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Review

Microcolumn liquid chromatography: instrumentation, detection and applications

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Abstract

This review discusses many different aspects of microcolumn liquid chromatography (LC) and reflects the areas of microcolumn LC research interest over the past decades. A brief theoretical discussion on a number of major issues, such as column characterisation, chromatographic dilution effects and extracolumn band broadening in microcolumn LC is given. Recent progress in column technology and the demands and developments of instrumentation and accessories for microcolumn LC are also reviewed. Besides that, the developments in a large number of established and also more recent detection techniques for microcolumn LC are also discussed. The potential of hyphenation of microcolumn LC with other techniques, more particularly of multidimensional chromatography and microcolumn LC coupled to mass spectrometry is reviewed. Finally, the perspective of microcolumn LC separation methods is stressed by a number of relevant applications. © 1997 Elsevier Science B.V.

Keywords: Microcolumn techniques; Reviews; Detection, LC; Instrumentation; Band broadening; Oils; Amino acids; Proteins; Bile acids; Indoleamines

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1. Introduction

The continuing interest in microcolumn liquid chromatography (LC) is indicated by the large number of review articles that have appeared in literature, covering the general aspects of microcolumn LC [1–10], column technology [11–15], detection in microcolumn LC [16–25], instrumentation [26,27] and multidimensional chromatography [28]. The introduction of microcolumn LC is attributed to Horváth and co-workers in 1967 [29,30], who used 0.5–1.0 mm inner diameter (I.D.) stainless steel columns packed with pellicular particles for the separation of ribonucleotides. During the following decades, high-performance liquid chromatography research was mainly focussed on packed columns with an I.D. of 4.6 mm. Therefore, it was not until the mid 1970s that Ishii et al. demonstrated the use of slurry-packed teflon microcolumns in a series of publications [31–36], which initiated a breakthrough in the development of microcolumn LC. Other type of microcolumns with capillary dimensions – i.e. smaller than 1.0 mm in I.D. – were introduced shortly thereafter [37–43]. In about the same time frame the group of Scott [44–48] reported on their work with packed 1.0 mm I.D. columns to achieve efficient, high-speed separations. The work of Novotny and co-workers [40–42], Yang [37] and

Scott et al. [44–48] are regarded as key publications in the field of microcolumn LC. The initial developments towards miniaturization in high-performance liquid chromatography were quickly followed by a number of laboratories worldwide [49–51].

Microcolumn LC has established itself as a complementary technique to conventional sized LC columns, which are more commonly used in high-performance liquid chromatography. This is due to certain advantages in an increasing number of applications where conventional high-performance liquid chromatography does not satisfy, or cannot compete with microcolumn LC. The most important advantages of microcolumn LC are the benefits that arise from the ability to work with minute sample sizes and small volumetric flow-rates, and the improvement in detection performance with the use of concentration-sensitive detection devices as a result of the strongly reduced chromatographic dilution [2–5]. Nowadays, microcolumn LC is almost exclusively performed – as a research tool and in routine analysis – with slurry-packed columns of various dimensions. This review article will, therefore, focus on recent technological advances with this type of microcolumns. Special attention will be given to the many detection techniques applied in microcolumn separations. In addition, the general advantages, and some applications are discussed.

Table 1
Suggested nomenclature for microcolumns for LC having different inner diameters

Column I.D.	Abbreviation
0.5–1.0 mm	Micro LC
100–500 μm	Capillary LC
10–100 μm	Nanoscale LC

2. Nomenclature

The use of the abbreviations microbore LC, micro LC and capillary LC are used interchangeably for packed microcolumns of different I.D. Definitions have been suggested in the literature [52,53], but have not found general acceptance. Microcolumn liquid chromatography is used frequently as the overall term to describe small-I.D. packed LC columns at different leading conferences and in most scientific journals, and will therefore be followed in this paper. Furthermore, there seems to be a tendency to describe 0.50–1.0 mm I.D. columns as micro LC, 100–500 μm I.D. columns as capillary LC, and 10–100 μm I.D. columns as nanoscale LC [54]. A summary of the suggested nomenclature is given in Table 1. As mentioned in Section 1, open tubular LC and the drawn-packed type of columns will not be considered in this paper, nor will the nomenclature for conventionally sized LC columns – i.e. LC columns with an I.D. of 2.0–4.6 mm – be addressed here.

3. Theoretical considerations

3.1. Column characterization

Column characterization in liquid chromatography is a very arbitrary matter, since it depends on the user's point of view. For instance, whether acceptable column lifetimes can be expected under certain separation conditions, or whether stationary phase and column manufacturers can supply columns of constant and reproducible quality are practical but certainly valid aspects. These points will, however, not be addressed in this paper. Here, only column performance – e.g. separation potential – and which

column performance criteria should be used will be discussed.

A number of column performance evaluation methods have been suggested [55–59]. Till now, however, little consensus exists between the different methods, with respect to test compounds, eluent, and other conditions to evaluate LC columns, and none of these methods has found general acceptance in chromatographic practice. Apart from this, the many available separation modes and columns in LC, and the different principles behind the suggested test methods, also have contributed to the present unsatisfactory and confusing situation for column test protocols. Generally, the retention factor k , selectivity α and the peak asymmetry A_s are believed to be representative parameters for the thermodynamic properties of a column.

The kinetic characteristics are often expressed in the dimensionless magnitudes of reduced plate height h , separation impedance E and flow resistance factor ϕ . The question whether small-I.D. LC columns show better kinetic properties compared to their conventional counterparts has been the subject of much discussion in the literature [60–65]. The results of these investigations covering different column I.D.s, and performed under test conditions which were not always similar, can be summarized as follows: h values are typically in the same range as conventional LC columns. However, at very small column I.D.s (<50 μm) a clear but steady decrease in the h -values can be observed, indicating the improved kinetics in microcolumn LC, which will be discussed in more detail in Section 4. At the same time, it was also observed that E and ϕ values tend to be somewhat higher at decreasing I.D.s, which is most likely caused by the less dense structure of the packed chromatographic bed.

3.2. Chromatographic dilution

A sample compound will be subjected to dilution during the chromatographic separation process. The chromatographic dilution D at the end of the column equals:

$$D = \frac{c_o}{c_{\max}} = \frac{\epsilon \pi r^2 (1+k) \sqrt{2\pi LH}}{V_{\text{inj}}} \quad (1)$$

where c_0 is the original compound concentration in a sample, c_{\max} is the final compound concentration at the peak maximum, r is the column radius, L is the column length, H is the column plate height, ϵ is the column porosity and V_{inj} is the injected sample volume. D will increase proportionally to the square of the column radius and to the square root of the length and plate height of the column. Under further identical chromatographic and injection conditions, and assuming Gaussian peak shapes, c_{\max} increases in inverse proportion to r^2 . For example, from Eq. (1) it can be calculated that this will result in a 235-fold increase in peak height and mass sensitivity for a reduction in the diameter of a column from 4.6 mm to 300 μm I.D. It must be stressed, however, that this advantage of small-I.D. columns can only be fully exploited when the same sample size can be loaded and the operating characteristics of such columns are identical compared to their conventional counterparts. In fact, since the sample size that can be loaded on a column is proportional to the amount of stationary phase in the column, this advantage is only valid in cases where the amounts of sample mass are restricted.

3.3. Extracolumn band broadening

Miniaturizing essentially comes down to the reduction of the I.D. of a column. At the same time the outer or extracolumn band broadening effects must be reduced accordingly, in order to achieve maximal performance of a miniaturized column. More specifically, this is of major importance to prevent considerable loss of efficiency, resulting in decreased resolution of the column.

Usually, the plate number of a column is calculated from, e.g. the standard deviation of a peak, resulting in the statement of column efficiency. From the point of view of principle, such a statement is doubtful, since virtually both the column and the instrumental band broadening contribute to the standard deviation of a chromatographic peak. Therefore, together with the presentation of column efficiencies, the applied equipment should be discussed, unless the extracolumn band broadening contribution is negligible compared to the peak broadening of a specific column. Since – for obvious reasons – extracolumn effects will always be manifest, this

matter comes down to the question of which extracolumn band broadening can be maximally accepted for an LC column under specific experimental conditions.

Starting from the generally accepted criterion that extracolumn band broadening may maximally reduce by 5% the resolution R of a chromatographic separation, it can be derived that:

$$R = \frac{\Delta t}{\sigma_t} = \frac{\Delta t}{1.05\sigma_c} \quad (2)$$

where Δt is the time difference between two closely migrating peaks, σ_c is the peak standard deviation caused by the chromatographic process, and σ_t is the peak standard deviation originating from column plus extracolumn effects.

Keeping in mind that $\sigma_t^2 = \sigma_c^2 + \sigma_e^2$ (volume or time units), this condition is satisfied when the extracolumn variance $\sigma_e^2 \leq 1.1025\sigma_c^2$ and, consequently, the loss in column plate number should not exceed 10% by extracolumn effects [23,25,27].

For a specific LC system the maximally acceptable variance $\sigma_{e(\text{acc})}^2$ equals:

$$\sigma_{e(\text{acc})}^2 \leq 0.10\sigma_c^2 \leq 0.10 \frac{\pi^2 L^2 r^4 \epsilon^2 (1+k)^2}{N} \quad (3)$$

where N is the plate number of the column [23–25]. Taking practical numerical values of, e.g. $L=0.15$ m, $\epsilon=0.75$ and $N=15\,000$ the corresponding $\sigma_{e(\text{acc})}^2$ values can easily be calculated as a function of column diameter and retention factor, as depicted in Fig. 1. From the results in Fig. 1, it is obvious that for smaller I.D. columns the corresponding acceptable extracolumn contributions are rapidly decreasing. Hence, the use of small-I.D. columns must be accompanied by a strong reduction of extracolumn effects. The approximation of the maximally allowed equipment band broadening outlined above can also be used to estimate the maximally acceptable contributions of the separate contributions of injector, detector, tubing and electronic time constants. For example, assuming arbitrarily that these latter four main effects contribute in equal ratio to $\sigma_{e(\text{acc})}^2$, it follows that $\sigma_D^2 = 1/4\sigma_{e(\text{acc})}^2$, where is σ_D^2 the contribution of the variance of the detector to $\sigma_{e(\text{acc})}^2$.

From $\sigma_D^2 = V_D^2/K_D^2$, where V_D is the detector volume and K_D^2 is the detector profile factor [23],

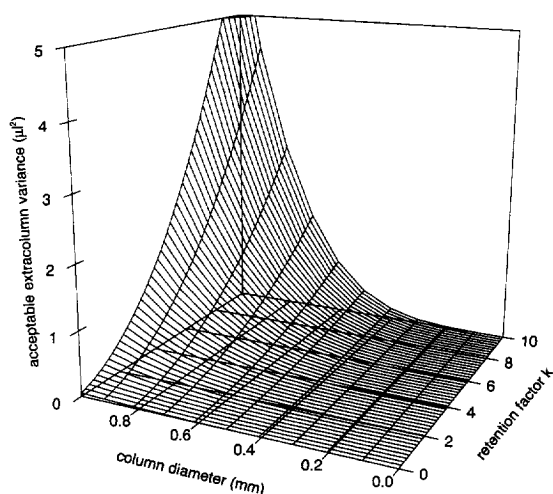


Fig. 1. Acceptable extracolumn variance $\sigma_{e(\text{acc})}^2$ as a function of the column diameter and the retention factor k . See text for details.

and taking the same numerical data of $L=0.15$ m, $\epsilon=0.75$ and $N=15\,000$ at constant linear eluent velocity, the resulting plot of the maximally allowed detection volume versus k and K_D^2 is for a column of $500\ \mu\text{m}$ I.D. (given in Fig. 2). K_D^2 – equalling 12 under ideal on-column detection conditions – accounts for dispersion, mixing and diffusion effects in the detection system. From the graph in Fig. 2 it can be seen that at low values of k a detector volume of

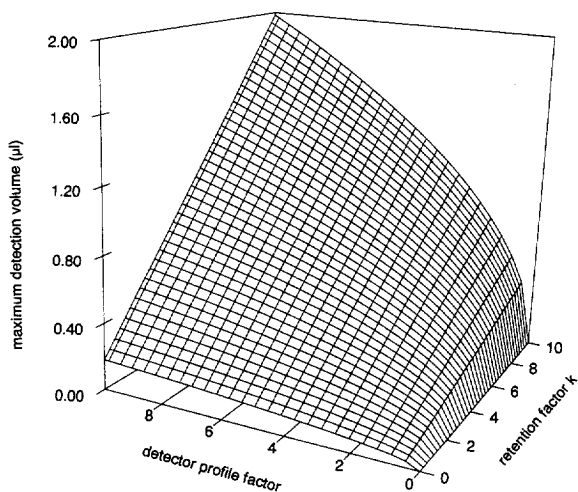


Fig. 2. Maximum allowed detection volume V_D as a function of the detector profile factor K_D^2 and the retention factor k . See text for details.

200 nl is maximally allowed. From this example it is obvious that severe reduction of extracolumn effects is mandatory in microcolumn LC. Such approximation as outlined above also allows a reasonable estimation of the other contributions to $\sigma_{e(\text{acc})}^2$, viz. injector, tubing and electronic time constant. This can be helpful in design and assembling equipment for microcolumn LC.

4. Column developments

Different filling techniques have been reported for the preparation of packed capillary LC columns, applying gases [66–68], supercritical fluids [67–71] or liquids [72,73] to transport the stationary phase particles into the column. For micro LC columns liquids are used exclusively to pack columns [74,75]. The basic concept of these packing techniques remains, however, the same: a frit – composed of a metal screen, glass wool, polymeric membranes or condensed silica particles – is mounted at one end of the column blank, and the particles are pushed into the columns from the other side. Basically, the packing process can be regarded as filtration.

The separation efficiency of microcolumns is influenced by a large number of parameters – not only so-called packing parameters (such as solvent selection, packing pressure, slurry concentration, the use of surfactants to stabilize the particle suspension, slurry vessel dimensions, etc. [76–82]), but also by column parameters (such as column blank material, frit selection, etc. [76,83–86]). The majority of these suggested packing techniques are empirically developed and mainly based on assumptions and trial and error methods. Nevertheless, good, efficient microcolumns can be produced by the packing methods described above, and their performance regarded as optimal – i.e. reduced plate heights near 2 – according to classical chromatographic theory.

Some research groups have tried to develop a better understanding of the packing processes – incorporating colloid chemical studies, filtration theories and stress behavior during bed compression [69,87–90]. Although certain trends could be assigned with these studies, a sound theoretical description and understanding of packing methods for microcolumns is not available yet. For example,

Vissers et al. [87,88] related the coagulating properties of a number of octadecyl reversed phases in different slurry and packing liquids with the final chromatographic performance of microcolumns. They found that coagulation of stationary phase particles is of less importance than normally is assumed. Tong et al. [69] developed a shear stress model to describe particle movement during column packing with supercritical fluids. It was found that relatively low packing pressures yielded more dense packing structures. However, no quantitative relationships were given. Furthermore, novel packing techniques – applying continuous vibration of the stationary phase slurry suspension with the aid of an ultra-sonification probe – do not seem to produce significantly better columns in terms of efficiency and column lifetime [91].

At present, columns with an I.D. of ~200–300 μm are the subject of many papers. There is, however, a tendency to use columns with an even smaller I.D. Driving forces towards this even further miniaturization are the use of such columns in capillary electrochromatography (CEC) [91–93] and electro-spray ionization-mass spectrometry (ESI-MS). In CEC, columns with an I.D. of 50–100 μm are required to prevent Joule heating of the mobile phase, whereas in LC-ESI-MS the use of 50–100 μm I.D. LC columns leads to a tremendous increase in sensitivity of the electrospray interface. A detailed description of both techniques is beyond the scope of this paper.

There is, however, also a scientific interest in the use of very narrow fused silica capillaries. Several research groups [63,65,94,95] reported that the plate height of packed columns reduces by a factor of approximately 2 when the column to particle diameter ratio – also known as the Knox–Parcher ratio – is smaller than 6. Classical plate height equations suggest, however, that the column diameter does not influence the plate height of packed columns – as indicated by the following simplified Horvath-Lin equation:

$$H = \frac{1.4D_m}{u} + 1.5d_p + \frac{2}{5} \frac{(k_0 + k + k_0k)^2}{(1 + k_0)^2(1 + k)^2} \frac{d_p^2}{D_m} u \quad (4)$$

where D_m the diffusion coefficient of the analyte in

the mobile phase, u the linear mobile phase velocity, k_0 the ratio of the interparticle void volume to the interstitial void volume in the column, and d_p the particle diameter.

This contradiction between theory and experimental observations was explained by Kennedy and Jorgenson [63] by the fact that inhomogeneities in mobile phase flow paths are strongly reduced due to the more uniform cross-sectional packing structures of such small-I.D. packed columns, resulting in flow paths of almost identical permeability. Peak dispersion is reduced since only wall-ordered packing structures are possible. The decrease in the packing structure variation gives rise also to more uniform retention factors, reducing column band broadening. Both effects contribute to the decrease to band broadening. Finally, small column I.D.s allow for more rapid transcolumn diffusion between all possible flow paths and retention regions, enabling the analyte to diffuse across the entire column cross-section. The smallest published reduced plate heights equal 1.0 for almost unretained compounds and 1.2–1.3 for slightly retained compounds using 5- μm reversed octadecyl modified particles [95].

5. Instrumentation

5.1. Solvent delivery and gradient systems

The flow spectrum for miniaturized high-performance liquid chromatography systems discussed in this paper runs from the nl to μl per minute scale for 50- μm up to 1.0-mm I.D. columns, respectively. At the present time and state of the technique, reciprocating and syringe pumps are favored solvent delivery systems for conventional and for micro-column LC. The reciprocating pumping systems are, however, favorable over syringe pumps for their large column back pressure compensation abilities, rapid flow equilibrium and stability, and good possibilities for micro-gradient delivery [96–102]. The rather time-consuming flow equilibrium, due to eluent compressibility and viscosity, and the difficulties in the mixing of micro-flows for gradient analysis, are especially inherent to syringe pumps. Moreover, column pressurization is a very time-

consuming task at low flow-rates, and can increase gradient delays significantly when syringe pumps are applied. For flows in the order of 50–150 $\mu\text{l min}^{-1}$, reciprocating and syringe pump systems are commercially available, including in most cases devices for binary, ternary or quaternary gradient formation. These delivery and gradient systems have proven to be useful down to column I.D.s of approximately 1.0 mm.

The delivery of flows in the nl and the low μl ranges for columns with I.D.s of approximately <500 μm cannot be readily performed by direct pumping. For such flows, the use of split-flow techniques can be an attractive alternative. These systems are based on the application of packed restrictor columns [96,103,104] or flow splitting devices based on a microflow processor concept [105]. Since this latter system compensates for viscosity changes of the eluent, e.g. during gradient analysis, it can be used in isocratic and gradient modes as well. For gradient analysis the microflow processor can be connected to a conventional gradient device, by which part of the flow is split in a constant adjustable ratio to the microcolumn.

5.2. Injection devices

Using Eq. (3), the maximally allowed injection volume for different I.D. and length of columns can be estimated. For 50–100 μm I.D. columns, injection volumes are in the order of a few nl up to approximately 1 μl for 1.0 mm I.D. columns. For manual and automated injection, the majority of the present injection systems in high-performance liquid chromatography consists of injection valves. Manual injections from the μl range down to approximately 20 nl can be routinely performed with (micro-)injection valves equipped with a replaceable internal loop. Below 20 nl, manual valve injections can be performed by positioning a split vent between the injector and the column [54]. Alternatively, for these very small volumes, the moving injection technique [106,107], the static split [108] or the pressure-pulse-driven stopped-flow injection technique [109] can be used too. These techniques have in common that, by controlling the injection time and flow through the injector, only a small part of the injection plug is

injected on the top of the column. Automated injection in the μl range can easily be performed by many of the autosamplers available on the market. Injection automation in the nl range usually requires a thorough adjustment of a conventional autosampler. Such modifications of conventional autosamplers for use in capillary LC have been described in literature [110]. Furthermore, the use of an automated micro-injector has been described [111]. With this latter system, however, only one sample at the time can be injected. Recently, an autosampler for the injection of very small samples has been described [112]. This commercially available injection device claims a reproducible injection of 50-nl to 5- μl sample volumes from minute samples with almost no loss of sample.

A general problem in microcolumn LC techniques is the loss of detection sensitivity due to the small injection volumes or masses. In a number of cases this problem can be overcome by the use of so-called on-column focusing techniques [113–116]. These techniques have in common that the sample solvent has a significantly lower eluent strength compared to the actual eluent. After arrival of the sample plug on the column top, the compounds will be focussed in a small plug. Focusing-enrichment factors of several hundreds have been reported, so significantly increasing detectability in microcolumn LC techniques. For example, for the separation of a test mixture consisting of resorcinol, benzaldehyde, phenol, nitrobenzene and toluene under reversed phase conditions, a focusing-enrichment factor of 200 in micro LC could be achieved, resulting in similar chromatograms compared to standard injections [114].

Since the loading time of a certain sample volume under focusing conditions increases at decreasing column I.D., this technique becomes impractical for capillary and nanoscale LC. This problem can be overcome by the use of packed precolumns, which must be properly connected to the analytical column and are essentially a part of the separation systems. The advantages of this approach are in the much larger sample loading flow-rate, which can be obtained during the sample focusing step. In addition, also the selectivity of the isolation of the sample compounds of interest can be controlled by the selection of a specific stationary phase in the precolumn [117–125].

5.3. Tubing and connections

Since the tubing which is used to connect the different parts of the equipment contributes linearly to its length, and to the power 4 of its radius to the extracolumn band-broadening variance, the selection of the connecting tube dimensions is extremely important. For columns of 500 μm –1.0 mm I.D. specially designed stainless steel tubing of 0.25 or 0.125 mm I.D. is usually applied. Alternatively, polyetheretherketone or fused silica tubing can be applied for this purpose. For smaller I.D. columns, the use of tubing is as much as possible avoided. Direct connections of the column to injector and detector are preferred or, alternatively, connecting tubing with I.D.s of $\leq 50 \mu\text{m}$ are applied. Apart from the proper selection of the connecting tube I.D., the length of these tubes should be as short as possible to prevent loss in resolution. In addition, the different parts connecting the column to the injector and detector must be properly designed to avoid dead volumes, and should be compatible with the applied devices in the microcolumn LC equipment.

5.4. Detectors

5.4.1. Refractive index detection

Refractive index (RI) detection has, in general, received only little attention in microcolumn LC and capillary separation techniques, although it is a widely applied detection technique in conventional high-performance liquid chromatography due to its universal character. Modifications to existing commercially available RI detectors have been reported for the use of RI detection with micro LC columns [126–129]. These studies mostly involved the reduction of extracolumn band-broadening effects and the demonstration of the separation of test mixtures. For instance, Fujimoto et al. [127] reported a limit of detection of 2–3 ng for dipentylphthalate separated isocratically on a reversed-phase column. Synovec [129] studied the theoretical aspects of RI detection in more detail. Tracing diagrams for cylindrical detector flow cell designs were studied. Furthermore, RI detection was optimized for different eluent and flow cell conditions.

With capillary LC, RI detection is even more difficult due to the fact that RI differences have to be

measured in extremely small volumes [130]. With the highly collimating nature of lasers, nl to pl volumes can easily be probed. Laser-based RI detection has, among others, been applied by Bornhop and co-workers [130–132] and Bruno et al. [133,134]. The capillary flow cell developed by Bruno et al. [134] is based on the so-called forward scatter, off-axis technique. With the use of an RI-matching fluid surrounding the capillary flow cell, and active temperature control of the flow cell assembly, improved performance is feasible. The limitations of this method are the off-axis alignment and the need to remove the polyimide coating of the fused silica capillary to calculate the refractive index.

A novel approach for measuring RI changes in capillary separation techniques is reported by Tarigan et al. [130]. With this technique, the interference-produced fringes are detected below the plane of excitation in a direct backscatter configuration. The spatial shift in the fringes is a measure of the refractive index for the fluid streaming through the capillary flow cell. Flow cells with I.D.s of 75–775 μm were probed with no modification to the polyimide-coated capillary flow cell, no changes in the optical set-up, and no reduction in the signal-to-noise ratio. The optical configuration of such a system is very straightforward. It consists of a He–Ne laser, a thermal-controlled flow cell assembly, and a silica photodetector to collect the reflected light. The flow cell assembly is tilted slightly to allow detection by the photodetector. With flow injection analysis, 3.2 pg of glycerol could be detected in a probed volume of approximately 2.6 nl. The change in RI at 3-times the standard deviation of the noise was, for this flow cell volume, equal to $1.9 \cdot 10^{-7}$ RI units. The detector response was found to be linear over a dynamic range of three decades.

5.4.2. UV absorbance detection

The most universally applied detector – in conventional and microcolumn LC – is the UV absorbance detector because of its ease of use and broad application area. To prevent extracolumn band broadening – i.e. keeping detection volumes at the sub- μl or nl level – on-column detection is often a first approach. A packing-free part of the column is used as the optical window – i.e. detector cell – and is brought into the light path of a UV absorbance

detector. The construction of such a cell has been discussed [135,136]. Vindevogel et al. [137] developed design guidelines for tubular UV absorbance detection flow cells. Their study involved the investigation of parameters, such as reflection of the incident light on the capillary wall, the distance between the photodetector and the flow cell, changes in the refractive index of the mobile phase, different cell designs, wavelength, and the linearity and noise. Due to the large variety of configurations no general recommendations were given. Further, it was suggested that not only the I.D. of the flow cell in this type of studies should be mentioned, but also parameters relating to light beam width and photocell distance should be used in the description of a flow cell.

The main disadvantage of on-column detection is the limited concentration sensitivity because of the limited path lengths. Limits of detection reported for on-column detection are typically around $1\text{--}5 \cdot 10^{-6} \text{ mol l}^{-1}$ for compounds such as uracil, cytosine and thymine, with a $100 \mu\text{m}$ I.D. flow cell [138]. Despite the extremely small path lengths, detection is feasible at the sub-ng level. Fiber optics have been suggested to collimate the excitation light onto the flow cell and for the collection of the UV light that has passed through the flow cell [139,140]. The limits of detection that were obtained with this system are, however, not favorable compared to on-column detection. A more successful approach towards improvement of the detection sensitivity with UV absorbance detection in microcolumn LC was the introduction of longitudinal flow cells with an optical path length up to 3–8 mm [141]. This type of flow cell and on-column flow cells have been studied extensively with respect to sensitivity, linear dynamic range, and its contribution to extracolumn band broadening and noise [138,142]. The sensitivity of the longitudinal type of cells is normally 50–100 times higher compared to on-column detection. However, the noise of longitudinal cells is generally somewhat higher than those with on-column UV detection. Consequently, limits of detection are about 25–50 times lower compared to on-column detection. For instance, the limit of detection for uracil with on-column detection was $3.1 \cdot 10^{-6} \text{ mol l}^{-1}$, and with the longitudinal flow cell it was equal to $9.8 \cdot 10^{-8} \text{ mol l}^{-1}$ [138]. The contribution of longitudinal

flow cells to extracolumn band broadening, i.e. chromatographic resolution, is generally negligible.

Laser-based UV absorption detection has been applied in capillary separation techniques too [131]. This system consisted of a dual laser set-up, optical devices such as polarizers, a quarter wave plate, lenses and objectives, and a photodiode. Simultaneous RI and UV absorbance detection was feasible. Limits of detection were $6 \cdot 10^{-6} M$ for a strongly absorbing compound. Considering the complexity, costs and sensitivity of such a set-up, the use of on-column detection of longitudinally shaped flow cells seems to be favorable.

Photodiode array (PDA) detection has been studied extensively in microcolumn LC research [143–147]. In practice it is, however, hardly ever applied for structural conformation detection. For example, Verzele et al. [144] adapted two commercially available PDA detectors for capillary LC by replacing the detector cell with a miniaturized detector cell. Cell designs – with respect to loss in spectral resolution due to extracolumn band broadening – were discussed and the sensitivity of conventional versus capillary LC compared. Ten-times lower limits of detection were found with capillary LC – despite the small optical path length of the PDA flow cell. Another example is the work of Sandra et al. [143], who applied PDA detection in capillary LC for the detection and identification of hop bitter acids from CO_2 -extracted hop samples. The recorded PDA UV spectra showed that two groups could be differentiated: α -acids (humulones) and β -acids (lupulones). This capillary LC separation proved to be favorable compared to conventional high-performance liquid chromatography and micellar electrokinetic chromatography, because of the insufficient resolution obtained on conventional columns and solubility problems in micellar electrokinetic chromatography.

5.4.3. Fluorescence detection

Among the various miniaturized detection techniques, fluorescence detection seems to be particularly attractive. Fluorescence emission provides more selectivity and increased sensitivity compared to UV absorption and RI detection. Further, emission spectra can be utilized to reveal structural information of

unknown compounds [148,149], and the ability to measure the packing of the column [150,151].

Straightforward on-column fluorescence detection is hardly ever applied in microcolumn LC. Most applications deal with laser-induced fluorescence detection or – as mentioned earlier – detection of fluorescence emission in the packing. Exceptions are the work of Gluckman and co-workers [148,149], who used a miniaturized fluorimetric array detector for the detection and identification of large polyaromatic compounds. The utility of fluorescence emission data was represented by comparing mass spectral data and the emission spectra from well-resolved peaks from a separation of neutral polycyclic aromatic compounds extracted from fuel oil. Based on the mass spectra alone the compounds could not be identified. Spectral subtraction was used to resolve coeluting compounds; this can be an important tool in the identification of compounds contained in complex sample mixtures or the determination of peak impurity. Takeuchi et al. [154] studied the mass detection limits achievable with a commercially available fluorescence detector in capillary LC. By tiling the photomultiplier, using cut-off filters and a packed flow cell, mass detection limits of 0.25–2.4 pg were obtained for aromatic hydrocarbons. In a separate paper the design of this packed flow cell was described [155]. Fluorescence detection has also been applied in environmental studies for the analysis of organotin species complexed with fluorescent tags [156], and for the analysis of water tracers to study distribution phenomena on the North Sea [122]. Another field where fluorescence detection is applied is in bioanalysis, in particular for the analysis of glutathione in human blood samples [157]. The latter application is of especially great interest. Due to the reduced chromatographic dilution on small-I.D. columns, less sample is required to achieve the same limits of detection as in conventional high-performance liquid chromatography. Hence, a patient can be sampled more often without putting too much burden on the patient.

In Section 5.4.2 the term on-column detection was introduced to describe the measurement of electromagnetic radiation in a detection window after the end-frit of the column. The same name has been used by others for the measurement of fluorescence emission in the packing [151–153]. Interchangeable

use of this term should be circumvented. Verzele and Dewaele [150] therefore suggested to use the term in-column detection for the measurement of emission light in the packed part of the column; this will be followed in this paper. With in-column detection the analyte is in a partitioning region. It can be deduced that the sensitivity of in-column detection will be $(1+k)$ times better than with on-column detection [153]. This effect was demonstrated by Verzele and Dewaele [150], who found limits of detection of 3 pg with on-column fluorescence detection and about 100–200 fg with in-column detection for the analysis of drugs. According to the authors, this improvement in sensitivity was in agreement with what theoretically had to be expected. However, the environment in which the analytes reside – e.g. in solution or in an absorbed state – are usually different, which results in different fluorescence characteristics. For instance, improvements in limits of detection of a factor of 100 were reported when the in-column detection principle was applied for the detection of pyrene [151], which was attributed to the higher fluorescence quantum yield in the absorbed state. Takeuchi et al. [158,159] reported similar effects in signal enhancement for in-column detection in capillary columns packed with cyclodextrin-bond stationary phases.

The major draw-back of in- and on-column fluorescence detection is the rather poor sensitivity compared to longitudinal UV-absorption flow cells and to conventional high-performance liquid chromatography with fluorescence detection. The reason for this limited detection performance is the short optical path length and small excitation area of on-column flow cells. It is obvious that for a higher excitation energy, a larger fluorescence intensity will be observed. This will, however, not immediately lead to better limits of detection, unless the noise – e.g. straight light, fluorescence from the cell wall or window and fluorescence or Raman scattering from the mobile phase – is independent on the intensity of the excitation source [160]. Due to the highly collimating nature of lasers, most of these noise sources – especially the fluorescence and Raman scattering of the solvent – can be strongly reduced. Furthermore, nl to pl volumes can be probed at the outlet of the column. Various flow cell designs and instrumental set-ups for laser-induced fluorescence

detection have been described in the literature. More detailed discussions about flow cells and illumination options can be found elsewhere [160,161]. More recent designs, properties and descriptions of laser-induced fluorescence detection for microcolumn LC can be found in the literature too [162,163]. Mass limits of detection – routinely obtained – are typically in the amol range.

Some interesting work – applying laser-induced fluorescence detection in microcolumn LC – has been published by Novotny and co-workers [164,165] and by McGuffin and Zare [166]. McGuffin and Zare [166] detected and analyzed carboxylic acids – after derivatization with 4-bromomethyl-7-methoxycoumarin to match the emission wavelength of a He–Cd laser (325 nm) – in saponified peanut and sesame oils. The compounds were identified on retention time. An almost similar tag was used by Gluckman et al. [165] for the analysis of bile acids

and solvolyzed plasma steroids. A steroid standard – containing nine standard bile acids of approximately 50 pg of each acid – was separated on a capillary LC column, of which a chromatogram is shown in Fig. 3. The experimental conditions are summarized in the caption of Fig. 3.

5.4.4. Electrochemical detection

The three basic detection modes of electrochemical detection are amperometric and potentiometric detection, and conductivity. The principle of these different detection modes can be found in many text books [23,167] and literature [16]. Amperometry is the most commonly used because of its ease of implementation. However, the design and application of conductivity and potentiometric detectors for microcolumn LC have been described too [168–172].

The initial developments in miniaturized electro-

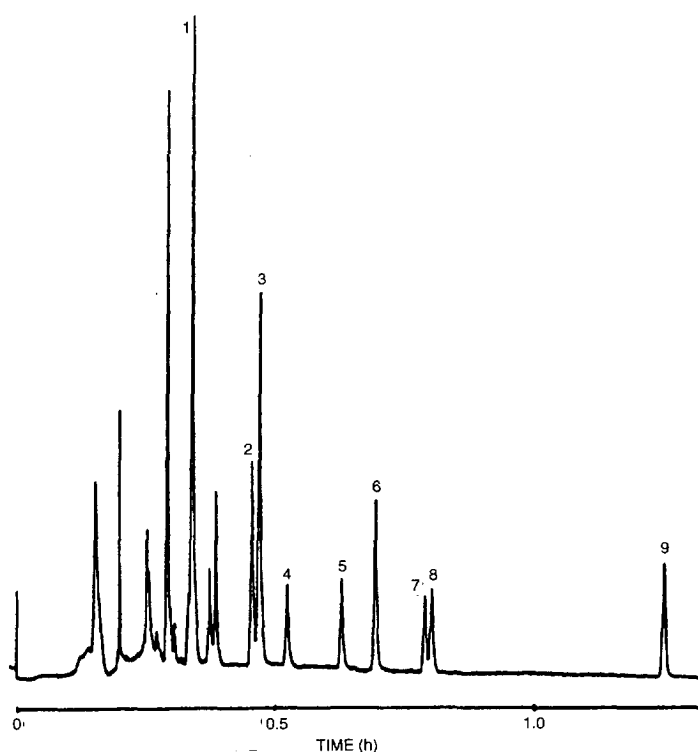


Fig. 3. Chromatogram of a standard mixture of bile acids derivatized with bromomethylcoumarin. Column, 0.66 m \times 250 μ m I.D. packed with 5 μ m C₁₈; continuous gradient from 75 to 100% aqueous acetonitrile at 1.5 μ l min⁻¹. Peak identification: (1) glycocholic acid; (2) glycocheno-deoxycholic acid; (3) glycodeoxycholic acid; (4) cholic acid; (5) urodeoxycholic acid; (6) glycolithocholic acid; (7) deoxycholic acid; (8) chenodeoxycholic acid; (9) lithocholic acid (reprinted with permission from [165]).

chemical detection – or the use of micro-electrodes – were reported for open tubular liquid chromatography by the groups of Manz [173,174] and Jorgenson [175,176], which were later also applied in microcolumn LC [63,95,177]. Such detectors typically consist of a small wire that is placed into the outlet of an open tubular column. For instance, Manz and Simon [173,174] used a 1- μm diameter ion-selective electrode for the potentiometric detection of K^+ ions. Jorgenson et al. constructed a 9- μm diameter carbon electrode for the amperometric detection of catechols and ascorbic acid [175], and for the voltammetric analysis of hydroquinone and catechol. Detection limits achievable with these detection schemes are in the fmol–pmol range.

Many different cell designs for electrochemical detection can be found in the literature. Very simple, cost-effective flow-through cell designs for amperometric detection in capillary LC were demonstrated by Ruban [178,179]. The cell construction of one of these detectors is depicted in Fig. 4. The proposed design permitted the use in capillary LC, e.g. no significant band broadening was observed. Mass limits of detection of 0.14 pg for adrenaline were reported. Other designs, such as wall-jet cells have been described too [180,181].

As stated before, amperometric response is the most commonly used detection mode in microcolumn LC. However, since the potential of the electrode is held at one value, only compounds that are easily oxidized and reduced at the set potential are detected. By scanning the potential – or by applying triangular potential waveform to the electrode – the

number of detectable components that are electroactive in the applied potential range may increase [16,182]. The latter technique is called voltammetric analysis and can provide – if the waveform is applied quickly enough – real-time voltammetric scans from components eluting from a micro or capillary LC column. Thus, analytes can be identified and co-eluting peaks resolved if their voltagrams are significantly different. The major limitation of voltammetry is the approximately 100-times worse mass sensitivity compared to amperometric detection [16].

Voltammetric detection has been applied in open tubular chromatography [175,176] and conventional liquid chromatography [183–185], but has found only little acceptance in microcolumn LC so far. An exception is the work of Goto and Shimada [182] who presented a rapid-scanning electrochemical voltammetric detector for capillary LC. Square-wave voltammeteries were applied because of their suitability for rapid-potential scanning.

The main application area of electrochemical detection in microcolumn LC is bioanalysis. The detection of serotonin and its metabolites in rat brain dialysate [186], the analysis of terbutaline in human plasma [187], and the detection of biogenic amines in brain tissue [188] are just a few examples. A more detailed description about the use of electrochemical detection in bioanalysis will be given in Section 7.

5.4.5. Gas chromatographic detectors

Microcolumns are typically operated – as shown earlier – at volumetric flow-rates of a few μl per minute for capillary LC columns and 30–50 μl min^{-1} for micro LC columns, and are therefore more suited to work with the detector systems currently used in gas chromatography (GC) than conventionally sized high-performance liquid chromatography columns. Obviously, the introduction of analytes dissolved into a detection zone at elevated temperatures – or a flame – is a fundamental problem. Although successfully applied in certain fields, compromises have to be made with respect to linearity, sensitivity and the capability to accept the complete effluent and all of the compounds, with GC detectors in microcolumn LC.

The LC eluent can either be transported into the GC detector, or be directly introduced [18]. The first approach has the main disadvantage of the very

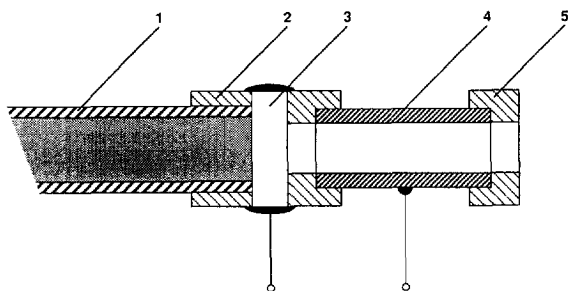


Fig. 4. Cell construction of an amperometric detector. (1) Capillary LC column; (2) and (5) fluoroplastic body; (3) working electrode; (4) reference electrode (adapted with permission from Ref. [178]).

likely risk of sample loss and is therefore hardly applied as an interface. Tsuda et al. [189] and Veening et al. [190] have reported on the use of a moving-wire flame ionization detector for capillary and micro LC, respectively. Typical mass limits of detection were 10–20 ng for triolin with the capillary LC system, and 160 and 400 ng for xylose and lactose with the micro LC system.

Shortly after the introduction of microcolumn LC, various types of direct introduction-based GC detectors – flame-based and flameless thermionic detection, flame photometric detection, and electron capture detection – were evaluated by different research groups.

5.4.5.1. Thermionic detection

Novotny and co-workers [191–193] introduced a flame-based thermionic detector (TID) as a detection technique in capillary LC. The microcolumn LC mobile phase is nebulized with a hydrogen/nitrogen mixture into a primary flame. The combustion products are combined with additional fuel gas and are transported into the secondary, analytical flame. This dual-flame thermionic detector was optimized for organophosphorus compounds, i.e. organophosphorus pesticides. The mass sensitivity of detection for phosphorus compounds was equal to 20 pg s^{-1} , and the response was found to be linear over at least three orders. The same thermionic detector has been used for selective nitrogen detection [194]. Mass detection limits were of the same order of magnitude as with selective phosphorus detection, i.e. 14 pg N s^{-1} , and also a linear response of three orders. The use of nitrogen-selective detection was demonstrated for the analysis of barbiturates.

Kientz et al. [195,196] demonstrated the use of thermionic detection for non-volatile polar compounds. The system performance was found to be dependent on the mobile phase modifier concentration. Further it was found that a relatively high buffer salt concentration could be tolerated. The introduction of non-volatile samples into the flame was achieved by plug-type solvent introduction. Detection limits were comparable to that published by Novotny and co-workers. Flameless thermionic detection was studied by Brinkman and co-workers [197–199] for trace-level detection of organopes-

ticides in environmental samples and different vegetables.

5.4.5.2. Flame photometric detection

Detector designs for flame photometric detection are very similar to those of thermionic detectors [191,200]. The main disadvantage of flame photometric detectors is the sensitivity dependence of modifier concentrations. Further, acetonitrile significantly increases the background noise and quenches the detector signal at even low percentages. Various interface designs have been proposed to overcome these problems [201–205]. Kientz and Brinkman [18] recently summarized and discussed the advantages and limitations of the use of flame photometric detection in microcolumn LC.

A very interesting application using a flame-based photometric type of detection has been published by Chang and Taylor [203]. Detection was based on the chemiluminescence reaction of ozone with sulfur monoxide – a combustion product when sulfur-containing compounds are introduced into a reducing hydrogen/air flame. The compounds were reduced in the flame of a commercially available flame ionization detector. Operated at optimized conditions, mass detection limits of 3 pg S s^{-1} at a mobile phase composition of methanol–water (50:50, v/v) are feasible. The performance of the detector was demonstrated by the analysis of PTH-amino acids and thiocarbamates, which are normally detected with UV absorption or PDA detection. The separation and detection of several PTH amino acids is shown in Fig. 5.

5.4.5.3. Electron capture detection

There are only a few publications that deal with electron capture detection in microcolumn LC. Brazhnikov et al. [206] used an electron capture detector to determine extracolumn band broadening to examine its use for 1.0-mm I.D. micro LC column testing under normal phase conditions. The application of electrocapture detection with reversed-phase analysis at flow-rates up to $50 \text{ } \mu\text{l min}^{-1}$ was demonstrated by Brinkman and co-workers [207]. The column effluent was evaporated with the aid of a miniaturized interface. However, clogging of the interface occurred occasionally when non-volatile acidic modifiers were applied. Furthermore, the

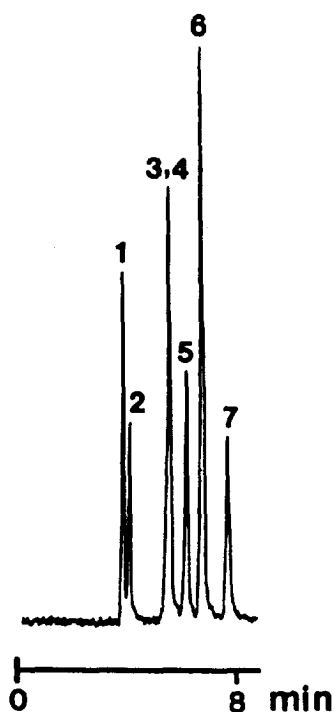


Fig. 5. Capillary LC–sulfur selective chemiluminescence detection analysis of PTH-amino acids. Mobile phase, methanol–water (50:50, v/v); column, 15 cm×320 μm I.D. 3 μm C₁₈; detector temperature, 375°C; sample, 3–6 ng of each compound. (1) PTH-alanine; (2) PTH-proline; (3) PTH-methionine; (4) PTH-phenylalanine; (5) PTH-leucine; (6) PTH-norleucine (reprinted with permission from Ref. [203]. Copyright (1991) American Chemical Society).

interface clogged more rapidly at lower mobile flow-rates. Hence, reversed-phase capillary LC with this type of interface is not feasible.

Zegers et al. [208] recently described an interface for the direct coupling of reversed-phase capillary LC with electron capture detection. The interface consists of a piece of fused silica capillary which is positioned in a stainless steel capillary that connects the capillary LC column with the detector. A make-up gas had to be used to cool the fused silica capillary. The interface allows for the detection of relatively non-volatile and polar compounds. Results were demonstrated for the analysis of chlorophenols, pesticides and benzodiazepines. The response of the system for all classes of compounds was linear over 2–3 orders of magnitude, and the limits of detection ranged from 10 to 440 pg. With large volume

injections, the concentration sensitivity could be increased by 2–3 orders of magnitude allowing for the detection of approximately 150 ng l⁻¹ of chlorophenols in river Meuse water.

5.4.6. Other detection principles

Besides the detection techniques discussed above, other principles of detection also have been studied for microcolumn LC, including (Fourier transform) infrared spectrometry – either measured on-line [209] or via deposition on thin-layer plates [210], chemiluminescence [211], indirect detection schemes [212–214], inductively coupled plasma atomic emission spectrometry [215], or evaporative light scattering [216]. These detection techniques have been applied with only limited success and have not found – mainly due to the fact that the detection technique is too selective or not robust enough – their way into the routine use of microcolumn LC.

A very interesting and promising novel approach towards detection miniaturization is the coupling between continuous-flow techniques – such as microcolumn LC and capillary electrophoresis – and nuclear magnetic resonance spectrometry (NMR) [217–219]. The hyphenation of microcolumn LC with NMR has a number of advantages compared to the coupling of conventional high-performance liquid chromatography with NMR spectroscopy. Fully deuterated solvents can be used due to the low solvent consumption. Suppression of the solvent signal is therefore not necessary, allowing the use of the complete chemical shift range for structural elucidation of the analytes. Furthermore, theoretical and feasibility studies have shown that a 400-fold reduction in cell volume only results in a 2-fold reduction in signal-to-noise ratio, allowing the use of 50-nl volume continuous-flow NMR detection cells. Shortcomings of continuous-flow NMR are the poor sensitivity due to the limited time to measure each individual analyte and the flow-rate dependency of the NMR line. Typical analyte concentrations are at the high mM level. Further, substantial band broadening – e.g. loss in chromatographic resolution – is observed in NMR flow-through cells. However, this is often compensated by the profits of structural information detection of NMR.

Continuous-flow detection allows for the use of proton-NMR chemical shift values as the second

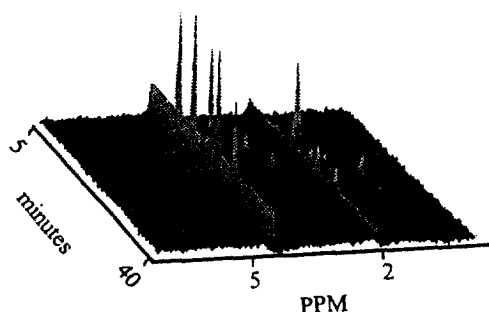


Fig. 6. Two-dimensional chromatogram of a mixture of 67 mM alanine, 33 mM arginine, 35 mM lysine, 14 mM leucine-arginine and 11 mM oxytocin-glycine (order of elution). A complete NMR spectrum was acquired every 9 s which was constructed from 64 consecutive scans with a 0.06-s pulse delay (512 point-free induction decays over a ± 4000 -Hz spectral window). The micro LC system comprised a 15-cm \times 1.0-mm I.D. column packed with 5 μ m C₁₈ that was operated at a flow-rate between 10 and 50 μ l min⁻¹. The mobile phase consisted of 2% TFA in 2H₂O (p2H 2.4)-C₂H₃CN (74:26, v/v) (adapted with permission from Ref. [218]. Copyright (1994) American Chemical Society).

dimension, which was demonstrated for the separation of amino acids and peptides [218], and vitamin A derivatives [219]. Two-dimensional NMR spectra can be recorded with stop-flow techniques. An example of a two-dimensional chromatogram of an amino acid/peptide mixture is given in Fig. 6. The number of theoretical plates that was observed for alanine at a flow-rate of 50 μ l min⁻¹ equalled 20 000 plates/m, indicating very poor separation efficiency or excessive extracolumn band broadening.

6. Hyphenation

6.1. Multidimensional chromatography

As with flame-based detectors, microcolumns are well suited for coupling with secondary separation techniques, i.e. multidimensional chromatography. Microcolumn LC has been interfaced with thin-layer chromatography applying infrared detection [220], conventional high-performance liquid chromatography [221], microcolumn LC [222–224], gas chromatography [225–231], supercritical fluid chromatography [232] and capillary electrophoresis [233]. The coupling of individual separation techniques

increases the total peak capacity of the chromatographic system, which is equal to the product of the peak capacities of the individual dimensions. The improved peak capacity allows for the separation of very complex samples.

The coupling of microcolumn LC with gas chromatographic (GC) techniques seems to be the most challenging since a liquid mobile phase has to be converted into a GC-compatible – i.e. gaseous – sample. Different type of interfaces have been developed. Among them are retention gap-based interfaces, such as on-column injectors [225] and loop-type interfaces [226], pyrolysis interfaces and (multi-capillary) stream splitters. The retention gap-based interfaces have been applied most extensively because of their ease of use. Retention gaps were originally developed for the introduction of large sample volumes onto GC columns. An uncoated inlet capillary – having negligible retention for the compounds of interest – is placed in front of the GC column. The large solvent volume is vaporized and the solute bands – that were spread out along the retention gap – are concentrated at the beginning of the separation column. An example of the successful application of an on-column injector as an on-line interface for microcolumn LC–GC is given in Fig. 7, illustrating the two-dimensional separation of a fuel oil sample. Heart-cutting was applied to introduce a part of the first dimension onto the secondary separation system. With heart-cutting techniques, only a fraction of the original sample is separated, with a peak capacity that is typical for a single dimension.

The full power of a two-dimensional separation system was used by Holland and Jorgenson [222] to separate biological amines with anion-exchange chromatography coupled to reversed-phase chromatography. Via a loop type interface, samples from the first dimension were temporarily stored, before they were transferred to the second dimension. To obtain a high sampling frequency, the 90 cm \times 100 μ m I.D. anion-exchange column was operated at a flow-rate of 33 nl min⁻¹, while the secondary 3 cm \times 100 μ m I.D. reversed phase column was maintained at 6 μ l min⁻¹. The peak capacity of the two-dimensional system was estimated to be 1400 peaks.

Another attractive way to achieve high sampling frequencies is to use capillary electrophoresis (CE)

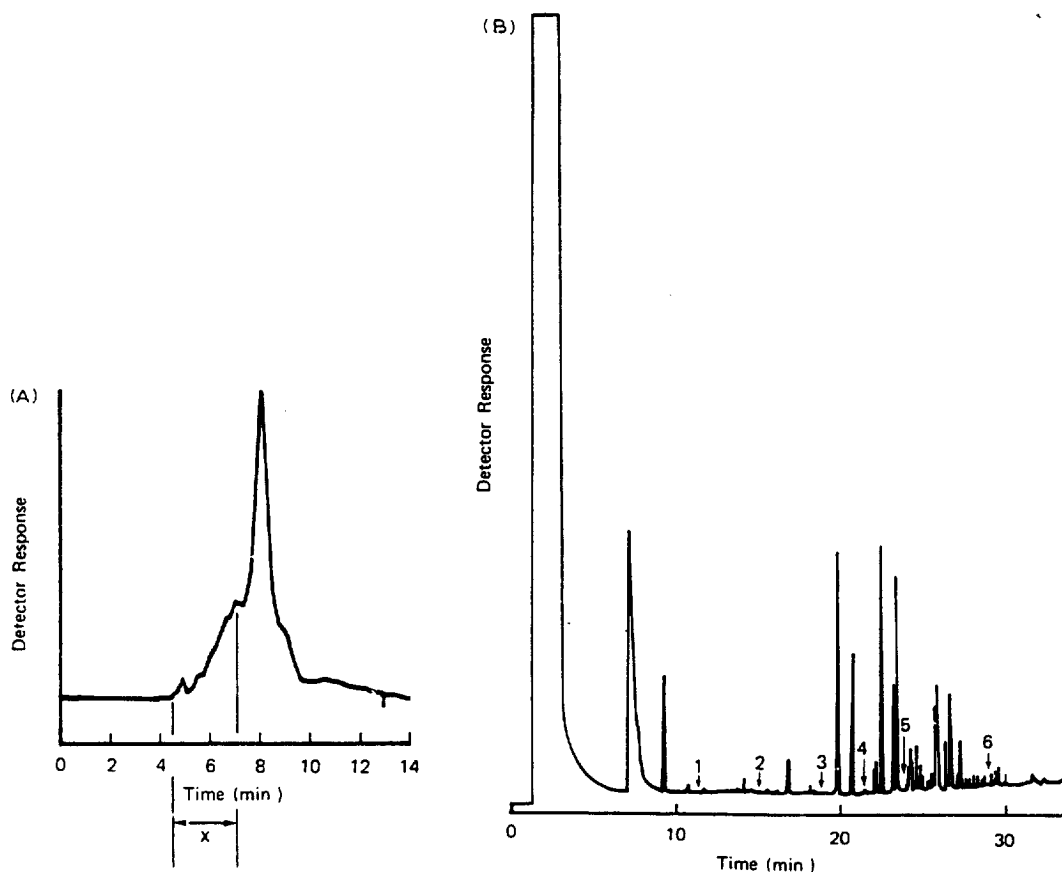


Fig. 7. Two-dimensional separation of a fuel oil sample by microcolumn LC–GC. (A) Capillary LC chromatogram; (B) gas chromatogram; x = fraction introduced into the GC. Conditions: capillary LC separation; column, $105\text{ cm} \times 250\text{ }\mu\text{m}$ I.D. packed with $7\text{ }\mu\text{m}$ silica; mobile phase, heptane; flow-rate, $10.6\text{ }\mu\text{l min}^{-1}$; UV absorption at 214 nm . GC conditions: column, $30\text{ m} \times 0.25\text{ mm}$ I.D.; retention gap, $15\text{ m} \times 250\text{ }\mu\text{m}$ fused silica; helium at 70 cm s^{-1} ; flame ionization detection at 275°C (make up gas, nitrogen at 30 ml min^{-1}); oven at 105°C for 9 min and programmed to 245°C at 5°C min^{-1} . Peak identification: (1) chlorobenzene; (2) 1,2-dichlorobenzene; (3) 1,2,4,5-tetrachlorobenzene; (4) 1,2,3,4-tetrachlorobenzene; (5) pentachlorobenzene; (6) hexachlorobenzene (reprinted with permission from Ref. [225]).

as the second dimension, which allows to obtain high efficiencies in short periods of time. Lemmo and Jorgenson [233] applied a two-dimensional separation system for the study of protein standards based on microcolumn size-exclusion chromatography (SEC) and CE. Two approaches were presented – a loop/valve interface and a so-called flow-gating interface. The chromatographic SEC dimension consisted of a $105\text{ cm} \times 250\text{ }\mu\text{m}$ I.D. or a $110\text{ cm} \times 100\text{ }\mu\text{m}$ I.D. column packed with a size-exclusion stationary phase. The flow through these columns was $235\text{--}360$ or 23 nl min^{-1} , respectively. CE was performed in untreated $50\text{ }\mu\text{m}$ I.D. fused silica

capillaries of different lengths. The applied voltage was $\pm 30\text{ kV}$. Because of the relatively large dead-volume of the bores of the loop/valve interface it was only applicable for the coupling with the $250\text{ }\mu\text{m}$ I.D. SEC column, i.e. too much extracolumn band broadening. Furthermore, the loop/valve interface hampered continuous sample collection. With the flow-gating interface – which basically consists of a PTFE gasket having a 1-mm channel that is sandwiched between two stainless steel plates – these problems were overcome. Injection into the CE capillary was achieved by selectively sending a transfer liquid flow through the PTFE channel or to

waste. When an injection was made, the SEC effluent was sent to waste. An example of the results obtained with the new type of interface is given in Fig. 8, which depicts the two-dimensional separation of protein standards by SEC–CZE.

6.2. Microcolumn LC–mass spectrometry

The introduction of continuous-flow fast atom bombardment and atmospheric pressure ionization techniques (electrospray and atmospheric pressure chemical ionization) have contributed to a very large extent to the current success of microcolumn LC, and have been one of the major driving forces behind the development of microcolumn LC. However, other types of interfaces also have been used for coupling with mass spectrometry (MS). Among them are electron impact and chemical ionization and the particle beam interface. Interfaces such as the moving belt interface [234] and the thermospray types of interface [235] are rarely applied. Microcolumns can

be either directly coupled to the ion source or via transfer lines. The latter will obviously increase band broadening, i.e. decreasing the chromatographic obtained resolution. On the other hand, the mass spectrometer also will add to the overall band broadening.

6.2.1. Electron impact/chemical ionization (direct liquid introduction)

Direct introduction of the microcolumn LC effluent into the source of the MS is sometimes applied. A recent study by Ranalder et al. [236] involved the separation and quantification of metabolites of retinoic acid in human plasma. The column was directly coupled to the source of the MS and negative ionization conditions were obtained by coaxial introduction of reagent gas. Albron and Stenhagen [237] reported on the connection of a 220 μm I.D. packed capillary LC column with an electron impact source for the analysis of plant extracts, phenolic acids and other polar compounds. Another interesting application of direct liquid introduction was published by Esmans et al. [238], who evaluated the system for the identification and detection of nucleosides in human urine after separation on a micro LC column. These are just a couple of examples of the use of direct liquid introduction interfaces for microcolumn LC–MS. Many others have been published. The advantages and limitations of microcolumn LC–MS with direct liquid introduction have been discussed by Lee and Henion [239].

6.2.2. Particle beam

Capiello's group has published a number of papers on the design and performance of a modified particle beam interface in conjunction with capillary LC [240–242]. By increasing the orifice diameter of the aerosol generator – i.e. the coaxial helium tubing – droplet formation of the mobile phase on the end of the capillary transfer tubing was circumvented and aerosol formation promoted. Further, a restriction was made in the capillary transfer tubing, which also aids the formation of an aerosol. Improved sensitivity of 1 order of magnitude was found compared to a standard particle beam interface with conventional liquid chromatography. The interface was applied for basic, neutral and acidic low volatile and thermally

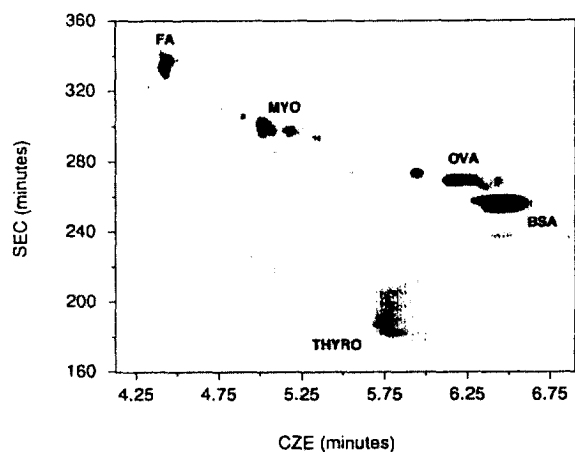


Fig. 8. Separation of protein standard by two-dimensional SEC–CZE with a flow-gating interface. Each protein was present at 0.5% (w/v) with 2.5% (w/v) formamide. THYRO, thyroglobulin; BSA, bovine serum albumin; OVA, chicken egg albumin; MYO, horse heart myoglobin; FA, formamide. The 110 $\text{cm} \times 110 \mu\text{m}$ I.D. SEC column was operated at a flow-rate of 20 nl min^{-1} . Injection was 8 min at 7 bar. The electrophoresis capillary had a length of 53 cm (33 cm to the detection window). CZE conditions: 30 s electromigration injection at 0 kV and 4 min overlapped runs at -11 kV. The actual CZE run time was 8 min. The buffer used for both separations was 10 mM tricine, 25 mM Na_2SO_4 , 0.005% sodium azide (w/v), pH 8.23 (reprinted with permission from Ref. [233]). Copyright (1993) American Chemical Society.

instable pesticides [242] and coumarins in plant extracts [240].

6.2.3. Continuous-flow fast atom bombardment

The first successfully applied microcolumn LC–MS interface was continuous-flow fast atom bombardment (CF-FAB), which has been applied mainly for the analysis and identification of biochemical compounds. This interface has been applied for coupling with microcolumn LC since the mid-1980s, but its breakthrough came some years later when a 220 cm × 50 μm I.D. packed capillary LC column was coaxially interfaced with CF-FAB [243]. The dead-time of such a system – operated at a flow-rate of ~30 nl min⁻¹ – is 79 min and found to be impractical. However, since then, the CF-FAB interface has undergone many modifications, making it a very attractive desorption ionization technique for polar and ionic compounds. CF-FAB is based on secondary ionization sputtering processes and requires a viscous matrix to produce ions. This matrix generally consists of an aqueous glycerol solution. The matrix solution can be either coaxially added to the post-column effluent [243,244] via an addition tee [245,246]. Alternatively, the glycerol matrix can be added to the mobile phase [247,248]. The latter will obviously affect chromatographic separation. Each interface design has its own advantages and limitations. The coaxial addition of matrix solution does not require any transfer tubing – therefore reducing extracolumn band broadening. The transfer line interface – where the glycerol is added via a tee – permits for simultaneous on-line UV absorption and MS detection. Optimization of the separation and ionization conditions can be optimized independently with both interfaces.

The number of applications dealing with microcolumn LC–CF-FAB is extensive. In a review paper in 1994, Tomer et al. [249] summarized a large number of publications dealing with capillary LC–MS. In this paper, two recently published examples will be discussed. Li et al. [250] applied capillary LC continuous-flow liquid secondary ion MS (CF-LSI-MS) – a technique very closely related to CF-FAB – for the rapid screening and identification of metabolites. Capillary LC–CF-LSI-MS was used as part of an integrated approach for discovery stage in vitro metabolites. Excellent sensitivity was obtained in

detecting model compounds in both the positive and the negative ion mode. Full-scan mass spectra could be obtained when 5 pmol of compounds was injected onto a 10 cm × 300 μm I.D. capillary LC column. Fig. 9 shows a typical example obtained with this system. Fig. 9 (top) corresponds to a reconstructed ion chromatogram of an oxidative metabolite that was – after treatment with CH₂N₂ – slowly converted into components A and B. In the bottom and middle traces of Fig. 9, the positive ion LSI-MS mass spectra of products A and B are given, respectively.

Li et al. [251] used cation-exchange capillary LC–FAB-MS in order to characterize reaction products after proteolytic cleavage of neuropeptides. A continuous bed, i.e. a gel that is covalently bonded to the capillary wall, was used as the stationary phase material. The 15% glycerol make-up solution was added to the post-column flow via a zero-dead-volume tee. The potential of such columns for on-line mass spectrometry and peptide analysis was investigated. The system allowed limits of detection at the pmol level.

6.2.4. Electrospray ionization

Electrospray ionization (ESI) was almost simultaneously introduced with CF-FAB and has become a very popular technique for the analysis of biochemical macromolecules. The coupling of an ESI interface to microcolumn LC is relatively easy. A schematic representation of one of the earliest developed ESI interfaces is given in Fig. 10 [252]. The liquid stream is introduced into the electrospray chamber at a typical flow-rate of 5–20 μl min⁻¹ through a stainless steel needle. This needle is grounded and the cylindrically shaped electrode is kept at –3.5 kV for positive ion detection. The metallized inlet and outlet of the glass capillary that passes ion-bearing gas into the first stage of the vacuum system are maintained at –4.5 kV and +40 V, respectively. The skimmer is set at –20 V and the ion lens in front of the quadrupole at –100 V. To produce negative ions, voltages of the same magnitude but of opposite sign have to be applied.

As a result of the applied electric field, some of the positive ions in the column effluent will drift towards the liquid surface and some of the negative ions will be pushed away from it, until near complete

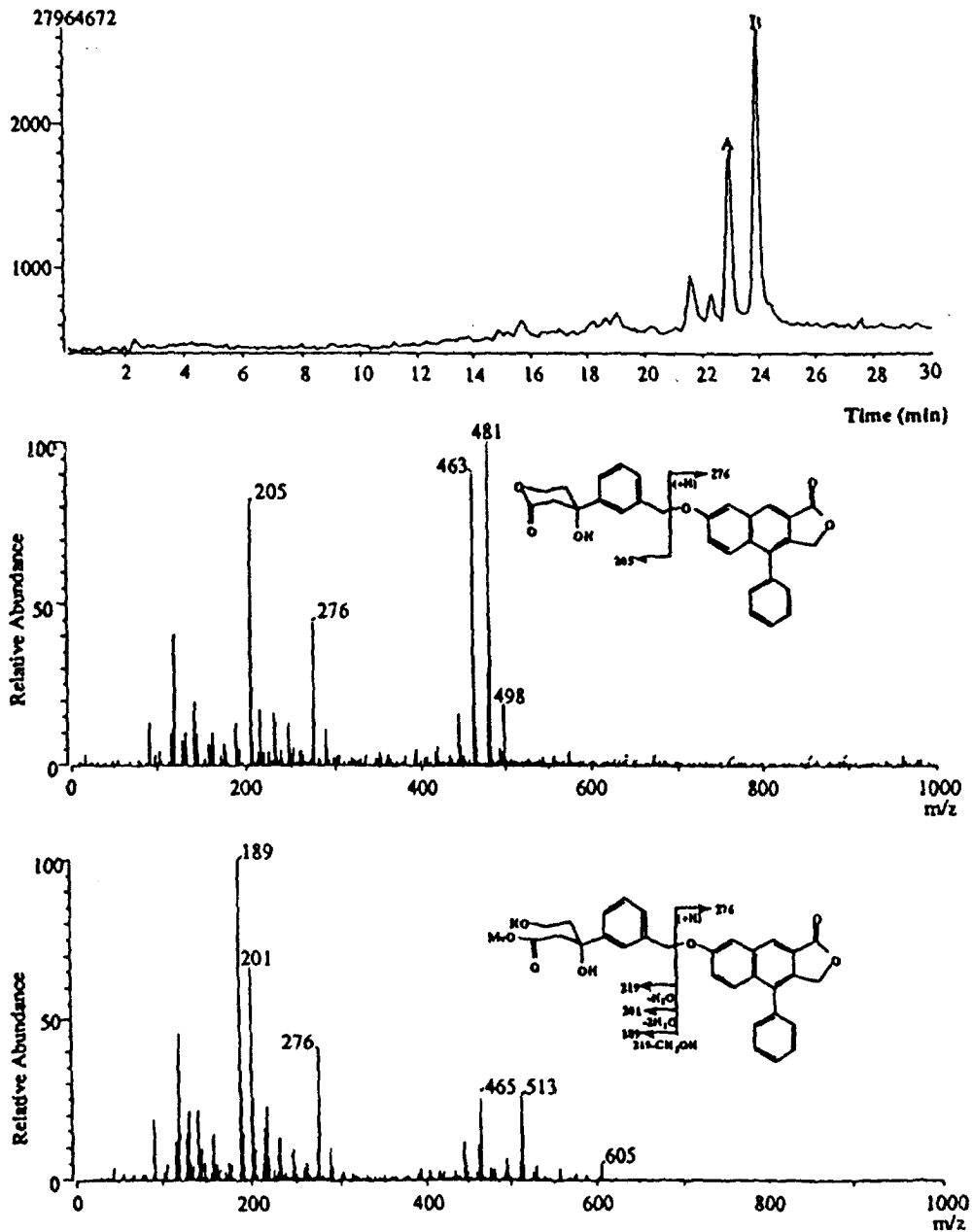


Fig. 9. Capillary LC–CF–LS–IMS analysis (reconstructed ion chromatogram; $m/z \sim 400\text{--}550$) of an isolated metabolite after treatment with CH_2N_2 (top) and positive ion LSI–MS spectra of product A (middle) and its methylester product B (bottom). See text for details (adapted with permission from Ref. [250]. Copyright (1995) American Chemical Society).

charge relaxation is achieved. The accumulating positive charge at the surface of the liquid leads to destabilization of the liquid surface, which is drawn

out of the capillary downfield, forming a stable, conically shaped fluid. At higher voltages, the tip of the fluid cone starts to emit very small positively

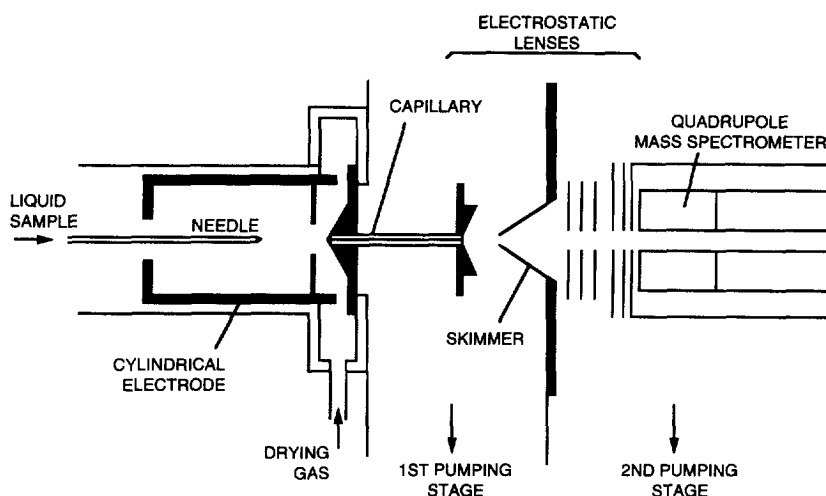


Fig. 10. Schematic representation of an electrospray interface (reprinted with permission from Ref. [252]. Copyright (1985) American Chemical Society).

charged droplets; the so-called stable jet mode. The next step in the ionization process is the evaporation of solvent molecules from the charged droplets, resulting in an increment in the charge-to-volume ratio, and to repeated disintegrations at the Rayleigh instability limit. Ultimately, positive gas-phase ions are produced. A detailed description of the mechanism of ESI is given in the literature [253]. However, the theory is not yet completely understood.

LC-ESI-MS interfaces have a near-linear relationship upon the concentration of the analyte, making it an important tool in quantitative analysis. For instance, Whitehouse et al. [252] reported a linear behavior of 4 orders of magnitude for their ESI interface. Furthermore, the mass spectrometer signal intensity was found to be almost independent of the liquid flow-rate. Hence, the ESI interface can be operated at extremely low mobile flow-rates that are typically used with nanoscale LC columns, resulting in extremely low mass sensitivity limits of detection.

As with CF-FAB interfaces, ESI interfaces are coupled with capillary LC either directly or via a transfer line. The advantages and limitations of transfer lines have been discussed earlier. Primarily, applications of capillary and nanoscale LC-ESI-MS have been in protein and peptide analysis. An extensive overview of ESI-MS in conjunction with capillary and nanoscale LC has been given in the literature [249]. In Section 7, some selected exam-

ples will be discussed, demonstrating the successful use of microcolumn LC-ESI-MS.

Microcolumn LC-MS is still under development. Current research is focussed on achieving even lower limits of detection. Just some examples are the coupling of microcolumn LC with matrix-assisted laser desorption ionization (MALDI) techniques, the coupling of nanoscale columns with an ESI interface to an orthogonal time-of-flight mass spectrometer, and the use of micro LC columns with an ESI source interfaced to an ion trap storage/reflectron time-of-flight mass spectrometer [254].

7. Applications

Microcolumn LC can be applied in many different fields. However, at present its main application areas are bioanalysis, neuroscience and protein/peptide research. In these cases, sample availability or mass spectrometric compatibility are often the driving force towards miniaturization. Other application areas are chiral separations and the analysis of industrial samples, e.g. polymers and additives. Some selected examples will be briefly discussed.

7.1. Protein/peptide research

The amount of applications that can be found in

the literature that deal with the analysis of biological compounds are numerous. Among the best examples of the use of microcolumn LC can be found in protein/peptide research. For example, Henzel and co-workers [255–257] reported on the identification of proteins from two-dimensional gels by means of capillary LC and mass spectrometry. Typically, a protein is characterized by means of an in situ digestion. The generated peptides are analyzed by means of MALDI-MS and with capillary LC followed by ESI-MS or by protein sequencing. The combination of the analysis results of these three techniques finally leads to the complete amino acid sequence of the protein. A typical example of a capillary LC separation of an in situ digested protein spot is given in Fig. 11. The most demanding and laborious steps in these types of protein characterization techniques are still the manual sample prepara-

tion steps required to recover the protein from the gel. However, this example shows that capillary LC has produced significant advances in the development of capillary LC-MS in protein/peptide research.

Other, similar examples have been published by Yates and co-workers [258,259]. The tandem mass spectra of modified and unmodified phosphorylated peptides, and high-molecular weight proteins isolated with two-dimensional gel electrophoresis were used to determine the amino acid sequence of the peptide and proteins, respectively. This method employs a reverse pseudo-mass spectral library search. For each amino acid sequence – that has some similarity to the sequence represented in the observed tandem mass spectrum – a library spectrum is predicted for the sequence and compared to the tandem mass spectrum.

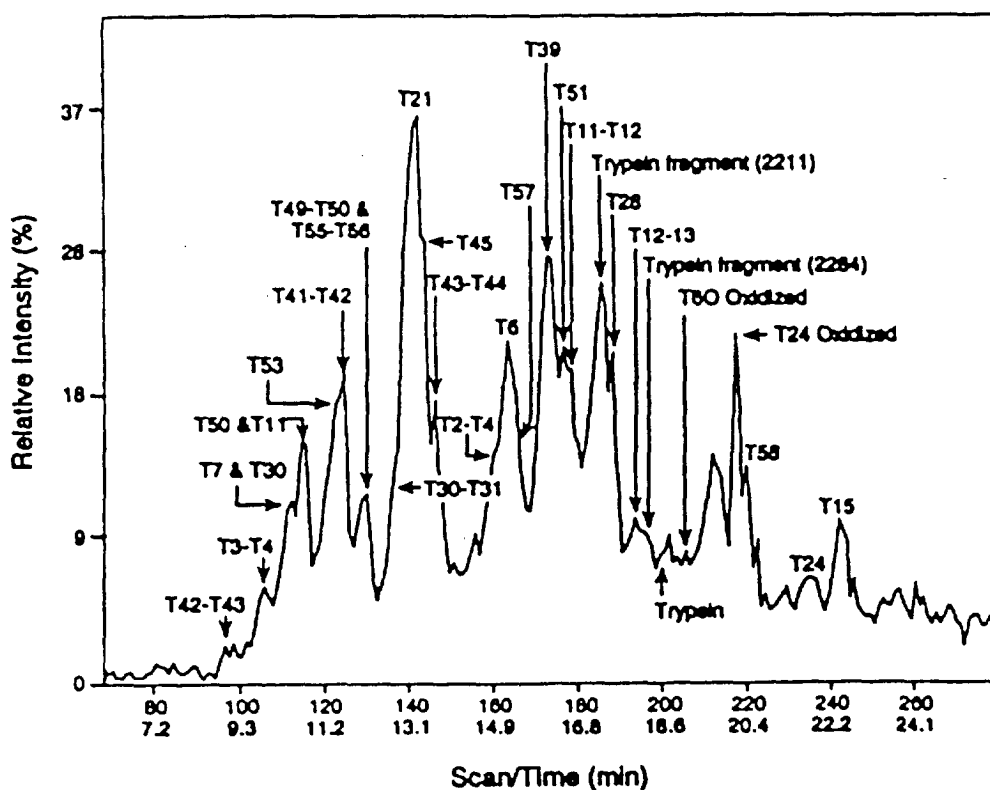


Fig. 11. Total ion current chromatogram obtained from an on-line capillary LC-ESI-MS analysis of an in situ digestion of a protein spot from a two-dimensional gel. The amount of protein was ~2 pmol. Peptide separation was conducted on a 180 μm i.d. capillary LC column (reprinted with permission from Ref. [257]).

Tempst et al. have published on the micro LC purification of peptides for combined chemical sequencing and MALDI-MS [260], and described in detail the microcolumn LC assembly and fraction collection, sample handling and peak selection for sequencing [261]. A very experimentally orientated paper, i.e. description of the capillary LC system and sample preparation, on protein isolation and peptide mapping with capillary LC has been published by Moritz et al. [262]. Tryptic peptide maps derived from *in situ* digested murine plasmacytoma were developed on 0.2 mm I.D. capillary columns. Limits of detection were not given. Kassel et al. [263] evaluated packed capillary perfusion columns for rapid sequencing of enzymatic digest with ESI-MS-MS. LC columns (180, 320 and 1000 μm I.D.) – either packed with small-pore materials or perfusion particles – were directly coupled to the ESI source of the mass spectrometer. Retention times were reduced by a factor of 3–5 with the capillary perfusion columns. Some loss in chromatographic resolution was found with the use of perfusion columns. However, capillary perfusion LC-MS permitted for the identification of the same peptide fragments at the 25–50 pmol level.

All previously discussed examples deal with MS as the identification method for the peptide fragments. Battersby et al. [264] demonstrated the characterization of recombinant DNA-derived human growth hormone (rhGH) isolated from an *in vivo* rat model using capillary LC. The chemical changes that occur in rhGH following intravenous administration were identified on retention time. Prior to characterization, the protein of interest was isolated with an affinity column. The recovered protein was then digested and analyzed on a capillary LC column. Deamination and oxidation of rhGH were found to occur *in vivo* and were identified at the sub-pmol level (<10 pmol).

7.2. Chiral separations

The chromatographic analysis of enantiomers is a rapidly growing research area, which also has been the subject of some microcolumn LC studies. Microcolumn LC is a very attractive technique for enantiomeric separations since it is possible to apply new types of stationary phases that are normally too

expensive, e.g. monoclonal antibodies or receptor proteins [265]. Furthermore, the consumption of expensive stereoselective mobile phase additives is lower, and the chromatographic efficiency and selectivity higher. The latter is not yet understood, but experimental data show improved plate numbers and chromatographic separation factors on capillary LC columns.

Chiral separations can be achieved by the use of chiral stationary phases [265–270], a chiral selector that is adsorbed on the packing material [265,271,272], or by using chiral mobile phase additives [265,273–275]. For instance, Wännman et al. [265] reported on the separation of chiral ethanol and propanol derivatives on a Pirkle phase. The authors concluded that every originally developed method for chiral separations on conventional high-performance liquid chromatography columns can be transferred to capillary LC systems with little effort. Kientz et al. [266] showed the analysis of chiral organophosphorus pesticides, and found that capillary LC has a 5-fold improved separation impedance, an approximately 2-fold increase in column permeability and better inertness compared to conventional LC. Cortes and Nicholson [267] showed, in a feasibility study, the potential of chiral separations on capillary-sized LC columns. Comparable results were found on conventionally sized columns at almost identical linear mobile phase velocities.

Takeuchi and co-workers [273–275] demonstrated the use of β - and γ -cyclodextrin as mobile phase additives for the separation of analogs of dansyl phenylalanine [273] and phosphate enantiomers [274,275]. The stationary phase was ordinary octadecyl silica. The use of small-I.D. microcolumns allowed for the use of these very expensive mobile-phase additives. The work of Vindevogel et al. [271] will be used as an example of the adsorption of a chiral selector on a stationary-phase surface. The elution order of *N*-nitroaroyl amino acid on bovine serum albumin-coated silica gel was determined and it was concluded that column-overloading effects on protein-based stationary phases occur at lower concentrations than on other stationary phases. Furthermore, as a result of this effect, the retention times of the chiral analytes shift non-predictably, especially for the last eluting compound. This effect was explained by blocking of the adsorption sides by the

first eluting compounds, or that enantioselectivity was obtained through adsorption sites that interact uniquely with one enantiomer. A quantitative description was not given.

7.3. Bioanalysis and neuroscience

On-line capillary LC – interfaced with either CF-FAB-MS-MS or ESI-MS-MS – was applied by Vouros and co-workers [276,277] for the detection of *in vivo*-formed DNA adducts. This technique was used for rapid screening of the reaction between carcinogenic adducts and calf thymus DNA in order to elucidate the biochemistry of the interaction [276]. Multiple reaction monitoring provided limits of detection below 50 fmol. Further, the technique was able to detect structural data of the adducts that were formed. In a successive paper a similar application was demonstrated [277]. In this case the study involved the detection of heterocyclic aromatic amine DNA adducts of food-derived carcinogenic compounds. The limit of detection of the target adduct was approximately 80 fmol, which was achieved by monitoring characteristic fragmentation patterns.

Vouros' group also reported on the analysis of vitamin D metabolites [278]. Vitamin D metabolites may have therapeutic effect in the treatment of leukemia. However, overdosage of this steroidal hormone can cause severe side effects. Capillary LC-tandem MS was employed to provide a high degree in sensitivity and selectivity at low levels. A derivatization step was introduced by reacting the vitamin D metabolites with 4-phenyl-1,2,4-triazoline-3,5-dione. The derivatives were characterized by continuous-flow FAB.

Femtogram detection limits for biogenic amines in rat brain tissues using micro LC with electrochemical detection was reported by Caliguri and Mefford [188]. The chromatographic separation of some indoleamine standards on a 25 cm×1.0 mm I.D. micro LC column is given in Fig. 12. The chromatogram on the right represents the quantity of two compounds present in 0.84 µg of tissue. Under the presented conditions it would be feasible to quantitate the indoles present in 90 ng of tissue. The obtained concentration values agreed with the literature. Besides the separation of indoleamines, the

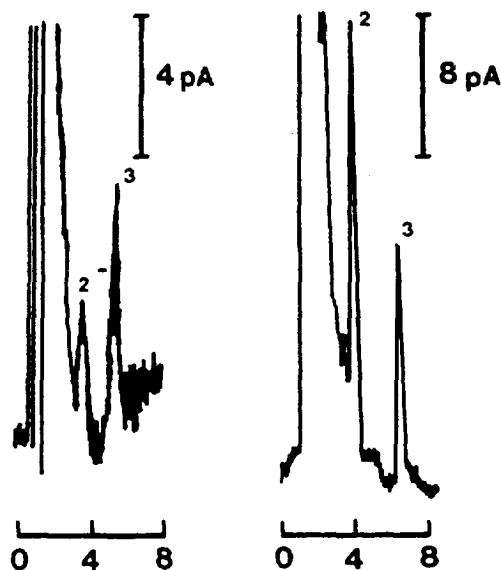


Fig. 12. Micro LC separation of an indoleamine standard at the limit of detection level (left) and indoleamines in rat hypothalamus extract (0.94 µg tissue, right). Column: 25 cm×1.0 mm I.D. packed with 5 µm C18; mobile phase, 0.1 M sodium acetate, 0.02 M acetate, 0.02 M citric acid, 50 mg l⁻¹ EDTA, 100 mg l⁻¹ sodium octyl sulphate and 4.5% (v/v) acetonitrile; flow-rate, 40–50 µl min⁻¹; detection, +0.6 V vs. Ag/AgCl. Compound identification: (2) 5-hydroxytryptamine-HCl; (3) 5-hydroxy-indoleacetic acid (adapted with permission from Ref. [188]).

authors also reported on the analysis of catecholamines.

Straub et al. [279] determined β-lactam residues in milk using perfusion capillary LC combined with ESI-MS. β-Lactam antibiotics are a widely used drug in veterinary medicine for the treatment of bacterial infections and are assigned as target drugs with high priority. The separation detection of six key components was presented. The ability to confirm these β-lactam residues at the 10-ppb level was regarded as a significant breakthrough. Further, the ability to concentrate and analyze the components in less than 13 min was also found to be of importance for repetitive screening.

The presented applications in this paper cover only a small part of microcolumn LC applications that can be found in literature. However, this section gives a flavor and a first impression of the application area of microcolumn LC and demonstrates the versatility of the technique.

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References

- [1] D. Ishii, T. Takeuchi, *Trends Anal. Chem.* 9 (1990) 152–157.
- [2] K. Jinno, C. Fujimoto, *LC·GC* 7 (1989) 328–336.
- [3] M. Novotny, *Anal. Chem.* 60 (1988) 502A–510A.
- [4] M. Verzele, C. Dewaele, M. De Weerd, *LC·GC* 6 (1988) 966–972.
- [5] K. Jinno, *Chromatographia* 25 (1988) 1004–1011.
- [6] M. Novotny, *J. High Resolut. Chromatogr. Chromatogr. Commun.* 10 (1987) 248–256.
- [7] M. Verzele, C. Dewaele, *The Science of Chromatography* (Journal of Chromatography Library, vol. 32), F. Bruner (Ed.), Elsevier, Amsterdam, 1985, pp. 435–447.
- [8] F.J. Yang, *J. High Resolut. Chromatogr. Chromatogr. Commun.* 6 (1983) 348–358.
- [9] D. Ishii, T. Takeuchi, *Rev. Anal. Chem.* 6 (1982) 87–112.
- [10] M. Novotny, *Anal. Chem.* 53 (1981) 1294A–1296A.
- [11] T. Takeuchi, *Fresenius' J. Anal. Chem.* 337 (1990) 631–637.
- [12] M. Verzele, C. Dewaele, *J. Chromatogr. Sci.* 45 (1989) 37–66.
- [13] M. Verzele, C. Dewaele, *J. High Resolut. Chromatogr. Chromatogr. Commun.* 10 (1987) 280–287.
- [14] D. Ishii, T. Takeuchi, *Adv. Chromatogr.* 21 (1983) 131–164.
- [15] M. Novotny, *J. Chromatogr. Sci.* 18 (1980) 473–478.
- [16] A.G. Ewing, J.M. Mesaros, P.G. Galvin, *Anal. Chem.* 66 (1994) 527A–537A.
- [17] J.W. Jorgenson, J. De Wit, *Chem. Anal.* 121 (1992) 395–425.
- [18] Ch.E. Kientz, U.A.Th. Brinkman, *Trends Anal. Chem.* 12 (1993) 363–373.
- [19] E.S. Yeung, W.G. Kuhr, *Anal. Chem.* 63 (1991) 275A–282A.
- [20] J. C. Gluckman, M.V. Novotny, *J. Chromatogr. Sci.* 45 (1989) 145–173.
- [21] B.G. Belenkii, *J. Chromatogr.* 434 (1988) 337–361.
- [22] N. Sagliano, R.A. Hartwick, *J. Chromatogr. Sci.* 24 (1986) 506–512.
- [23] M. Goto, *Microcolumn Separations* (Journal of Chromatography Library, vol. 30), M.V. Novotny, D. Ishii (Eds.), Elsevier, Amsterdam, 1985, pp. 309–331.
- [24] K. Jinno, C. Fujimoto, Y. Hirata, D. Ishii, *Microcolumn Separations* (Journal of Chromatography Library, vol. 30), M.V. Novotny, D. Ishii (Eds.), Elsevier, Amsterdam, 1985, pp. 177–196.
- [25] E.S. Yeung, *Microcolumn Separations* (Journal of Chromatography Library, vol. 30), M.V. Novotny, D. Ishii (Eds.), Elsevier, Amsterdam, 1985, pp. 117–143.
- [26] V.V. Berry, H.E. Schwartz, *Chromatogr. Sci.* 45 (1989) 67–116.
- [27] J.C. Gluckman, M.V. Novotny, *Microcolumn Separations* (Journal of Chromatography Library, vol. 30), M.V. Novotny, D. Ishii (Eds.), Elsevier, Amsterdam, 1985, pp. 57–72.
- [28] H.J. Cortes, *Chromatogr. Sci.* 45 (1989) 211–237.
- [29] C.G. Horváth, B.A. Preiss, S.R. Lipsky, *Anal. Chem.* 39 (1967) 1422–1428.
- [30] C.G. Horváth, S.R. Lipsky, *Anal. Chem.* 41 (1969) 1227–1234.
- [31] D. Ishii, K. Asai, K. Hibi, T. Jonokuchi, M. Nagaya, *J. Chromatogr.* 144 (1977) 157–168.
- [32] D. Ishii, K. Hibi, K. Asai, T. Jonokuchi, *J. Chromatogr.* 151 (1978) 147–154.
- [33] D. Ishii, K. Hibi, K. Asai, M. Nagaya, *J. Chromatogr.* 152 (1978) 341–348.
- [34] D. Ishii, K. Hibi, K. Asai, M. Nagaya, K. Mochizuki, Y. Mochida, *J. Chromatogr.* 156 (1978) 173–180.
- [35] D. Ishii, A. Hirose, K. Hibi, Y. Iwasaki, *J. Chromatogr.* 157 (1978) 43–50.
- [36] D. Ishii, A. Hirose, I. Horiuchi, *J. Radioanal. Chem.* 45 (1978) 7–14.
- [37] F.J. Yang, *J. Chromatogr.* 236 (1982) 265–277.
- [38] T. Takeuchi, D. Ishii, *J. Chromatogr.* 213 (1981) 25–32.
- [39] T. Takeuchi, D. Ishii, *J. High Resolut. Chromatogr. Chromatogr. Commun.* 49 (1981) 469–470.
- [40] Y. Hirata, M. Novotny, *J. Chromatogr.* 186 (1979) 521–528.
- [41] T. Tsuda, M. Novotny, *Anal. Chem.* 50 (1978) 632–634.
- [42] T. Tsuda, M. Novotny, *Anal. Chem.* 50 (1978) 271–275.
- [43] C. Dewaele, M. Verzele, *J. High Resolut. Chromatogr. Chromatogr. Commun.* 1 (1978) 174–176.
- [44] C.E. Reese, R.P.W. Scott, *J. Chromatogr. Sci.* 18 (1980) 479–486.
- [45] R.P.W. Scott, *J. Chromatogr. Sci.* 18 (1980) 49–54.
- [46] P. Kucera, *J. Chromatogr.* 198 (1980) 93–109.
- [47] R.P.W. Scott, P. Kuchera, *J. Chromatogr.* 169 (1979) 51–72.
- [48] R.P.W. Scott, P. Kuchera, M. Munroe, *J. Chromatogr.* 186 (1979) 475–487.
- [49] C. Eckers, K.K. Cuddy, J.D. Henion, *J. Liq. Chromatogr.* 6 (1983) 2383–2409.
- [50] Y. Hirata, K. Jinno, *J. High Resolut. Chromatogr. Chromatogr. Commun.* 6 (1983) 96–199.
- [51] W.T. Kok, U.A.Th. Brinkman, R.W. Frei, H.B. Hanekamp, F. Nootgedacht, H. Poppe, *J. Chromatogr.* 237 (1982) 357–369.
- [52] H.G. Barth, W.E. Barber, C.H. Lochmüller, R.E. Majors, F.E. Reguier, *Anal. Chem.* 60 (1988) 387R–435R.
- [53] M. Verzele, C. Dewaele, *J. High Resolut. Chromatogr. Chromatogr. Commun.* 10 (1987) 280–287.
- [54] J.P. Chervet, M. Ursem, J.P. Salzmänn, *Anal. Chem.* 68 (1996) 1507–1512.
- [55] P. Jandera, J. Roskosná, *J. Chromatogr.* 556 (1991) 145–158.
- [56] C. Horváth, W. Melander, J. Molnár, *Anal. Chem.* 49 (1977) 142–154.
- [57] S.V. Galushko, *Chromatographia* 36 (1993) 39–42.
- [58] M.J. Walters, *J. Assoc. Off. Anal. Chem.* 70 (1987) 465.
- [59] H. Engelhardt, H. Löw, W. Götzinger, *J. Chromatogr.* 544 (1991) 371–379.
- [60] M. Ghijs, P. Sandra, *J. Microcol. Sep.* 3 (1991) 443–448.

- [61] C. Borra, S.M. Han, M. Novotny, *J. Chromatogr.* 385 (1987) 75–85.
- [62] V.L. McGuffin, M. Novotny, *J. Chromatogr.* 255 (1983) 381–393.
- [63] R.T. Kennedy, J.W. Jorgenson, *Anal. Chem.* 61 (1989) 1128–1135.
- [64] P.A. Bristow, J.H. Knox, *Chromatographia* 10 (1977) 279–289.
- [65] K.E. Karlsson, M. Novotny, *Anal. Chem.* 60 (1988) 1662–1665.
- [66] Y. Guan, L. Zhou, Z. Shang, *J. High Resolut. Chromatogr.* 15 (1992) 434–464.
- [67] G. Crescentini, A.R. Mastrogioacomo, *J. Microcol. Sep.* 3 (1991) 539–545.
- [68] G. Crescentini, F. Bruner, F. Mangani, Y. Guan, *Anal. Chem.* 60 (1988) 1659–1662.
- [69] D. Tong, K.D. Bartle, A.A. Clifford, A.M. Edge, *J. Microcol. Sep.* 7 (1995) 265–278.
- [70] D. Tong, K.D. Bartle, A.A. Clifford, *J. Microcol. Sep.* 6 (1994) 249–255.
- [71] A. Malik, W. Li, M.L. Lee, *J. Microcol. Sep.* 5 (1993) 361–369.
- [72] S. Hoffmann, L. Blomberg, *Chromatographia* 24 (1987) 416–420.
- [73] J.C. Gluckman, A. Hirose, V.L. McGuffin, M. Novotny, *Chromatographia* 17 (1983) 303–309.
- [74] R.F. Meyer, R.A. Hartwick, *Anal. Chem.* 56 (1984) 2211–2214.
- [75] P. Roumeliotism, N. Chatziathanassiou, K.K. Unger, *Chromatographia* 19 (1984) 145–150.
- [76] T.M. Zimina, R.M. Smith, P. Meyers, B.W. King, *Chromatographia* 40 (1995) 662–668.
- [77] K. Baechmann, I. Haag, T. Prokop, *Fresenius' J. Anal. Chem.* 345 (1993) 545–546.
- [78] F. Francolini, C. Borra, M. Novotny, *Anal. Chem.* 59 (1987) 2428–2432.
- [79] P. Welling, H. Poppe, J.C. Kraak, *J. Chromatogr.* 321 (1985) 450–457.
- [80] J.C. Kraak, *LC Mag.* 3 (1985) 88–90.
- [81] H. Menet, P. Gareil, M. Caude, R. Rosset, *Chromatographia* 18 (1984) 73–80.
- [82] J.C. Gluckman, J.C. Hirose, V.L. McGuffin, M. Novotny, *Chromatographia* 17 (1983) 303–309.
- [83] A. Capiello, P. Palma, F. Mangani, *Chromatographia* 32 (1991) 389–391.
- [84] M. Verzele, C. Dewaele, M. De Weerd, S. Abbott, *J. High Resolut. Chromatogr.* 12 (1989) 164–168.
- [85] S.M. Han, D.W. Armstrong, *Anal. Chem.* 59 (1987) 1583–1584.
- [86] K. Masaharu, Y. Mori, T. Amano, *Anal. Chem.* 57 (1985) 2235–2239.
- [87] J.P.C. Vissers, H.A. Claessens, J. Laven, C.A. Cramers, *Anal. Chem.* 67 (1995) 2103–2109.
- [88] J.P.C. Vissers, E.C.J. van den Hoef, H.A. Claessens, J. Laven, C.A. Cramers, *J. Microcol. Sep.* 7 (1995) 239–245.
- [89] D.C. Shelly, T.J. Edkins, *J. Chromatogr.* 411 (1987) 185–199.
- [90] D.C. Shelly, V.L. Antonucci, T.J. Edkins, T.J. Dalton, *J. Chromatogr.* 458 (1989) 267–279.
- [91] R.J. Boughtflower, T. Underwood, J. Maddin, *Chromatographia* 41 (1995) 398–402.
- [92] M.M. Dittmann, K. Wienand, F. Bek, G.P. Rozing, *LC-GC* 13 (1995) 800–804.
- [93] J.H. Knox, I.H. Grant, *Chromatographia* 32 (1991) 317–328.
- [94] W.H. Wilson, H.M. McNair, Y.F. Man, K.F. Hyver, *J. High Resolut. Chromatogr.* 13 (1990) 18–21.
- [95] S. Hsieh, J.W. Jorgenson, *Anal. Chem.* 68 (1996) 1212–1217.
- [96] V. Berry, H. Schwartz, in: F.J. Yang (Ed.), *Microbore Column Chromatography: A Unified Approach to Chromatography*, Marcel Dekker, New York, 1989, pp. 67–116.
- [97] M. Saito, K. Hibi, D. Ishii, T. Takeuchi, in: D. Ishii (Ed.), *Introduction to Microscale High Performance Liquid Chromatography*, VCH, Weinheim, Germany, 1988, pp. 7–31.
- [98] E.C. Huang, T. Wachs, J. Conboy, J.D. Henion, *Anal. Chem.* 62 (1990) 713A–725A.
- [99] P.R. Brown, *Anal. Chem.* 62 (1990) 995A–1008A.
- [100] D. Ishii, in: K. Jinno and P. Sandra (Eds.), *12th International Symposium on Capillary Chromatography*, Industrial Publishing & Consulting, Tokyo, Japan, 1990, p. 14.
- [101] C.L. Flurer, C. Borra, F. Andreolini, M.V. Novotny, *J. Chromatogr.* 448 (1988) 73–86.
- [102] J.F. Banks Jr., M.V. Novotny, *J. Microcol. Sep.* 2 (1990) 84–87.
- [103] S.J. Van der Wal, F.J. Yang, *J. High Resolut. Chromatogr. Chromatogr. Commun.* 6 (1983) 216–217.
- [104] N. Nagae, H. Itoh, N. Nimura, T. Kinoshita, T. Takeuchi, *J. Microcol. Sep.* 3 (1991) 5–9.
- [105] J.P. Chervet, C.J. Meijvogel, M. Ursem, J.P. Salzman, *LC-GC* 10 (1992) 140–148.
- [106] H.A. Claessens, A. Burcinova, C.A. Cramers, Ph. Mussche, C.E. van Tilburg, *J. Microcol. Sep.* 2 (1990) 132–137.
- [107] M.C. Harvey, S.D. Stearns, J.P. Averette, *LC Liq. Chromatogr. HPLC Mag.* 3 (1985) 5.
- [108] J.W. Jorgenson, E.J. Guthrie, *J. Chromatogr.* 255 (1983) 335–348.
- [109] A. Manz, W. Simon, *J. Chromatogr.* 387 (1987) 187–196.
- [110] R.C. Simpson, *J. Chromatogr. A* 691 (1995) 163–170.
- [111] H.J. Cortes, J.R. Larson, G.M. McGowan, *J. Chromatogr.* 607 (1992) 131–134.
- [112] J.P.C. Vissers, J.P. Chervet, J.P. Salzman, *Int. Lab.* January 1996, 12F–12G
- [113] G. Kamperman, J.C. Kraak, *J. Chromatogr.* 337 (1985) 384–390.
- [114] H.A. Claessens, M.A.J. Kuyken, *Chromatographia* 23 (1987) 331–336.
- [115] Takeuchi, D. Ishii, in: T. Hanai, H. Hatano (Ed.), *Advances in Liquid Chromatography, Methods in Chromatography, Vol. 1*, World Scientific, Singapore, 1996, pp. 169–198.
- [116] M.J. Mills, J. Maltas, W.J. Lough, *J. Chromatogr. A* 759 (1997) 1–11.
- [117] T. Takeuchi, Y. Jin, Y.Y.Y. Yuye, D. Ishii, *J. Chromatogr.* 321 (1985) 159–164.
- [118] M.W.F. Nielen, R.C.A. Koordes, R.W. Frei, U.A.Th. Brinkman, *J. Chromatogr.* 330 (1985) 113–119.

- [119] E. Noroozian, F.A. Maris, M.W.F. Nielen, R.W. Frei, G.J. de Jong, U.A.Th. Brinkman, *J. High Resolut. Chromatogr. Chromatogr. Commun.* 10 (1987) 17–24.
- [120] Ch.E. Kientz, A. Verweij, G.J. de Jong, U.A.Th. Brinkman, *J. High Resolut. Chromatogr.* 12 (1989) 793–796.
- [121] C. Moore, K. Sato, Y. Katsumata, *J. Chromatogr.* 539 (1991) 215–220.
- [122] R.E.J. van Soest, J.P. Chervet, M. Ursem, J.M. Suijlen, *LC·GC* 9 (1996) 586–593.
- [123] J. Cai, J. Henion, *Anal. Chem.* 56 (1996) 72–78.
- [124] J.P.C. Vissers, W.P. Hulst, J.P. Chervet, H.M.J. Snijders, C.A. Cramers, *J. Chromatogr. B* 686 (1996) 119–128.
- [125] H. Shen, C.A. Aspinwall, R.T. Kennedy, *J. Chromatogr. B* 689 (1997) 295–303.
- [126] C. Fujimoto, T. Morita, K. Jinno, *Chromatographia* 22 (1986) 91–94.
- [127] C. Fujimoto, T. Morita, K. Jinno, *J. High Resolut. Chromatogr. Chromatogr. Commun.* 9 (1986) 623–626.
- [128] G.D. Reed, *J. High Resolut. Chromatogr. Chromatogr. Commun.* 9 (1986) 250–252.
- [129] R.E. Synovec, *Anal. Chem.* 59 (1987) 2877–2884.
- [130] H.J. Tarigan, P. Neill, C.K. Kenmore, D.J. Bornhop, *Anal. Chem.* 68 (1996) 1762–1770.
- [131] D.J. Bornhop, N.J. Dovichi, *Anal. Chem.* 59 (1987) 1632–1636.
- [132] D.J. Bornhop, T.G. Nolan, N.J. Dovichi, *J. Chromatogr.* 384 (1987) 181–187.
- [133] A.E. Bruno, K. Kuhn, H.M. Widmer, *Anal. Chim. Acta* 234 (1990) 259–262.
- [134] A.E. Bruno, B. Krattinger, F. Maystre, H.M. Widmer, *Anal. Chem.* 63 (1991) 2689–2697.
- [135] M. Kamahori, Y. Watanabe, J. Muri, M. Taki, H. Miyagi, *J. Chromatogr.* 465 (1989) 227–232.
- [136] C. Kientz, A. Verweij, *J. High Resolut. Chromatogr. Chromatogr. Commun.* 11 (1988) 294–296.
- [137] J. Vindeogel, G. Schuddinck, C. Dewaele, M. Verzele, *J. High Resolut. Chromatogr. Chromatogr. Commun.* 11 (1988) 317–321.
- [138] G.J.M. Bruin, G. Stegeman, A.C. van Asten, X. Xu, J.C. Kraak, H. Poppe, *J. Chromatogr.* 559 (1991) 163–181.
- [139] M. Janecek, V. Kahle, M. Krejci, *J. Chromatogr.* 438 (1988) 409–413.
- [140] M. Janecek, F. Foret, K. Slais, P. Bocek, *Chromatographia* 25 (1988) 815–816.
- [141] J.P. Chervet, M. Ursem, J.P. Salzmänn, R.W. Vannoort, *J. High Resolut. Chromatogr.* 12 (1989) 278–281.
- [142] J.P. Chervet, M. Ursem, J.P. Salzmänn, R.W. Vannoort, *LC·GC* 7 (1989) 514–517.
- [143] P. Sandra, G. Steenbeke, M. Ghys, G. Schomburg, *J. High Resolut. Chromatogr.* 13 (1990) 527–528.
- [144] M. Verzele, G. Steenbeke, J. Vindeogel, *J. Chromatogr.* 477 (1989) 87–93.
- [145] T. Takeuchi, D. Ishii, *J. Chromatogr.* 288 (1984) 451–456.
- [146] D. Ishii, M. Goto, T. Takeuchi, *J. Chromatogr.* 316 (1984) 441–449.
- [147] T. Takeuchi, D. Ishii, *J. High Resolut. Chromatogr. Chromatogr. Commun.* 7 (1984) 151–152.
- [148] J.C. Gluckman, M. Novotny, *J. High Resolut. Chromatogr. Chromatogr. Commun.* 8 (1985) 672–677.
- [149] J.C. Gluckman, D.C. Shelly, M. Novotny, *Anal. Chem.* 57 (1985) 1546–1552.
- [150] M. Verzele, C. Dewaele, *J. Chromatogr.* 395 (1987) 85–89.
- [151] T. Takeuchi, D. Ishii, *Chromatographia* 25 (1988) 697–700.
- [152] D. Shelly, J. Gluckman, M. Novotny, *Anal. Chem.* 56 (1984) 483–486.
- [153] E.J. Guthrie, J.W. Jorgenson, *Anal. Chem.* 56 (1984) 483–486.
- [154] T. Takeuchi, T. Asano, D. Ishii, *J. Chromatogr.* 471 (1989) 297–302.
- [155] T. Takeuchi, D. Ishii, *Chromatographia* 25 (1988) 697–700.
- [156] W.R. Blair, E.J. Parks, G.J. Olsen, F.E. Brinckman, M.C. Valeiras-Price, J.M. Bellama, *J. Chromatogr.* 410 (1987) 383–394.
- [157] B. Lin Ling, W.R.G. Baeyens, C. Dewaele, B. Del Castillo, *J. Pharm. Biomed. Anal.* 10 (1992) 985–988.
- [158] T. Takeuchi, D. Ishii, *J. High Resolut. Chromatogr. Chromatogr. Commun.* 11 (1988) 841–843.
- [159] T. Takeuchi, T. Miwa, *Chromatographia* 38 (1994) 555–558.
- [160] E.S. Yeung, *Microcolumn Separations (Journal of Chromatography Library, vol. 30)*, M.V. Novotny, D. Ishii (Eds.), Elsevier, Amsterdam, 1985, pp. 135–158.
- [161] T.J. Edkins and D.C. Shelly, in: G. Patonay (Ed.), *HPLC Detector: Newer Methods*, VCH, New York, 1992, pp. 1–26.
- [162] Z. Rosenzweig, E.S. Yeung, *Appl. Spectrosc.* 47 (1993) 1175–1179.
- [163] A.A. Abbas, D.C. Shelly, *J. Chromatogr. A* 691 (1995) 37–53.
- [164] F. Adreolini, S.C. Beale, M. Novotny, *J. High Resolut. Chromatogr. Chromatogr. Commun.* 11 (1988) 20–24.
- [165] J. Gluckman, D. Shelly, M. Novotny, *J. Chromatogr.* 317 (1984) 443–453.
- [166] V.L. McGuffin, R.N. Zare, *Appl. Spectrosc.* 39 (1985) 847–853.
- [167] A. Manz, Z. Fröbe, W. Simon, *Microcolumn Separations (Journal of Chromatography Library, vol. 30)*, M.V. Novotny, D. Ishii (Eds.), Elsevier, Amsterdam, 1985, pp. 297–308.
- [168] J.E. Baur, E.W. Kristensen, R.W. Wightman, *Anal. Chem.* 60 (1988) 2334–2338.
- [169] K. Slais, B. Oscik-Mendyk, *J. Chromatogr.* 456 (1988) 370–374.
- [170] K. Slais, *J. Chromatogr.* 436 (1988) 413–420.
- [171] M. Janecek, K. Slais, *J. Chromatogr.* 471 (1989) 303–309.
- [172] T. Takeuchi, T. Miwa, A. Hemmi, T. Maeda, *Chromatographia* 37 (1993) 173–175.
- [173] A. Manz, W. Simon, *J. Chromatogr. Sci.* 21 (1983) 326–333.
- [174] A. Manz, W. Simon, *Anal. Chem.* 59 (1987) 74–79.
- [175] L.A. Knecht, E.J. Guthrie, J.W. Jorgenson, *Anal. Chem.* 56 (1984) 479–482.
- [176] J.C. White, R.L. St. Claire, III, J.W. Jorgenson, *Anal. Chem.* 58 (1986) 293–298.

- [177] M. Goto, K. Shimada, T. Takeuchi, D. Ishii, *Anal. Sci.* 4 (1988) 17–20.
- [178] V.F. Ruban, *J. High Resolut. Chromatogr.* 13 (1990) 112–115.
- [179] V.F. Ruban, *J. High Resolut. Chromatogr.* 16 (1993) 663–665.
- [180] K. Slais, D. Kourilova, *Chromatographia* 16 (1982) 265–266.
- [181] L.J. Nagels, J.M. Kaufman, G. Schuddink, C. Dewaele, G.J. Patriarcho, M. Verzele, *J. Chromatogr.* 459 (1988) 163–172.
- [182] M. Goto, K. Shimada, *Chromatographia* 21 (1986) 631–634.
- [183] C.N. Svendsen, *Analyst* 18 (1993) 123.
- [184] A. Aoki, T. Matsue, I. Uchia, *Anal. Chem.* 64 (1992) 44–49.
- [185] M. Takahashi, M. Morita, O. Niwa, H. Tabei, *J. Electroanal. Chem.* 335 (1992) 253.
- [186] S. Sarre, Y. Michotte, C.A. Marvin, G. Ebiger, *J. Chromatogr.* 582 (1992) 29–34.
- [187] K.A. Sager, M.T. Kelly, T. Mary, M.R. Smith, *J. Chromatogr.* 577 (1992) 109–116.
- [188] E.J. Caliguri, I.N. Mefford, *Brain Res.* 296 (1984) 156–159.
- [189] T. Tsuda, A. Nago, G. Nakagawe, M. Maseki, *J. High Resolut. Chromatogr. Chromatogr. Commun.* 6 (1983) 694–695.
- [190] H. Veening, P.P. Tock, J.H. Kraak, H. Poppe, *J. Chromatogr.* 352 (1986) 345–350.
- [191] V.L. McGuffin, M. Novotny, *J. Chromatogr.* 218 (1981) 179–187.
- [192] V.L. McGuffin, M. Novotny, *Anal. Chem.* 55 (1983) 2296–2302.
- [193] J. C. Gluckman, M. Novotny, *J. Chromatogr.* 314 (1984) 103–110.
- [194] J.C. Gluckman, M. Novotny, *J. Chromatogr.* 333 (1985) 291–300.
- [195] Ch.E. Kientz, A. Verweij, G.J. de Jong, U.A.Th. Brinkman, *J. Chromatogr.* 626 (1992) 59–69.
- [196] Ch.E. Kientz, A. Verweij, G.J. de Jong, U.A.Th. Brinkman, *J. Chromatogr.* 626 (1992) 71–80.
- [197] U.A.Th. Brinkman, F.A. Maris, *LC-GC* 1 (1987) 45.
- [198] F.A. Maris, R.J. van Delft, R.W. Frei, R.B. Geerdink, U.A.Th. Brinkman, *Anal. Chem.* 58 (1996) 1634–1638.
- [199] J.C. Gluckman, D. Barcelo, G.J. de Jong, R.W. Frei, F.A. Maris, U.A.Th. Brinkman, *J. Chromatogr.* 367 (1986) 35–44.
- [200] V.L. McGuffin, M. Novotny, *Anal. Chem.* 53 (1981) 946–951.
- [201] S. Folestad, B. Josefsson, P. Marstop, *Anal. Chem.* 59 (1987) 334–339.
- [202] Ch.E. Kientz, A. Verweij, H.L. Boter, A. Poppema, R.W. Frei, G.J. de Jong, U.A.Th. Brinkman, *J. Chromatogr.* 467 (1989) 385–394.
- [203] H.-C.K. Chang, L.T. Taylor, *Anal. Chem.* 63 (1991) 486–490.
- [204] A.L. Howard, C.L.B. Thomas, L.T. Taylor, *Anal. Chem.* 66 (1994) 1432–1437.
- [205] J.F. Karnicky, L.T. Zitelli, S.J. van der Wal, *Anal. Chem.* 59 (1987) 327–333.
- [206] V.V. Brazhnikov, L.M. Yakashina, E.M. Syanova, Sh.A. Karaptyan, *J. High Resolut. Chromatogr. Chromatogr. Commun.* 6 (1983) 451.
- [207] F.A. Maris, J.A. Stäb, G.J. de Jong, U.A.Th. Brinkman, *J. Chromatogr.* 445 (1988) 129–138.
- [208] B.N. Zegers, J.F.C. de Brouwer, A. Poopema, H. Lingeman, U.A.Th. Brinkman, *Anal. Chim. Acta* 304 (1995) 47–56.
- [209] A.J. Lange, P.R. Griffiths, D.J.J. Fraser, *Anal. Chem.* 63 (1991) 782–787.
- [210] C. Fujimoto, T. Morita, K. Jinno, K.H. Shafer, *J. High Resolut. Chromatogr. Chromatogr. Commun.* 11 (1988) 810–814.
- [211] G.J. De Jong, N. Lammers, F.J. Spruit, C. Dewaele, M. Verzele, *Anal. Chem.* 59 (1987) 1458–1461.
- [212] T. Takeuchi, T. Miwa, *Chromatographia* 37 (1993) 281–283.
- [213] S.H. Chen, C.E. Evans, V.L. McGuffin, *Anal. Chim. Acta* 246 (1991) 65–74.
- [214] J. Chu, R. Hu, T. Miwa, T. Takeuchi, *Chromatographia* 40 (1995) 379–381.
- [215] R. Gotz, J.W. Elgersma, J.C. Kraak, H. Poppe, *Spectrochim. Acta B* 49B (1994) 761–768.
- [216] S. Hoffmann, H.R. Norli, T. Greibrokk, *J. High Resolut. Chromatogr.* 12 (1989) 260–264.
- [217] H. Wu, T.L. Peck, A.G. Webb, R.L. Magin, J.V. Sweedler, *Anal. Chem.* 66 (1994) 3849–3857.
- [218] H. Wu, A. Webb, T.L. Peck, J.V. Sweedler, *Anal. Chem.* 67 (1995) 3101–3107.
- [219] K. Albert, G. Schlotterback, L.-H. Tseng, U. Braumann, *J. Chromatogr. A* 750 (1996) 303–309.
- [220] C. Fujimoto, T. Morita, K. Jinno, *J. Chromatogr.* 438 (1988) 329–337.
- [221] T. Takeuchi, M. Asai, H. Haraguchi, D. Ishii, *J. Chromatogr.* 599 (1990) 549–556.
- [222] L.A. Holland, J.W. Jorgenson, *Anal. Chem.* 67 (1995) 3275–3283.
- [223] D.B. Kassel, T.G. Cousler, M. Shalaby, P. Selchri, N. Gorden, T. Nadler, in: R.J.W. Crabb (Ed.), *Tech. Protein. Chem. VI*, Academic Press, San Diego, 1995, pp. 39–46.
- [224] T.E. Mulligan, R.W. Blain, N.F. Oldfield, B.A. Mico, *J. Liq. Chromatogr.* 17 (1994) 133–150.
- [225] H.J. Cortes, B.E. Richter, C.D. Pfeiffer, D.E. Jansen, *J. Chromatogr.* 349 (1985) 55–61.
- [226] K. Grob, J.-M. Stoll, *J. High Resolut. Chromatogr. Chromatogr. Commun.* 9 (1986) 518–523.
- [227] H.J. Cortes, L. Green, C. Shayne, M. Robert, *Anal. Chem.* 62 (1991) 2719–2724.
- [228] F. Munari, A. Frisciani, G. Mapelli, S. Tristianu, K. Grob, J.M. Colin, *J. High Resolut. Chromatogr. Chromatogr. Commun.* 8 (1985) 601–606.
- [229] I.L. Davis, K.D. Bartle, G.E. Andrews, P.T. Williams, *J. Chromatogr. Sci.* 26 (1988) 125–130.
- [230] D. Duquet, C. Dewaele, M. Verzele, S. McKinley, *J. High Resolut. Chromatogr. Chromatogr. Commun.* 11 (1988) 824–829.

- [231] K. Welch, N.E. Hoffman, J. High Resolut. Chromatogr. 15 (1992) 171–175.
- [232] R. Moulder, K.D. Bartle, A.A. Clifford, Analyst 116 (1991) 1293–1298.
- [233] A.V. Lemmo, J.W. Jorgenson, Anal. Chem. 65 (1993) 1576–1581.
- [234] A.C. Barefoot, R.W. Reiser, Biomed. Environ. Mass Spectrom. 19 (1989) 77–82.
- [235] A. Carrier, L. Varfalvy, M.J. Berthrand, J. Chromatogr. A 705 (1995) 205–217.
- [236] U.B. Ranalder, B.B. Lausecker, C. Huselton, J. Chromatogr. 617 (1993) 129–135.
- [237] H. Albron, G. Stenhagen, J. Chromatogr. 394 (1987) 35–49.
- [238] E.L. Esmans, P. Geboes, Y. Luyten, F.C. Alderweirelt, Biomed. Mass Spectrom. 12 (1985) 241–245.
- [239] E.D. Lee, J.D. Henion, J. Chromatogr. Sci. 23 (1985) 253–264.
- [240] A. Capiello, G. Famigliani, F. Mangani, B. Tirillini, J. Am. Soc. Mass Spectrom. 6 (1995) 132–139.
- [241] A. Capiello, G. Famigliani, Anal. Chem. 66 (1994) 3970–3976.
- [242] A. Capiello, G. Famigliani, F. Bruner, Anal. Chem. 66 (1994) 1416–1423.
- [243] M.A. Moseley, L.J. Deterding, J.S.M. de Wit, K.B. Tomer, R.T. Kennedy, N. Brag, J.W. Jorgenson, Anal. Chem. 61 (1989) 1577–1584.
- [244] M.A. Moseley, L.J. Deterding, K.B. Tomer, J.W. Jorgenson, Anal. Chem. 61 (1991) 1467–1473.
- [245] J.E. Coutant, T.M. Chen, B.L. Ackermann, J. Chromatogr. 529 (1990) 265–275.
- [246] S. Pleasence, P. Thibault, M.A. Moseley, L.J. Deterding, K.B. Tomer, J.W. Jorgenson, J. Am. Soc. Mass Spectrom. 1 (1990) 312–319.
- [247] J.P. Gagné, A. Carrier, L. Varfalvy, M.J. Berthrand, J. Chromatogr. 647 (1993) 13–20.
- [248] J.P. Gagné, A. Carrier, L. Varfalvy, M.J. Berthrand, J. Chromatogr. 647 (1993) 21–29.
- [249] K.B. Tomer, M.A. Moseley, L.J. Deterding, C.E. Parker, Mass Spectrom. Rev. 13 (1994) 431–457.
- [250] C. Li, N. Chaurut, Y. Ducharme, L.A. Trimble, D.A. Nicoll-Griffith, J.A. Yergey, Anal. Chem. 67 (1995) 2931–2936.
- [251] Y.-M. Li, P. Hjerten, N. Stellen, F. Nyberg, J. Silberring, J. Chromatogr. B 664 (1995) 426–430.
- [252] C.M. Whitehouse, R.N. Dreyer, M. Yamashita, J.B. Fenn, Anal. Chem. 57 (1985) 675–679.
- [253] M.G. Ikonou, A.T. Blades, P. Kebarle, Anal. Chem. 63 (1991) 1989–1998.
- [254] M.G. Qian, D.M. Lubman, Anal. Chem. 67 (1995) 2870–2877.
- [255] S.C. Wong, C. Grimley, A. Oadau, J.H. Bourell and W.J. Henzel, in: R.H. Angeletti (Ed.), Tech. Protein. Chem. IV, Academic Press, San Diego, CA, 1993, pp. 371–378.
- [256] W.J. Henzel, T.M. Billeci, J.T. Stults, S.C. Wong, C. Grimley, C. Watanabe, Proc. Natl. Acad. Sci. USA 90 (1993) 5011–5015.
- [257] W.J. Henzel, C. Grimely, J.H. Bourell, T.M. Billeci, S.C. Wong, J.T. Stults, Methods Comparison Methods Enzymol. 6 (1994) 239–247.
- [258] A.J. Link, J. Eng, J.R. Yates III, Am. Lab. 8 (1996) 27–30.
- [259] J.R. Yates III, J.K. Eng, A.L. McCormack, Anal. Chem. 67 (1995) 3203–3210.
- [260] P. Tempst, S. Geromanos, C. Elicone, H. Erdjument-Bromage, Methods Comparison Methods Enzymol. 6 (1994) 252–261.
- [261] C. Elicone, M. Lui, S. Geromanos, H. Erdjument-Bromage, P. Tempst, J. Chromatogr. A 676 (1994) 121–137.
- [262] R.L. Moritz, G.E. Reid, L.D. Ward, R.J. Simpson, Methods Comparison Methods Enzymol. 6 (1994) 213–226.
- [263] D.B. Kassel, B. Sushan, T. Sakuma, J.P. Salzmann, Anal. Chem. 66 (1994) 236–243.
- [264] J.E. Battersby, V.R. Mukku, R.S. Clark, W.S. Hancock, Anal. Chem. 57 (1995) 447–455.
- [265] H. Wännman, A. Walhagen, P. Erlandson, J. Chromatogr. 603 (1992) 121–131.
- [266] Ch.E. Kientz, J. Langenberg, G.J. De Jong, U.A.Th. Brinkman, J. High Resolut. Chromatogr. 14 (1991) 460–464.
- [267] H.J. Cortes, L.W. Nicholson, J. Microcol. Sep. 6 (1994) 257–262.
- [268] V. Schurig, M. Jung, S. Mayer, M. Fluck, S. Negura, H. Jacubetz, J. Chromatogr. A 694 (1995) 119–128.
- [269] J. Hermansson, I. Hermansson, J. Nordin, J. Chromatogr. 631 (1993) 79–90.
- [270] T. Takagi, Y. Itabashi, T. Tsuda, J. Chromatogr. Sci. 27 (1989) 574–577.
- [271] J. Vindevogel, J. van Dijck, M. Verzele, J. Chromatogr. 447 (1988) 297–303.
- [272] S.M. Han, D.W. Armstrong, J. Chromatogr. 389 (1987) 256–260.
- [273] T. Takeuchi, N. Nagae, J. High Resolut. Chromatogr. 15 (1992) 121–123.
- [274] T. Takeuchi, J. High Resolut. Chromatogr. 14 (1991) 560–561.
- [275] R. Hu, T. Takeuchi, J.-Y. Jin, T. Miwa, Anal. Chim. Acta 295 (1994) 173–179.
- [276] D. Rindgen, R.J. Turesky, P. Vouros, Chem. Res. Toxicol. 8 (1995) 1005–1013.
- [277] S.M. Wolf, P. Vouros, Chem. Res. Toxicol. 7 (1994) 82–88.
- [278] B. Yeung, P. Vouros, G. Reddy, Am. Lab. 7 (1994) 12–16.
- [279] R. Straub, M. Linder, R.D. Voyksner, Anal. Chem. 66 (1994) 3651–3658.