The use of electrospray mass spectrometry in the detection and determination of molecules of biological significance

W. Franklin Smyth*
ABCS School, University of Ulster, Coleraine BT52 1SA, N.I., UK

This review will briefly explain the mechanism of electrospray ionisation, discuss the molecules and species that can be ionised by this technique such as proteins, nucleotides, carbohydrates, drugs, pollutants, organometallic and inorganic species. It will also select relevant examples from the recent literature that illustrate the wide applicability of electrospray mass spectrometry when interfaced to HPLC and CE for the detection and determination of such biologically significant molecules. ©1999 Elsevier Science B.V. All rights reserved.

Keywords: Electrospray mass spectrometry; Molecules of biological significance; Analysis using HPLC–ESMS and CE–ESMS

1. Introduction

Electrospray mass spectrometry (ESMS) was introduced by Yamashita and Fenn [1] in 1984 and has made a significant commercial impact in the last decade [2]. The mechanisms of the transformation of ions in solution to ions in the gas phase prior to their mass analysis in a mass spectrometer has been reviewed by, among others, Kebarle and Tang [3], Bruins [4] and Gaskell [5] with Cole [6] recently editing a book on the fundamentals, instrumentation and applications of ESMS. Along with matrix-assisted laser desorption ionisation (MALDI), it has widened the scope of MS analysis of high molecular mass compounds such as proteins, nucleotides and synthetic polymers by virtue of the detailed information regarding molecular mass and structure from extremely small quantities of material that can be provided. ESMS relies, in these cases, on the production of multiply charged ions whose \( m/z \) values can be analysed on virtually all types of mass spectrometers. The ‘softness’ of the ES technique means that not only can the molecular ion or its modifications (e.g. \([\text{M+H}]^+\), \([\text{M-H}]^-\)) be monitored to reveal the molecular mass but also noncovalent interactions between molecules in solution can be preserved in the gas phase, as well as permitting the study of three-dimensional molecular conformations. Detailed structural information on molecules of a wide range of molecular masses can be obtained by resort to transport region collisionally induced dissociation (CID) with a single MS instrument, collisionally induced dissociation with triple quadrupole MS instruments, and MS\(^n\) techniques using quadrupole ion-trap instrumentation. ES has become one of the most important ionisation techniques for the on-line coupling of liquid phase separation techniques such as high performance liquid chromatography (HPLC) and capillary electrophoresis (CE) with mass spectrometry (MS). Perusal of a recent volume of J. Chromatogr. A [7], primarily dealing with HPLC–MS, illustrates how HPLC–ESMS is being increasingly used for the identification and determination of organic analytes of biological significance.

2. Mechanism of electrospray

Electrospray, as a method of transfer of ions from the solution phase to the gas phase, is a strongly endothermic process since in solution the ion is solvated and is not in the gas phase. For the process

\[
\text{Na}^+(\text{aq}) \rightarrow \text{Na}^+(\text{g})
\]
where $\Delta G_{\text{sol}}^0$ and $\Delta H_{\text{sol}}^0$ stand for the reverse process, i.e. the transfer of the ion from gas phase to solution. It should be noted that this $\Delta H^0$ value is greater than that required to break a carbon C–C bond which is of the order of 85 kcal mol$^{-1}$. Therefore if the energy is supplied in one package over a short period of time, the act of freeing an organic ion from solvent molecules can also lead to fragmentation. This is more likely to occur in ionisation techniques such as fast atom bombardment (FAB) rather than the ‘softer’ ES where desolvation is achieved gradually by thermal energy at relatively low temperatures. There are three major steps in the production by electrospray of gas phase ions from solution phase ions as illustrated in Fig. 1, i.e. (1) the production of charged droplets at the ES capillary tip (the electrophoretic mechanism), (2) the shrinkage of the charged droplets by solvent evaporation and repeated droplet disintegrations, leading ultimately to very small highly charged droplets capable of producing gas phase ions, and (3) production of gas phase ions from the very small and highly charged droplets.

In step 1, a voltage $V_c$ of 2–4 kV is applied to the metal capillary which is typically 0.2 mm o.d. and 0.1 mm i.d. and located 1–3 cm from the counter electrode. Because the electrospray capillary tip is very thin, the electric field $E_c$ in the air at the capillary tip is very high and can be evaluated by the approximate relationship as follows, when the counter electrode is large and planar

\[
E_c = \frac{2V_c}{r_c} \ln \left( \frac{4d}{r_c} \right)
\]

where $r_c$ is the capillary outer radius (say $10^{-4}$ m) and $d$ is the distance from the capillary tip to the counter electrode (say 0.02 m). This leads to a value of $E_c = 6 \times 10^6$ V m$^{-1}$ for $V_c = 2$ kV and results, in the positive ion ES mode (i.e. the capillary is the positive electrode) in a charge separation with the positive ions having moved towards the meniscus of the liquid in the capillary and the negative ions having moved away from the surface. This is known as the electrophoretic mechanism. The mutual repulsion between the positive ions at the surface overcomes the surface tension of the liquid to form a cone, followed by the emergence of a fine jet from the cone tip and the production of charged droplets. This process of nebulisation generally uses the assistance of a high velocity gas flow with the pneumatic nebulisation forming the droplets and the electric field charging them in a simple approximation. When compared to electrospray without pneumatic nebulisation, aqueous solutions and higher flow rates can be used at lower field strengths, thus eliminating electrical discharge phenomena. Such electrical discharges are particularly a problem in the negative ion mode with field emission of electrons.

Fig. 1. Major processes occurring in electrospray ionisation. Reprinted from [3] with permission.
coming from the spray needle or the sharp tip of the liquid emerging from the capillary. This results in significant reduction of analyte signals ultimately detected by the mass spectrometer and can be ameliorated by the capture of these electrons by oxygen, a freon or the vapour of a chlorinated solvent. In addition to the use of pneumatic nebulisation, dilution of the aqueous solutions subjected to electrospray with organic solvents desirably reduces surface tension. Coaxial addition of a sheath flow of methanol, acetonitrile, ethanol, etc. to the sample solution at the tip of the spray capillary can also used as with CE–ESMS applications.

In addition to this electrophoretic mechanism in step 1, Blades et al. [8] have stated that the ES process (in the positive ion mode) has also to involve electrochemical conversion of ions to neutrals to electrons (within the metal ES capillary) when charge balance considerations and the fact that only electrons can flow through the metal wire supplying the electric potential to the electrodes are taken into account. When positively charged droplets hit the negatively charged plate in Fig. 1, electrons are consumed in a neutralisation reaction. The ES device therefore can be viewed as ‘an electrolytic cell of a somewhat special kind’ insofar as part of the ion transport (between electrodes) does not occur in solution (as in a conventional electrolytic cell) but through the gas phase. Blades et al. [8] surmised that the redox reactions with the lowest redox potentials would predominate in this charge balancing process and the actual reactions occurring would depend on the particular solvent and solution composition. Given ‘wet methanol’ containing a chloride salt as an electrolyte, they postulated that when the ES capillary was held at a high positive voltage (i.e. positive ion mode) the build-up of negative ions in the capillary might be counterbalanced by electrochemical oxidation reactions that result in the neutralisation of negative ions (Eqs. a and b), the production of positive ions (Eqs. c and d) or both:

\[
\begin{align*}
2\text{Cl}^- (aq) & = \text{Cl}_2 (g) + 2e^- \\
4\text{OH}^- (aq) & = \text{O}_2 + 2\text{H}_2\text{O}(l) + 4e^- \\
2\text{H}_2\text{O}(l) & = \text{O}_2 (g) + 4\text{H}^+(aq) + 4e^- \\
\text{M (capillary metal)} & = \text{M}^{n+} + ne^- 
\end{align*}
\]

Bruins [4] has stated that under the majority of experimental HPLC–ESMS and CE–ESMS conditions, positive charge on droplets is generated by the removal of negative charge via electrochemical discharge of negative ions against the metal wall of the spray capillary. These electrode reactions can also be used to produce ions of special interest by removal of electrons from analyte molecules such as porphyrins having low ionisation energies and structural characteristics that aid stabilisation of the positive ion formed (often a radical cation) [9].

The ES current \(I_{\text{spray}}\) generated by these electrochemical reactions, as required by charge balance considerations, can result in significant changes in solution composition. For example, consider the oxidation of water (Eq. c) to be the major charge balancing redox reaction to occur when spraying a weakly basic analyte from an aqueous solution whose pH, measured prior to entering the capillary, was 7.0. If it is assumed that a constant value of \(I_{\text{spray}} = 100 \text{nA}\) is observed (not an uncommon current for say a \(10^{-6} \text{ mol dm}^{-3}\) concentration of an analyte in a mobile phase containing an excess concentration of other electrolytes in an HPLC–ESMS assay – solvents such as MeOH and acetonitrile can contain significant concentrations of impurity electrolyte ions such as \(\text{NH}_4^+ , \text{Na}^+ \)) the pH of the solution exiting the capillary for flow rates of 10, 1.0 and 0.1 \(\mu\text{l min}^{-1}\) would be 5.2, 4.2 and 3.2 respectively. These changes in pH are quite dramatic and would be expected to alter the ES response of a weakly basic analyte through an increase in the degree of protonation in solution ([6], p. 87). In direct analogy to two electrode controlled current electrolysis, where in this case the ES ion source is the controlled current device, it is expected that the potential at the metal/solution interface in the ES capillary, which ultimately determines which redox reactions can occur, will be that value for a given magnitude of \(I_{\text{spray}}\) necessary to oxidise sufficient species in the solution within the ES capillary to maintain that current.

Step 2 involves the disintegration of droplets. After a droplet separates from the liquid front at the tip of the spray capillary, electrical repulsion has become larger than the cohesive force that keeps the liquid together. During its flight through the gas at atmospheric pressure, the droplet undergoes size reduction by evaporation of solvent, so that charge density at the droplet surface increases. When electrostatic repulsion exceeds the surface tension, the droplet becomes unstable and falls apart. The radius of primary droplets in ES is of the order of 0.5–1 \(\mu\text{m}\) and this will reduce ultimately to a radius of approximately 10 nm, prior to release of sample ions (ion evaporation, step 3) which can then be taken into the mass spectrometer itself.
The number of ions that so escape from droplets is related to the charge on droplets, which can be derived from spray current measurement. Kebarle et al. ([6], chapter 1) have proposed equations for a two electrolyte system.

\[ I_A = f p \frac{k_A[A^+]}{k_A[A^+]+k_B[B^+]} I_{spray} \]  
(2)

\[ I_B = f p \frac{k_B[B^+]}{k_A[A^+]+k_B[B^+]} I_{spray} \]  
(3)

where \( I_A = A^+ \) ion signal at the MS detector, \( I_B = B^+ \) ion signal at the MS detector, \( f = \) fraction of charges on droplets that are converted to gas phase ions, \( p = \) fraction of gas phase ions transported into the mass analyser, \( k_A = \) sensitivity coefficient for \( A^+ \), \( k_B = \) sensitivity coefficient for \( B^+ \) and \( I_{spray} = \) total droplet current (electrospray current).

According to these equations, the abundance of a sample ion is proportional to the amount of charge on the droplets and proportional to a sensitivity coefficient \( k \) which is dependent on ion structure. Ionic surface active species have a high \( k \) value (ca 10) and are observed with high sensitivity in ESMS. Alkali metal ions have a low \( k \) value (ca 1) and the \( k \) values for protonated organic bases are in between (ca 3–6) ([6], chapter 1). No data are available for peptides and other biomolecules. Eq. 2 can be extended for a multi-electrolyte system by extending the denominator with the appropriate number of \( k[A^+] \) terms. The significance of Eq. 2 in HPLC–ESMS and CE–ESMS is that at constant \( I_{spray} \), the \( A^+ \) ion signal is proportional to \( [A^+] \) if \( [A^+] \ll [B^+] \). This condition is usually met in HPLC–ESMS if \( B^+ \) is an electrolyte present in the eluent at the mM level while the analyte concentration is at the \( \mu M \) level.

The ES process, based as it is on the release of sample ions from the surface of a charged droplet, does not necessarily give a true picture of bulk solution chemistry as it exists in the HPLC or CE eluent. The state of protonation for proteins, for example, as observed by the mass spectrometer in ESMS, can only in some cases reflect the number of protonated basic sites present in the protein when in solution (i.e. due to protonation of arginine, lysine, histidine and terminal amino group) [10]. At a given acidic pH of 3.5, multiply charged negative ions have been observed due to deprotonated acidic residues when operating in the negative ion mode. At a pH of 3.5, these acidic groups are not deprotonated in solution [11]. Also, polyprotonated protein ions can be observed by ESMS in the positive ion mode from solutions of pH 10 even though the protein is deprotonated in the original solution. In these latter two cases, proton transfer reactions must be occurring in response to the change of environment from solution to gas phase. With respect to smaller molecular mass analytes, McClean et al. [12] have observed [M+H]^+ molecular ions for a selection of 1,4-benzodiazepines after HPLC separation on a reversed phase C18 column with a mobile phase of 65% methanol, 5% acetonitrile and 30% water. Although the 1,4-benzodiazepines are eluted after HPLC separation as neutral molecules, they arrive at the MS detector as protonated species. It can be surmised that they become protonated in the electrospray process by the protons produced in Eq. c in the electrospray capillary and/or by protons present as impurities in the methanol (0.0003% HCOOH) and acetonitrile (0.002% CH3COOH) solvents which increase in concentration with solvent evaporation.

3. Candidate molecules/species for electrospray

3.1. Proteins/peptides

ESMS can be applied to the determination of the molecular mass of large molecules because of the tendency for multiply charged ions to be formed. Since mass spectrometers display spectra according to the mass/charge ratio \((m/z)\), a protein of molecular mass 30 000 with 20 protonated basic sites will give a peak corresponding to \([M+20H]^+\) at an \(m/z\) value of \((M+20)/20\), i.e., 1501. Hence mass spectra from molecules whose molecular mass would greatly exceed the range of normal sector and quadrupole instruments can be observed. A positive ion ESMS of myoglobin (molecular mass 16 951.5) gives rise to an envelope of peaks corresponding to the intact molecule carrying different numbers of charges from 23 at \(m/z\) 738 to 12 at \(m/z\) 1413. Transformation software exists that presents the data as a reconstructed mass spectrum as if the sample had yielded a singly charged molecular ion. Oliver and Green [13] and others have applied such ESMS to the analysis of abnormal or variant haemoglobins. This is illustrated in Fig. 2 where samples were directly injected into ESMS in water and methanol (50+50) containing 0.1% formic acid. Fig. 2a shows peaks for \(\alpha\)- and \(\beta\)-globin from normal HbA but Fig. 2b, obtained from an adult human, shows two \(\beta\)-globin peaks, labelled S and B and mass measured at 15 837.5 and 15 867.1 respec-
This indicates that the human subject is heterozygous for the sickle cell anaemia gene. The presence of the second β-globin (S) arises from the substitution of glutamic acid by valine at position 6 of the β-chain. Thus the sample contains both normal and abnormal β-chains and the subject is a carrier for sickle cell anaemia. Fig. 2c shows a homozygous case with all of the β-globin present in the sickle form. This subject is affected by sickle cell anaemia and would be expected to show clinical symptoms. The single β-chain is easily observed and its shift in mass from the normal HbA β-chain is clearly revealed. The MS characterisation of abnormal haemoglobins (ca 600 identified to date) is currently an area of considerable development. Performing mathematical deconvolution using maximum entropy software allows for a minimum measurable mass difference of 6 Da for proteins in the 15–16kDa range [14]. Tryptic digestion of proteins followed by separation procedures and ESMS can help to deduce which amino acid has changed, with peptide sequencing used to locate its position.

The ESMS of peptides and proteins has recently been reviewed by Loo and Loo ([6], chapter 11) and Gaskell has included some such material in his review of the principles and practice of electrospray [5]. In the former review, molecular mass determination, aspects of protein structure (primary, secondary, tertiary and quaternary), sequencing by tandem mass
spectrometry, determination of metal-binding stoichiometry and subunit stoichiometry of the quaternary structure are all included.

3.2. Carbohydrates and lipids

Ohashi has recently reviewed this subject ([6], chapter 13) and has stated that CID-MS/MS has been found an indispensable technique in ESMS to elucidate structures and is complementary to information obtained by FAB.

3.3. Nucleic acids and their constituents

Crain has recently reviewed this subject ([6], chapter 12). Careful sample preparation has been found critical for analysis of these strongly anionic molecules and this review pays particular attention to the practical aspects of sample manipulation prior to analysis. The extent to which sequence can be derived by collision-induced dissociation (CID) of multiply charged oligonucleotide ions has also been reviewed for both ion-trap and triple quadrupole mass analysers, as has the use of ESMS to detect bimolecular non-covalent interactions in which one participant is a nucleic acid.

3.4. Molecular that already exist as ions in solution

For instance, quaternary ammonium salts, phosphonium salts and salts of strong acids such as phosphates, sulphates and sulphphonates.

3.5. Low molecular mass organic bases

For instance, amines, alkaloids, etc. can form singly protonated species. HCOOH and CH₃COOH are commonly used to generate these species for direct injection ESMS.

3.6. Low molecular mass organic acids

For instance, phenols, carboxylic acids, phosphonic acids and sulphonic acids can form anionic species suitable for ESMS. NH₂OH is commonly used to generate these species prior to direct injection ESMS. Straub and Voyksner [15] have reviewed negative ion formation in ESMS with particular reference to β-lactam antibiotics, aminoglycosides, aminocyclitols, tetracyclines, sulphonamides, explosives and other molecules.

3.7. Molecules that do not contain basic or acidic functional groups

These molecules can be ‘ionised’ by association with another ion in solution, e.g. NH₄⁺ and Na⁺ for positive ion detection and Cl⁻, CH₃COO⁻, HCOO⁻, CF₃COO⁻ for negative ion detection. Examples of this class of molecules are amides, polyhydroxy compounds, esters and ethers.

Van Berkel et al. have used quaternary ammonium derivatives and ferrocene-based ‘electrochemically ionisable’ derivatives [16] to enhance the ESMS analysis of simple alcohols and sterols. Ferrocenyl carboxylate derivatives, such as Fe-NHCOOR (where ROH designates the alcohol or sterol) were prepared and analysed by ESMS. Limits of detection (LODs) for the derivatised sterols were significantly better than those reported for the underivatised sterols with atmospheric pressure chemical ionisation mass spectrometry (APCIMS). Van Berkel et al. [16] demonstrated the utility of this derivatisation approach for the selective detection of alcohols in complex matrices using a saw palmetto (Serenoa repens) fruit extract known to contain a variety of alcohols at low levels.

3.8. Organometallic complexes

Gatlin and Turecek ([6], chapter 15, p. 527) have recently reviewed the ESMS of these species. Alkali metal complexes of a variety of organic ligands, including polyethylene glycols, coronands, cryptands, ionophore antibiotics, etc. have been obtained by ESMS and their gas phase properties have been correlated with their chemistries in solution. Transition metal complexes of biological molecules such as metalloporphyrins that have low ionisation energies can be detected as radical cations by ESMS after oxidation at the tip of the ES capillary.

3.9. Inorganic species

Alkali metal ions (Li⁺, Na⁺, K⁺, Cs⁺ and Rb⁺), alkaline earth ions (Mg²⁺, Ca²⁺, Sr²⁺, Ba²⁺) and singly and doubly charged transition metal ions together with their complexes with mono- and polydentate ligands can all be detected by ESMS. Anions of inorganic acids such as NO₃⁻, Cl⁻, H₂PO₄⁻ and SO₄²⁻ can also be detected by ESMS. Although ICP-MS is the technique of choice for the analysis of inorganic solutions, based on its superior sensitivity and a wide linear dynamic range, ESMS has, in principle, the ability to provide extra information on the analyte’s
valence state, molecular form and counteranions ([6], Chapter 15, p. 533).

4. Selection of applications of HPLC–ESMS and CE–ESMS from the recent literature

4.1. Proteins/peptides/carbohydrates

The covalent attachment of oligosaccharides to proteins is one of the most common post-translational modifications of eukaryote cells. Glycoproteins are fundamental to many biological processes, including fertilisation, immune defence, viral replication, cell growth, inflammation, etc. These molecules contain oligosaccharides that are attached to a protein at the hydroxyl group of serine or threonine (O-linked) or to the amide side chain of asparagine (N-linked). Variable oligosaccharide structures can be found at each linkage site and further complexity arises from the partial occupancy at each glycosylation site. Mass spectrometry has played a valuable role in the elucidation of these complex glycoproteins, including carbohydrate structure and amino acid sequence. Bateman et al. [17] have described the use of nano-electrospray CZE–MS for the study of N- and O-linked glycoprotein digests at sample loadings of high femtomoles to low picomoles using capillaries modified with [(acryloylamino)propyl]trimethylammonium chloride and dilute formic acid electrolyte for high resolution of the analytes. The technique of stepped-orifice voltage scanning [18] was used to identify glycopeptides in complex proteolytic digests. Further structural information was obtained using CE–MS–MS to elucidate the composition of both N- and O-linked glycopeptide oligosaccharides. Collisional activation in the orifice/skimmer region was also used to generate first generation fragment ions (e.g. [peptide+GlcNAc]+) which underwent subsequent dissociation in the r.f-only collision cell of the triple quadrupole mass spectrometer. This provided informative peptide backbone fragment ions usually not available from fragment ion spectra of multiply protonated glycopeptide ions. These methods were applied to the characterisation of α-amylase inhibitor 1, a lectin from Lotus tetragonolobus, two N-linked glycoproteins and to K-casein, a glycoprotein comprising O-linked sialylated glycans. The former case of α-amylase inhibitor 1 is taken as an example. The analysis of the peptides arising from mild acid cleavage of the α-chain from α-amylase inhibitor 1 can be achieved using CZE and total ion current detection in the region m/z 500–1600. Identification of the glycopeptides is then possible using selective ion monitoring at m/z = 204, corresponding to the GlcNAc (N-acetylhexosamine) oxonium ion generated by CID in the orifice/skimmer region of the mass spectrometer. For example, the first family of glycopeptides are spaced by m/z 54 consistent with triply protonated ions and correspond to the peptide A1–20 to which is bonded a variable oligosaccharide composed of GlcNAc2Man3–6. Based on the relative peak intensities, the predominant glycoform for this glycopeptide is GlcNAc2Man3. The peptide sequence ATETS-FIIDAFKTNLILQG contains two asparagine residues but only the underlined one has the tripeptide sequon characteristic of an N-linked glycopeptide.

4.2. Nucleic acids and their constituents

In their review of the 1990–96 literature of HPLC–MS, Esmans et al. [19] regard ESMS as becoming the HPLC detector of choice for the analysis of both high and low molecular mass nucleic acid material in biological samples. A section of this paper is devoted to the detection and determination of DNA adducts resulting from DNA’s reaction with chemical carcinogens. This is a particularly difficult task in vivo samples where the adduct (in which a new covalent bond is formed) may be present in only minute amounts (1 base modified out of 106–1011 bases, i.e. ca 10 pg of adduct in 1 mg of DNA). As an example, Vanhouette et al. [20] have investigated the use of HPLC–ESMS and HPLC–tandem ESMS for the structural identification of the 2'-deoxyribonucleotide/bisphenol A diglycidyl ether adducts. Negative ion ESMS low energy collision activated decomposition (CAD) spectra allowed the differentiation between phosphate alklylation and base alkylation. Among other adducts discussed are phenylglycidyl ether adducts (such as (I)), N6-oxopropenyl-21-deoxyadenosine, N2-3-etheno-thymine isolated in vivo from human and rat liver, hedamycin-d(CACGTG)2 and DNA adducts of 2-amino-1-methyl-6-phenylimidazo[4,5,6]pyridine and malondialdehyde.

4.3. Molecules that already exist as ions in solution, e.g. sulphonates

Sulphonated azo dyes are widely used in the textile industry to colour natural fibres. Manufacture and use of these dyes cause an environmental problem if the effluent is not carefully monitored. Azo dyes have
been shown to undergo reduction in natural waterways and the degradation products include amines which are known to be carcinogenic. There is therefore an interest in sensitive techniques to monitor and identify low levels of azo dyes. Although the sulphonic acid group(s) provide these dyestuffs with essential water solubility they also render them involatile and hence GLC cannot be used for their determination. Oxspring et al. [21] have reported the separation and determination of reactive textile dyes, which included sulphonated azo dyes, by HPLC and CE using visible spectrometric detection. LODs by HPLC were generally 1–2 orders of magnitude superior to CE and of the order of \(10^{-7}\) mol dm\(^{-3}\). CE, however, showed a higher efficiency of separation with up to \(4.25 \times 10^5\) theoretical plates and could satisfactorily resolve impurities in the dye preparations and also the hydrolysis products of these sulphonated azo dyes. The interfacing of HPLC and CE with a sensitive mass spectrometric detector is therefore seen as viable hyphenated techniques for the identification and quantification of such sulphonated azo dyes, their degradation and metabolic products in samples such as effluents, river waters, etc.

Bruins et al. [22] have separated such sulphonated azo dyes by HPLC and introduced them into the atmospheric pressure ion source of a triple quadrupole mass spectrometer using pneumatic nebulisers. A heated nebuliser together with corona discharge produced ions by gas phase chemical ionisation. CID of \([M-H]^-\) ions gave \(SO_3^-\) as a fragment characteristic of sulphonated azo dyes and parent ion scans on \(SO_3^-\) were used for identification of azo dyes in environmental samples. CE–ESMS has been evaluated for dyes other than sulphonated azo dyes, e.g. cationic laser dyes such as Rhodamine 6G [23], and CEC–negative ion ESMS has been applied to the determination of food colours [24].

Several studies of the ESMS behaviour of sulphonated azo dyes, a necessary precursor to ESMS coupling to HPLC and CE, have appeared in the literature. Smyth et al. [25] have examined the negative ion ESMS of four Remazol textile dyes and their hydrolysis products. They have assigned the major MS signals to species such as \([M-Na^-]\), \([M-2Na+1H]^+\) and \([M-2Na]^{2-}\). It was possible to use this information to directly monitor some of these dyes in an effluent sample although separation procedures such as HPLC and CE coupled with ESMS were recommended for a more complete analysis of such samples. Ballantine et al. [26] have also investigated the negative ion ESMS of polysulphonated azo dyes and have found that certain amine bases such as diethylamine enhance the sensitivity of ESMS towards these molecules. Polysulphonated azo dyes were observed to produce two or more ion series, each being the result of varied amounts of cation–proton exchange during the ionisation process. For example, reactive red 120 (II) of molecular mass 1469 Da is a hexasulphonated diazo dye and produced a strong \((z = -2)\) ion series when dissolved in a 50:50 mix of MeOH and H\(_2\)O.
Fig. 3. Full scan MS–MS spectra of glyphosate–FMOC (a; precursor ion m/z 390) and AMPA–FMOC (b; precursor ion m/z 332) derivatives with a low (5 eV) and high (25 eV) collision energy (2.0 mTorr Ar) at concentrations of 0.2 μg dm$^{-3}$. Included in the spectra are the proposed structures of the observed ions. Reprinted from [29] with permission.
words, for the $\varepsilon = -2$ series the single acid ion peak was observed at 667.9.

### 4.4. Low molecular mass organic bases

HPLC–ESMS is now widely used in the pharmaceutical industry for the detection and determination of drugs and their metabolites. This is because many drugs contain basic nitrogen atoms that can be protonated in the electrospray process and hence are detectable by ESMS. Smyth et al. [25] have surveyed recent applications of HPLC–ESMS and CE–ESMS to drug analysis.

Zavitsanos and Alebic-Kolbah [27] have developed a sensitive and selective HPLC–ESMS method for the enantioselective determination of the $\alpha$-adrenoceptor antagonist terazosin (III) in human plasma following a 5 mg single oral dose. The chromatography was based on normal phase chiral separation with post-column solvent addition of 2-propanol–5 mM ammonium acetate (3:1 v/v). Positive ion ESMS utilised the intense $[M+H]^+$ peak at $m/z$ 388.2 and there was a relatively small peak at $m/z$ 410.2 corresponding to $[M+Na]^+$. The method was compared to HPLC–fluorescence detection and was found to be more sensitive allowing useful information to be obtained about enantiomeric ratios from subject plasma samples at later time points post dose.

McClean et al. [12] have recently compared CE–ESMS and HPLC–ESMS for the detection and determination of 15 1,4-benzodiazepines and their metabolites. The former technique displayed a superior selectivity but HPLC–ESMS utilising a quadrupole ion trap mass spectrometer possessed superior LODs and was able to detect and quantify diazepam and its metabolites in a hair sample of a patient on therapeutic dosage of this drug.

### 4.5. Low molecular mass organic acids

Chiron et al. [28] have used automated on-line liquid–solid extraction followed by HPLC–ESMS for the determination of acidic herbicides such as 2,4-D, MCPA, MCPP, MCPB, benzoicin, benzatone and hydroxybentazones. Their proposed method required only 50 cm$^3$ water, had an LOD of 0.01–0.03 µg dm$^{-3}$ and utilised SIM on the $[M–H]^-$ species.

The maximum allowable concentration of the herbicide glyphosate (IV), molecular mass 169, in drinking water set by the EC is 0.1 µg dm$^{-3}$ and can be assayed by derivatisation with 9-fluorenyl methoxy-carbonyl chloride (FMOC-Cl) (V) followed by solid phase extraction and finally determination by HPLC with fluorescent detection. The structure of the derivative is given in (VI), MW391. The major degradation product, aminomethylphosphonic acid (AMPA) (VII) (MW111), can also be analysed by this method. This method, however, is relatively non-specific and requires MS confirmation to ascertain the identity of the glyphosate and AMPA peaks. Vreeken et al. [29] have recently published a paper with this end in mind producing a fully automated procedure using HPLC–MS and HPLC–MS–MS. Their scan routine resulted
in three analytically usable signals, i.e. the [M−H]− ion signal from the derivative during the HPLC–MS scan and two products ion signals formed upon CID. Quantification and identification based on the ratios of these three signals and the retention time were very specific thus reducing the number of false positives. The appropriate product ion spectra are given in Fig. 3 for (VI) and the AMPA–FMOC derivative.

4.6. Molecules that do not contain acidic or basic functional groups

Molina et al. [30] have determined organophosphorus pesticides in water by solid phase extraction followed by HPLC–ESMS using SIM of the [M+Na]+ ion with a method detection limit of 0.01 µg dm−3.

Hydrocarbon carotenes are another example of a class of compounds that will not ionise under ESMS conditions. However, because of the low ionisation potential of the π electrons in the conjugated chain of carotenoids, molecular ion radicals, M+·, have been generated for carotenes during ESMS by adjustment of the electrospray conditions to facilitate electrophoretic charging and electrochemical ionisation at the metal–liquid interface of the electrospray capillary [31]. Ionisation was enhanced by post-column addition of a halogenated oxidant (0.1% heptafluorobutanol). Such HPLC–ESMS analysis is illustrated in Fig. 4 where the MS detector was found ca 100 times more sensitive than the photodiode array absorbance detector and showed an LOD for α-carotene of 28 pmol.

5. Conclusions

ESMS is now a widely used technique, particularly when linked to HPLC, for the identification and determination of a wide range of molecules of high and low molecular mass such as proteins and drugs respectively. Sample pretreatment/analyte concentration such as an immunoaffinity process prior to HPLC–ESMS has been demonstrated to provide significant LOD improvements [32] and time of analysis for proteins and peptides has been reduced using perfusion chromatography [33]. HPLC–ESMS has also been used successfully in an automated mode for the handling of large numbers of samples in drug metabolism and pharmacokinetic studies as well as in screening for active compounds in combinatorial libraries. In addition, low-flow rate or ‘nano-ES’ ion sources have been developed [34] for low sample flow rates (down to 10–20 nl min−1 which are well suited to capillary HPLC (0.18 mm i.d. columns and smaller)) or capillary electrophoresis where the flow rates often do not exceed 1 µl min−1. Capillary electrophoresis, with the need to provide both an electrical contact and a stable electrospray at the capillary tip, will additionally benefit from improvements in interface durability and reproducibility. Routine separations and protein/peptide sequence determination at femtomole/atto-mole levels are distinct possibilities.

References

Sequential injection analysis: an alternative approach to process analytical chemistry

Neil W. Barnett, Claire E. Lenehan, Simon W. Lewis*
School of Biological and Chemical Sciences, Deakin University, Geelong, Vic. 3217, Australia

Flow injection analysis (FIA) is well established as a powerful sample handling procedure for laboratory and process analytical chemistry, but the latter application is often hampered by the complexity of the required manifolds particularly for multicomponent analyses. In certain circumstances multicomponent process analytical chemistry is only feasible by utilising several independent FIA systems. This review presents an overview of sequential injection analysis (SIA), which addresses these limitations, and applications of the methodology to the challenging area of process analytical chemistry will be discussed. ©1999 Elsevier Science B.V. All rights reserved.

Keywords: Sequential injection analysis; Process analytical chemistry; On-line analysis

1. Introduction

High quality chemical information attainable in ‘real time’ is vital for the control of modern chemical manufacturing facilities. The provision of this information by process analytical chemistry (PAC) is essential not only for monitoring and controlling a process, but also for the efficient use of energy, time and raw materials [1]. Given the considerable community concern over the impact of the chemical industry on the environment, the information generated by PAC is increasingly used to minimise plant effluent in addition to improving product quality, quantity and consistency [2].

Traditionally PAC has been performed remote from the process line in a centralised laboratory [3]. Offline chemical measurement in this manner may lead to a significant time delay between submission of a sample and analysis result, particularly when demands upon staff and instrumentation are great. The limited number of samples that can be analysed in any particular time can result in an incomplete chemical profile and therefore only a partial understanding of the process being monitored. An alternative approach is to utilise automated, unattended, analytical instrumentation sampling directly from the process line. With such