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# Considerations on Column Selection and Operating Conditions for LC–MS

**Pat Sandra[1,2],** with **Gerd Vanhoenacker[2], Fréderic Lynen[2], Liu Li[2] and Marc Schelfaut[2],** [1]Research Institute for Chromatography, Kortrijk, Belgium, [2]Dept of Organic Chemistry, University of Ghent, Belgium.

Mass spectrometry (MS) is slowly but surely becoming the detection system of choice for liquid chromatography (LC) analysis in many areas. Translating methods from conventional LC–ultraviolet (UV) detection to LC–MS is often required because optimized LC–UV mobile phases usually contain buffers and additives that have a negative effect on MS ionization, MS performance and maintenance of the mass spectrometer. Moreover, depending on the LC–MS configuration, all or part of the effluent is directed towards the MS optics. In addition, because of the high selectivity of MS, column dimensions have changed drastically over recent years leading to high-throughput screening in, for example, combinatorial chemistry, clinical/pharmaceutical studies, bioanalysis etc. In this section, an overview is presented of column and mobile phase selection for LC–MS. In the framework of this guide it is impossible to strive for completeness. However, relatively simple guidelines are advanced to successfully optimize LC–MS for several applications. Most illustrations are from the laboratories of the authors.

## Fundamentals of LC Related to MS Hyphenation

In order to fully understand column selection for LC–MS, some simplified equations are discussed. For more details we refer the reader to the "Starting Out Right" article series by J.W. Dolan (1).

Chromatographic resolution is given in Equation 1, and analysis time in Equation 2, where $N$ is the plate number, $H$ the plate height, $d_p$ the particle diameter (µm), $\gamma$ a dispersion factor, $\lambda$ the packing factor, $\alpha$ the selectivity factor, $k$ the retention factor and $u$ the average linear velocity (mm/s). In the optimal case, that is, reversed-phase separations on C18, $\gamma$ and $\lambda$ are close to 1. In other separation modes such as size exclusion chromatography (SEC), $\gamma$ can be as high as 10. It is important to note that in both the resolution and analysis time equations, the internal diameter (i.d.) of the LC column is not included, and both are thus independent of column i.d.

The average linear velocity is related to the column pressure drop $\Delta P$ (in Pa) and the particle diameter of the packing material (Equation 3), where $\eta$ is the liquid viscosity (in Pa.s). The value "1000" is an empirical factor called the *specific permeability coefficient*. In LC, high pressure drops are required and LC instrumentation is capable of operating at 40 MPa.

These fundamental equations mean that a 25 cm × 4.6 mm i.d. column packed with 5 µm spherical particles is nowadays "the workhorse" in LC. Pressure drop is not a problem with commonly used mobile phases and velocities ranging from 1 mm/s (~0.8 mL/min) to 3 mm/s (~2.5 mL/min) can easily be obtained giving plate numbers in the order of 10 to 20 × $10^3$.

The present trends are, however, to reduce column length and/or internal diameter.

Reducing the column length is often accompanied with a decrease in particle diameter to keep the efficiency constant. A 15 cm × 4.6 mm i.d. column with 3 µm particles gives the same plate number for nearly the same pressure drop as a 25 cm × 4.6 mm i.d. column with 5 µm particles. The slope of the efficiency versus velocity curve becomes flatter for the small-particle column and higher velocities can be applied. This, together with the shorter column length, reduces the analysis time substantially. Decreasing the column length further for the same particles will reduce the efficiency and thus the "chromatographic" resolution. This is illustrated in Figure 1, which shows the analysis of some analgesics on different column lengths.

The sample was analysed on Zorbax SB-$C_{18}$ columns (various lengths, 4.6 mm i.d., $d_p$ 3.5 µm) with a 1 mL/min flow of the mobile phase: 1 mM sodium octane sulfonate (pH 2.5)/$CH_3CN$ in ratio 80/20 at 70 °C, and with UV detection at 275 nm. On the 3 cm column, the solutes are

$$Rs = {}^1/_4 N^{0.5} \cdot \alpha - 1/\alpha \cdot k/k + 1 = {}^1/_4 (L/H)^{0.5} \cdot \alpha - 1/\alpha \cdot k/k + 1 = {}^1/_4 (L/2\gamma\lambda d_p)^{0.5} \cdot \alpha - 1/\alpha \cdot k/k + 1 \quad [1]$$

$$t_R = L/u \cdot (1 + k) = 16\, R_s^2 \cdot (\alpha/\alpha - 1)^2 \cdot (1 + k)^3/k^2 \cdot \gamma\lambda d_p/u \quad [2]$$

$$u = \Delta P . d_p^2 / 1000\, \eta . L \quad [3]$$

baseline separated in less than 2 min. Because of the flat nature of the efficiency versus velocity plot, further decrease in analysis time can be performed by increasing the flow-rate as shown in Figure 2.

No loss in resolution is observed between 1 (Figure 1), 2, 3 and 4 mL/min while analysis time decreases from 2 to 0.5 min. Such speeds are very favourable for high-throughput screening even if the solutes are not completely resolved or not resolved at all! In those instances, the "mass spectrometric" selectivity is fully exploited by selecting specific target ions. In high-throughput screening using MS, MS–MS or high-resolution MS different approaches are presently used. The fastest cycle times are obviously obtained by flow injection analysis (FIA) and automated systems are commercially available for unattended analysis of over 4600 samples using 384-well plates. FIA, however, requires relatively simple samples and no interference of matrix solutes. The technique is, therefore, mainly restricted to applications of combinatorial chemistry and drug discovery. For other applications, such as pharmacokinetic studies, medicinal chemistry and natural product analysis, interfering solutes (proteins, sugars, etc.) are separated via a short LC column, the efficiency (length) of which is dictated by the problem at hand. Typically, columns of 1 to 5 cm are applied with particles ranging from 1.5–4 µm to 50 µm (Turbo flow LC, Cohesive Technologies).

For 4.6 mm i.d. columns operated at high speed, the flows generated are generally too high to be directly introduced into the MS. The classic way to bypass this is to use a flow splitter after the column (see later). This, however, is accompanied by loss in sensitivity. A better way is to reduce the internal diameter of the LC column.

This can be deduced from the flow equation

$$\text{flow}_{MC} = \text{flow}_{CC} \cdot (\text{i.d.}_{MC}/\text{i.d.}_{CC})^2 \quad [4]$$

where MC stands for a microbore column (typically 2 mm i.d.) and CC for a conventional column of 4.6 mm, such that by decreasing the i.d. by a factor 2, flow is reduced by a factor 4 for the same velocity. These low flows (typically 0.2 mL/min for a 2 mm i.d. column ) are compatible with state-of-the-art LC–MS configurations using, for example, orthogonal or Z-spraying.

A typical application, namely the sequencing by LC–$MS^3$ of two peptides obtained by combinatorial chemistry is discussed (Figure 3).

Two strains of amino acids are attached to a steroid-like (Scaffold) structure with MW 1143.7 dalton. The sequence of one strain is constant (Leu-His-Ala), while the sequence of the other strain is variable ($AA_1$-$AA_2$-$AA_3$) but consists of Phe, Ser and Gly. FIA cannot determine the sequences of isomeric peptides and a fast chromatographic step was implemented to partially separate the two isomers. The column (3 cm × 2.1 mm i.d., 3.5 µm $d_p$ C18) was operated at 0.3 mL/min MeOH/$H_2O$ (60/40). Both spectra are given in Figure 4 and elucidation of the fragments in $MS^3$ in Table 1. The sequence in compound 1 is Phe-Ser-Gly and in compound 2 Ser-Gly-Phe.

Further miniaturization of the column has led to capillary LC–MS. This technique is widely accepted when only limited sample quantities are available, for example, in peptide mapping. Typically, stainless steel columns of 1 mm or fused-silica capillaries with 300 to 180 µm i.d. are used at flow-rates of 50, 4 and 2 µL/min, respectively. Concerning the MS site, columns can be coupled directly to the MS via a nanospray interface followed by atmospheric pressure electrospray ionization (APESI) or atmospheric pressure chemical ionization (APCI), or chromatographic peaks can be collected by automated techniques directly onto matrix assisted laser desorption ionization (MALDI)

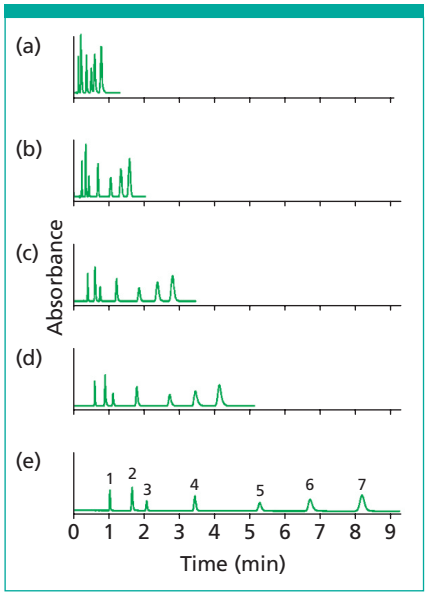


**Figure 1:** *LC analysis of several analgesics on columns of various lengths, as follows: (a) 15 mm, (b) 30 mm, (c) 50 mm, (d) 75 mm and (e) 150 mm. For chromatograpic conditions, see text. Peaks: 1 = 4-acetamido-phenol, 2 = caffeine, 3 = 2-acetamido-phenol, 4 = acetanilide, 5 = acetosalicylic acid, 6 = salicylic acid, 7 = acetophenetidin. (Figure courtesy of Agilent Technologies.)*

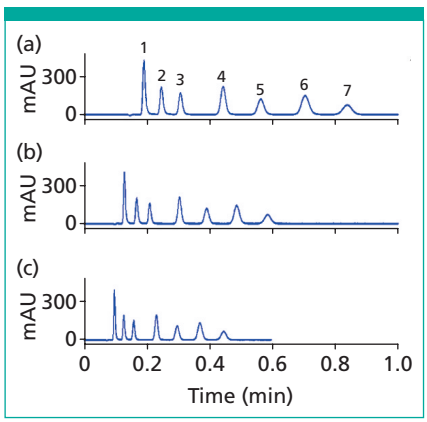


**Figure 2:** *LC analysis of several analgesics on a 3 cm length column at the following mobile-phase velocities: (a) 2 mL/min, (b) 3 mL/min and (c) 4 mL/min. Pressure (a) 90 bar, (b) 133 bar and (c) 181 bar. For peak identification, see Figure 1. (Figure courtesy of Agilent Technologies.)*

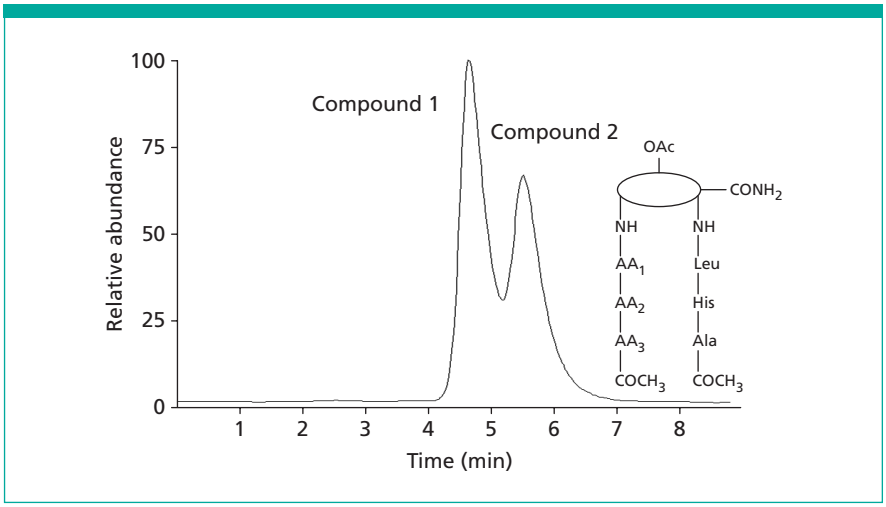


**Figure 3:** *LC–$MS^3$ of two isomeric peptides.*

targets having sizes in the range of a few microlitres. For an overview we refer the reader to reference 2 and to specific capillary LC–MS manufacturers' literature.

In conclusion, 15 cm × 2 mm i.d. LC columns packed with 3 to 4 µm particles are recommended for high-efficiency separations in LC–MS, and shorter columns for combinatorial chemistry and high-throughput screening analyses.

## Hyphenating LC to MS

Much of the advancement in LC–MS over recent years has been in the development of atmospheric pressure ionization (API) techniques and the application of off-axis nebulizers. Most mass spectrometers can nowadays accept flow-rates of up to 1–2 mL/min for APESI and of 4 mL/min for APCI. The LC column outlet can therefore be directly coupled to the nebulizer. This is commonly done using polyetheretherketone (PEEK) tubing and fittings. This material is, however, not resistant to all chemicals and for some applications such as SEC with tetrahydrofuran (THF), PEEK should be replaced by fused-silica tubing.

Notwithstanding the high flow-rates that state-of-the-art LC–MS instrumentation can handle, lower flow-rates are often preferred, on the one hand, because some instruments operate better at lower flow-rates and, on the other hand, because less maintenance is required, especially when involatile additives are included in the mobile phase (see later). Lower flow-rates are obtained by applying 2 mm i.d. LC columns or by splitting the flow of conventional columns, thus diverting part of the column flow away from the MS. This is performed by placing a T-piece (PEEK or stainless steel) after the column (Figure 5(a)). A similar device is used for postcolumn addition (Figure 5(b)). One arm of the T-piece is connected to an LC pump to deliver a make-up liquid composed of MS-friendly constituents to give good and controlled ionization. Several applications will be presented to illustrate this principle.

With minor modifications modern LC–MS systems can generate good results at microlitre (capillary LC) and nanolitre (electrodriven methods) flow-rates.

## Adapting LC to LC–MS Methods

Successful use of LC–MS often involves changes in sample preparation and especially in solution chemistry compared with, for example, an LC–UV method. During the sample preparation and/or chromatographic process compounds that can cause background ions, suppress ionization or give rise to several molecular weight adducts should be removed. Desalting can reduce the sodium or potassium adduct formation that commonly occurs in APESI. This is less critical for APCI. Solution chemistry is of utmost importance in APESI because formation of ions in solution is essential. If mobile-phase adjustment for MS interferes with the chromatographic performance, postcolumn modification of the mobile phase may offer a good solution. Solution chemistry is less critical for APCI because ionization occurs in the gas phase. Nevertheless, the solvent can still have a significant effect on ionization. For the positive APCI mode protic solvents work better than aprotic solvents, while for the negative mode, solvents that capture an electron should be used. Last but not least, volatile constituents (solvents, buffers, additives) in the mobile phase should be selected whenever possible. This does not mean that involatile buffers cannot be applied, but at the expense of robustness and maintenance time.

Very important is making a good selection of ionization modes: APESI or APCI, and the polarity: positive or negative mode. This is not always straightforward.

APESI is useful for polar and ionic solutes ranging in molecular weight from 100 to 150 × $10^3$ dalton. Large molecules acquire more than one charge allowing their

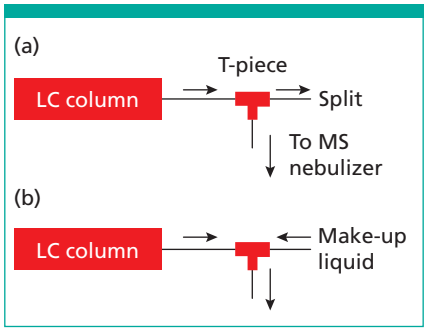


**Figure 5:** *(a) Postcolumn splitting and (b) make-up fluid addition.*

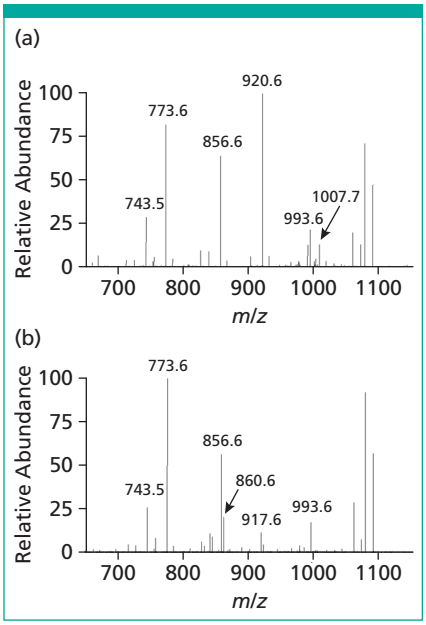


**Figure 4:** *$MS^3$ for (a) compound 1 and (b) compound 2 from Figure 3.*

**Table 1:** *Interpretation of the Fragmentation for Compounds 1 and 2 from Figures 3 and 4.*

| | | | |
|---|---|---|---|
| **MS** | $[M + Na]^+$ | 1166.7 | |
| **MS–MS on 1166.7** | $[M - CH_3COOH + Na]^+$ | 1106.7 | |
| **MS–MS–MS on 1106.7** | $[A + Na]^+$ | 773.6 | |
| | $[A + AA_1 + Na]^+$ | | Variable |
| | $[A + AA_1 + AA_2 + Na]^+$ | | Variable |
| | $([A + AA_1 + AA_2 + AA_3\text{-Ac} + Na]^+$ | | 1106.7) |
| | $[B + Na]^+$ | 743.5 | |
| | $[B + Leu + Na]^+$ | | 856.5 |
| | $[B + Leu + His + Na]^+$ | | 993.6 |
| | $([B + Leu + His + Ala\text{-Ac} + Na]^+$ | | 1106.7) |
| | Residue weight of Phe = 147.2, Ser = 87.1, Gly = 57.1 | | |
| **Sequence:** | | | |
| **Compound 1** | | | |
| $AA_1$ | **Phe** | $[A + Phe + Na]^+$ | 920.6 amu |
| $AA_2$ | **Ser** | $[A + Phe + Ser + Na]^+$ | 1007.6 amu |
| $AA_3$ | **Gly** | | |
| **Compound 2** | | | |
| $AA_1$ | **Ser** | $[A + Ser + Na]^+$ | 860.5 amu |
| $AA_2$ | **Gly** | $[A + Ser + Gly + Na]^+$ | 917.6 amu |
| $AA_3$ | **Phe** | | |

analysis by MS instruments with a mass range of 3000 *m/z*. With the help of deconvolution programs the molecular weight can then be determined.

APCI is applicable to non-polar and medium polarity molecules with a molecular weight ranging from 100 to 2000 dalton.

The recently introduced atmospheric pressure photoionization (APPI) technique is applicable to many of the same solutes as APCI but can give better response for highly apolar solutes.

Notwithstanding these rules of thumb, it is advisable to check, eventually via FIA, what mode gives the best response taking the signal-to-noise ratio into consideration.

As an illustration, some medium polarity, small molecular weight testosterone esters that were supposed to give the best ionization in APCI, based on the fact that they could be analysed with a particle beam interface and electon impact ionization (3), could be ionized using APESI with higher response and better baseline stability (Figure 6). The ODS column (12.5 cm × 4.6 mm i.d., $d_p$ 5 µm) was operated at a flow-rate of 1 mL/min in a gradient (A: $H_2O$, B: MeOH) from 0 to 12 min from 82% to 95% B. The MS spectra of peak 2, testosterone phenylpropionate (*m/z* 420), in positive APCI and APESI modes are given in Figure 6. As can be seen, APESI gives the proton, sodium and potassium adduct ions. It is of utmost importance to evaluate the relative distribution of adducts for quantification and validation (4).

Desalting is normally not a problem for large (bio)molecules but for small molecules better data are obtained when adduct formation is forced in a specific direction by adding a selected cation postcolumn; for example, sodium as a sodium acetate solution (5).

The selection of a correct detection polarity (positive and negative mode) is based mostly on the characteristics of the solutes. Sometimes acidic and basic solutes are present in the same sample and both modes should be evaluated (4). At present, by continuously alternating the voltage on the capillary of the MS inlet, both positive and negative ions can be recorded in the same chromatographic run.

Sometimes all different modes have been evaluated and ionization still does not occur. This was the situation for the analysis of the long-chain esters in jojoba oil. The problem could be solved by applying coordination ion spray (CIS) MS. CIS was pioneered by Karlsson (5) for the analysis of carbohydrates and further developed by the group of Bayer (6). In CIS, positively or negatively charged complexes are formed by addition, preferably postcolumn, of a suitable ion to the analytes followed by APESI analysis. As both polar and non-polar organic compounds can form coordination compounds with an appropriate central atom, this form of ionization is highly versatile. Moreover, the sample is subjected to less thermal stress than when APCI is used. For the jojoba oil esters, $Ag^+$ ions (10 µL/min of a 1.6 mg/mL $AgNO_3$ aqueous solution) were added postcolumn. The separation was performed on a Spherisorb BDS column (25 cm × 2.0 mm i.d., $d_p$ 5 µm) with solvent A: MeOH and solvent B consisting of a mixture of MeOH/acetone/hexane (1/1/1). The flow-rate used was 0.2 mL/min with isocratic conditions at 70% B for 25 min. Figure 7(a) clearly shows the different esters in jojoba oil and as an illustration the MS of the peak eluting at 15.42 min is shown in Figure 7(b).

The pseudo molecular ion $[M + Ag]^+$ is clearly observed with good sensitivity. The Ag atom gives rise to isotopic peaks at Δ*m/z* 2 with equal intensities (107/109). With proper selection of the collision-induced dissociation (CID) voltage the acidic and alcoholic part can be elucidated; that is, the peak eluting at 15.42 min is $C_{19}H_{37}COOC_{20}H_{39}$.

Selection of the ionization technique is closely linked to the applied mode of LC. The properties of the most prominent separation mechanisms in LC are discussed. Considerations, difficulties and solutions for the hyphenation with MS are summarized. The LC modes with their preferred ionization technique are shown in Figure 8.

**Normal-Phase Liquid Chromatography**

In normal-phase liquid chromatography (NPLC), the stationary phase is polar and the mobile phase has a weaker polarity

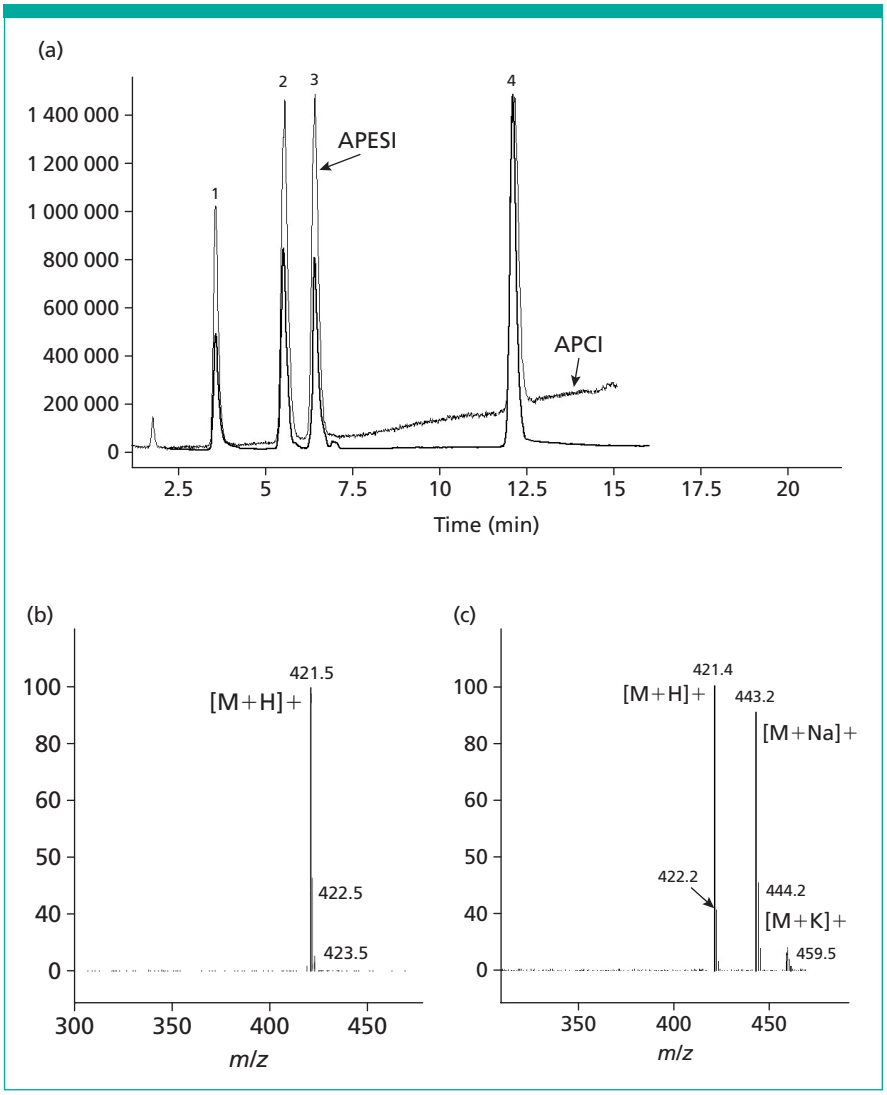


**Figure 6:** *(a) LC–APCI-MS and LC–APESI-MS analysis of some testosterone esters. Peaks: 1 = testosterone propionate, 2 = testosterone phenylpropionate, 3 = testosterone caproate, 4 = testosterone decanoate. (b) Peak 2 in APCI mode. (c) Peak 2 in APESI mode.*

than the stationary phase. NPLC is used to separate apolar to medium polarity solutes and, especially, isomers. The separation mechanism is a result of electrostatic forces between the permanent dipole of the stationary phase and the permanent or induced dipole of the analytes. Stationary phases are inorganic in nature (silica, alumina, porous graphitized carbon), or polar groups such as diol, aminopropyl and cyanoproyl are chemically bonded to a silica support. Bonded phases are increasingly used because of improved reproducibilities compared with inorganic phases that, moreover, require very long equilibration times. Mobile phases range from weak (e.g., hexane), to medium strong (e.g., chloroform, acetonitrile, tetrahydrofuran) to strong (e.g., isopropanol, methanol), and mixtures of any of these. Retention increases with increasing polarity of the solutes. For polar compounds asymmetrical peak shapes can be corrected by addition of acetic or formic acid (for acids), and ammonia or triethylamine (for bases) to the mobile phase. NPLC mobile phases are quite MS-friendly and easily evaporate in the ion source. Consequently, flow-rates as high as 4 mL/min can be applied. Because of the polarity of the analytes APCI (or APPI) appears to be the ionization technique of choice and addition of buffers or acidic and basic solutes is not required. However, for solutes that easily ionize the application of APESI often results in improved sensitivity. This was the situation for the determination of some pesticides in water samples. Although reversed-phase LC (RPLC) could be applied, NPLC was selected because it was more compatible with the sample preparation procedure. Figure 9 shows the selected ion chromatograms on a diol column (25 cm × 4.6 mm i.d., $d_p$ 5 µm) with a mobile-phase gradient from 50:50 *n*-hexane/isopropanol containing 0.1% $NH_4Oac$ (50:50) to 30:70 in 20 min. The triazines (propazine, atrazine, simazine) and phenylurea (isoproturon, diuron) pesticides were monitored in the positive APESI mode while the chlorophenol pesticides were detected in the negative APESI mode. This was performed in the same run by alternating the voltage on the MS inlet capillary.

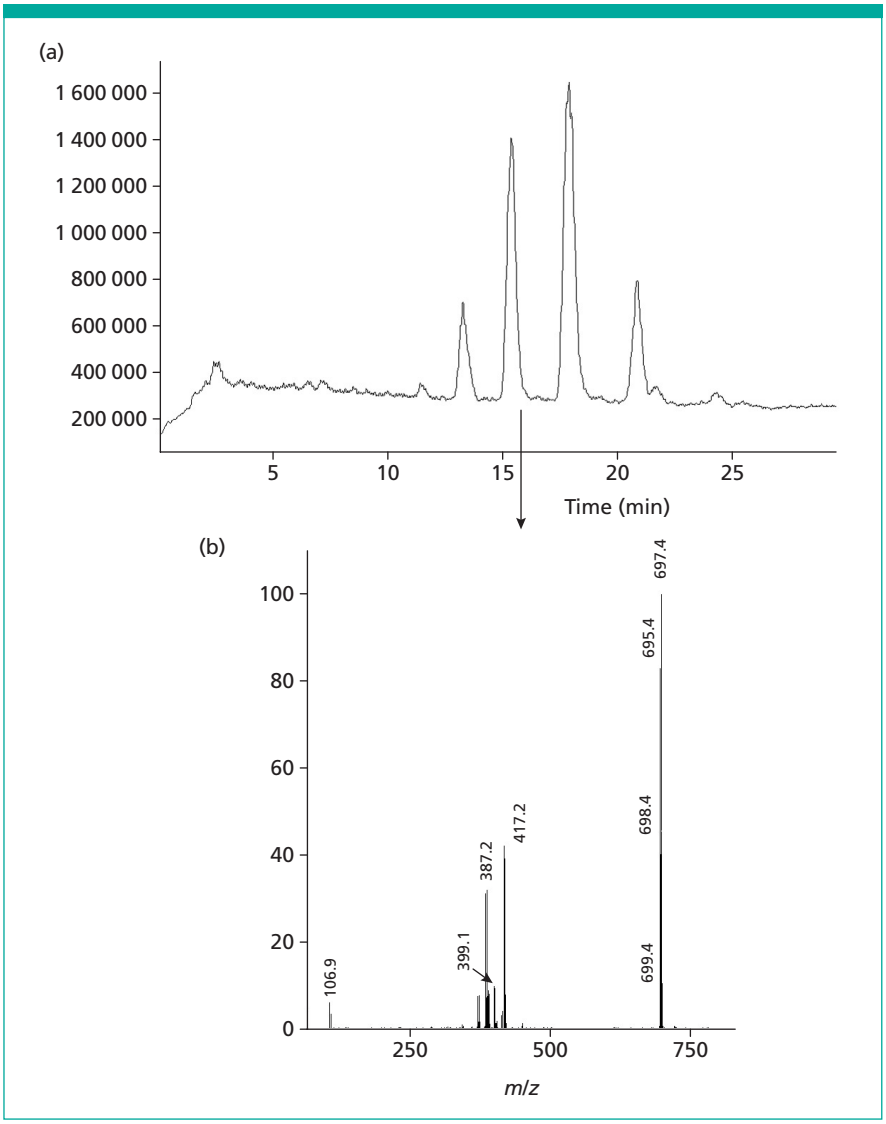


**Figure 7:** *(a) Analysis of jojoba oil with LC–CIS-APESI and (b) the mass spectrum of the peak eluting at 15.42 min.*

**Supercritical Fluid Chromatography**

In supercritical fluid chromatography (SFC) a "fluid", either a gas or a liquid, above its critical temperature and pressure, is used as mobile phase. $CO_2$ is applied in most instances because of its favourable critical parameters; that is, a critical temperature of 31 °C and a critical pressure of 7.3 MPa. Moreover, it is cheap, non-toxic and inflammable. The mobile phase is kept under supercritical conditions via an electronically controlled variable restrictor positioned after a spectroscopic detector, such as UV (packed column SFC or pSFC) or via a fixed restrictor positioned before a gas-phase detector (capillary SFC or cSFC). Only pSFC will be discussed as it is more useful for routine use, robust and easier to couple to MS than cSFC. The retention characteristics of the analytes are influenced by the properties of the stationary phase, and also by the polarity, selectivity and density of the mobile phase ($CO_2$). The density is controlled by variation of temperature and pressure of the supercritical medium. Furthermore, by addition of polar modifiers, such as methanol, elution of medium polarity compounds under high densities can be achieved. Strictly speaking, pSFC is NPLC; the mobile phase is apolar to medium polarity and the stationary phase is polar (e.g., silica, diol-, aminopropyl- or cyanopropyl silica). In the absence of a modifier, $CO_2$ can act as reagent gas giving real molecular ion spectra (Equation 5).

$$[CO_2^{+\circ} + M \rightarrow CO_2 + M^{+\circ}] \qquad [5]$$

Alternatively, with a modifier such as MeOH and under APCI conditions clear $[M + H]^+$ ions are observed. Several SFC–MS interfaces have been described but for pSFC they are too complicated. Commercial SFC instruments can easily be coupled to MS with only a very minor modification, namely by positioning a zero-dead-volume stainless steel T-piece before the restrictor for postcolumn make-up liquid addition. pSFC–MS is illustrated with the analysis of triglycerides (TGs) in soya oil.

Until now, the most successful chromatographic approach to separate TGs has been silver-ion chromatography (SIC), a form of NPLC. TGs are separated according to the number, position and geometrical configuration of the double bonds. Silver ions have the ability to form reversible complexes with unsaturated compounds. The stability of the complexes is low and the formation depends upon the number, position, configuration and steric hindrance of the double bond. In state-of-the-art SIC, the silver ions are loaded in situ onto a cation exchange stationary phase; that is, via ionic bonds to phenylsulfonic acid connected to the silica support (Figure 10).

A Nucleosil 100-5 SA column (25 cm × 4.6 mm i.d., $d_p$ 5 µm) was flushed with an aqueous 1% ammonium acetate solution at a flow-rate of 0.5 mL/min for 1 h, followed by distilled water for another 60 min. A silver nitrate aqueous solution (20% w/v) was then injected via a Rheodyne valve in 50 µL aliquots at 1 min intervals (20 successive injections). The column was finally washed for 20 min with water and for 1 h with methanol. The TG analysis was performed at 65 °C, a flow-rate of 1 mL/min $CO_2$ containing a mixture of acetonitrile/isopropanol (6/4) as modifier. The pressure was programmed from 150 bar (2 min) to 300 bar at 1.5 bar/min. The modifier was programmed from 1.2% (2 min) to 7.2% (28 min) at 0.3%/min, and then to 12.2% at 0.54%/min. The injection volume was 5 µL of a 0.2% solution of soya oil. The make-up solvent was 1.5 mL/min methanol. The separation of TGs according to their degree of unsaturation and carbon number is shown in Figure 11. When two solutes are characterized by the same degree of unsaturation, higher retention is related to a higher carbon number; for example, PLL (peak 12) elutes before SLL (peak 13). The abbreviations used are P for palmitic acid, S for stearic acid, O for oleic acid, L for linoleic acid and Ln for linolenic acid. Some representative spectra: PLP (peak 4), PLL (peak 12), PLLn (peak 16) and LLLn (peak 21) are shown in Figure 12. APCI is able to produce molecular ion signals $[M + H]^+$ and fragments related to the fatty acids linked to the glycerol backbone acid $[M-(R-COO)]^+$. The higher the degree of unsaturation, the more intense is $[M + H]^+$ and the less intense are the fragment ions.

Table 2 identifies the triglycerides and their specific fragments obtained at 100 V CID.

**Reversed-Phase Liquid Chromatography**

Because of its robustness and ease of method development, RPLC in its different forms covers more than 80% of LC applications. Besides classic RPLC, hydrophobic interaction chromatography (HIC) and ion pair chromatography (IPC) make use of the same separation mechanism. In RPLC medium polarity, polar and ionogenic compounds are separated according to the difference in hydrophobicity by partitioning between an apolar stationary phase and a polar mobile phase. Most stationary phases are based on silica chemically modified with

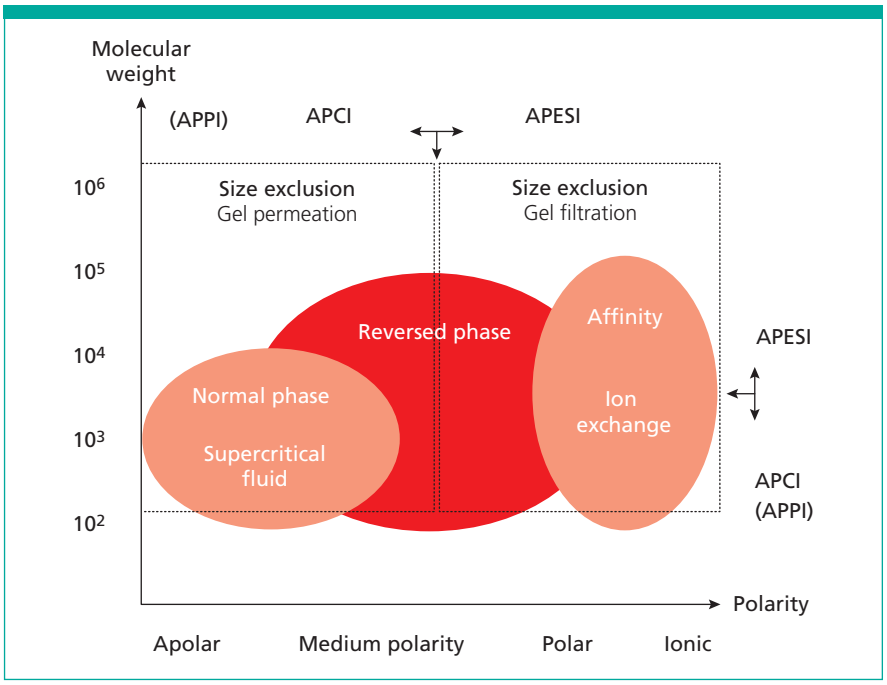


**Figure 8:** *LC modes linked to ionization techniques.*

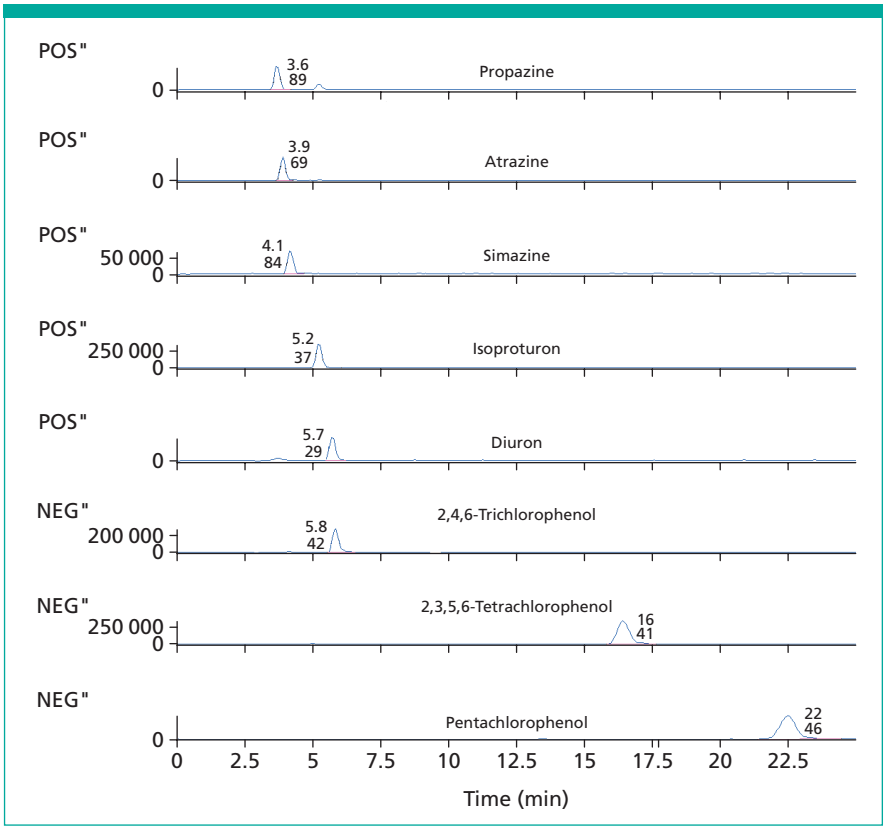


**Figure 9:** *Analysis of some pesticides by NPLC–APESI-MS in the alternating positive and negative mode.*

octadecyl (C18 or ODS), octyl (C8) or phenyl. Also cyanopropyl silica can be used for reversed-phase-type separations. Other apolar stationary phases are based on synthetic polymers such as the styrene-divinylbenzene copolymer. The elution strength of the mobile phase is increased according to the hydrophobicity of its constituents; that is, water<methanol< acetonitrile<ethanol<isopropanol< tetrahydrofuran. Mixtures of the organic modifiers, mostly methanol and acetonitrile, in water or aqueous buffers are commonly applied. Buffering is presently the subject of controversial discussions. In the past (and unfortunately still very often the case) a buffer was needed first to neutralize the analytes because ions show poor retention on RP columns and second to block unwanted activity of the stationary phase. Involatile buffers, for example, are very popular because of their good buffering capacity and the wide pH range over which they can be applied. Fortunately, the characteristics in terms of inertness of silica-based stationary phases have improved tremendously in recent years and buffering with involatile substances can be omitted. This is illustrated with the analysis of the analgesics discussed previously (Figures 1 and 2). IPC was applied through addition of sodium octane sulfonate to the mobile phase (Figure 13). The analysis was performed on a 3 cm column at a 2 mL/min flow of the mobile phase: 1 mM sodium octane sulfonate (pH 2.5)/$CH_3CN$ in the ratio 80/20 at 70 °C and with UV detection at 275 nm. This could easily be translated to the application of the mobile phase $H_2O$ (pH 2.5)/$CH_3CN$ (1% formic acid overall). Another application of the translation involatile to volatile can be found in reference 4.

As a rule of thumb, an RPLC–MS method will always be developed in our laboratory starting from volatile substances in the mobile phase. Only when the required chromatographic selectivity cannot be obtained involatile buffers will be used. However, microbore columns will be selected to reduce the flow and this accompanied with postcolumn addition of an acid (positive ion mode) or a base (negative ion mode) to enhance ionization (Equation 6).

Moreover, buffers will be evaluated in which only the anionic or cationic part is involatile, for example, ammonium phosphate instead of sodium phosphate.

The selection of mobile-phase constituents in RPLC–MS using state-of-the-art RP columns is not an easy task and conditions proposed for a number of applications are not always optimized for others. Nevertheless some guidelines for APESI and, to a lesser extent, for APCI are

- methanol is to be preferred above acetonitrile as organic modifier, definitely in the positive polarity mode
- ammonium acetate ($NH_4OAc$) and ammonium formate ($NH_4OCOH$) are generally applicable (pH ~7): concentration for APESI ≤ 10 mM and for APCI < 100 mM
- for the positive ion mode a pH < 7 should be used (5 is preferred). $CH_3COOH$, HCOOH, as such or in combination with $NH_4OAc$ or $NH_4OCOH$. The use of $CF_3COOH$ will be discussed later.
- for the negative ion mode a pH > 7 should be used (9 is preferred). $NH_4OH$, TEA (triethylamine), DEA (diethylamine), as such or in combination with $NH_4OAc$ or $NH_4OCOH$.

Literature data on the analysis of therapeutic drugs (7, 8), drugs of abuse and poisons (8) supports these guidelines.

This does not mean that buffering or addition of additives is always required for MS reasons! FIA analysis, if possible, is helpful in deciding whether additives must be added to obtain good ionization. This is illustrated with the analysis of some degradation products in an oestradiol drug formulation (Figure 14(a)). The sample was analysed in the negative APESI mode on an

| | | |
|---|---|---|
| positive ion mode | $Solute + HA \longleftrightarrow SoluteH^+ + A^-$ | [6] |
| negative ion mode | $Solute + B \longleftrightarrow Solute^- + HB$ | |

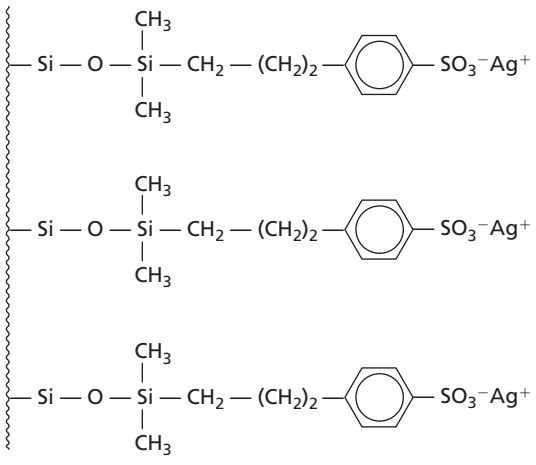


**Figure 10:** *Silver ions loaded on a cation exchange stationary phase.*

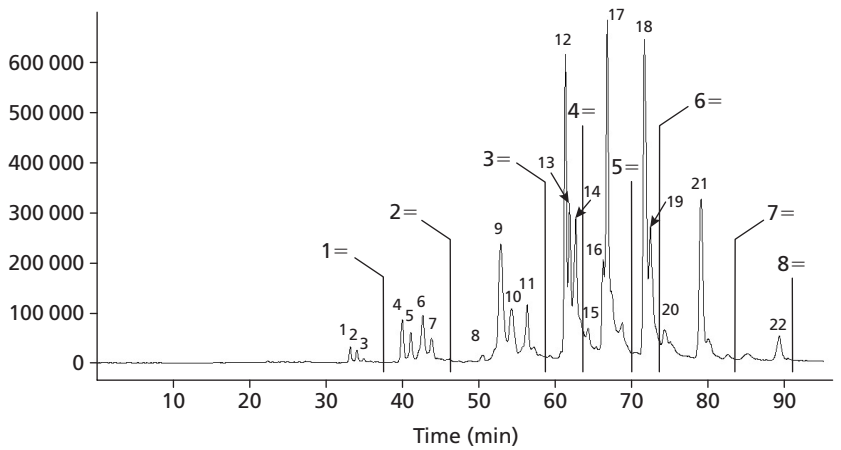


**Figure 11:** *SI-pSFC–APCI-MS analysis of the triglycerides in soya oil.*

ODS column (25 cm × 4.6 mm i.d., $d_p$ 5 µm) with a 0.7 mL/min flow of the mobile phase: MeOH/$CH_3CN$/$H_2O$ in a ratio of 5/40/60. There are no special reasons why additives have not been added and why APESI in the negative mode gives the best results. The spectra related with oestradiol and its degradation products are shown in Figures 14(b–d). The structures could be identified as 6-keto-oestradiol and 9,11-dehydro-oestradiol.

Concerning IPC, the first observation to be made is that because of the improved inertness of RPLC columns, developed IPC methods can often be translated in conventional RPLC separations (see Figure 13). If ion-pair formation is necessary for LC–MS then heptafluorobutyric acid (HFBA) as an anionic reagent and tetraethylammonium hydroxide (TEAH) as a cationic reagent should be used. Long-chain hydrophobic ions such as cetyltrimethylammonium chloride for anions and sodium dodecylsulfate for cations often interfere with the mass spectral data.

In HIC, proteins and peptides are induced to bind to weakly hydrophobic macroporous stationary phases such as phenyl or butyl (C4) silica or synthetic polymers using a buffered mobile phase at high ionic strength. The biomolecules are then selectively desorbed using a decreasing salt concentration gradient. Typical HIC conditions are a gradient from 1 to 3 M ammonium sulfate to 0 M at neutral pH (0.1 mM sodium phosphate) over a 15 to 20 min gradient. These conditions are definitely not compatible with APESI-MS and desalting prior to MS is required. For identification purposes using MS, HIC is, therefore, generally performed in the off-line mode (fraction collection), with desalting and FIA analysis. Another alternative involves conventional RPLC on macroporous stationary phases using a gradient between 0.1% TFA (trifluoro-acetic acid) $H_2O$ and 0.1% TFA $CH_3CN$. Good ionization is only obtained when the ion pairing and surface-tension effect of TFA is suppressed by postcolumn addition of a propionic acid/isopropanol (75:25 v/v) solution (9). This is illustrated in Figure 15 which shows part of the tryptic digest map by RPLC–APESI-MS of 1 nmol chicken lysozyme on an ODS column (25 cm × 2.1 mm i.d., $d_p$ 5 µm) at 0.2 mL/min of the gradient A: 0.1% TFA/ $H_2O$ to B 0.1% TFA/$CH_3CN$, 0 to 60% B in 60 min, and at 50 °C. The make-up liquid flow was 0.4 mL/min. Figure 15(a) shows the profile without postcolumn addition of the TFA fix and Figure 15(b) with postcolumn addition.

**Size Exclusion Chromatography**

In SEC molecules are separated according to their size (Stokes radius). The stationary phases are porous particles and the pore size determines the molecular weight range that can be separated on a particular stationary phase. A whole range of stationary phases with pore sizes from 50 to 100 000 Å are commercially available.

The actual separation occurs by diffusion of the solutes into the pores. Sample molecules with sizes greater than the pores cannot enter, are excluded and elute with the void volume of the column. Molecules with sizes smaller than the pore size enter the pores and elute in order of decreasing size. For biomolecules (water-soluble synthetic polymers) the mobile phase is

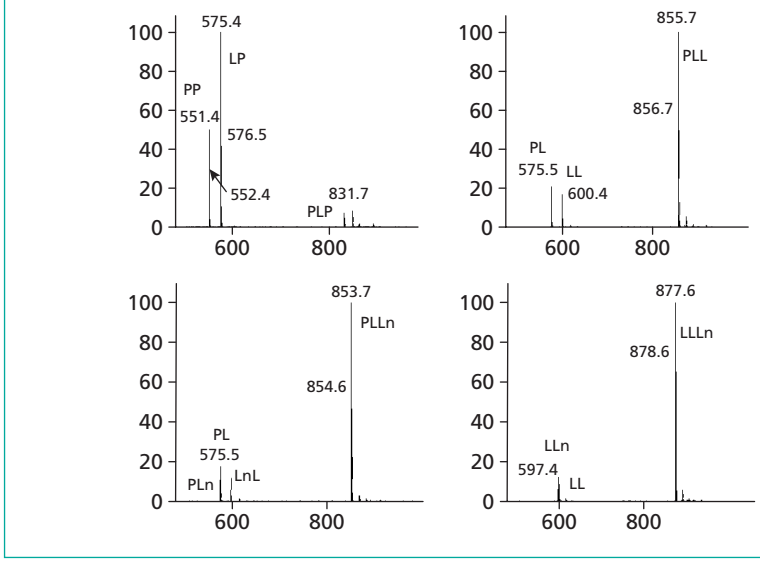


**Figure 12:** *Some representative spectra of the SI-pSFC–APCI-MS analysis of the triglycerides in soya oil.*

**Table 2:** *Triglycerides Identified in Soya Oil by SI-pSFC-APCI-MS.*

| Peak | No. '=' | ID | $[M+H]^+$ | Fragment 1 | | Fragment 2 | | Fragment 3 | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | ID | *m/z* | ID | *m/z* | ID | *m/z* |
| 1 | 1 | POP | 833.7 | PP | 551.5 | OP | 577.5 | — | — |
| 2 | 1 | POS | 861.7 | PO | 577.5 | SO | 605.5 | PS | 579.5 |
| 3 | 1 | SOS | 889.7 | SO | 605.5 | SS | 607.6 | — | — |
| 4 | 2 | PLP | 831.7 | PP | 551.5 | PL | 575.5 | — | — |
| 5 | 2 | PSL | 859.7 | PL | 575.5 | SL | 603.5 | PS | 579.5 |
| 6 | 2 | POO | 859.7 | PO | 577.5 | OO | 603.5 | — | — |
| 7 | 2 | SOO | 887.8 | OO | 603.5 | SO | 605.5 | — | — |
| 8 | 3 | PPLn | 829.7 | PP | 551.5 | PLn | 573.5 | — | — |
| 9 | 3 | POL | 857.7 | PL | 575.5 | LO | 601.5 | PO | 577.5 |
| 10 | 3 | SLO | 885.7 | LO | 601.5 | SL | 603.5 | SO | 605.5 |
| 11 | 3 | OOO | 885.7 | OO | 603.5 | — | — | — | — |
| 12 | 4 | PLL | 855.7 | PL | 575.5 | LL | 599.5 | — | — |
| 13 | 4 | SLL | 883.7 | LL | 599.5 | SL | 603.5 | — | — |
| 14 | 4 | LOO | 883.7 | LO | 601.5 | OO | 603.5 | — | — |
| 15 | 5 | OLnO | 881.7 | OLn | 599.5 | — | — | — | — |
| 16 | 5 | PLLn | 853.7 | PL | 573.5 | LLn | 597.5 | PL | 575.5 |
| 17 | 5 | OLL | 881.7 | LL | 599.5 | OL | 601.5 | — | — |
| 18 | 6 | LLL | 879.7 | LL | 599.5 | — | — | — | — |
| 19 | 6 | LnLO | 879.7 | LLn | 597.5 | LO | 601.5 | OLn | 599.5 |
| 20 | 7 | OLnLn | 877.7 | OLn | 599.5 | — | — | — | — |
| 21 | 7 | LLLn | 877.7 | LnL | 597.5 | LL | 599.5 | — | — |
| 22 | 8 | LnLLn | 875.7 | LnL | 597.5 | — | — | — | — |

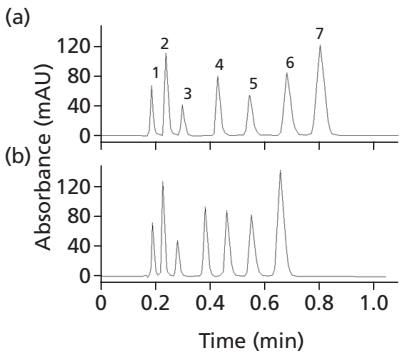


**Figure 13:** *LC analysis of analgesics with a (a) volatile mobile phase and (b) non-volatile mobile phase.*

aqueous and the technique is called gel filtration chromatography (GFC). In order to avoid interaction of biomolecules with the stationary phase high ionic strength buffers are commonly used. For GFC crosslinked polydextran or polysaccharide gels constitute the stationary phase. For polymers soluble in organic solvents the mobile phase is generally tetrahydrofuran (THF), dichloromethane or xylene and the technique is called gel permeation chromatography (GPC). The stationary phases are synthetic polymers such as polystyrenedivinylbenzene or polyacrylamide.

The major drawback of most conventional LC–MS techniques is the limited mass range (2000–3000) they can handle. Biopolymers can be successfully analysed by SEC–APESI techniques because they are ionized through acid-base equilibria and they acquire more than one single charge and, typically, a mixture of differently charged ions is obtained. By deconvolution programs the molar mass can then be reconstituted. Typical mobile phases for GFC contain involatile buffers, such as 100 mM sodium sulfate and phosphate and they should be replaced by 100 mM ammonium acetate or ammonium phosphate.

Unfortunately, APESI-MS has limited application for synthetic polymer analysis because they do not possess acidic or basic functional groups that can be used for ion formation. Moreover, each molecule gives rise to a charge distribution envelope further complicating spectral interpretation. Polymer species up to 3000 dalton could be successfully analysed by SEC–APESI-MS using THF as mobile phase. Important to note is that the commonly used PEEK tubing, unions and fittings should be replaced by metal pieces and fused-silica tubing because PEEK is not resistant to THF. Based on the work of Prokai and Simonsick (10) it is now common practice to add sodium ions to facilitate ionization. This is preferably done postcolumn with a 250 µM sodium iodide in THF/1-propanol solution in the ratio 9:1. Applications include the analysis of poly(ethylene oxides), aliphatic polyesters, phenolic resins and polysulfides. For high molecular weight polymers, MALDI TOF-MS after off-line SEC with fraction collection is now the method of choice. Different options for off-line SEC–MALDI TOF have been discussed by Pasch and Rode (11). It has been shown that polymers can be analysed to molar masses of 500 000 dalton. To bypass the laborious and time-consuming off-line approach, direct deposition methods on the MALDI target have been developed and recently become commercially available (LC-Transform Series 500, Lab Connections, Inc., Malborough, Massachusetts, USA). Miniaturization of the column has broadened and simplified the on-line combination SEC–MALDI TOF-MS (12). The recent introduction of ESI TOF with a typical mass range of 12 000 dalton can cover the characterization of medium molecular weight polymers (13). Polymer chemists should also consider the application of gradient polymer elution chromatography (GPEC) and LC at the critical point of adsorption (LCCC). Both methods detail chemical heterogeneity within oligomer groups and use MS-friendly mobile phases. We refer the reader to the

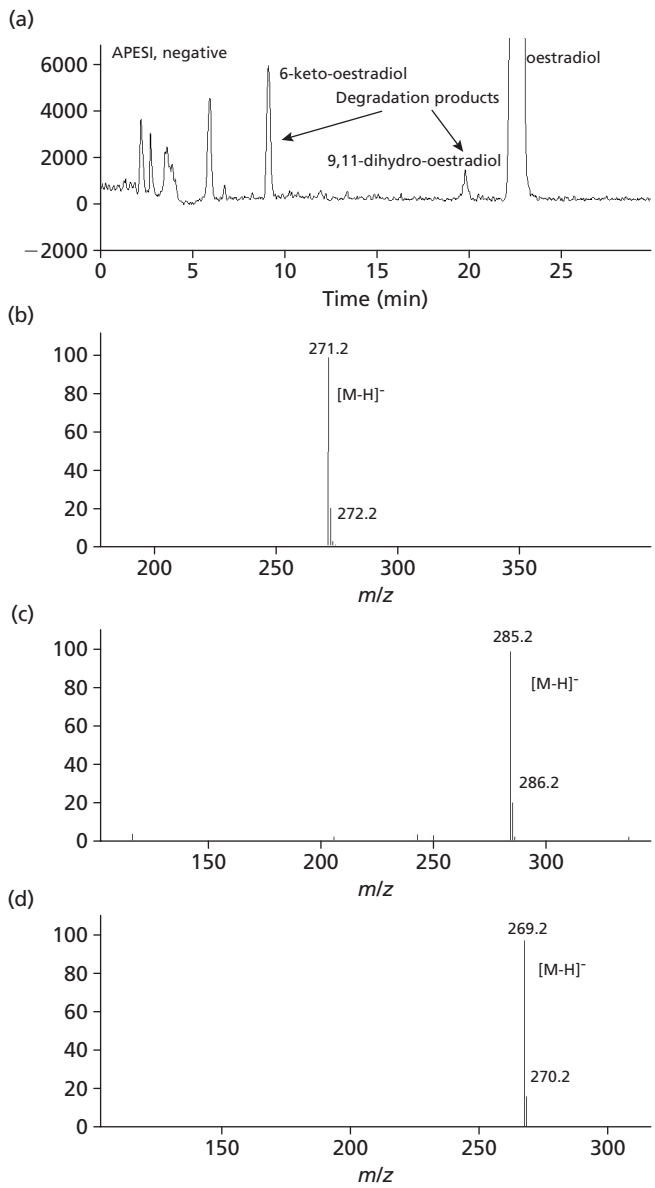


**Figure 14:** *RPLC–APESI-MS analysis of oestradiol and degradation products.*

specialized literature for more details on both separation modes (14). A typical profile of GPEC–APCI-MS is given in Figure 16. Polyethylene glycol monomethylether 750 was analysed in the positive mode with an $H_2O$/MeOH gradient from 55/45 to 40/60 in 60 min. The % diol could be calculated using the MS data.

**Ion-Exchange Chromatography**

Ion exchange chromatography (IEC) is mainly used for the separation of organic ions or easily ionizable solutes, that is, most biomolecules. The stationary phase is either silica gel or a styrene-divinylbenzene copolymer to which ionic groups are chemically bound. For anion separations quaternary ammonium groups (strong anion exchange or SAX) or amino groups (weak anion exchange or WAX) are connected to the packing material. For cation separations the packing material bears either sulfonic acid (strong cation exchange or SCX) or carboxylic acid (weak cation exchange or WCX) side groups. For strong exchange packing materials, the ionization of the stationary phase is almost pH-independent, whereas for weak ion exchange packing materials the pH should preferably be below 9 for WAX and above 5 for WCX. Mobile phases are aqueous electrolyte solutions. Organic modifiers are often added to suppress hydrophobic interaction that can significantly contribute to retention. Important parameters for the mobile phase are the type of counter-ion, the ionic strength of the electrolyte and the pH. Typical counter-ions for AIEC–MS are formate>acetate>$OH^-$, and for CIEC–MS $NH_4^+$>$H^+$ Also, temperature plays an important role because the chemical balances, responsible for the separation, are affected by temperature variations. Figure 17 shows the CIEC–MS analysis of chlormequat in a methanol extract of pears. The use of chlormequat results in an intensification of chlorophyll formation and increased flowering, and thus fruit set. Its use has been restricted because of toxicity to humans. Analysis was performed on Macrosphere WCX 7 µm columns (two 15 cm × 2.1 mm i.d.) with the mobile phase MeOH (90%)/ 100 mM $NH_4OAc$ (10%) at a flow of 0.25 mL/min and at 25 °C. The MS was operated in the positive APESI mode. The Cl-isotope ions 122/124 were monitored.

SCX in combination with MS was applied to the analysis of amphetamines and metabolites in urine with $CH_3CN$ (65%)/5 mM $NH_4OAc$ adjusted to pH 4 (35%) (15).

In conventional IEC and also in ion

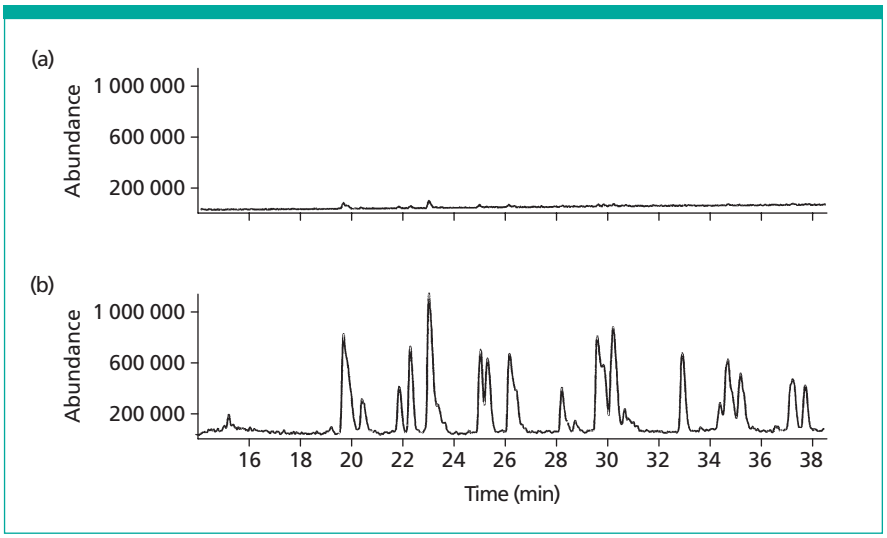


**Figure 15:** *Effect of TFA suppression in RPLC–APESI-MS (a) without and (b) with postcolumn addition of TFA fix.*

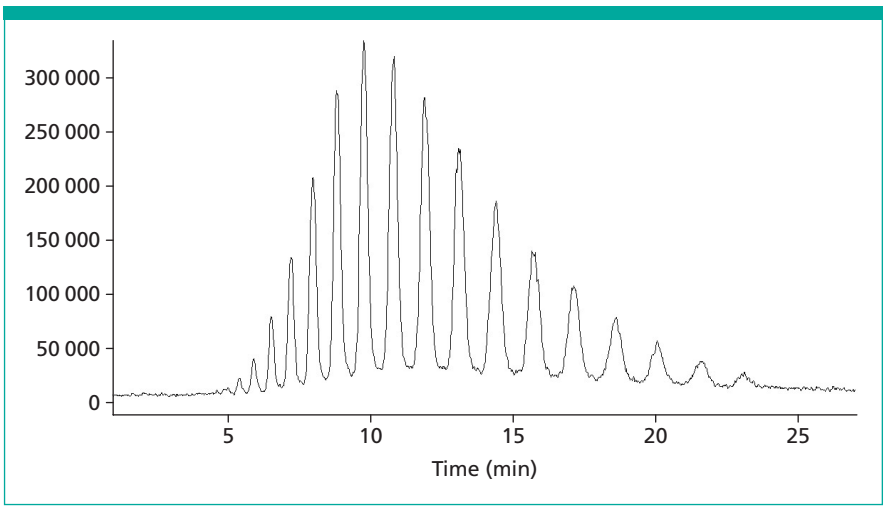


**Figure 16:** *GPEC–APCI-MS analysis of PEG monomethylether 750.*

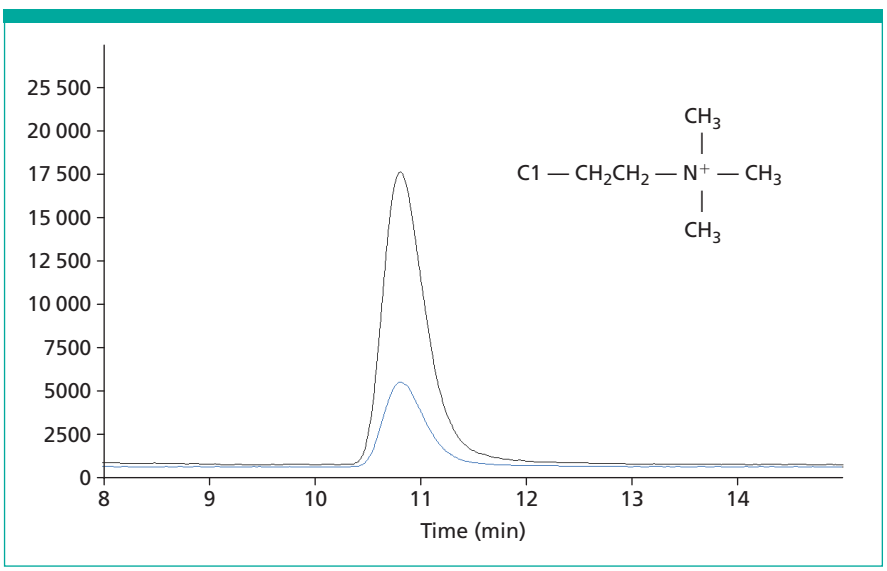


**Figure 17:** *CIEC–APESI-MS of 50 ppb chlormequat in peers. The Cl-isotope ions 122 (—)/124 (—) were monitored.*

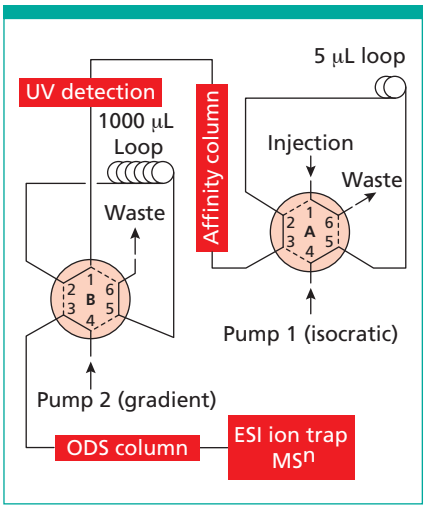


**Figure 18:** *AC–RPLC–APESI-MS$^n$ combination.*

chromatography (IC), high concentrations (0.1–1 M) of involatile additives such as salts, acids and bases are often used. This has a dramatic effect on the sensitivity in MS. These ions should be removed on-line by ion-suppressors. A typical illustration can be found in reference 16. Neutral and acidic sugars in bacterial cell hydrolysates were analysed on a Carbopac PA-1 pellicular anion exchange column using a gradient of 20 mM NaOH (15 min) to 120 mM NaOAc in 100 mM NaOH (10 min). The eluate was neutralized in a 2 mm anion suppressor en route to APESI.

### Affinity Chromatography

Affinity chromatography (AC) is based on highly specific interactions between a ligand, bound to a matrix; that is, the packing material, and a specific substance or substances (e.g., an enzyme and its substrate, an antibody for the antigen that stimulated its response, etc.). Other solutes that may be present in the sample are unadsorbed and can be washed out of the system. The target compound(s) can then be selectively removed from the matrix by passing a compound with larger affinity for the ligand through the column or by changing the mobile-phase composition (pH, salt concentration, ion strength, etc.). A wide variety of support materials can be employed, for example, natural polymers such as cellulose, synthetic polymers such as polyacrylate and inorganic material such as silicagel.

AC is mainly used for the analysis and purification of bioactive compounds but recently, since the development of combinatorial chemistry, actively participates in the drug lead discovery process. The direct combination of affinity chromatography with APESI-MS could, however, only be achieved for a limited number of model separations (17). The mobile-phase composition normally used in AC does not give good ionization in MS. Elution of strongly interacting compounds needs a low pH, high ion strengths and addition of organic modifiers or chaotropic agents to the mobile phase. These compositions lead to precipitation of salts in the ESI source, decreasing the sensitivity of detection and the reproducibility of analysis. A solution can be found using a second column in which the separation is performed under favourable APESI-MS conditions (18, 19). This also increases the analytical "space" with the advantage that if several compounds elute together from the affinity column, they can be separated on the second column. We recently reported on the screening of a combinatorial library by AC coupled to RPLC with APESI-MS$^n$ analysis (20). The ligand selected, namely vancomycin, interacts specifically with peptides terminating with (D)-Ala-(D)-Ala or with (D)-Ala in the ultimate and an aromatic (D)-amino acid in the penultimate position. A library of 36 tetrapeptides of the type Fmoc-(L)-Asp-(L)-Asp-(D)-Xaa-(D)-Xaa (Xaa = Gly, Ala, Phe, His, Ser, Tyr) was synthesized and screened.

A schematic of the AC–RPLC–APESI-MS$^n$ combination is shown in Figure 18.

The effluent of the AC separation is sent through a UV/vis detector to monitor the strongly retained solutes and then via a 1 mL loop to waste. The AC separation was performed on a Chirobiotic V (vancomycin) column (25 cm × 4.6 mm i.d., $d_p$ 10 µm). The mobile phase was 0.1 M NaOAc, pH 3.35 (60%)/$CH_3CN$ (40%) at 1 mL/min. The chromatogram is shown in Figure 19(a). For transfer of a 1 mL fraction of a retained peak, the 6-way valve B is switched and the content of the loop is transferred to the reversed-phase column and detected by ion trap MS. The RPLC

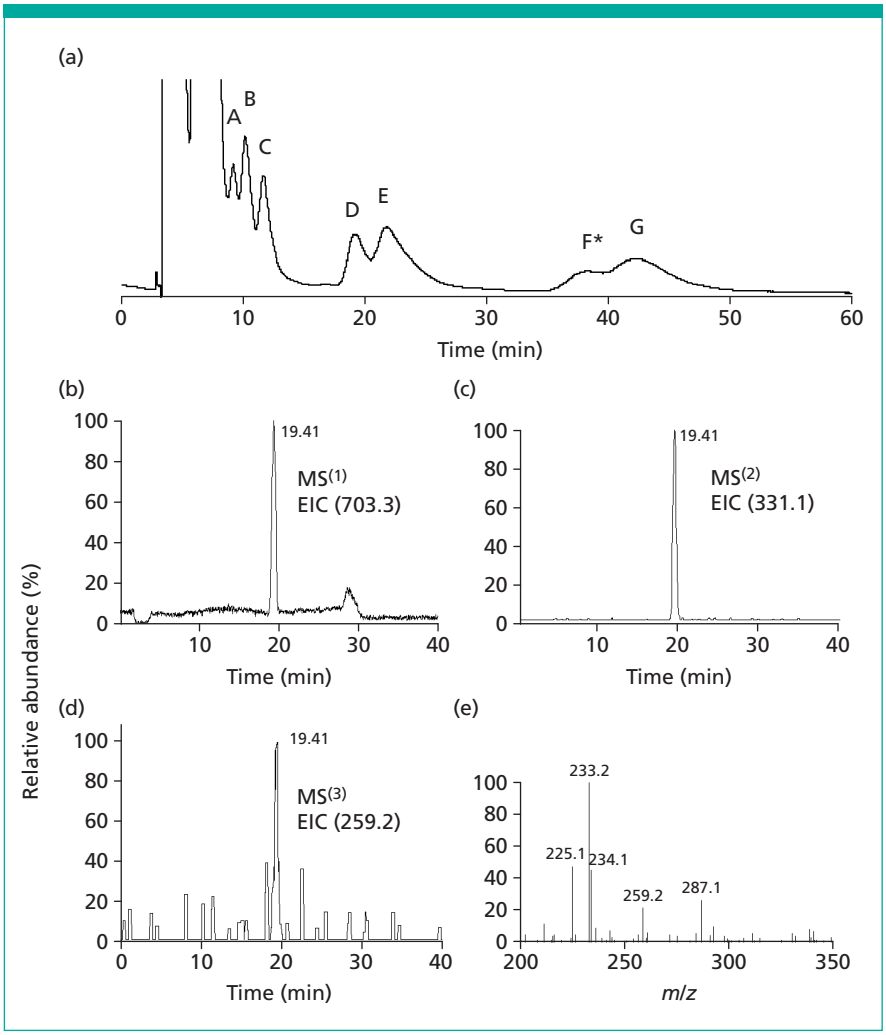


**Figure 19:** *(a) AC separation of the library. 1 mL of the signals indicated from A to G were sampled and analysed on the reversed-phase column. Corresponding extracted ion chromatography of ions (b) 703.3, (c) 331.1 and (d) 259.2 in the MS$^1$, MS$^2$ and MS$^3$ modes, respectively, for 1 mL between 37 and 38 min. (e) MS$^3$ spectrum upon fragmenting ion 331.1.*

column was a Luna C18 (25 cm × 4.6 mm i.d., $d_p$ 5 µm). The mobile phase was delivered in a focusing solvent consisting of A: $H_2O$ (1% HOAc), B: MeOH and with the following gradient from 0 to 2 min 5% B, from 2 to 15 min to 90% B, from 15 to 25 min at 90% B and from 25 to 26 min to 5% B. As an example, 1 mL (between 37 and 38 min) of fraction F in Figure 19(a) gives 703.3 as $[M-H]^-$ ion in the reversed-phase LC extracted ion chromatogram (Figure 19(b)). This ion can originate from four peptides, namely Fmoc-DDSF, Fmoc-DDFS, Fmoc-DDAY and Fmoc-DDYA. Upon fragmentation of ion 703.3 ($MS^2$), fragment α (mass 240 dalton) containing the Fmoc-group is lost, leading to ion 463 that immediately decomposes into the neutral fragment β (mass 132 dalton) and the stable ion 331.1. This ion still corresponds to the same four peptide sequences and further fragmention is needed ($MS^3$) (Figure 19(e)). Ion 259.2, corresponding to DY ($-2H_2O-H^+$) unequivocally elucidates the sequence Fmoc-DDYA. Ion 287.0 is a result of the loss of one $CO_2$ and two $H_2O$ molecules of ion 331.1. Ion 233.2 corresponds to the fragment YA ($-2H_2O$).

The MS interpretation for the retained solutes is summarized in Table 3.

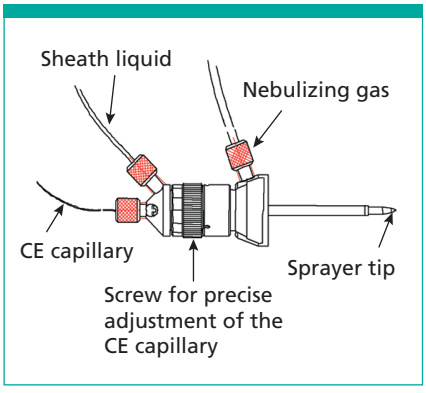


**Figure 20:** *CE–MS interface.*

## Capillay Electrophoresis

In capillary electrophoresis (CE), differential migration of solutes in an electric field, is performed in narrow-bore, fused-silica capillaries, typically 25 to 100 µm i.d. and in lengths of 20 to 100 cm, filled with an electrolyte solution. By applying high electrical fields, high efficiency and resolution is obtained in short analysis times. Moreover, the numerous separation modes of CE offer separation mechanisms for the analysis of small and large molecules, charged or uncharged. For the basic principles of CE and the different modes we refer the reader to some books (21, 22).

Considering the high performance of CE in terms of efficiency and speed, a logical development is its combination with MS and because CE is a liquid-phase separation technique interfacing is required. CE–MS interfacing has been reviewed in several articles and books (e.g., 23).

Typical flow-rates in CE are in the range 10 to 100 nL/min and addition of a make-up liquid is required. The design of the interface used in our laboratory is shown in Figure 20.

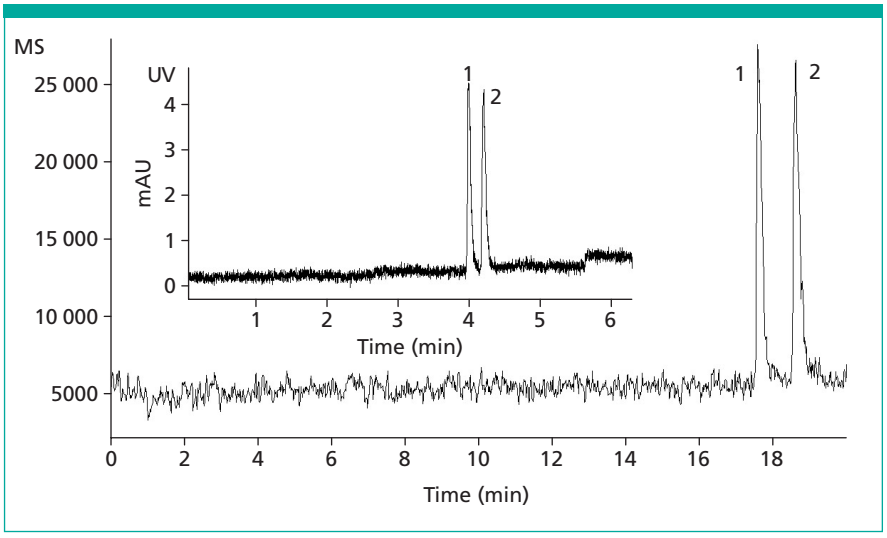


**Figure 21:** *CE–UV–ESI-MS analysis of berberine and palmatine. Peaks 1 = berberine, 2 = palmatine.*

**Table 3:** *Peptides Retained in the Affinity Separation and Determined with MS and $MS^n$.*

| AC | RPLC | | MS | MS (2) | | MS (3) | | MS (4) | Sequence |
|---|---|---|---|---|---|---|---|---|---|
| Peak | Elution time (min) | $[M-H]^-$ | Corresponding compositions | Fragment ion | Composition (Y) | Fragment Ion | Composition (Z) | Fragment ion | |
| A | 19.31 | 719.1 | Fmoc-DDSY<br>Fmoc-DDYS | 347.0 | DSY<br>DYS | 259.2 | DY | | Fmoc-DDYS |
| B | 20.49 | 703.3 | Fmoc-DDSF<br>Fmoc-DDFS<br>Fmoc-DDAY<br>Fmoc-DDYA | 331.1 | DSF<br>DFS<br>DAY<br>DYA | 243.2 | DF | | Fmoc-DDFS |
| C | 18.79 | 627.2 | Fmoc-DDAS<br>Fmoc-DDSA | 255.0 | DAS<br>DSA | 225.0 | DAS<br>DSA | 181.0 | Fmoc-DDSA |
| D | 11.98 | 611.2 | Fmoc-DDAA | — | — | — | — | — | Fmoc-DDAA |
| E | 17.01 | 677.3 | Fmoc-DDAH<br>Fmoc-DDHA | 305.1 | DAH<br>DHA | 233.3 | DH | | Fmoc-DDHA |
| F | 19.41 | 703.3 | Fmoc-DDSF<br>Fmoc-DDFS<br>Fmoc-DDAY<br>Fmoc-DDYA | 331.1 | DSF<br>DFS<br>DAY<br>DYA | 259.2 | DY | | Fmoc-DDYA |
| G | 20.45 | 687.1 | Fmoc-DDFA<br>Fmoc-DDAF | 315.1 | DFA<br>DAF | 243.2 | DF | | Fmoc-DDFA |

**Table 4:** *Validation for CE–ESI-MS of Two Alkaloids.*

| Validation parameters | Berberine (336 *m/z*) | Palmatine (352 *m/z*) |
|---|---|---|
| Repeatability of injection (RSD %, n = 6, 1 ppm, one day) | 6.7 | 7.0 |
| Linearity (n = 5, range : 160–1000 ppb) | r = 0.9998 | r = 0.9991 |
| Limit of quantification (10 S/N) | 15 ppb | 15 ppb |

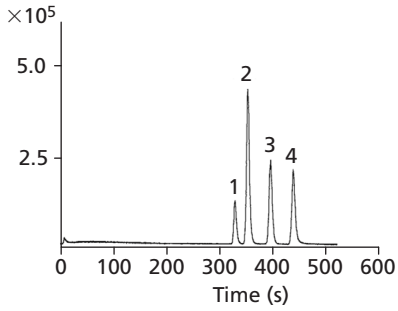


**Figure 22:** *CE–ICPMS analysis of anionic arsenic species. Peaks: 1 = As(V), 2 = MMA, 3 = DMA, 4 = As (III).*

With this interface, shifting from LC–MS to CE–MS can be performed in minutes and the capillary can be properly adjusted via the screw. Figure 21 shows the CE–UV–ESI-MS analysis of two alkaloids, namely berberine and palmatine. The capillary of 50 µm i.d. was 95 cm in length to the MS and 22 cm to the on-column UV detection. The electrolyte was 30 mM $NH_4OAC$ at pH 4 with 20% MeOH. The applied voltage was 25 kV. The sheath liquid was 4 µL/min MeOH/$H_2O$ (90/10) and the nebulizing gas pressure was 10 psi. Some validation data are given in Table 4. As for LC, the composition of the sheath fluid must be fine-tuned and can drastically improve ionization.

As an example, for the CE–MS analysis of heterocyclic amines the sheath liquid MeOH/$H_2O$ (90/10) containing 0.1% $CH_3COOH$ gave a 20-fold increase in ionization compared with 0.1% HCOOH. CE–ESI-MS has been applied to the analysis of quaternary ammonium pesticides, sulfonated dyes, antibiotics, steroids, benzodiazepines, toxins, neuropeptides, proteins, glycoproteins, oligonucleotides, etc.

Today, the combinations LC–ICP-MS (24) and CE–ICP-MS have also been introduced, mainly for metal speciation. For the latter, the interface is very similar to the CE interface but a microconcentric nebulizer is used. Figure 22 shows the analysis of a standard solution of 10 to 20 ppb arsenite As(V), monomethylarsonic acid (MMA), dimethylarsinic acid (DMA) and arsenate As (III) on an 88 cm × 75 µm i.d. capillary column. The electrolyte was composed of 20 mM borate pH 9.4 with 2% osmotic flow modifier (OFM). The applied voltage was –25 kV, the sheath liquid 0.14 M $HNO_3$ + 1% MeOH at 80 µL/min and the nebulizer gas was at 0.5 L/min. The $^{75}As^+$ ion was monitored.

## Conclusion

Recent developments in LC–MS, mainly atmospheric pressure ionization and off-axis spraying, have made the technique as reliable, versatile and easy-to-use as capillary GC–MS. Interpretation of the spectral data in LC–MS, and even in LC–$MS^n$, generally requires some background information on the nature of the solutes, for example, a peptide, a benzodiazepine, a flavanoid, etc. Compared with capillary GC–MS with electron impact ionization or chemical ionization under vacuum, LC–MS does not offer the same identification possibilities because of the different ionization mechanisms. Libraries can be constructed but only for use in a specific field, for example, steroids, a class of therapeutic drugs etc. Nevertheless, LC–MS has became an invaluable tool to selectively quantify solutes, and to confirm structures or to elucidate structural characteristics. This is confirmed with thousands of publications in recent years which unfortunately could not be referenced in the framework of this guide. W.M.A. Niessen reviewed the literature data up until 1999 in his excellent book *Liquid Chromatography–Mass Spectroscopy* (25).

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*Professor P. Sandra is Director of the Research Institute for Chromatography in Kortrijk, Belgium and professor in separation sciences at the University of Ghent, Belgium and at the University of Stellenbosch, South Africa.*
*G. Vanhoenacker, F. Lynen and Liu Li are PhD students at the University of Ghent.*
*M. Schelfaut is MS operator in the Separation Sciences Group at the University of Ghent.*