

**SPECIAL FEATURE:
TUTORIAL**

Comparison of Conventional, Narrow-bore and Capillary Liquid Chromatography/Mass Spectrometry for Electrospray Ionization Mass Spectrometry: Practical Considerations†

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Electrospray ionization (ESI) is nowadays the most important technique for on-line liquid chromatography/mass spectrometry (LC/MS) coupling. Different ESI probe designs including the microESI and pneumatically assisted ESI (ionspray) interfaces allow the introduction of sample at flow-rates ranging from a few hundred nl min^{-1} to $1\text{--}2 \text{ ml min}^{-1}$. In this paper, an overview of the different LC/ESIMS devices is presented from the point of view of their flow compatibility. Several parameters for LC/ESIMS miniaturization and its effects on sensitivity are considered from the practical point of view. Low-flow ESI requires the use of narrow-bore and capillary columns. Sample preconcentration microdevices are recommended in order to circumvent some miniaturization drawbacks such as the low optimum injection volume and the low column capacity. Some considerations on fused-silica micro-column and microESI needle construction are also presented. Copyright © 1999 John Wiley & Sons, Ltd.

KEYWORDS: liquid chromatography/mass spectrometry; electrospray ionization mass spectrometry

INTRODUCTION

The on-line combination of liquid chromatography with mass spectrometry (LC/MS) has been a long-term goal with origins that can be traced back to the early 1960s.¹ The first attempts were based on mechanical transport of the evaporated eluent into the ion source, such as in the moving belt interface,^{2,3} or alternatively on the use of very low flows compatible with the MS vacuum, as in the direct liquid introduction (DLI)^{4,5} and continuous-flow fast atom bombardment (CF-FAB) interfaces.⁶ Several developments, especially in vacuum technology, and the advent of new interfaces such as thermospray (TSP)⁷ and particle beam (PB),⁸ have greatly enhanced the compatibility of LC operating conditions and flow-rates with MS requirements. However, nowadays, the most important revolution in this field has been the development of modern atmospheric pressure ionization (API) sources^{9,10} based on electrospray ionization

(ESI)^{11,12} and discharge-induced atmospheric pressure chemical ionization (APCI).^{13–15}

In the 1980s, TSP was the dominant ionization technique for effective on-line LC/MS. At that time and until recently, the capability of LC/MS interfaces to cope with conventional chromatographic flow-rates was seen as one of the more important requirements for popular acceptance. TSP, in addition to the PB interfaces, with optimum flow-rates in the range $0.5\text{--}2 \text{ ml min}^{-1}$, fulfil this requirement and over the years these techniques have shown their capability to deal with a wide range of applications. Although TSP has gradually given way to the new API-based technology, it can be considered as the technique that narrowed the gap between practical LC/MS and bioanalysis. Because it resulted from a quest for compatibility, its success has also been a strong driving force and a touchstone for newer developments. The first ESI interfaces were reported independently by Fenn and co-workers^{11,16,17} and by Alexandrov *et al.*¹² in the mid-1980s. ESI proved to be a much more efficient ionization technique for high-mass, highly polar compounds than TSP and was compatible with common reversed-phase LC solvent mixtures. However, the best performance of early ESI interfaces was found at flow-rates around $0.5\text{--}5 \text{ } \mu\text{l min}^{-1}$. In terms of its coupling to separation

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techniques, this flow-rate range was in fact good for nothing: too low for conventional LC, too high for capillary electrophoresis (CE). Coupling with chromatography required the use of post-column flow splitters or fused-silica capillary columns. Additionally, the physical requirements for electrospraying made it difficult to work with some common chromatographic eluents of high conductivity and/or high surface tension.

For LC coupling, practical solutions rapidly came in the form of the assisted electrospray interfaces. Pneumatically assisted electrospray (ionspray, ISP)¹⁸ and ultrasonically assisted electrospray¹⁹ raised flow-rate limits to 1 ml min⁻¹. At the same time, CE was coupled to MS by using either a coaxially added sheath liquid²⁰ or a liquid junction device.²¹ This device allowed the electric circuit of the electrophoretic system to be closed and it served as a post-column T-piece for solvent addition in order to reach the optimum flow rate for the electrospray process.

Current ISP probes can be fitted in the same API sources as APCI probes, and in most cases both ionization methods can be purchased together. Modern APCI probes, developed on the basis of the early API sources,¹³ produce gas-phase ionization of the eluent by a corona discharge at atmospheric pressure. The consequent gas-phase ion-molecule reactions give rise to soft ionization of the analytes. In practical terms, the wide range of compound types that can be analysed by the combined APCI/ISP sources and the capability of these sources to access conventional flow-rates have led to the displacement of the older TSP instruments. Some APCI features such as gas-phase chemical ionization processes and the use of a heated nebulizer are common to APCI and TSP. The similarity of APCI spectra with those obtained by TSP expedited the acceptance of the new sources.

More recent ESI developments brought about the microelectrospray²² and nanospray devices.²³ The first microelectrospray interface developed by Emmett and Caprioli²² used 50 µm i.d. needles and worked in the 0.3–0.8 µl min⁻¹ range. This design allowed for the detection of 50 fmol of enkephalin (full scan) by using an integrated concentration-desalting device in the form of a small length of capillary packed with C₁₈ solid phase. The success of this configuration brought back the early ESI probe configuration in the form of several optimized designs, sometimes also called microelectrospray interfaces. These were able to cope with flow-rates in the range from ~200 nl min⁻¹ to 4 µl min⁻¹, with optima depending on the particular design. The nanospray ion source employs capillaries with spraying orifices of 1–2 µm i.d. and is able to produce a stable electrospray signal with flow-rates in the low nl min⁻¹ range. As will be shown later, nanospray emitters afford enhanced mass and concentration sensitivity over other ESI interfaces. Nanospray interfaces have found their most popular applications in the peptide analysis field.^{24–28} A few microlitres of sample can be electrosprayed for more than 1 h. During this time, full-scan spectra and tandem mass spectra can be obtained with a minimum consumption of sample. Nanoelectrospray ion sources, as described by Mann's group,^{23,29} are off-line devices and have not been used for LC/MS coupling. These nanosources are still a reference for LC/MS

interfaces development from both practical and theoretical points of view.

HPLC COLUMNS FOR LC/MS

Classification

HPLC instrumentation has also evolved to meet the low-flow requirements of the new interfaces. Commercially available HPLC columns have diameters that result in an impressive range of working flow-rates. Several classifications of the HPLC columns types can be found in the literature.^{30,31} For the purpose of this paper, we shall use an alternative classification based strictly on columns diameters (Table 1).

The classification in Table 1 groups chromatographic columns in three main classes: conventional, narrow-bore and capillary. Very different technical specifications are required for the chromatographic system (liquid chromatograph and detector) used with each class of column. In the case of liquid chromatographs it is possible to build or to buy flow-splitting devices that allow the use of conventional HPLC pumps and gradient systems with narrow-bore or capillary columns.³⁰ UV detector cells, however, always have to be selected to fit the corresponding flow-rate range. This classification preserves the common terms *preparative* and *analytical* columns for *wide-* and *normal-bore* columns and, although not indicated, medium-large-scale preparative columns (> 10 mm i.d.) that have their own specific instrumental requirements are not considered because they have no significance within the scope of this paper. The same has to be said relative to open-tubular columns. The applicability of these columns has been shown by several workers³² and they have been coupled to ESIMS³³ and APCI.³⁴ However, open-tubular columns show important drawbacks for practical extended application, especially in terms of reduced loading capacity and capacity factors and the number of its applications in the literature is very small. For this reason we shall exclusively cover packed columns below.

Operational characteristics and usage

The use of analytical (3–4.6 mm i.d.) and narrow-bore (1–2 mm i.d.) columns for LC/ESIMS is common prac-

Table 1. Classification of LC columns by column internal diameter

Column diameter	Flow-rate
Conventional:	
Wide-bore (preparative)	>4.6 mm >3 ml min ⁻¹
Normal-bore (analytical)	3–4.6 mm 0.5–3 ml min ⁻¹
Narrow Bore	1–2 mm 0.02–0.3 ml min ⁻¹
Capillary:	
Microbore	0.15–0.8 mm 2–20 µl min ⁻¹
Nanobore	20–100 µm 0.1–1 µl min ⁻¹

tice in most laboratories. For example, a literature search for the period 1992–97,³⁵ shows that out of a total of 697 abstracts on LC/ESIMS, 558 (80%) could be found to use these column diameters. The data from the literature search are summarized in Table 2 and Fig. 1. Typical operational characteristics of these column types are summarized in Table 3. The wide acceptance of narrow-bore columns for this work (50% of the total number of applications) relies on two main characteristics: (a) they can be used with common modern HPLC pumps and (b) flow-rates (50–500 $\mu\text{l min}^{-1}$) are compatible with ISP sources, obviating the need for flow splitting, which remains common practice with conventional columns.

Figure 1 shows the trends for the period 1992–97. As illustrated, there is a noticeable increase in the use of

conventional and narrow-bore columns in the period 1995–96, probably reflecting the maturity and success of ISP sources. Within the searched period, and especially within the last year, narrow-bore columns were those most often used for ESI coupling. Narrow-bore columns have optimum flow-rates that fit the ISP source requirements and could also be a better approach for ESI sources than conventional columns because a smaller split ratio is required. Nevertheless, the reduced optimum ESI flow-rates (1–10 $\mu\text{l min}^{-1}$ range) can be seen as a clear driving force towards the use of capillary LC columns, as reflected in their comparatively low but steadily increasing use in this 5-year period. It is interesting to note that even though the relative number of applications of capillary columns grew in the years 1993 and 1994, in the following years the increasing popularity of narrow-bore columns for on-line LC/MS raises the ratio 'narrow-bore applications *vs.* capillary applications.' The reason for the combined dominance of conventional and narrow-bore columns can mainly be found in the ease of larger bore columns. These do not require any major modification of standard equipment and facilitate large-volume injections of diluted samples in cases where the concentration of the analyte is low and sample availability is not limited.

Capillary columns were already applied in early LC/MS experiments using interfaces such as the moving

Table 2. Literature search. LC/MS update (1992–97)³⁵

Mode of operation	No. of abstracts in database
LC/MS (All)	2027
LC/ESIMS	697
Conventional LC/ESIMS	235
Narrow-bore LC/ESIMS	323
Capillary LC/ESIMS	139

Table 3. HPLC column characteristics (adapted from Tomer *et al.*³¹)

Column I.D. ^a	Volume	Flow-rate	Injection volume	Relative concentration at detector	Relative loading capacity
4.6 mm	4.1 ml	1 ml min^{-1}	100 μl	1	8469
2.0 mm	783 μl	0.2 ml min^{-1}	19 μl	5.3	1598
1.0 mm	196 μl	47 $\mu\text{l min}^{-1}$	4.7 μl	21.2	400
320 μm	20 μl	4.9 $\mu\text{l min}^{-1}$	485 nl	206	41
50 μm	490 nl	120 nl min^{-1}	12 nl	8459	1

^a All columns are 25 cm long.

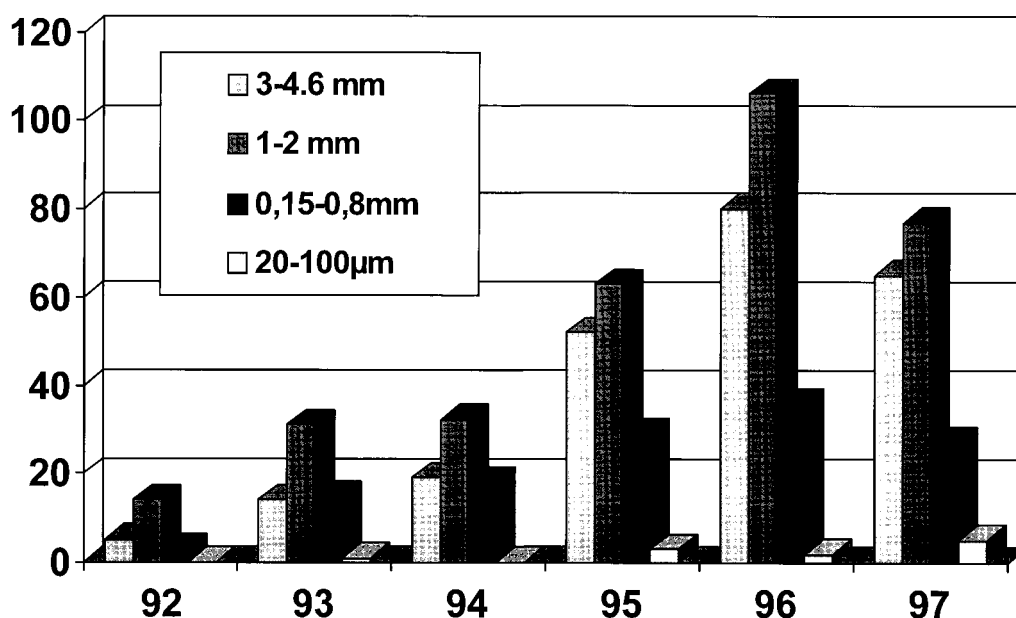


Figure 1. Relative use of different bore columns in LC/ESIMS applications from 1992 to 1997. 3–4.6 mm, conventional; 1–2 mm, narrow-bore; 0.15–0.8 mm and 20–100 μm , capillary. The relatively lower values for 1997 can be explained by the fact that the database for 1997 is not yet complete.

belt,³⁶ DLI^{37,38} or continuous-flow FAB^{39,40} and their characteristics and advantages are now well documented.^{31,41–45} Despite this, the above-indicated considerations explain in part their relatively small number of applications, especially in the case of nanobore capillaries. For some years the commercial availability of these columns was limited. For this reason, in the past and even now in most cases, capillary columns were laboratory-built by using common stationary phases and relatively simple slurry packing procedures.³¹

The specialized instrumentation requirements have also acted as a barrier towards the laboratory implementation of this kind of miniaturized application. However, it has been shown that capillary separations can be performed with normal LC equipment after some easy adaptations. This comprises the use of flow splitters, miniaturized injectors and miniaturized detection devices. Kits to upgrade normal-bore HPLC equipment to micro- and/or nanoscale separation are commercially available. An extensive review of the specific needs and possible solutions for capillary separations has been published recently⁴⁶ and Chervet *et al.*³⁰ discussed the development of nanoscale columns.³⁰

LOW-FLOW SYSTEMS

Chromatographic considerations

Conventional LC can be coupled with ESI and ISP interfaces by using either post-column splitting or optimized ESI interfaces capable of attaining flows of up to 2 ml min⁻¹.⁴⁷ However, the first choice results in significant sample losses and the latter has not entailed noticeable sensitivity gains. Although in practice conventional and narrow-bore columns account for 82% of the reported applications of LC/ESIMS in the period 1992–97 (Fig. 1), there are clear reasons for the development of low-flow systems that can be directly coupled to ESIMS. It has been repeatedly mentioned in the literature that miniaturization of chromatographic column parameters brings advantages in terms of solvent consumption, improved detection sensitivities and hence reduced sample consumption.^{30,46} This is why, despite the difficulties that can be encountered in capillary LC work, there is an increasing number of publications using small-bore columns coupled to microESI devices with no splitting.

A reduction in column diameter produces a higher sample peak concentration in the detector. The maximum peak concentration of the sample in the column eluate (C_{\max}) is given⁴⁷ by

$$C_{\max} = \frac{mN^{1/2}}{(2\pi)^{1/2}V_0(1+k)}$$

C_{\max} depends on the absolute amount of sample loaded on the column (m) and the column efficiency (N) and it is inversely proportional to the column dead volume (V_0) and the retention factor (k). Since V_0 is a function of the column dimensions (internal diameter), it can be calculated that the C_{\max} ratio for two different i.d. columns would be equal to the ratio of the squares of their

respective i.d. values. This would correspond to the values shown in the last but one column in Table 3. Thus, for the same mass of sample injected on-column, miniaturization from a 4.6 mm i.d. analytical column to a 320 μ m i.d. capillary column would result in a theoretical concentration gain at the detector of more than two orders of magnitude, simply by diminishing the column diameter by about one tenth.

Mass spectrometric considerations

Mass-versus concentration-sensitive detection. The above gain in peak concentration would result in an improvement in response when using detectors such as the UV or the refractive index detector where the signal obtained within the working range is proportional to the concentration of the sample. Thus, the response (R) of a concentration-sensitive detector can be expressed in terms of sample concentration (C_{\max}) as

$$R \propto C_{\max}$$

If we continuously introduce a solution of an absorbing compound into a UV detector, we will observe the same absorbance independently of the flow-rate.

On the other hand, mass flow-dependent detectors such as the flame ionization detector or the electrochemical detector for gas and liquid chromatography, respectively, have a response proportional to the total number of molecules being detected per unit time. Accordingly, the equation for a mass flow-sensitive detector includes the terms for flow-rate F and the split ratio S :

$$R \propto C_{\max} FS$$

Mass spectrometers are mass flow-dependent detectors. Therefore, if we continuously introduce ions into a mass spectrometer, the signal will decrease as the flow-rate decreases. As the gain in analyte concentration obtained by using small-diameter columns occurs at the expense of a reduced flow-rate entering the detector, the higher peak concentration does not necessarily result in a greater response in the mass spectrometer.

The extension of the flow-rate effect on the mass spectrometer response is very dependent on the interface characteristics. In Fig 2 and 3 the effects of flow-rate on response for a conventional ESI and a megafLOW ISP⁴⁸ and a laboratory-made microESI⁴⁹ interface, respectively, are depicted. As can be seen, within a given flow range, the ISP and microESI signals increase with increase in flow-rate. This dependence is more pronounced in the case of the microESI interface (Fig. 3).

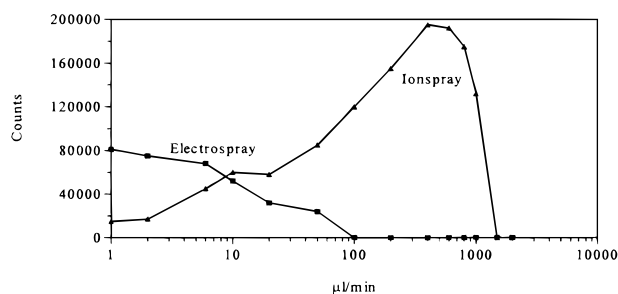


Figure 2. Conventional electrospray and ionspray SIM ion current signals versus flow-rate.⁴⁸

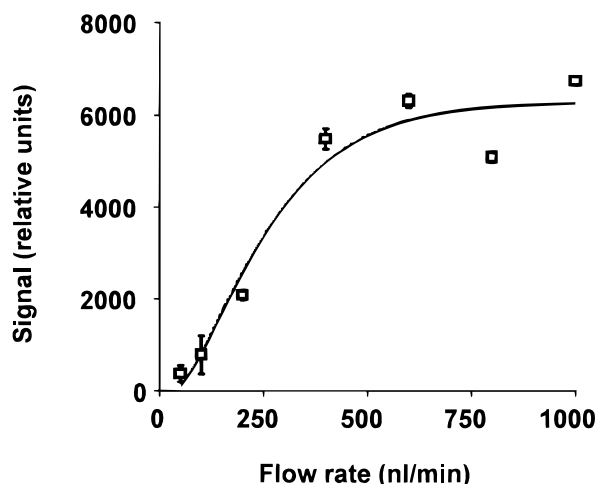


Figure 3. Signal versus flow-rate for a laboratory-made micro-electrospray interface.⁴⁹

In other cases, a reverse mass-flow dependence behaviour can be observed, such as depicted in Fig. 2 for the ESI interface. In this example, the signal diminishes when the flow-rate is increased. Similar behaviour has been reported by Banks,⁵⁰ who showed how the ESI ion signal obtained across a wide range of LC/MS flow-rates actually decreases by about 30% when the flow-rate is increased from 1 $\mu\text{l}/\text{min}$ to 1 ml/min , even though in the latter case 1000 times more sample is consumed. Also, with both the microESI and the high-flow ESI interfaces, a plateau is reached where the response is not mass flow dependent. The explanation for these disparate behaviours resides in the different efficiencies of the diverse ESI source designs in ion production and sampling.⁵¹ It has been shown that ionization and ion sampling efficiency increase inversely with flow-rate, counteracting the direct dependence of detector signal on absolute mass. As a result, ISP and ESI mass spectrometers can behave as if they were concentration-sensitive detectors^{9,48,51} or even show an enhanced response at lower flow-rates. The latter effect is generally observed with micro- and nanoESI interfaces made of very narrow capillaries which are optimized to produce stable electrospray at flow-rates in the mid-high nl min^{-1} range.

Increased performance of very low-flow spraying devices. In electrospray ionization, the quality of ion sampling from the atmospheric pressure plume strongly affects the total number of ions reaching the MS detector. Droplet space charge repulsion puts a limit on the maximum droplet density in the spray, resulting in a wide spray plume. Consequently, only a small part is sampled by the mass spectrometer. For a conventional ESI interface, Smith *et al.*⁵² estimated that the overall efficiency for ion sampling and detection using a triple-quadrupole TAGA instrument with an ESI source working at 3–6 $\mu\text{l min}^{-1}$ was about one ion in 100 000. In terms of ion transfer to the quadrupole analyser, only one ion in 10 000 is introduced for MS analysis and detection.

Several parameters of the ESI emitter, e.g. the inner and outer diameters of the spray tip and its position

relative to the sampling orifice, can be optimized to improve ionization efficiency and ion sampling. It has been noted that the production of microsperms with small plumes enhances sampling efficiency.^{9,23} Low-flow ESI capillaries can be placed very close to the inlet of the mass spectrometer and thus a larger part of the ESI plume leaving the ESI needle is focused into the analyser. Another way to increase the number of ions sampled to the mass spectrometer is by using larger i.d. entrance skimmers or capillaries. This implies also a higher gas flow entering the mass spectrometer and therefore additional pumping capacity has to be provided.

The spray performance can be improved by adjusting the flow-rate and tip diameters, which determine the formation and size of the droplets from which ions will be desorbed.⁵³ An equation that describes droplet formation in ESI has been presented by Wilm and Mann:²³

$$r_e = \left\{ \frac{\rho}{4\pi^2\gamma \tan\left(\frac{\pi}{2} - \theta\right) \left[\left(\frac{U_T}{U_A}\right)^2 - 1 \right]} \right\}^{1/3} \left(\frac{dV}{dt}\right)^{2/3}$$

where r_e is the radius of the emission region for droplets at the tip of the Taylor cone, γ is the surface tension of the liquid, θ the liquid cone angle (for the classical Taylor cone model $\theta = 49.3^\circ$), ρ is the density of the liquid, U_T is the threshold voltage, U_A is the applied voltage and dV/dt is the flow-rate. From this equation, it follows that droplet formation at the Taylor cone is dependent on the flow-rate. By decreasing the flow-rate, smaller droplets will be emitted from the ESI point. Small droplets possess a higher surface-to-volume ratio, enhancing the desorption of ions into the gas phase. To stabilize the Taylor cone, the capillary diameter has to be reduced in parallel with the flow-rate reduction. Early conventional Analytica of Branford ESI interfaces operating at 1–5 $\mu\text{l min}^{-1}$ were made of stainless-steel capillaries of about 500 μm i.d. MicroESI needles, as developed by Caprioli and co-workers, use fused-silica capillaries with 20–50 μm i.d. and show optimum flow-rates in the range 200–800 nl min^{-1} ; finally, the fused-silica or glass capillaries used for nanospray have diameters at the tip exit of only 1–2 μm and produce stable sprays in the range 20–200 nl min^{-1} .^{23,25,29} Conventional 500 μm i.d. interfaces produce initial droplets with average sizes in the μm range.⁵³ Nanoelectrospray tips produce much smaller droplets which a radius of ~ 200 nm. Wilm and Mann²³ estimated that at a concentration of 1 $\text{pmol } \mu\text{l}^{-1}$ such small droplets would each contain only one analyte molecule on average.

The combination of improved ion sampling efficiency and spray performance has a dramatic effect on the performance of the ESI source. Emmett and co-workers^{22,54} placed a 20 μm ESI capillary (flow-rate 200 nl min^{-1}) a few mm from the MS inlet to obtain attomole detection limits. Wilm and Mann²⁹ estimated the efficiency of an ESI source fitted with a 1–2 μm i.d. nanoESI tip operated at ~ 20 nl min^{-1} and placed 1–2 mm in front of the MS entrance. In a conventional interface, only one in 200 800 analyte molecules in the solvent were detected by the MS detector, similar to the one in 100 000 value estimated by Smith *et al.*⁵² (see

above). The nanoESI tip improved this ratio to one in 390 molecules detected by the mass spectrometer, this being 510-fold better. With these miniaturized emitters, mass sensitivity is greatly enhanced and spectra have been obtained with low femtomole amounts of sample and attomole and zeptomole detection limits have been reached.⁵⁵ Despite the much lower flow-rates, the concentration sensitivity is also enhanced: 2–3 times higher ion currents are reported for a given analyte concentration relative to a conventional ESI source.

Nowadays, it is generally recognized that the design of miniaturized systems with reduced flow-rates, that use small diameter columns and an optimized interface for this flow, yields enhanced mass and concentration sensitivities.

Sample loading considerations

Concentration detection limits attainable by diminishing the column diameter are, however, limited by the optimum injection volume and loading capacity of the specific column. In principle, the optimum injection volume and column loading capacity are reduced proportionally to the square of the column diameter. For column diameters <1 mm, injection volumes lie in the submicrolitre range (Table 3). For the same sample concentration, this proportionally reduces the total amount of sample that can be injected. Although it is possible to concentrate the sample by solvent evaporation, there are practical difficulties in dealing efficiently with small injection volumes. In most cases, the initial sample volume is also small, so that volume reduction and sample handling become unmanageable. For these reasons, with narrow-bore and especially with capillary columns, some kind of on-line sample preconcentration becomes necessary.

Simple solutions are either to inject in a solvent with lower eluotropic strength, to achieve on-column analyte focusing, or to use a solvent gradient to preconcentrate the sample at the head of the column, before elution. In this way, it is possible to inject a relatively large volume of sample on to a capillary column without deleterious chromatographic effects, provided that the sample components are strongly retained under the initial conditions.

However, owing to the diminished loading capacity of the capillary column, high sample volumes can produce column overloading, especially when working with complex matrices where several components contribute to the total mass injected. To prevent this problem, a precolumn could be used in the same way as used in a conventional chromatographic system. By using a narrow-bore precolumn, preconcentration and washing steps can be carried out effectively before desorbing the retained material in a small volume to the capillary column.^{49,56}

MINIATURIZATION

Capillary columns

First attempts to develop LC capillary columns were made at the beginning of the 1980s.^{32,41,57–60} Nowa-

days, capillary columns can be bought from various manufacturers. Because they are still in the developmental phase, however, many groups pack their own miniaturized columns. This is also the result of the high market price of some of these columns and the ease of preparing high-quality columns in the laboratory.

There are several protocols for the packing of micro- and nanobore columns.^{31,41,61–65} Usually, the body of the capillary column is made of commercially available untreated fused silica. The first step in column packing is to build a frit at the exit end of the capillary. Subsequently, the capillary column is coupled to a reservoir filled with a slurry of the packing material. This is pushed under pressure into the column inlet by a flowing stream. The frit allows the solvent to pass through the column and the solid phase fills the column in a process similar to filtration. A practical guide for this packing procedure was given by Tomer *et al.*³¹

The frit in fused-silica capillaries is made by tapping the analytical column into a pile of silica particles, filling ~1 mm of the outlet of the column.⁴¹ The end is then heated with a torch that sinters the silica particles and forms a frit which is stable to relatively high pressures.

Alternatively, the frit can be made by pushing the capillary column through a porous membrane sheet two or three times, resulting in a stable plug at the outlet of the column. Robins and Guido⁶⁶ used Teflon membranes as a frit and Davis and Lee⁶⁵ used PVDF sheets. Oosterkamp *et al.*⁴⁹ used two glass-fibre filters which were sandwiched between two capillaries (the column and the outlet capillary) inside a holder capillary. Both frits were shown to be stable at the pressure necessary for analysis. Lubman and co-workers⁶⁷ used a Valco microbore column fitting with a very small amount of glass-wool as a frit. The thickness was 0.1 mm and it was found to be more stable than glass frits.

Of these methods, the sintered-glass method is the most commonly employed. Sintered-glass frits are stable when using capillaries of >300 µm o.d. In our experience, columns with smaller outer diameters (180 µm) are more vulnerable to the heating process, making the capillary too fragile to handle.

Columns with diameters as small as 20 µm have been packed successfully by using these procedures.⁴¹ However, the smaller sizes are difficult to fill. Consequently, the smallest common diameters found in the literature are around 50–75 µm. Capillary columns usually show efficiencies and overall performance comparable to or better than those of conventional columns. Kennedy and Jorgenson⁴¹ reported 40 000–65 000 plates for a 25 µm i.d. column of 33 cm length filled with 5 µm particles. They observed that the column efficiency improves on diminishing the column diameters from 50 to 20 µm. This improvement was explained by a decrease in flow dispersion and in resistance to mass transfer with column diameter. In capillary chromatography, wall effects make a greater contribution than in conventional LC columns, leading to relatively more band broadening at high flow-rates. Band broadening can be diminished by using electro-driven flows, as performed in capillary electrochromatography (CEC), instead of pressure forces. CEC uses an electrical field across the column length,

resulting in electroosmotic transport of the solvent, similar to that in CE.⁶⁸⁻⁷⁰ At the very low flow-rates employed, electro-driven flow improves the performance of the column. CEC columns can reach efficiencies up to 200 000 plates, greatly exceeding the efficiency of capillary LC. It is believed that this is due to improved mass transport between stationary phase and liquid phase. An intermediate between capillary LC and CEC is the so-called pseudo-electrochromatography, which is a pressurized CEC mode.^{71,72}

NanoESI and microESI sprayers and interfaces

Emitter construction. Unprocessed commercially available stainless-steel and fused-silica capillaries of adequate internal diameters have been used for ionspray and for conventional and microESI. To optimize spray formation at the lower flow-rates used in microelectrospray interfaces, some changes are usually made to the capillary tip. Fused-silica capillaries with small inner diameters are commonly etched with HF²² or the tip is rubbed using sandpaper, resulting in very sharp ESI tips. Electropolishing has also been used with metal capillaries to produce similarly sharp tips.⁷³ ESI tips have been constructed with inner diameters down to 10 μm . By using a micropipette puller, spray tips as small as a few μm can be prepared from glass or fused-silica capillaries.^{23,55} We use the same approach in our laboratory to obtain microspray capillaries by using a torch and letting the capillary stretch under gravity. Although not used for LC/MS, it is interesting to note the approach of Valaskovic *et al.*⁵⁵ They obtain reproducible tip diameters down to 2 μm by first pulling capillaries and later etching them with HF. These tips can be operated at flow-rates from 0.1 to 20 nl min^{-1} , depending on the diameter.

Voltage application. In metallic needles, the electrical voltage can be applied directly to the spraying needle. When using non-conductive glass or fused-silica capillaries, the voltage at the spray tip is applied in two different ways, either by liquid contact with an electrode at

some point before the needle tip^{49,55,74a} or by coating of the capillary with gold^{23,29,74b} or silver.⁷⁵ In tips employing a liquid contact, the voltage is conducted through the mobile phase to the ESI tip. For example, as shown in Fig. 4, an operationally simple microESI interface can be put together with a 50 μm i.d. inlet capillary connected to a 25 μm i.d. outlet spray tip. The electrical contact is made at the stainless-steel holder and the frit helps to produce an effective liquid contact in the gap. This custom-made emitter assembly is mounted on a commercially available micropositioner as shown in Fig. 5. Also, microwires have been used to produce the electrical contact but only for CE/ESIMS coupling.⁷⁶ Gold-coated ESI tips have been produced by using a gold sputtering procedure. Subsequently, a clamp is used to attach an electrical voltage to the spray. The latter method is less sensitive to different mobile phases, as has been shown recently by Vanhoutte *et al.*⁷⁷ The connection of nanoscale separations to MS has been accomplished using different interfaces. Hunt and co-workers^{78,79} used nanoLC columns in combination with a normal ESI interface for structural elucidation of subpicomole amounts of peptides bound to the Major Histocompatibility Complex (MHC) molecules. Also on-line CEC/MS has been accomplished using normal-scale ESI interfaces.⁸⁰⁻⁸² By modifying the electrospray interface, an improvement of the detection limits down to 2 fmol protein digest can be obtained.⁸³ Further downscaling of the LC/MS method by coupling of nanoscale LC to dedicated microESI interfaces has resulted in detection limits down to the attomole range.^{30,49,84}

Preconcentration (PC) in Micro- and NanoLC/MS

The main limitation of micro- and nanoscale separation techniques is that only very small sample volumes can be applied (see Table 1). To be able to inject higher sample volumes, column switching techniques have been employed.⁸⁴ Thus, a given sample can be concentrated on a precolumn. By switching the precolumn

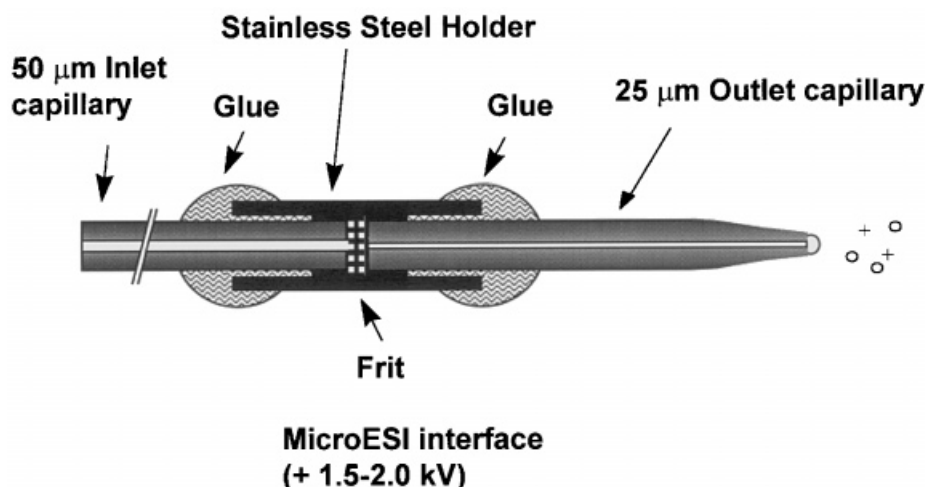


Figure 4. MicroESI needle construction.⁴⁹

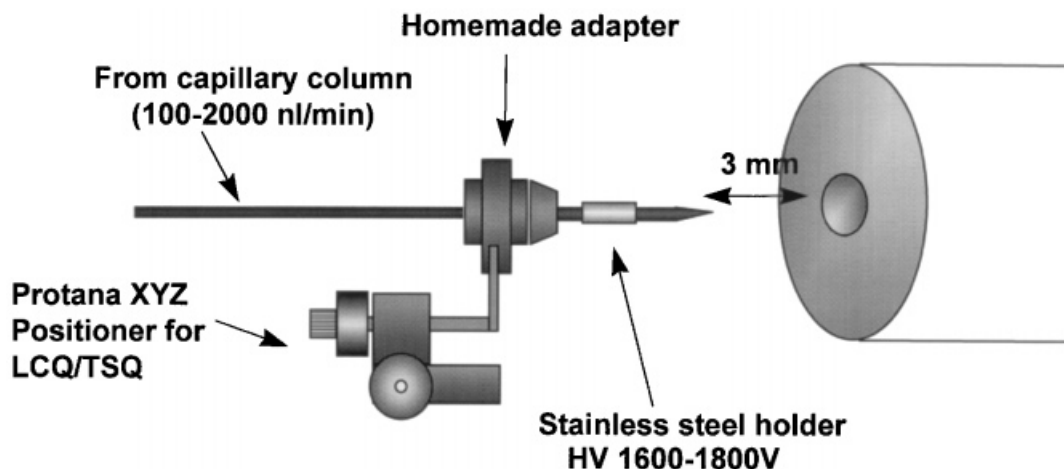


Figure 5. MicroESI configuration.

on-line to the analytical column, the analytes can be desorbed by the mobile phase of the analytical column. On-line preconcentration prior to micro- or nanoLC/MS has been shown to improve concentration detection limits.^{56,49,85} Thus, much lower concentration detection limits than for off-line continuous infusion nanoelectrospray experiments can be obtained.

Microbore LC-ESI/MS in combination with on-line preconcentration (PC) has shown its value in bioanalysis.^{49,56,85} Cai and Henion⁸⁵ combined immunoextraction with a PC/microLC/MS method. An immunoaffinity column was used to preconcentrate LSD and its metabolites in diluted urine samples from volumes as large as 50 ml. The bound compounds were then released into a reversed-phase trapping column with dimensions of 15 × 0.5 mm i.d. From this column, the compounds were eluted in the backflush mode towards the analytical column (150 mm × 300 μm i.d.) and analysed using a conventional ESI interface. Thus,

LSD and its analogue could be detected at the 2.5 pg ml⁻¹ level.

Zell *et al.*⁵⁶ described a validated method for the determination of pharmaceutical compounds in blood. Here, 1 ml of blood was extracted with dichloromethane. After evaporation to dryness, the sample was reconstituted in 70 μl of 20% methanol. The sample was then injected into an analytical system consisting of a 5 mm × 0.8 mm i.d. reversed-phase trapping column, a 150 mm × 300 μm i.d. analytical column and an ESI interface with a spray needle of small internal diameter. With the use of such a PC/microLC/MS method, detection limits of 1 pg ml⁻¹ in 1 ml of blood were obtained (limit of detection 1 pg).

Oosterkamp *et al.*⁴⁹ have recently reported a PC/nanoLC/MS method for the detection of the peptide endothelin in cell culture supernatants (Fig. 6). For this purpose, an analytical method was developed using custom-made nanoLC columns (75 μm i.d.) and a

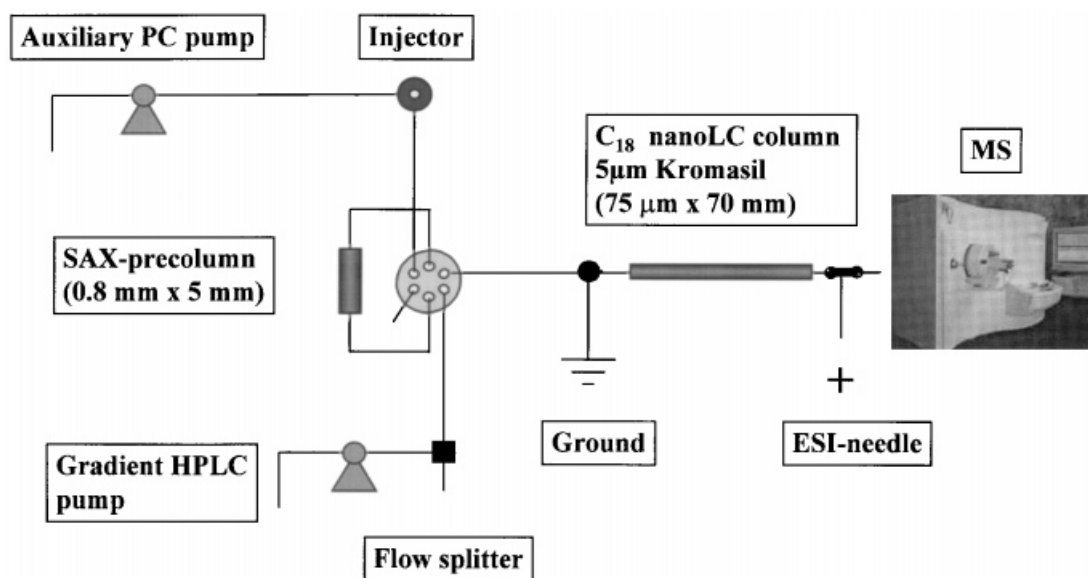


Figure 6. Micropreconcentration device for a microbore LC system coupled to a microESI source.

microelectrospray interface. Thus, to be able to analyse large sample volumes in order to cope with low biological analyte concentrations, the nanoLC/ESIMS method was coupled to an on-line preconcentration system based on a strong anion-exchange material (5 mm × 0.8 mm i.d.). The cell supernatant was deproteinated with acetonitrile and the supernatant was evaporated to dryness. In this way, up to 200 µl of the reconstituted sample (equivalent to 50 µl of supernatant) can be injected into the analytical system.

These methods show that the use of on-line microPC/microLC/MS can reduce concentration detection limits to levels much lower than those that can be obtained with normal-bore LC methods, as shown below.

Practical advantages of microPC with miniaturized LC/MS systems. Table 4 illustrates, in a comparative manner, the step-by-step increase in sensitivity that can be achieved by stepwise reduction in column inner diameter and application of sample preconcentration techniques. For this purpose, we have taken our own data generated in the course of the above-mentioned study on the LC/ESIMS detection of low endogenous levels of endothelins.⁴⁹ By using 1 mm i.d. columns operated at a flow-rate of 100 µl min⁻¹, we could achieve 50 fmol limits of detection (LOD) for 100 µl injections of standard solutions. This corresponds to a concentration sensitivity of 0.5 nmol l⁻¹. However, when working with real samples, the detection limit was up to 500 fmol and for this we had to process 15 ml samples of HUVEC supernatants, with a resulting concentration sensitivity of 5 nmol l⁻¹.⁸⁶ The application of the concepts considered above led us to try the same approach but using instead 250 µm i.d. capillary columns at a flow-rate of 5 µl min⁻¹. The LOD for standards was reduced to 2 fmol and any further gains were limited by the 10 µl maximum injection permitted on these columns (data not shown).

In a further scale-down to nanoLC, we used 75 µm i.d. columns operated at a flow-rate of 0.5 µl min⁻¹ and found a higher mass sensitivity with an LOD of only 0.5 fmol but low concentration sensitivity (0.2 nmol l⁻¹) owing to the restricted 2.5 µl sample injection. This, however, could be readily increased to 200 µl injected by applying sample preconcentration techniques, as we have recently described. Although the mass sensitivity is of the same order as that obtained with capillary

columns (1.5 fmol), the concentration sensitivity improves significantly in this way to 30 pmol l⁻¹ for real samples. In the latter case, only 500 µl are processed as opposed to the 15 ml needed initially with the microbore system.⁴⁹

CONCLUSIONS AND PERSPECTIVES

The mass spectrometer is by definition a mass flow-sensitive detector where the signal varies with the number of analyte molecules reaching the ion source per unit time. However, in the case of the electrospray ion sources, the flow split term *S* in the corresponding equation is magnified by the basic characteristics of the ionization mechanism since it is not only flow but also design dependent. In other words, *S* is a function of the combined mechanisms of ion production and ion sampling efficiency. In practice, the spray tip of the interface becomes a splitter since only a portion of the spray plume has access to the MS inlet capillary or skimmer. Thus, it has been discussed that the size and positioning of the spray cone determine the number of ions actually entering the mass spectrometer, whereas the ionization efficiency is directly related to the size of the microdroplets generated by the interface. The smaller the plume of microdroplets and the closer it is to the inlet orifice, the higher is the sampling ratio. Likewise, the smaller the microdroplets actually sprayed, the higher is the ion production rate. Both conditions are readily achieved at the micro- to nano-flow ranges.

These considerations lead to the conclusion that, contrary to what one could expect of a mass flow-sensitive detector, the miniaturization of the ESI interface should result in increased sensitivity. This has been clearly demonstrated in a stepwise fashion by the data given in Table 4. These results together with those in the literature lead us to believe that micro- and nanoelectrospray will be the methods of choice when sensitivity considerations become important.

Nevertheless, the combination of LC and ESIMS has not yet resulted in detection limits at the sub-attomolar level, such as those obtained in off-line nanoelectrospray analysis (see above). One of the reasons is that

Table 4. Detection sensitivity as a function of column inner diameter and sample preconcentration procedures

System	Column i.d.	Flow-rate (µl min ⁻¹)	Sample volume (ml)	Injection volume (µl)	LOD (fmol)	Concentration LOD (nmol l ⁻¹)
Narrow-bore LC/MS	1 mm	100	Std	100	50	0.5
	1 mm	100	15 ml	100	500	5
Narrow-bore LC/MS/MS	1 mm	100	Std	100	100	—
µLC/MS/MS	250 µm	5	Std	10	2	0.2
µPC/µLC/MS/MS	250 µm	5	Std	100	2	0.02
NanoLC/MS/MS	75 µm	0.5	Std	2.5	0.5	0.2
µPC/nanoLC/MS/MS	75 µm	0.5	500 µl	200	1.5	0.03

there are no routine LC methods with flow-rates down to the low nl min^{-1} level. Therefore, LC separation methods are operated at relative high flow-rates (100–500 nl min^{-1}) compared with the 1–20 nl min^{-1} for off-line infusion nanoelectrospray. Accordingly, on-line nano-separation MS techniques appear less sensitive than continuous infusion nanoelectrospray tips when absolute detection limits are compared.

The practical benefits of low-flow LC/ESIMS systems can be summarized by their very low limits of detection (needed for the analysis of samples available in either limited amounts or in very low concentrations). On the other hand, their perceived drawbacks in terms of com-

mercial availability are not that significant in view of the relative simplicity of the production of custom-made capillary columns and low-flow spray devices. On this account, although reproducibility and ruggedness have still to be proved, it can be predicted that the use of miniaturized LC/MS systems will steadily increase in the near future.

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