include ammonium acetate and ammonium formate (Voyksner, 1997).

Strong acids, such as trifluoroacetic (TFA), heptafluorobutyric, and hydrochloric acid, which are used as ion-pairing agents in HPLC, also tend to mask the analyte signal in ESI-MS (Apffel et al., 1995; Kuhlmann et al., 1995; Temesi & Law, 1999). This masking of the response may be a result of the aforementioned ion-pairing effects; TFA (pK_a 0.23) is significantly more acidic than weak acids like acetic acid (pK_a 4.72), which don't cause the same signal suppression. Kuhlmann et al. supported the hypothesis that ion-pairing is responsible for the signal suppression observed with TFA by showing that, whereas the ion signal is severely suppressed for strongly basic analyte species (those that would be expected to pair most strongly with the TFA), weakly basic analytes are hardly affected.

In cases, where adequate chromatographic separation requires strongly acidic ion-pairing agents like TFA, it is possible to overcome the resulting signal suppression through post-column addition of a weak acid such as propionic acid (Apffel et al., 1995; Kuhlmann et al., 1995). Signal enhancement by as much as 50-times, as compared to that with just TFA, can be achieved with this technique. Kuhlmann et al. propose that this signal enhancement is a result of the volatile TFA (bp 72°C) evaporating quickly from the ESI droplets, and of the analyte/TFA pairs being replaced with analyte/propionic acid pairs, which are less tightly bound.

VII. SUMMARY

In this review, we have presented some important factors to be considered in performing analysis with ESI-MS. One consideration is the characteristics of the analyte. For an analyte to be suitable for analysis with ESI-MS, it must either exist as a preformed ion in solution or be chargeable

through protonation or deprotonation, adduct formation, or electrochemical oxidation or reduction. Analyte pK_a has less of an effect on ESI responsiveness than might be imagined, due to energy considerations in the ESI process and differences in pH between the ESI droplet and the bulk solution.

The structure of the analyte can also influence its ESI response. Analytes with significant nonpolar (hydrophobic) portions will generally have a higher electrospray response than more polar analytes. This higher response arises because hydrophobic analytes have higher affinities for electrospray droplet surfaces and, thus, tend to carry a greater fraction of the excess charge produced in the electrospray process.

Knowledge of the factors that affect ESI responsiveness can help the user to predict the suitability of a given analyte for analysis with ESI-MS. This knowledge can also provide insights into methods of improving the responsiveness of analytes poorly suited for the technique. When an analyte is poorly responsive to analysis with ESI-MS, its response can be improved through derivatization. Derivatization reactions are performed so that the derivatized analyte will have an increased chargeability, a greater nonpolar character, or a combination of both.

Sources of the limitation at the upper and lower ends of the calibration curve are important when performing quantitative analysis with ESI-MS. The detection limit in ESI is usually determined by the presence of chemical interference due to clusters of solvent molecules with ionic impurities. When chemical interference is not limiting, the sensitivity of the instrument may determine the detection limit. It is generally believed that ion transmission through the mass spectrometer is the key factor that governs sensitivity. Therefore, most research to improve sensitivity of ESI-MS focuses on improvements of source and instrument design for enhanced ion transmission.

There is still some uncertainty as to the source of saturation in the electrospray response at high concentrations. This saturation may arise due to inherent limitations in the number of ions that can be produced in the ESI process. An alternative explanation is that instrumental factors, such as transmission of ions from atmospheric pressure into the mass spectrometer, are limiting. Regardless of the mechanism of saturation, it is important to realize that competition effects are common when working in the saturation region of the calibration curve, and cause a suppression in response for some analytes.

The success of analysis with ESI-MS depends not only on the characteristics of the analyte and its concentration, but also on instrumental parameters. Stable ESI operation can be achieved by balancing the solution flow rate and applied voltage with the surface tension of the liquid. If the flow rate of the solution or its surface tension is too high, then it is impossible to accomplish

stable ESI operation. The balance of the parameters of voltage, flow rate, and surface tension can be facilitated with the use of self-stabilization, either by the addition of a large series resistor or by allowing the ESI process to set the solution flow rate.

Lastly, characteristics of the solvent used for the ESI analysis can greatly influence its success. For analysis in the positive ion mode, solutions of water with a high percentage of either methanol or acetonitrile are generally preferred. A volatile, weak acid, such as acetic acid, is added to this solvent to increase conductivity and to facilitate the protonation of the analyte. This solvent system has advantages of conductivity and surface tension that are compatible with ESI analysis, and low levels of background solvent clusters. Solvent choice for analysis in the negative ion mode is complicated by the fact that it is difficult to produce stable, negative ions to carry the excess negative charge at low analyte concentrations. Furthermore, corona discharge often occurs in the negative ion mode, the results of which are fluctuations in spray stability and background in the form of ionized solvent. The use of a solvent system that contains a small percentage of some halogenated solvent (such as chloroform, hexafluoroisopropanol, or 2,2,2-trifluoroethanol) has the dual benefits of suppressing corona discharge and facilitating the formation of stable negative ions through the electrochemical reduction inherent to the electrospray process.

When interfacing ESI-MS to HPLC, additional issues must be considered in solvent selection. It is necessary to modify separation protocols to eliminate non-volatile buffers and ion-pairing reagents, which may cause background interference and signal suppression. It is also often necessary to deviate from the ideal solvent composition and flow rate for ESI in order to accomplish successful separation of the analytes. If highly aqueous solvents or high flow rates are necessary for the separation, then pneumatically assisted ESI is employed to aid in droplet formation and desolvation.

This review presents some fundamental studies of the ESI-MS process that shed light on issues of interest to the ESI-MS user. However, this review is by no means the end of the story. Further research in ESI-MS fundamentals will undoubtedly lead to an improved understanding of the process. The results of such research may include improvements in quantitative capabilities, increases in the dynamic range and detection limit, and enhanced performance in the negative ion mode.

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REFERENCES

- Ackloo SZ, Smith RW, Terlouw JK, McCarry BE. 2000. Characterization of Ginseng Saponins using electrospray mass spectrometry and collision-induced dissociation experiments of metal-attachment ions. *Analyst* 125:591–597.
- Alomirah HF, Alli I, Konishi Y. 2000. Applications of mass spectrometry to food proteins and peptides. *J Chrom A* 893:1–21.
- Amad MH, Cech NB, Jackson GS, Enke CG. 2000. Importance of gas-phase proton affinities in determining the electrospray ionization response for analytes and solvents. *J Mass Spectrom* 35:784–789.
- Amad MH, Cech NB, Enke CG. 2000. Characterizing the constant current and constant voltage modes of electrospray ionization. 48th Conference on Mass Spectrometry and Allied Topics, June 2000, Long Beach, California.
- Apffel A, Fisher S, Goldberg G, Goodley PC, Kuhlmann FE. 1995. Enhanced sensitivity for peptide-mapping with electrospray liquid-chromatography mass-spectrometry in the presence of signal suppression due to trifluoroacetic acid-containing mobile phases. *J Chrom A* 712:177–190.
- Apffel A, Chakel JA, Fischer S, Lichtenwalter K, Hancock WS. 1997. Analysis of oligonucleotides by HPLC-electrospray ionization mass spectrometry. *Anal Chem* 69:1320–1325.
- Apffel A, Chakel J, Udiavar S, Swedberg S, Hancock WS, Souders C, Pungor E. 1998. Application of new analytical technology to the production of a “well-characterized biological”. *Dev Biol Stand* 96:11–25.
- Bahr U, Pfenninger A, Karas M, Stahl B. 1997. High-sensitivity analysis of neutral underivatized oligosaccharides by nano-electrospray mass spectrometry. *Anal Chem* 69:4530–4535.
- Belov ME, Gorshkov MV, Udseth HR, Anderson GA, Smith RD. 2000. Zeptomole-sensitivity electrospray ionization: Fourier transform ion cyclotron resonance mass spectrometry of proteins. *Anal Chem* 10:2271–2279.
- Bonfiglio R, King RC, Olah TV, Merkle K. 1999. The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds. *Rapid Commun in Mass Spectrom* 13:1175–1185.
- Bowers MT (Ed.). 1973. Gas phase ion chemistry. vol 2. New York: Academic Press. pp 1–14.
- Bruins AP. 1997. ESI source design and dynamic range considerations In: Cole RB (Ed.). *Electrospray ionization mass spectrometry*. New York: John Wiley and Sons, Inc. pp 107–135.
- Bruins AP, Henion JD, Covey TR. 1987. Ion spray interface for combined liquid chromatography/atmospheric pressure ionization mass spectrometry. *Anal Chem* 59:2642–2646.
- Burlingame AL. 1996. Characterization of protein glycosylation by mass spectrometry. *Curr Opin Biotechnol* 7:4–10.
- Cao P, Moini M. 1998. Capillary electrophoresis electrospray ionization high mass accuracy time-of-flight mass spectrometry for protein identification using peptide mapping. *Rapid Commun Mass Spectrom* 12:864–870.

- Cech NB, Enke CG. 2000. Relating electrospray ionization response to non-polar character of small peptides. *Anal Chem* 72:2717–2723.
- Cech NB, Enke CG. 2001. The effect of affinity for charged droplet surfaces on the fraction of analyte charged in the electrospray process. *Anal Chem* 73:4632–4639.
- Cech NB, Krone JR, Enke CG. 2001a. Predicting electrospray response from chromatographic retention time. *Anal Chem* 73:208–213.
- Cech NB, Krone JR, Enke CG. 2001b. Electrospray ionization detection of an inherently nonresponsive epoxide by peptide binding. *Rapid Commun Mass Spectrom* 15:1040–1044.
- Che FY, Song JF, Aeng R, Wang KY, Xia QC. 1999. Analysis of 8-aminonaphthalene-1,3,6-trisulfonate-derivatized oligosaccharides by capillary electrophoresis-electrospray ionization quadrupole ion trap mass spectrometry. *J Chromatogr A* 858:229–238.
- Chen DR, Pui DYH. 1997. Experimental investigation of scaling laws for electrospraying: dielectric constant effect. *Aerosol Science and Technology* 27:367–380.
- Cheng ZL, Siu KW, Guevremont R, Bergman SS. 1992. Electrospray mass spectrometry: a study of some aqueous-solutions of metal-salts. *J Am Soc Mass Spectrom* 3:281.
- Cloupeau M, Prunet-Foch B. 1989. Electrostatic spraying of liquids in cone-jet mode. *J Electrostat* 22:135–159.
- Cole RB, Harrata AK. 1992. Charge-state distribution and electric-discharge suppression in negative-ion electrospray mass-spectrometry using chlorinated solvents. *Rapid Commun Mass Spectrom* 6:536–539.
- Cole RB, Harrata AK. 1993. Solvent effect on analyte charge state, signal intensity, and stability in negative ion electrospray mass spectrometry; implications for the mechanism of negative ion formation. *J Am Soc Mass Spectrom* 4:546–556.
- Cole RB, Zhu JH. 1999. Chloride ion attachment in negative ion electrospray ionization mass spectrometry. *Rapid Commun Mass Spectrom* 13:607–611.
- Colton R, Traeger JC, Harvey J. 1992. Electrospray mass spectrometric studies of some phosphonium cations. *Org Mass Spectrom* 27:1030–1033.
- Constantopoulos TL, Jackson GS, Enke CG. 1999. Effects of salt concentration on analyte response using electrospray ionization mass spectrometry. *J Am Soc Mass Spectrom* 10:625–634.
- Constantopoulos TL, Jackson GS, Enke CG. 2000. Challenges in achieving a fundamental model for ESI. *Anal Chim Acta* 406:37–52.
- De La Mora JF, Loscertales JG. 1994. The current emitted by highly conducting Taylor Cones. *J Fluid Mech* 260:155–184.
- Dole M, Mack LL, Hines RL, Mobley RC, Ferguson LD, Alice MB. 1968. Molecular beams of macroions. *J Chem Phys* 49:2240–2249.
- Drozin VG. 1955. The electrical dispersion of liquids as aerosols. *J Colloid Sci* 7:616.
- Emmett MR, Caprioli RM. 1994. Micro electrospray mass spectrometry: ultra-high-sensitivity analysis of peptides and proteins. *J Am Soc Mass Spectrom* 5:605–613.
- Enke CG. 1997. A predictive model for matrix and analyte effects in electrospray ionization of singly-charged ionic analytes. *Anal Chem* 69:4885–4893.
- Fenn JB. 1993. Ion formation from charged droplets: roles of geometry, energy and time. *J Am Soc Mass Spectrom* 4:524–535.
- Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM. 1989. Electrospray ionization for mass spectrometry of large biomolecules. *Science* 246:64–71.
- Gelpi E. 1995. Biomedical and biochemical applications of liquid chromatography-mass spectrometry. *J Chrom A* 703:59–80.
- Geremanos S, Freckleton G, Tempst P. 2000. Tuning of an electrospray ionization source for maximum peptide-ion transmission into a mass spectrometer. *Anal Chem* 4:777–790.
- Gomez A, Tang K. 1994. Charge and fission of droplets in electrostatic sprays. *Phys Fluids* 6:404–414.
- Griffey RH, Greig MJ, Gaus HJ, Liu K, Monteith D, Winniman M, Cummins LL. 1997. Characterization of oligonucleotide metabolism in vivo via liquid chromatography/electrospray tandem mass spectrometry with a quadrupole ion trap mass spectrometer. *J Mass Spectrom* 32:305–313.
- Guevremont R, Barnett DA, Purves RW, Vandermeij J. 2000. Analysis of a tryptic digest of pig hemoglobin using ESI-FAIMS-MS. *Anal Chem* 72:4577–4584.
- Hager DB, Dovichi NJ, Klassen J, Kebarle P. 1994. Droplet electrospray mass-spectrometry. *Anal Chem* 66:3944–3949.
- Herderich M, Richling E, Roscher R, Schneider C, Schwab W, Humph HU, Schreier P. 1997. Application of atmospheric pressure ionization HPLC-MS-MS for the analysis of natural products. *Chromatographia* 45:127–132.
- Hiraoka K, Kudaka I, Fujimaki S, Shinohara H. 1992. Observation of the fullerene anions C-60(–) and C-70(–) by electrospray ionization. *Rapid Commun Mass Spectrom* 6:254–256.
- Hopfgartner G, Wachs T, Bean K, Henion J. 1993. High flow ion spray liquid-chromatography mass-spectrometry. *Anal Chem* 65:439–446.
- Huber CG, Krajete A. 2000. Sheath liquid effects in capillary high-performance liquid chromatography-electrospray mass spectrometry of oligonucleotides. *J Chrom A* 870:413–424.
- Iribarne JV, Thomson BA. 1976. On the evaporation of charged ions from small droplets. *J Chem Phys* 64:2287–2294.
- Iribarne JV, Dziedzic PJ, Thomson BA. 1983. Atmospheric pressure ion evaporation-mass spectrometry. *Int J Mass Spectrom Ion Phys* 50:331–347.
- Jackson GS, Enke CG. 1999. The electrical equivalence of electrospray ionization mass spectrometry. *Anal Chem* 71:3777–3784.

- Jeannot R, Sauvard E. 1999. High-performance liquid chromatography coupled with mass spectrometry applied to analyses of pesticides in water. Results obtained in HPLC/MS/APCI in positive mode. *Analisis* 27:271–280.
- Karas M, Bahr U, Dulcks T. 2000. Nano-electrospray ionization mass spectrometry: addressing analytical problems beyond routine. *Fresenius J Anal Chem* 366:669–676.
- Karplus PA. 1996. Hydrophobicity regained. *Protein Sci* 6:1302–1307.
- Kebarle P, Ho Y. 1997. On the mechanism of electrospray mass spectrometry. In: Cole RB (Ed.) *Electrospray ionization mass spectrometry*. New York: Wiley. p 14.
- Kebarle P, Peschke M. 1999. On the mechanisms by which the charged droplets produced by electrospray lead to gas phase ions. *Analytica Chimica Acta* 20070:1–25.
- Kelly MA, Vestling MM, Fenselau CC, Smith PB. 1992. Electrospray analysis of proteins: a comparison of positive-ion and negative-ion mass spectra at high and low pH. *Org Mass Spectrom* 27:1143–1147.
- Kertesz V, Van Berkel GJ. 2001. Minimizing analyte electrolysis in an electrospray emitter. *J Mass Spectrom* 36:204–210.
- Kim T, Tolmachev AV, Harkewicz R, Prior DC, Anderson GA, Udseth HR, Smith RD, Bailey TH, Rakov S, Futrell JH. 2000a. Design and implementation of a new electrohydrodynamic ion funnel. *Anal Chem* 72:2247–2255.
- Kim T, Udseth HR, Smith RD. 2000b. Improved ion transmission from atmospheric pressure to high vacuum using a multicapillary inlet and electrodynamic ion funnel interface. *Anal Chem* 72:5014–5019.
- Kostiainen R, Bruins AP. 1994. Effect of multiple sprayers on dynamic range and flow rate limitations in electrospray and ionspray mass spectrometry. *Rapid Commun Mass Spectrom* 8:549–558.
- Kostiainen R, Bruins AP. 1996. Effect of solvent composition on dynamic range and sensitivity of pneumatically-assisted electrospray (ion spray) mass spectrometry. *Rapid Commun Mass Spectrom* 10:1393–1399.
- Kuhlmann FE, Apffel A, Fisher SM, Goldberg G, Goodley PC. 1995. Signal enhancement for gradient reverse-phase high-performance liquid-chromatography electrospray-ionization mass spectrometry analysis with trifluoroacetic and other strong acid modifiers by postcolumn addition of propionic-acid and isopropanol. *J Am Soc Mass Spectrom* 6:1221–1225.
- Le Bihan T, Pinto D, Figeys D. 2001. Nanoflow gradient generator coupled with μ -LC-ESI-MS/MS for protein identification. *Anal Chem* 73:1307–1315.
- Liu C, Wu Q, Harms AC, Smith RD. 1996. On-line microdialysis sample cleanup for electrospray ionization mass spectrometry of nucleic acid samples. *Anal Chem* 68:3295–3299.
- Liu CL, Muddiman DC, Tang KQ, Smith RD. 1997. Improving the microdialysis procedure for electrospray ionization mass spectrometry of biological samples. *J Mass Spectrom* 36:425–431.
- Loo JA, Loo RRO, Light KJ, Edmonds CG, Smith RD. 1992. Multiply charged negative-ions by electrospray ionization of polypeptides and proteins. *Anal Chem* 64:81–88.
- Lu WZ, Poon GK, Carmichael PL, Cole RB. 1996. Analysis of tamoxifen and its metabolites by on-line capillary electrophoresis-electrospray ionization mass spectrometry employing nonaqueous media containing surfactants. *Anal Chem* 68:668–674.
- Mann M, Fenn JB, Wong SF. 1990. Where do all the charges go in electrospray ionization? In: Hedin A, Sundqvist B, Benninghoven A (Eds.) *Ion formation from organic solids*. New York: John Wiley & Sons. pp 139–144.
- Matsuura K, Takashina H. 1998. Effects of functional groups of acrylic acid derivatives as derivatization reagents for thiol compounds on molecular ion responses in electrospray ionization mass spectrometry. *J Mass Spectrom* 33:1199–1208.
- Matz LM, Clowers BH, Steiner WE, Siems WF, Hill HH. 2001. Liquid-sheath-flow electrospray ionization feasibility study of direct water analysis with the use of high-resolution ion-mobility spectrometry. *Field Analytical Chemistry and Technology* 5:91–96.
- Mehlis B, Kertscher U. 1997. Liquid chromatography mass spectrometry of peptides of biological samples. *Analytica Chimica Acta* 352:71–83.
- Miketova P, Schram KH. 1997. Mass spectrometry of nucleotides and oligonucleotides. *Mol Biotechnol* 8:249–253.
- Muddiman DC, Smith RD. 1998. Sequencing and characterization of larger oligonucleotides by electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry. *Rev Anal Chem* 17:1–68.
- Niessen WMA, Tinke AP. 1995. *Liquid chromatography-mass spectrometry general principles and instrumentation*. *J Chrom A* 703:37–57.
- Ogorzalek Loo RR, Smith RD. 1994. Investigations of the gas-phase structure of electrosprayed proteins using ion-molecule reactions. *J Am Soc Mass Spectrom* 5:221–229.
- Okamoto M, Takahashi K, Doi T. 1995. Sensitive detection and structural characterization of trimethyl(*p*-aminophenyl)-ammonium derivatized oligosaccharides by electrospray ionization mass spectrometry and tandem mass spectrometry. *Rapid Commun Mass Spectrom* 9:641.
- Owens DR, Bothner B, Phung Q, Harris K, Siuzdak G. 1998. Aspects of oligonucleotide and peptide sequencing with MALDI and electrospray mass spectrometry. *Bioorg Med Chem* 6:1547–1554.
- Purves RW, Gabryelske W, Li L. 1998. Investigation of the quantitative capabilities of an electrospray ionization ion trap linear time-of-flight mass spectrometer. *Rapid Commun Mass Spectrom* 12:695–700.
- Quirke JME, Adams CL, Van Berkel GJ. 1994. Chemical derivatization for electrospray-ionization mass-spectrometry. 1. Alkyl-halides, alcohols, phenols, thiols and amines. *Anal Chem* 66:1302–1315.

- Raffaelli A, Bruins AP. 1991. Factors affecting the ionization efficiency of quaternary ammonium compounds in electrospray/ion spray mass spectrometry. *Rapid Commun Mass Spectrom* 5:269–275.
- Ramsey RS, Goeringer DE, McLuckey SA. 1993. Active-chemical background-noise reduction in capillary electrophoresis/ion trap mass-spectrometry. *Anal Chem* 65:3521–3524.
- Rollgen FW, Juraschek R. 1998. Pulsation phenomenon during electrospray ionization. *Int J Mass Spectrom* 177:1–5.
- Saf R, Mirtl C, Hummel K. 1994. Electrospray mass-spectrometry using potassium-iodide in aprotic organic solvents for the ion formation by cation attachment. *Tetrahedron Lett* 35:6653–6656.
- Shaffer SA, Tolmachev A, Prior DC, Anderson GA, Udseth HR, Smith RD. 1999. Characterization of an improved electrodynamic ion funnel interface for electrospray ionization mass spectrometry. *Anal Chem* 71:2957–2964.
- Shen XD, Perreault H. 1998. Characterization of carbohydrates using a combination of derivatization, high-performance liquid chromatography, and mass spectrometry. *J Chrom A* 811:47–59.
- Sjoberg PJR, Bokman CF, Bylund D, Markides KE. 2001. A method for determination of ion distribution within electrosprayed droplets. *Anal Chem* 73:23–28.
- Slobodnik J, van Barr BLM, Brinkman UAT. 1995. Column liquid chromatography-mass spectrometry: selected techniques in environmental applications for polar pesticides and related compounds. *J Chrom A* 703:81–121.
- Steen RJCA, Hogenboom AC, Leonards PEG, Pererboom RAL, Cofino WP, Brinkman UAT. 1999. Ultra-trace-level determination of polar pesticides and their transformation products in surface and estuarine water samples using column liquid chromatography-electrospray tandem mass spectrometry. *J Chrom A* 857:157–166.
- Stephenson JL, McLuckey SA. 1996. Ion/ion proton transfer reactions for protein mixture analysis. *Anal Chem* 68:4026.
- Straub RF, Voyksner RD. 1993. Negative ion formation in electrospray mass spectrometry. *J Am Soc Mass Spectrom* 4:578–587.
- Strege MA. 1999. High-performance liquid chromatographic electrospray ionization mass spectrometric analyses for the integration of natural products with modern high-throughput screening. *J Chrom B* 725:67–78.
- Taffin DC, Ward TL, Davis EJ. 1989. Electrified droplet fission and the Rayleigh limit. *Langmuir* 5:376–384.
- Tang L, Kebarle P. 1991. Effect of the conductivity of the electrosprayed solution on the electrospray current. Factors determining analyte sensitivity in electrospray mass spectrometry. *Anal Chem* 63:2709–2715.
- Tang L, Kebarle P. 1993a. Dependence of ion intensity in electrospray mass spectrometry on the concentration of analytes in the electrosprayed solution. *Anal Chem* 65:3654–3668.
- Tang L, Kebarle P. 1993b. From ions in solution to ions in the gas phase. *Anal Chem* 65:972A–985A.
- Tang K, Smith RD. 1999. Theoretical prediction of charged droplet evaporation and fission in electrospray ionization. *Int J Mass Spectrom* 185/186/187:97–105.
- Tang K, Smith RD. 2001. Physical/chemical separations in the break-up of highly charged droplets from electrosprays. *J Am Chem Soc* 123:343–347.
- Tang K, Lin Y, Matson DW, Kim T, Smith RD. 2001. Generation of multiple electrosprays using microfabricated emitter arrays for improved mass spectrometric sensitivity. *Anal Chem* 73:1658–1663.
- Temesi D, Law B. 1999. The effect of LC eluent composition on MS response using electrospray ionization. *LC–GC* 17:627–632.
- Troxler H, Kuster T, Rhyner JA, Gehrig P, Heizmann CW. 1999. Electrospray ionization mass spectrometry: analysis of the Ca²⁺-binding properties of human recombinant alpha-parvalbumin and nine mutant proteins. *Anal Biochem* 268:64–71.
- Van Berkel GJ. 1997. The electrolytic nature of electrospray ionization. In: Cole RB (Ed.). *Electrospray ionization mass spectrometry*. New York: Wiley. pp 65–105.
- Van Berkel GJ. 1998. Electrolytic corrosion of a stainless-steel electrospray emitter monitored using an electrospray-photodiode array system. *J Anal At Spectrosc* 13:603–607.
- Van Berkel GJ. 2000. Insights into analyte electrolysis in an electrospray emitter from chronopotentiometry experiments and mass transport calculations. *J Am Soc Mass Spectrom* 11:951–960.
- Van Berkel GJ, Asano KG. 1994. Chemical derivatization for electrospray ionization mass spectrometry. 2. Aromatic and highly conjugated molecules. *Anal Chem* 66:2096–2102.
- Van Berkel GJ, Zhou F. 1995. Characterization of an electrospray ion source as a controlled-current electrolytic cell. *Anal Chem* 67:2916–2923.
- Van Berkel GJ, McLuckey SA, Glish GL. 1992. Electrochemical origin of radical cations observed in electrospray ionization mass spectra. *Anal Chem* 64:1586–1593.
- Van Berkel GJ, Zhou F, Aronson JT. 1997. Changes in bulk solution pH caused by the inherent controlled-current electrolytic process of an electrospray source. *Int J Mass Spectrom Ion Proc* 162:55–67.
- Van Berkel GJ, Quirke JM, Tigani RA, Dilley AS. 1998. Derivatization for electrospray ionization mass spectrometry. 3. Electrochemically ionizable derivatives. *Anal Chem* 70:1544–1554.
- Van Berkel GJ, Quirke JM, Adams CL. 2000. Derivatization for electrospray ionization mass spectrometry. 4. Alkenes and alkynes. *Rapid Commun Mass Spectrom* 14:849–858.
- Voyksner RD. 1996. Atmospheric pressure ionization liquid chromatography mass spectrometry for environmental analysis. *Biochem Biotech App Electrospray Ionization Mass Spectrom* 619:565–582.

- Voyksner RD. 1997. Combining liquid chromatography with electrospray mass spectrometry. In: Cole RB (Ed.). *Electrospray ionization mass spectrometry*. New York: Wiley. pp 323–341.
- Voyksner RD. 2001. (personal communication).
- Wang G, Cole RB. 1994. Disparity between solution-phase equilibria and charge state distributions in positive-ion electrospray mass spectrometry. *Organic Mass Spectrometry* 29:419–427.
- Wang G, Cole RB. 1997. Solution, gas-phase, and instrumental parameter influences on charge-state distributions in electrospray ionization mass spectrometry. In: Cole RB (Ed.). *Electrospray ionization mass spectrometry*. New York: Wiley. pp 137–174.
- Waterval JCM, Lingeman H, Bult A, Underberg WJM. 2000. Derivatization trends in capillary electrophoresis. *Electrophoresis* 21:4029–4055.
- Whitelegge JP, Gunderson CB, Faull KF. 2001. Electrospray ionization mass spectrometry: analysis of the Ca^{2+} -binding properties of human recombinant alpha-parvalbumin and nine mutant proteins. *Protein Science* 7:1423–1430.
- Wilm M, Mann M. 1996. Analytical properties of the nanoelectrospray source. *Anal Chem* 68:1–8.
- Wilson SR, Wu Y. 1993. Applications of electrospray ionization mass spectrometry to neutral organic molecules including fullerenes. *J Am Soc Mass Spectrom* 4:596–603.
- Yen T-Y, Charles MJ, Voyksner RD. 1996. Processes that affect electrospray ionization-mass spectrometry of nucleobases and nucleosides. *J Am Soc Mass Spectrom* 7:1106–1108.
- Yeung B, Porter TJ, Vath JE. 1997. Direct isoform analysis of high-mannose containing glycoproteins by on-line capillary electrophoresis electrospray mass spectrometry. *Anal Chem* 69:2510–2516.
- Yoshino K, Takao T, Murata H, Shimomshi Y. 1995. Use of derivitizing agent 4-aminobenzoic acid 2-(diethylamino) ethyl ester for high-sensitivity detection of oligosaccharids by electrospray ionization mass spectrometry. *Anal Chem* 67:4028.
- Zhang JC, Cech NB, Enke CG. 2002. 2,2,2-Trifluoroethanol as an additive for low-noise negative ion electrospray mass spectrometry. *Anal Chem* (submitted).
- Zhou S, Cook KD. 2000. Protonation in electrospray mass spectrometry: wrong way round or right-way-round? *J Am Soc Mass Spectrom* 11:961–966.
- Zhou S, Cook KD. 2001. A mechanistic study of electrospray mass spectrometry: charge gradients within electrospray droplets and their influence on ion response. *J Am Soc Mass Spectrom* 12:206–214.
- Zhou S, Hamburger M. 1995. Effects of solvent composition of molecular ion response in electrospray mass spectrometry: investigation of the ionization process. *Rapid Comm Mass Spectrom* 9:1516–1521.
- Zhu JH, Cole RB. 2000. Formation and decompositions of chloride adduct ions, $[\text{M} + \text{Cl}]^-$, in negative ion electrospray ionization mass spectrometry. *J Am Soc Mass Spectrom* 11:932–941.
- Zook DR, Bruins AP. 1997. On cluster ions, ion transmission, and linear dynamic range limitations in electrospray (ionspray) mass spectrometry. *Int J Mass Spectrom Ion Process* 162:129–147.
- Zook DR, Forsmo-Bruce H, Briem S. 2000. ESI-MS response characteristics of a synthetic peptide. *Chromatographia* 52:S60–S64.