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Review

Recent developments in microcolumn liquid chromatography

Johannes P.C. Vissers*

LC Packings, Baarsjesweg 154, 1057 HM Amsterdam, The Netherlands

Abstract

An overview of the most recent developments in microcolumn liquid chromatography (LC) is presented. A short theoretical discussion on chromatographic dilution and extracolumn bandbroadening is given and also the recent progress and advances in column technology and instrumentation are reviewed. However, the emphasis of this review is on miniaturized sample clean-up, sample introduction techniques and on both established and more recent detection techniques for microcolumn LC. The hyphenation of miniaturized LC columns with other techniques, specifically on multidimensional chromatography and the coupling of microcolumn LC to mass spectrometry is discussed in detail. Both the on-line and automated off-line interfacing to other separation and detection techniques will also be addressed. Finally, a number of typical microcolumn LC applications are presented in order to demonstrate the potential of microcolumn LC methods in a variety of scientific areas.

Keywords: Reviews; Microcolumn; Extracolumn bandbroadening; Bandbroadening; Clean-up methods; Proteins; Peptides

Contents

*Tel.: +31-20-683-9768; fax: +31-20-685-3452.

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1. Introduction

The continuing interest in microcolumn liquid chromatography (LC) is shown by the increasing number of papers that are being published on this technique and by the large number of review articles that have recently appeared in the literature. Specific reviews include: general aspects of microcolumn LC [1–11], column technology [12–16], detection in microcolumn LC [17–26], instrumentation [27,28] and multidimensional chromatography [29]. The introduction of microcolumn LC is attributed to Horváth et al. who in 1967 [30,31], used 0.5–1.0-mm inner diameter (I.D.) stainless steel columns packed with pellicular particles for the separation of ribonucleotides. In the years following, high-performance liquid chromatography research was mainly focused on packed columns with an I.D. of 4.6 mm. Therefore, it was not until the mid 1970's before Ishii et al. demonstrated the use of slurry packed Teflon microcolumns in a series of publications [32–37], which initiated a breakthrough in the development of microcolumn LC. Other types of microcolumns with capillary dimensions – i.e. smaller than 1.0 mm I.D. – were introduced shortly thereafter [38–44]. At the same time Scott et al. [45–49] reported their work with packed 1.0 mm I.D. columns to achieve efficient, high-speed separations. The work of Novotny and co-workers [41–43], Yang [38] and Scott et al. [45–49] are regarded as key publications in the field of microcolumn LC. The initial developments towards miniaturization in high-performance liquid chromatography were soon adapted by a number of laboratories worldwide [50–52].

Microcolumn LC has established itself as a complementary technique to conventional sized LC columns, which are more routinely used in high-performance liquid chromatography. This is mainly due to the advantages found in an increasing number of applications of microcolumn LC where conventional high-performance liquid chromatography falls short or can not compete. The most important advantages of microcolumn LC are the ability to work with minute sample sizes, small volumetric flow-rates, and the enhanced detection performance with the use of concentration sensitive detection devices due to reduced chromatographic dilution [2–5]. Presently, 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 focus on recent technological advances with these slurry-packed microcolumns. Consideration will be given to miniaturized sample preparation techniques and detection in microcolumn LC. In addition, the general advantages of multidimensional separation techniques using miniaturized LC columns and applications relating to the hyphenation to mass spectrometry and some applications are discussed. The nomenclature used in this paper is as previously described [11].

2. Theoretical considerations

2.1. 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 is given by:

$$D = \frac{c_0}{c_{max}} = \frac{\epsilon_t \pi r^2 (1+k)\sqrt{2\pi LH}}{V_{inj}} \quad (1)$$

where c_0 is the initial compound concentration in a sample, c_{max} is the final compound concentration at the peak maximum, r is the column radius, k is the retention factor, L is the column length, H is the column plate height, ϵ_t is the column porosity and V_{inj} is the injected sample volume. D increases proportionally with the square of the column radius and also with the square root of the column length and plate height of the column. Hence, under identical chromatographic and injection conditions c_{max} increases inversely to r^2. From Eq. (1) a 235-fold increase in peak height and mass sensitivity for a reduction in the diameter of a column from 4.6 to 300 μm I.D. can be calculated. It must be stressed, however, that this advantage of smaller 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 as compared to conventional columns. Even though the sample size is proportional to the amount of stationary phase in one plate, this advantage is only valid in cases where the sample mass is restricted.

2.2. Extracolumn bandbroadening

Miniaturization is essentially the reduction of the I.D. of a column. At the same time the outer or extracolumn bandbroadening effects must be reduced accordingly in order to achieve maximal performance. This is of major importance to prevent a considerable loss of efficiency, which may result in decreased resolution of the chromatographic system.

Typically the plate number of a column is calculated from the standard deviation of a peak, which yields the column efficiency. However, this condition is often not met, since both the column and the instrumental bandbroadening contribute to the standard deviation of a chromatographic peak. Therefore, together with the presentation of column efficiencies, the applied instrumentation and hardware should be discussed, unless the extracolumn bandbroadening contribution is negligible compared to the peak broadening of a specific column. Since extracolumn effects will always be present, it is more a question of what is an acceptable level of extracolumn bandbroadening for an LC column under specific experimental conditions.

Generally, a 5% reduction in chromatographic resolution is accepted as the maximum allowable instrumental contribution to the overall variance. Consequently, the loss in column plate based on extracolumn effects number should not exceed 10% [11,28]. For a specific LC system the maximum acceptable $\sigma^2_{e(acc)}$ variance is described as:

$$\sigma^2_{e(acc)} \leq 0.10\sigma^2_c \leq 0.10(\pi r^2 \epsilon_t)^2 (1+k)^2 HL \quad (2)$$

where σ^2_c is the volumetric column variance. Representative column dimensions and the maximum allowable variance for a non-retained ($k=0$) and a slightly retained compound ($k=1$ or $k=2$) for three different type of microcolumns are shown in Table 1.

Table 1
Maximum acceptable extracolumn variance $\sigma^2_{e(acc)}$ for different microcolumns for non-retained and slightly retained components[a]

Column type	Column I.D.	Column length (cm)	$\sigma^2_{e(acc)}$ (nl^2)		
			$k=0$	$k=1$	$k=2$
Micro LC	1.0 mm	15.0	45 300	181 000	408 000
		25.0	75 600	302 000	680 000
Capillary LC	300 μm	15.0	370	1470	3310
		25.0	610	2450	5510
Nanoscale LC	75 μm	15.0	1.4	5.7	13
		25.0	2.4	9.6	22
		40.0	3.8	15	34

[a] The total porosity ϵ_t was taken as 0.70 and a 5% loss in column efficiency was permitted ($\theta^2=0.05$). Ideal injection profiles ($K^2=12$), and optimum mobile phase flow-rate and plate height ($2d_p$) were assumed. The particle diameter was taken as 5 μm.

3. Microcolumn packing and monolithic supports

3.1. Filling techniques

Different filling techniques have been reported for the preparation of packed capillary LC columns, which include applying gases [53–55], supercritical fluids [56–61] or liquids [62,63] to transport the stationary phase particles into the column. For micro LC columns liquids are used exclusively to pack the columns [64,65]. The basic concept of all of the major packing techniques however remains the same: a frit – composed of a metal screen, glass wool, polymeric membranes or condensed silica particles – is mounted at one of an unfilled column. The particles are then pushed into the columns from the opposite side. Filtration simply describes the entire packing process.

The separation efficiency of microcolumns is influenced by a large number of parameters, not only by packing parameters such as solvent selection, packing pressure, slurry concentration, the use of surfactants to stabilize the particle suspension, slurry vessel dimensions, etc. [66–72], but also by column parameters like column blank material, frit selection, etc. [66,73–77]. The majority of these particular 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 can be regarded as optimal – i.e. reduced plate heights near two – according to classical chromatographic theory.

Some research groups have tried to develop a better understanding of the packing processes by incorporating colloid chemical studies, filtration theories and stress behavior during bed compression [59,78–81]. Although certain trends could be assigned from these studies, a sound theoretical description and understanding of packing methods for microcolumns remains unavailable. Vissers et al. [78,79] related the coagulating properties of a number of octadecyl reversed-phase materials in different slurry and packing liquids to the final chromatographic performance of microcolumns. They found that coagulation of stationary phase particles has less of an impact than previously was assumed. Tong et al. [59] 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 shown. Furthermore, novel packing techniques that apply 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 [82].

At present, extensive studies of columns with an I.D. of ~200–300 μm appear in the literature. There is also a growing trend in column usage with an even smaller I.D. The driving force towards further miniaturization can be attributed to the use of such columns in capillary electrochromatography (CEC) [82–84] and electrospray ionization mass spectrometry (ESI-MS). CEC columns with an I.D. of 50–100 μm are required for prevention of 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 however beyond the scope of this paper.

There is a scientific interest in the use of very narrow fused-silica capillaries. Several research groups [85–88] reported that the plate height of packed columns reduces by a factor of approximately two when the column to particle diameter ratio – also known as the Knox–Parcher ratio – is smaller than six. None of the well-known classical plate height equations suggest however that the column diameter d_c does not influence the plate height of packed columns, whereas only the particle diameter d_p does.

This contradiction between theory and experimental observations was explained by Kennedy and Jorgenson [85] 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 also gives rise to more uniform retention factors, which in turn reduces column bandbroadening. Both effects contribute to a com-

bined decrease to bandbroadening. 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 are 1.0 for non-retained compounds and 1.2–1.3 for slightly retained compounds using 5 μm reversed octadecyl modified particles [88].

In a recent publication, MacNair et al. [89] reported on the preparation and performance of ultrahigh-pressure packed reversed-phase nanoscale LC columns. Although basically a slurry packing technique, MacNair and co-workers introduced some novel aspects of column packing including the effect of the column inlet pressure on the efficiency of the column, analysis time and retention factor in nanoscale LC. The use of high column inlet-pressures requires the design of special packing and pumping equipment plus custom connection and injection devices. Typical inlet pressures during column packing have been reported at 4100 bar and operating pressure in between 1500–4100 bars. Justification of these high inlet pressures was the use of 1.5 μm non-porous stationary phase particles and column lengths up to 70 cm. To obtain such high inlet pressures, two pneumatic pumps were placed in series – a single-stage and a triple-stage air-actuated pneumatic amplifier respectively – the schematics of which are shown in Fig. 1. When the column is operated at maximum mobile phase velocity, the theoretical height of a plate that can be generated with this a system was 5 μm for slightly retained compounds. Analysis times were on the order of 30 min for hydroxylated polycyclic aromatic hydrocarbons with a retention factor $k \leq 2$. Retention factors were found to be linearly dependent on the column inlet pressure since no heat dissipation is anticipated when using small I.D. columns.

3.2. Monolithic supports

The introduction of CEC has stimulated the development of monolithic column packings. A chromatographic bed that is pH-stable over a very broad range – preferably up to pH values of 8 or higher – and to reduce negative side-effects that arise from sintered frits, i.e. bubble formation, adsorption-induced tailing and extracolumn bandbroadening. Monolithic column packings are not exclusively used for CEC. Hjertén et al. [90] demonstrated that a monolithic LC column could be constructed by

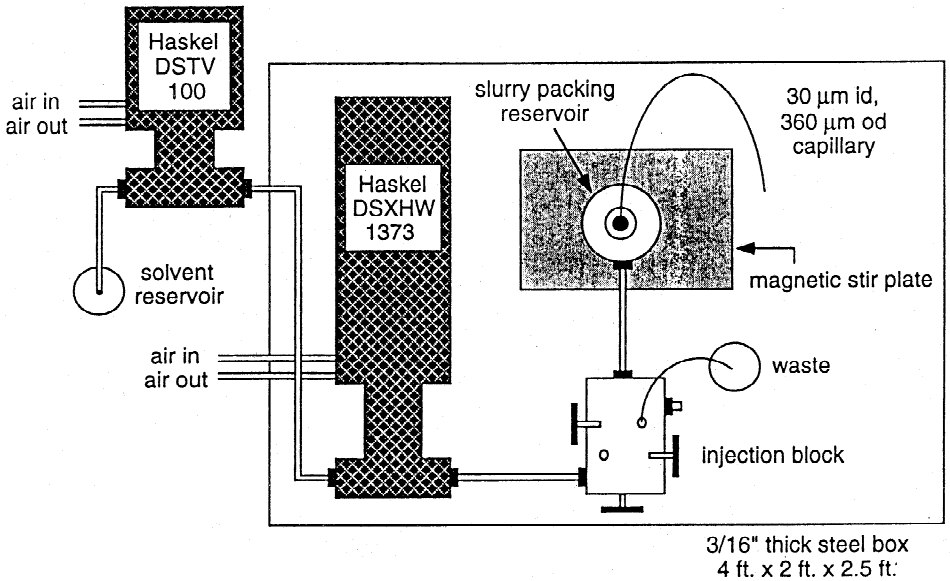

Fig. 1. General experimental layout for packing column for ultrahigh pressure liquid chromatography (reprinted with permission from Ref. [89]. Copyright (1997) American Chemical Society).

compressing a solvent-swollen copolymer. Despite the lack of a continuous pore size distribution, it was shown that this material could be used for ion-exchange separations of proteins. Svec and Fréchet [91] reported on the preparation of a continuous rod of porous polymer. After molding a polymeric rod by an in-situ polymerization in the tube of a chromatographic column, the polymer was functionalized with either a diol or an amino group. The performance of this monolithic bed was further demonstrated by the ionic exchange separation of proteins. Another approach towards the production of a monolithic chromatographic bed is the construction of a functionalized silica skeleton, which has been demonstrated by Minikucho et al. [92]. The chromatographic performance in terms of plate height was found to be comparable with 5 μm stationary phase particles. Fields [93] demonstrated a similar approach for the development of a continuous column support for capillary LC columns. Minimum plate heights were found to be close to 75 μm, which is considerably higher than the plate heights for silica particle packed columns.

The application of LC column approaches to the development of a monolithic bed have been extended and optimized for CEC columns. Palm and Novotny [94] and Ericson et al. [95] reported on gel-matrices for use as the monolithic support. Both groups conducted the polymerization of a gel in-situ in the chromatographic tube. First, the capillary wall was pretreated with a reagent that allows a covalent bond to form between the gel and the fused-silica capillary. After in-situ polymerization of the gel, the matrix was functionalized with a C_4, C_6 or C_{12} ligand [94] or with a C_{18} ligand [95]. The plate heights for the C_4, C_6 and C_{12} functionalized matrices were 2.8 μm, 3.3 μm and 4.2 μm respectively under CEC conditions [94]. The plate height for the C_{18} functionalized columns was about 8.3 μm [95]. No explanations for these apparent ligand-dependant plate counts were given. Peters et al. [96–98] have published a series of papers on a one-step in-situ copolymerization in 100 and 150 μm I.D. fused-silica capillaries. The monolithic columns obtained have plate heights better than 8.3 μm under CEC conditions. These plate heights can also be achieved in capillary LC. However, many types of monolithic columns – including gel-matrix monolithic columns – do not suffer from limited bed stability or the formation of bubbles within the column during analysis. Furthermore, since no supporting frits are required, no unwanted interactions arise from the interaction of the sample with the frit material are to be expected. Horváth et al. produced a monolithic silica column by sintering bare 6 μm silica particles along the complete column length [99]. After sintering, the fused particles were functionalized with a C_{18} ligand. The plate height was estimated to be 16 μm in the pressure driven LC mode and 8 μm in the electrokinetic CEC mode. Plate heights reported by Dualy et al. [100] were around 12.5 μm in the CEC mode, using a similar sol-gel technology approach.

4. Miniaturized instrumentation

Decreasing the column volume puts stringent requirements on the instrumentation applied in microcolumn LC. All volumetric extracolumn dispersion sources have to be scaled down according to the volume of the separation column. This is particularly important for the injection and detection volume. This section addresses these topics, as well as miniaturized sample clean-up and preconcentration techniques. Besides reducing the extracolumn sources of dispersion, the volumetric flow-rate through the column must also be scaled down. Until recently, μl min^{-1} and nl min^{-1} flow rates could only be achieved by means of flow splitting. However, commercial instrumentation has become available to perform micro and capillary LC, using either reciprocating or syringe pumps. The advantages and limitations of both techniques were discussed in a previous paper [11]. Nanoscale column flows are limited to flow splitting methods. It is important to mention that the linear flow through a packed column is independent of the column diameter and therefore the time of analysis is also independent of the column diameter. This is of course only true if microcolumns are as densely packed as conventional LC columns. As a result, methods developed on conventional LC columns should be directly transferable to microcolumns. Although the latter seems difficult to achieve since delay times in gradients are not comparable in practice.

4.1. Injection devices and sample clean-up techniques

As discussed in section 2.2 of this paper, extracolumn bandbroadening is generally regarded as a source of volumetric dispersion. In the case of extracolumn bandbroadening originating from the injection valve – the loop volume of the injection valve is usually taken into account [2,7,101]. The maximum allowable injection volume V_{inj} for a non-retained compound – which generates a fractional loss θ^2 in column efficiency – can be estimated by:

$$V_{inj}^2 = K^2\sigma_{inj}^2 = \theta^2(K\pi r^2\epsilon_t)^2 HL \quad (3)$$

where K^2 is a constant that characterizes the injection profile, i.e. the dispersion introduced by the injection valve. Note that Eq. (3) is only valid for small values of θ^2. For an ideal delta function K^2 equals 12. Eq. (3) does not take into account post-injection extra-column bandbroadening processes, thus requires more complex expressions and will not be addressed in this paper [28].

From Eq. (3) the maximum allowable injection volume can be calculated when a 5% loss in column efficiency ($\theta^2 = 0.05$) is permitted. The results for micro, capillary and nanoscale LC columns are given in Table 2. Besides the injection volume, the injection time and the I.D. of the injection loop may contribute to sample dispersion. The effect of the injection time on peak dispersion was described by Colin et al. [102]. Slais et al. [103] have given a detailed description concerning the effect of the injection loop I.D. on extra-column dispersion, the importance of which is clearly shown by their calculations. Both of these studies concerned the extracolumn effects in conventional high-performance liquid chromatography.

Table 2
Maximum injection volumes calculated for microcolumns of different I.D. and length[c]

Column I.D.	Column length (cm)	V_{inj} (nl)[a]	
		$d_p = 3$ μm	$d_p = 5$ μm
1.0 mm	15.0	400	520
	25.0	–[b]	670
300 μm	15.0	36	47
	25.0	–[b]	61
75 μm	15.0	2.3	2.9
	25.0	–[b]	3.8
	40.0	–[b]	4.8

[a] A 5% loss in column efficiency was allowed ($\theta^2 = 0.05$).

[b] In practice 3 μm particles are only applied in 15 cm columns or shorter due to the generated hydrodynamic resistance.

[c] The total porosity ϵ_t was taken as 0.70. Ideal injection profiles ($K^2 = 12$), and optimum mobile phase flow-rate and plate height ($2d_p$) were assumed. The retention factor k equalled 0.

4.1.1. Small volume injections

As shown in Table 2, for 50–100 μm I.D. nanoscale LC columns injection volumes are in the order of a few nl up to approximately 1 μl for 1.0 mm I.D. columns. Manual injections ranging from a few μl down to approximately 20 nl can be routinely performed with injection valves equipped with an exchangable internal loop. Below 20 nl, manual valve injections can be performed by positioning a split vent between the injector and the column [104]. Alternatively, for these very small volumes the moving injection technique [105,106], the static split [107] or the pressure-pulse-driven stopped-flow injection technique [108] can be used, although they remain unpopular. What these techniques have in common is 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 be easily performed by many commercially available autosamplers. However, injection automation in the nl range usually requires thorough hardware adjustments of a conventional autosampler. Such modifications of conventional autosamplers for use with capillary LC have been described in the literature [109]. Furthermore, the use of an automated microinjector has been described [110]. However, with this latter system multiple sample analysis was not possible. Recently, autosampler technology was introduced for injection from very small sample amounts and small quantities [111].

4.1.2. On-column focussing (large volume injections)

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 [112–116]. These techniques commonly have the sample solvent at significantly lower eluent strength compared to the actual eluent. After arrival of the sample plug onto the top of the column, the compounds will be focused in a small plug. Focusing enrichment factors of several hundreds have been reported, which significantly increased 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 comparable chromatograms to standard injections [113].

Large volume injections are routinely applied for the analysis of proteins and peptides, especially in the case of in-gel digested proteins where the peptides present have to be extracted in several tenths of microliters resulting in very low sample concentrations. Since most gel-extracts contain moderate amounts of organic modifiers and most peptides are soluble in aqueous solutions, sample focussing is easily achieved on reversed-phase microcolumns. Specific examples will be presented in section 6. Recently, on-column focussing has been applied to environmental trace analysis for polycyclic aromatic hydrocarbons and acidic pesticides [117,118]. The latter application is very challenging, since acidic pesticides are extremely soluble in water and with polycyclic aromatic hydrocarbons the solubility in the sample solvent should also be considered. As a rule of thumb, the sample solvent should contain 20–30% less organic modifier in order to focus the sample on top of the column [116,118]. If experimental conditions are sufficiently addressed, the limits of detection can be improved significantly. For example, a focusing enrichment factor of 800 in capillary LC was achieved for the separation and identification of acidic pesticides under reversed-phase conditions [117].

4.1.3. Miniaturized precolumn and sample enrichment techniques

The loading time of a given sample volume under focusing conditions increases at decreasing column flow-rate. This technique is rendered impractical for capillary and nanoscale LC, yet it can be performed by the use of packed precolumns. These must be properly connected to the analytical column and are then essentially a part of the separation system. The advantages of this approach are that much larger sample flow-rates can be obtained during the sample-focusing step. In addition, the selectivity and isolation of the sample components of interest can be controlled by selecting a specific stationary phase for the precolumn [119–135].

Several groups reported on the construction of micro-precolumns and their use in capillary [128,131] and nanoscale LC [128]. Capillary LC precolumns are also commercially available. Most groups however reported on the use of a precolumn in microcolumn LC to enhance sample loading and for clean-up. Exceptions are the work of both Cai and Henion [124,134] and Miller and Herman [132], who used on-line immunoaffinity extraction incorporated in a microcolumn switching setup to selectively trap the target analytes. The outline of such a miniaturized immunoaffinity extraction setup is given in Fig. 2. All elements in this setup are representative of other microcolumn switching setups. In some cases – however – an additional precolumn is placed in front of the analytical column to trap the analytes [125,134]. Vissers et al. [125] used a microprecolumn switching system to remove sodium dodecyl sulphate from tryptic digested protein samples. The use of microprecolumns in nanoscale LC has also been described [129,130,135]. Oosterkamp et al. [135] studied the linearity and limits of detection of a nanoscale system for the analysis of peptides, Vanhoutte et al. [130] studied the sensitivity of a nanoscale column switching setup for the detection and identification of DNA adducts and van der Heeft et al. [129] applied it to the direct identification of peptides presented by major histocompatibility complex molecules.

Thordason et al. [136] reported on the use of a miniaturized supported liquid membrane device for sample preparation in capillary LC. The device consists of a hollow fiber that is placed into a KelF block. The acceptor solution was kept stagnant and the donor (sample) solution transferred through the device at a flow-rate between 10–20 μl min^{-1}. After sample enrichment, the acceptor solution was transferred into the sample loop of an injection valve, from where it was transferred to the analytical

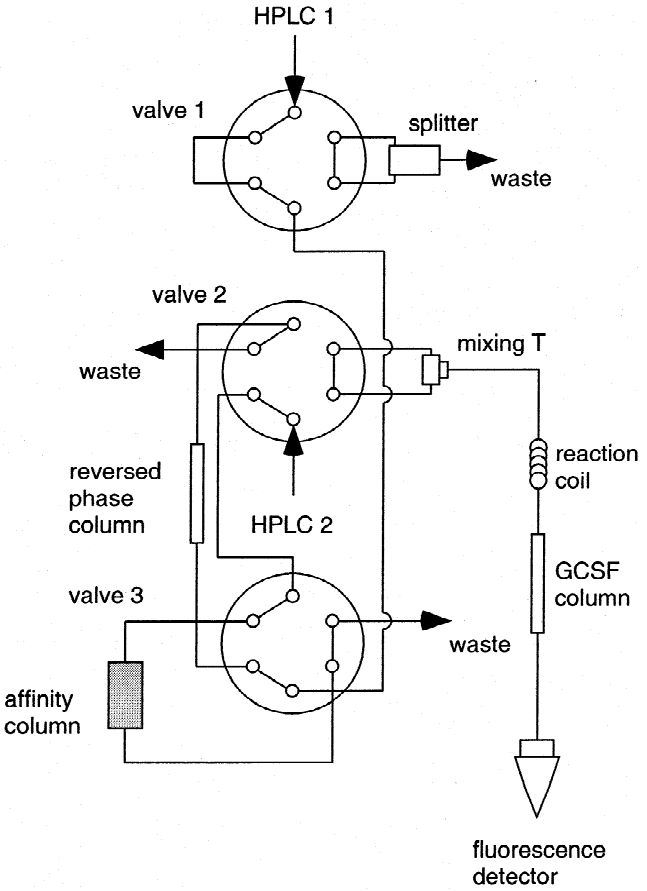

Fig. 2. Column switching setup for capillary affinity chromatography with immunochemical detection. The reverse phase column was constructed from 20 cm×500 μm I.D. PEEK packed with a perfusion stationary phase. The affinity column was 1 cm×0.5 cm I.D. packed with polyclonal anti-granulocyte colony stimulating factor (GCSF) antibody that was immobilized on a cyanogen bromine-activated Sepharose gel. The GCSF column was constructed from 20 cm×800 μm I.D. Teflon tubing. GCSF was immobilized on 20 μm epoxide activated affinity media. (Adapted with permission from Ref. [132]. Copyright (1996) American Chemical Society.)

capillary LC column. The system was tested with a secondary amine as a model substance in aqueous solutions as well as in plasma. The extraction efficiency was found to be 32.5% and the enrichment per unit time was reported to be constant at approximately 0.9 times min^{-1}.

4.2. Detectors

UV absorption detection is common to both conventional and microcolumn LC as the most widely applied detection technique. Other types of detectors have been investigated in great detail by many research groups; specifically fluorescence, electrochemical and gas chromatographic detectors. However, the number of published papers on the latter type of mentioned detection techniques has been declining rapidly after the introduction of microcolumn LC. This was not only due to the ubiquitous nature of UV absorption detection, but principally due to the successful introduction of continuous-flow fast atom bombardment (FAB) and electrospray ionization mass spectrometry (ESI-MS). However, occasionally some papers are still published on fluorescence detection [137], refractive index detection [138,139] and flame based detectors [140]. Previously, these types of detectors are discussed extensively and typical applications presented

[11,17–26,141]. In this paper, only UV absorption detection, fluorescence detection, electrochemical detection and some recent developments in detectors for miniaturized flow and separation systems will be addressed.

4.2.1. UV absorbance and photodiode array detection

The most universal detector is the UV absorbance detector because of its ease of use and broad application. To prevent extracolumn bandbroadening – i.e. keeping detection volumes at the submicroliter or nanoliter 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 an UV absorbance detector. The construction of such a cell has been discussed [142,143]. Vindevogel et al. [144] attempted to develop 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 both the linearity and noise. Due to the large variety of configurations no general recommendations could be given. It was suggested, however, that not only the I.D. of the flow cell in this type of studies should be mentioned, but also parameters relating to the light beam width and photocell distance should be included in the full description of a flow cell.

The main disadvantage of on-column detection is the limited concentration sensitivity, which is a function of the flow cell path length. Limits of detection reported for on-column detection for compounds like uracil, cytosine and thymine with a 100 μm I.D. flow cell are typically around $1–5 \cdot 10^{-6}$ mol l^{-1} [145]. Despite the extremely small path lengths, detection is possible at the subnanogram level. Fiber optics have been suggested to collimate the excitation light onto the flow cell and also collect the UV light that has passed through the flow cell [146,147]. The limits of detection that were obtained with this system are however not as favorable in comparison 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 [148]. This type of flow cell and on-column flow cells have been studied extensively with respect to sensitivity, linear dynamic range, and any contribution to extracolumn bandbroadening and noise [145,149]. The sensitivity of longitudinal type of cells is normally 50–100 times greater versus on-column detection. However, the noise of longitudinal cells is generally somewhat higher than 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 $3.1 \cdot 10^{-6}$ mol l^{-1} and with the longitudinal flow cell equal to $9.8 \cdot 10^{-8}$ mol l^{-1} [145]. The contribution of longitudinal flow cells to extracolumn bandbroadening, i.e. chromatographic resolution, is typically negligible.

Another approach that leads to an increase in path length of the detector flow cell is to increase the diameter of the capillary outlet of a column. In this case however only a limited region of the capillary is utilized [150]. This results in a larger detection region, which is explained by the 3–5 times I.D. increase of the capillary. Due to its shape – there is a smooth transition in diameter from the capillary to the widest portion of the cell – it is often referred to as a bubble cell [150]. Sensitivities with this type of flow cell are 3–5 times greater than compared to on-column detection. Furthermore, the bubble flow cell can be employed for single wavelength and diode array detection. The linear range for this cell was equal to 3–4 orders of magnitude for benzoic acid and thiourea. It must be noted that these results were obtained only using capillary electrophoresis and few applications exist for bubble cells in-line with microcolumn LC.

Photodiode array (PDA) detection has been studied extensively in regards to microcolumn LC research [151–155]. In practice it is however very rarely applied for structural conformation. For example, Verzele et al. [152] 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 bandbroadening – were discussed along with the sensitivity of conventional versus capillary LC. Limits of detection were

found to be ten times lower with capillary LC – despite the small optical path length of the PDA flow cell. Another example is the work of Sandra et al. [151], who applied PDA detection in capillary LC for the detection and identification of hop bitter acids form CO_2 extracted hop samples. The recorded PDA UV spectra showed that two families of compounds could be differentiated: α-acids (humulones) and β-acids (lupulones). Capillary LC separation was favorable compared to conventional high-performance liquid chromatography and micellar elektrokinetic chromatography. Insufficient resolution obtained on conventional columns and solubility problems in micellar elektrokinetic chromatography contribute to these shortcomings.

4.2.2. Fluorescence detection

Fluorescence detection in microcolumn LC has been studied in great detail as a consequence of microcolumns being introduced. The increase in sensitivity with fluorescence detection in microcolumn LC is rather low compared to conventional high-performance liquid chromatography. This limited detection performance is due to the short optical path length and small excitation area of on-column flow cells. If higher excitation energies are obtained, larger fluorescence emission intensity will result. This however will not immediately lead to lower limits of detection, unless the noise – i.e. stray light, fluorescence from the wall of the cell or window, and fluorescence and Raman scattering of the mobile phase – is independent of the intensity of the excitation source [26]. With the use of collimating lasers, most of the noise sources – especially the fluorescence and Raman scattering originating from the mobile phase – can be reduced.

Various flow cell designs and instrumental set-ups for laser-induced fluorescence detection in conventional [26,156] and microcolumn LC [157,158] have been described. Mass limits of detection routinely observed are typically in the amol range. Often derivatization of the compounds of interest is required, which has been demonstrated by Novotny and co-workers [159,160] and McGuffin and Zare [161] for the analysis of carboxylic and bile acids respectively.

One unique feature of fluorescence detection is that emission spectra can be utilized to reveal structural information of unknown compounds, as demonstrated by Gluckman et al. [162,163] for the identification of very large polycyclic hydrocarbons from fuel oil extracts. Spectral subtraction was used to resolve co-eluting compounds that could not have been identified by mass spectrometry.

With fluorescence detection it is also possible to measure in the packing of the column, so-called in-column detection [164]. With in-column detection the analyte is in a partition region. It can be deduced that the sensitivity of in-column detection will be $(1+k)$ times better as compared to on-column detection. This effect was demonstrated and found to be in agreement with theory by Verzele and Dewaele [164] for the analysis of drugs. However, other groups [165–167] have reported much higher signal-to-noise improvements, which was attributed to higher fluorescence quantum yields in the absorbed state. This effect has been observed for both reversed-phase [165] and chiral separations [166,167].

4.2.3. Electrochemical detection

The three basic detection modes of electrochemical detection are conductivity and amperometric and potentiometric detection. The principles of these different detection modes can be found in textbooks [24,168] and literature [17]. Amperometry is commonly employed because of its ease of use and versatility. The design and application of conductivity and potentiometric detectors for microcolumn LC have also been described for routine use [169–173].

The initial developments in miniaturized electrochemical detection – and the use of micro-electrodes – were reported for open tubular liquid chromatography by the groups of Manz [174,175] and Jorgenson [176,177], which were also applied to microcolumn LC [85,88,89,165,178]. These detectors typically consist of a small wire that is placed into an outlet of an open tubular column. For instance, Manz and Simon [174,175] 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 [176], and for the voltametric analysis of hydroquinone and catechol. Detection limits obtained with these detection systems 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 designed by Ruban [179,180]. Other designs, including wall-jet cells [181,182], platinum coated tubular electrodes [183], platinum electrodes [184] and a carbon interdigeted array microelectrodes [185] have also been described. The simplicity of the design of an electrochemical detector for capillary LC separations is shown in Fig. 3.

As mentioned before, amperometric detection is the most commonly used electrochemical detection mode in microcolumn LC. However, since the potential of the electrode is held constant, 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 selectivity and the information content about the components which are electroactive in the applied potential range may increase [17,186]. The latter technique – voltametric analysis – can provide real-time voltametric scans from components eluting from a micro or capillary LC column only when the waveform is applied quickly. Thus, analytes can be identified and co-eluting peaks resolved if their voltagrams are significantly different. The major limitation of voltametry is its lower mass sensitivity in comparison to amperometric detection [17].

Voltametric detection has been applied in open tubular chromatography [176,177] and conventional liquid chromatography [187–189], yet for microcolumn LC it is generally limiting. One exception was presented by Goto and Shimada [186]. A rapid-scanning electrochemical voltametric detector for capillary LC was introduced. Square-wave voltammetries were applied because of their suitability with rapid-potential scanning. It should be mentioned that scanning is the only viable option for ultra-micro wire detectors. With larger voltametric detection systems the diffusional delay virtually destroys the complete structure of the voltagram.

The main application area of electrochemical detection in microcolumn LC is in bioanalysis, especially the separation and detection of cathacholamines. A few examples are the detection of seratonin and its metabolites in rat brain dialysate [190], the analysis of terbutaline in human plasma [191], the detection of biogenic amines in brain tissue [192] and the in-vivo monitoring of neuropeptides using microdialysis sampling [193].

4.2.4. Other detection principles

Besides the detection techniques discussed above, other detection method have been studied for microcolumn LC. These include (Fourier transform) infrared spectrometry – either measured on-line [194] or via deposition on thin layer plates [195], chemiluminescence [196], indirect detection schemes [197–199], inductively coupled plasma atomic emission spectrometry [200], or evaporative light scattering [201]. These detection techniques have been applied with limited success and have not found for use with routine microcolumn LC due to the fact that the detection technique is either too selective or not robust enough.

Lewis et al. [202,203] studied the possibilities and usefulness of electrospray condensation particle counting detection as a sensitive and universal detector for miniaturized separation techniques. Although very promising results were reported in the size-exclusion chromatography mode, the detector was found to have nonlinear behavior in the re-

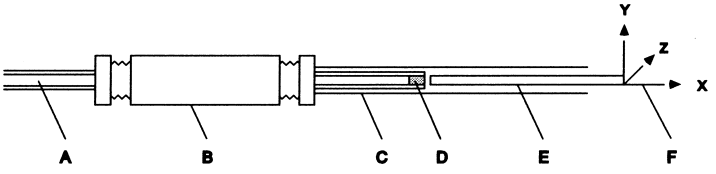

Fig. 3. The coupling of an electrochemical detector and a capillary LC column. (A) capillary LC column; (B) stainless steel union; (C) fused-silica cell; (D) end-column frit; (E) microelectrode assembly and (F) *xyz*-positioner. (Reprinted from Anal. Chim. Acta, 344, Wallenborg et al., A microchemical detector for use at low linear velocities in capillary column systems, 77–85, Ref. [184], Copyright (1997) with kind permission from Elsevier Science.)

versed-phase LC mode for the separation of proteins. This lack of linearity for the proteins investigated was attributed to the non-constant ratio of x-mers produced by the electrospray interface.

4.3. Temperature programming

A few research groups have looked into the possibilities of using elevated temperatures [204] or a temperature gradient in capillary LC [205,206]. Microcolumn separations were conducted by either superimposing a flow and temperature gradient [205] or by combining temperature programming and gradient elution [206]. Bio- and synthetic macromolecules have shown promise in this area in terms of increasing resolution. Although the presented results are in favor of conducting separation at high temperatures or with temperature gradients for test-mixtures, no workable applications have been demonstrated yet.

5. Hyphenation

5.1. Multidimensional chromatography

As with flame-based detection systems, microcolumns are well suited for the coupling with secondary separation techniques, i.e. multidimensional chromatography. Microcolumn LC has been interfaced with thin layer chromatography applying infrared detection [207], conventional high-performance liquid chromatography [208], microcolumn LC [209–212], gas chromatography [213–219], supercritical chromatography [220] and capillary electrophoresis [221]. The coupling of individual separation techniques increases the total peak capacity of the chromatographic system, which is the product of the peak capacities of the individual dimensions. An overall improvement in the peak capacity allows for the separation of very complex samples.

The coupling of microcolumn LC with gas chromatographic (GC) techniques appears to be quite challenging since a liquid mobile phase has to be converted into a GC compatible vapor phase. Among the different type of interfaces, which have been developed, are retention gap based interfaces like on-column injectors [213] and loop-type interfaces [214], pyrolysis interfaces and (multi-capillary) stream splitters. The retention gap based interfaces have been extensively applied which can be attributed to 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 spread out along the retention gap are concentrated at the top of the separation column. An example of a successful application of an on-column injector as an on-line interface for microcolumn LC–GC is given in Fig. 4, which illustrates the two-dimensional separation of a fuel oil sample. Heart-cutting was applied to introduce a part of the first dimension onto the secondary dimension separation system. Heart-cutting techniques allow only a fraction of the original sample to be separated and a total peak capacity that is typical for a single dimension.

The full separation power of a two-dimensional separation system was used by Holland and Jorgenson [209] 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 transferring to the second dimension. To obtain a high sampling frequency – i.e. the rate at which the effluent of the first dimension separation column could be sampled – the 90 cm$\times$100 μm I.D. anion-exchange column was operated at a flow-rate of 33 nl min^{-1}, while the secondary 3 cm$\times$100 μm I.D. reversed-phase column was maintained at a flow-rate of 6 μl min^{-1}. The peak capacity of the two-dimensional system was estimated to be close to 1400 peaks.

Another interesting method to achieve high sampling frequencies is the use of capillary electrophoresis (CE) as the second dimension, which allows for high efficiencies in a short time. Lemmo et al. [221] 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 cm$\times$250 μm I.D. or a 110 cm$\times$100 μm I.D. column

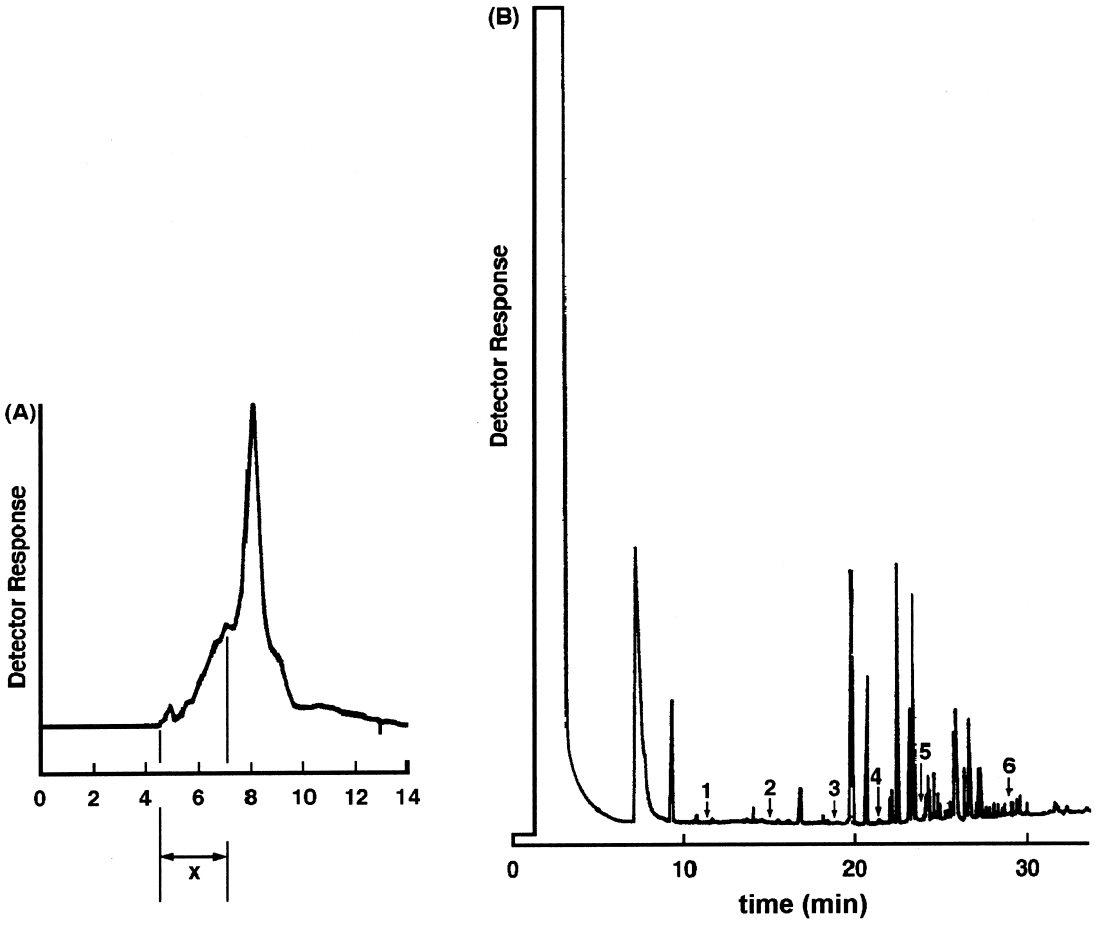

Fig. 4. Two-dimensional separation of a fuel oil sample by microcolumn LC–GC. (A) Capillary LC chromatogram; (B) gas chromatogram; x = fraction introduced into the GC. Conditions capillary LC separation: column: 105 cm×250 μm I.D. packed with 7 μm silica; mobile phase: heptane; flow-rate: 10.6 μl.min^{-1}; UV absorption at 214 nm. GC conditions: column: 30 m×0.25 mm I.D.; retention gap 15 m×250 μm 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-tetrachlorobenzenen; (4) 1,2,3,4-tetrachlorobenzene; (5) pentachlorobenzene; (6) hexachlorobenzene (reprinted from J. Chromatogr., 296, Cortes et al., Determination of trace chlorinated on-line multidimensional chromatography using packed-capillary liquid chromatography and capillary gas chromatography, 55–61, Ref. [213], Copyright (1985) with kind permission from Elsevier Science).

packed with a size exclusion stationary phase. The flow through these columns was 235–360 nl min^{-1} or 23 nl min^{-1} respectively. CE was performed in untreated 50 μm I.D. fused-silica capillaries of different lengths with an applied voltage of ±30 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 μm I.D. SEC column, i.e. too much extracolumn bandbroadening. Furthermore, the loop/valve interface hampered continuous sample collection. With the flow gating interface – which basically consists of a Teflon gasket having a 1 mm channel that is sandwiched in 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 Teflon channel or to waste. When the sample was transferred from the flow-gating interface to the CE capillary – i.e. when an injection was made onto the second dimension – the SEC effluent

was sent to waste. An example of the results obtained with the new type of interface is given in Fig. 5, which depicts the two dimensional separation of protein standards by SEC–CZE.

5.2. Microcolumn LC–mass spectrometry

The introduction of continuous flow fast atom bombardment (FAB) and atmospheric pressure ionization (API) techniques such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) have contributed greatly to the current success of microcolumn LC. One of the major driving forces behind the development of microcolumn LC is the availability of these different ionization techniques for mass spectrometry (MS). In fact, the first successfully applied microcolumn LC–MS interface was continuous flow FAB ionization. Other types of interfaces have been used for the coupling with mass spectrometry (MS) including electron impact and chemical ionization [222–226] and the particle beam interface [227–230]. Interfaces like the moving belt interface [231] and thermospray-type of interfaces [232] are rarely applied. These types of interfaces and their use in microcolumn LC–MS have been reviewed and summarized recently by Tomer et al. [233]. Only atmospheric based interfaces and the recent developments in automated off-line and on-line coupling of matrix-assisted laser desorption ionization (MALDI) mass spectrometry with microcolumn LC will be discussed in this review, even though on-line use of continuous flow FAB occasionally still appears in literature [234–238]. Microcolumns can either be directly coupled to the ion source or more generally via transfer lines. The latter will obviously increase bandbroadening and decrease the chromatographic resolution.

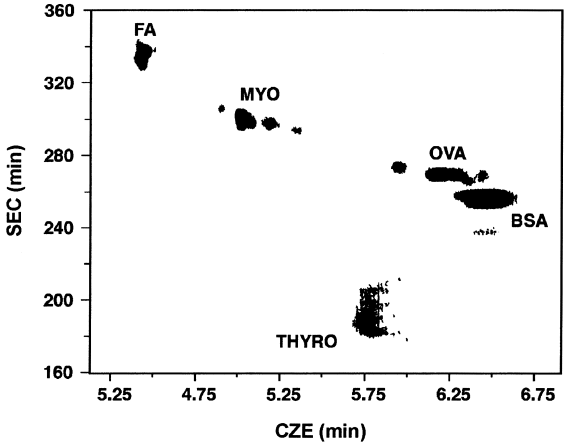

Fig. 5. Separation of protein standard by two-dimensional SEC–CZE with a flow gating interfaces. Each protein was present at 0.5% (w/v) with 2.5% (w/v) formamide. THYRO = thyroglobulin; BSA = bovine serum albumin; OVA = chicken egg albumin; MYO = horse hart myoglobulin; FA = formamide. The 110 cm × 110 μm 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 m*M* tricine, 25 m*M* Na_2SO_4, 0.005% sodium azide (w/v), pH 8.23 (reprinted with permission from reference [221]. Copyright (1993) American Chemical Society).

5.2.1. Electrospray ionization

Electrospray ionization (ESI) was almost simultaneously introduced with CF–FAB and has become a very routinely used technique for the analysis of biochemical macromolecules. ESI is by far the most popular and most widespread ionization technique in on-line LC–MS. It is both a simple and an elegant method; it can handle small and large molecules, operates at atmospheric pressure and relatively low temperatures, and provides soft molecule ionization. The mechanistic aspects of electrospray ionization have been recently discussed by Bruins [239].

The first electrospray interface for LC–MS was introduced by Whitehouse et al. and reported to have a linear behavior of four orders of magnitude [240]. Furthermore, the mass spectrometer signal intensity was found to be almost independent of the liquid flow-rate. Hence, 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. LC–ESI-MS interfaces have also a near-linear relationship upon the concentration of the analyte, making it an important tool in quantitative analysis.

Since the introduction of electrospray, the design of the interface has undergone many changes and has been the subject of a number of research papers. Recently, Alexander et al. [241] described a nano-electrospray source for nanoscale LC and CE without

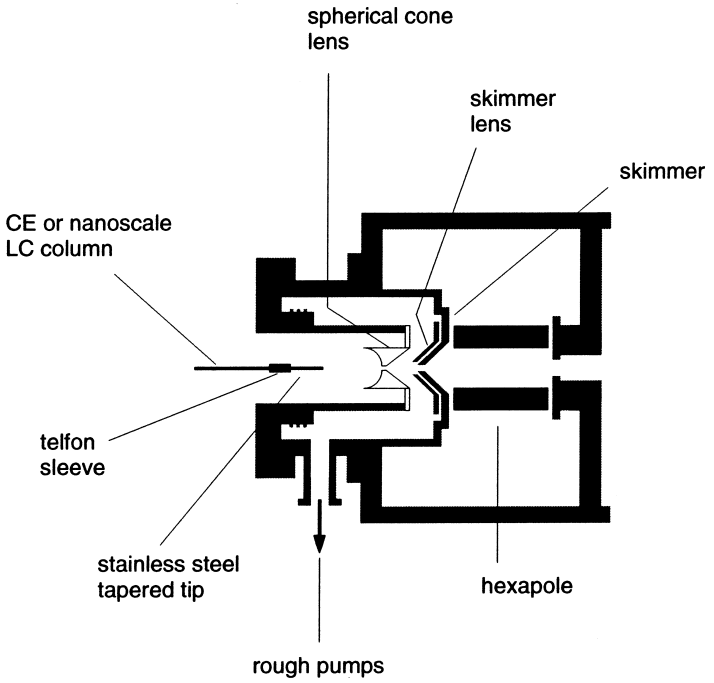

Fig. 6. Schematic diagram of an electrospray interface used in nanoscale–LC–ESI-MS experiments (adapted with permission from Ref. [241]. Copyright (1998) John Wiley & Sons, Ltd.).

the need of make-up flows. A schematic diagram of this interface design is given in Fig. 6. Unique to this design was the use of a spherical stainless steel lens with a 200 μm orifice. Dry nitrogen gas was introduced at 5–10 l min^{-1} to assist in droplet solvation. The tapered ESI tip was prepared from 50 μm I.D.×302 μm O.D. stainless steel tube. The capillary voltage was held at +3.6 kV, the cone voltage at 40 V and the source temperature was 23°C. The flow-rate going through the interface ranged between 50–200 nl min^{-1}. At a flow-rate between 100 and 200 nl min^{-1} stable ion intensity in the infusion mode was observed for poly-propyleneglycol in methanol/water (50:50, v/v).

As a result of the applied electric field, charged droplets will be produced. Details on the ionization process can be found elsewhere. Theory is however not completely understood [239,242]. ESI interfaces as with many other MS interfaces can be coupled with capillary LC either directly or via a transfer line, the advantages and limitations of which have been mentioned earlier. The primary applications of capillary and nanoscale LC–ESI-MS have been in protein and peptide analysis. An extensive literature overview of ESI-MS in conjunction with capillary and nanoscale LC has been published [233]. In the application section some selected examples will be discussed, demonstrating the successful use of microcolumn LC–ESI-MS.

Microcolumn LC–ESI-MS is still under development. Current research is focussed on achieving even lower limits of detection. Examples are the coupling of micro and nanoscale LC columns with an ESI interface to an orthogonal time-of-flight mass spectrometer [243], the use of capillary LC columns with an ESI source interfaced to ion trap mass spectrometers [244–246] and ion trap storage/reflectron time-of-flight mass spectrometer [247,248], and the application of collision induced dissociation in capillary LC–ESI-MS for the analysis of complex sample mixtures [249].

5.2.2. Automated off-line matrix-assisted laser desorption ionization mass spectrometry

Matrix assisted laser desorption ionization (MALDI)-MS is regarded as a complementary technique to ESI-MS, even though many compounds can

be analyzed with both ionization techniques. The main advantage of ESI lies in the ease of interfacing with liquid based separation techniques, where MALDI is somewhat more straightforward for the analysis of complex biological samples because of its high sensitivity, easily interpretable mass spectra and its relatively low suspension to salts and detergents.

To overcome ''the on-line problem'', a number of researchers have developed automated fractionation techniques to collect directly onto the MALDI–target. MALDI–target sizes are usually in the range of a few microliters. Hence, the application of conventional high-performance liquid chromatography would overflow the target, which can be circumvented by postcolumn splitting techniques. However, this will reduce the overall sensitivity of the technique and the sample is often unnecessarily consumed. Matrix-solution is either placed on the target prior or after collection of the fractions onto the target, or alternatively, the matrix-solution is added postcolumn via a tee to the mobile phase.

Micro and capillary size exclusion chromatography have been combined in an automated off-line fashion by Nielen [250] and Yun et al. [251], respectively, for the analysis of polymer samples. Nielen [250] used MALDI-MS in combination with UV absorption detection for the absolute mass calibration of polymers with a very narrow molecular weight distribution. Yun et al. [251] used the MALDI-MS data as complementary information to the data generated by capillary size exclusion chromatography. For the separation of proteins, Grimm and Grasser [252] applied capillary LC-automated off-line MALDI-MS. Separation and identification of the proteins from an extract of immature maize kernels was conducted at the nanogram scale. The same fraction collection device was used to collect onto inert membranes for protein sequence analysis by Edman degradation and onto nitrocellulose membranes for Western blot analysis, which is experimentally interesting. Information obtained from randomly selected protein fractions by means of MALDI-MS analysis was found to be in good agreement with sequencing data. The MALDI-MS data also confirmed the results of Western-blot analysis to selectively identify the DNA-binding protein. Ground breaking work on nanoscale LC–MALDI-MS for the analysis of peptides in single neurons has been published by Hsieh et al. [253]. Identification of peptides present in the brain of a snail was achieved on basis of retention time and mass spectral information. In addition, sequencing of one of the native peptides was conducted by means of post-source decay techniques. Grimm et al. [254] used capillary LC–MALDI-MS for the analysis of linear and branched oligosaccharides. MALDI-MS reduced sample handling and provided molecular weight information for neutral and silylated oligosaccharides. Otherwise derivatization of the saccharides had to be performed to be able to conduct UV absorption detection. Fig. 7 shows the sample information that can be attained with such a system.

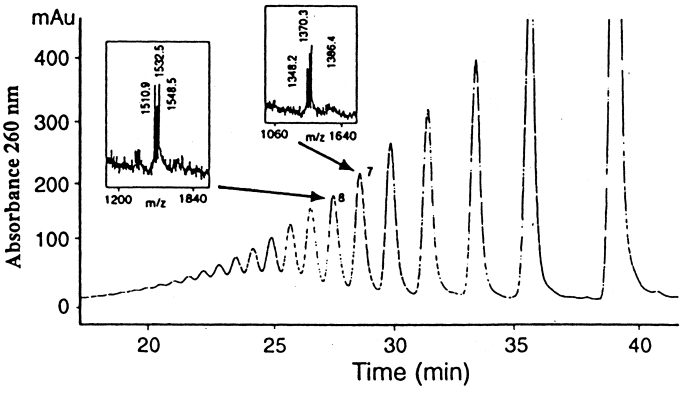

Fig. 7. Capillary LC separation of the components of a dextran ladder. The inserts show the MALDI spectra of components 7 and 8 respectively. The separation was conducted on a 25 cm×300 μm I.D. column packed with a C_{18} reversed-phase with a flow-rate of 2 μl min^{-1}, using a gradient from 10 to 25% B in 35 min and remaining at 25% B for a further 20 min. Solvent A was 100 m*M* ammonium acetate pH 6.5 and solvent B 100% acetonitrile (adapted with permission from Ref. [254]. Copyright (1998) American Chemical Society).

5.2.3. On-line matrix-assisted laser desorption ionization mass spectrometry

Other approaches of microcolumn LC–MALDI-MS include the on-line coupling of microcolumn separation techniques coupled to MALDI-MS. On-line MALDI-MS requires that the liquid samples be directly analyzed in the vacuum of the mass spectrometer, making microcolumn separation techniques a very attractive tool to perform on-line analysis. At present no universal on-line MALDI interface for simple and sensitive analysis of minute samples is commercially available. However, a variety of designs are being evaluated. For example, a continuous flow probe similar to a continuous flow FAB interfaces has been described for the analysis of flowing samples with a liquid matrix [255]. In another design, the exit of a capillary zone electrophoresis column was placed directly in the vacuum region of a time-of-flight mass spectrometer [256]. The sample ions – eluting in a solution of $CuCl_2$ were desorbed/ionized by laser irradiating at the end of the separation capillary. In a recent publication, an interface is described that consists of the deposition of a sample liquid stream at flow-rates of 100 – 400 nl min^{-1} onto a rotating surface inside the vacuum region of the mass spectrometer [257]. Even with the design adjustments, the interface still require the analytes stream to be premixed with a suitable matrix.

5.3. Microcolumn LC–nuclear magnectic resonance

A novel approach towards detection miniaturization is the coupling between continuous-flow techniques – such as microcolumn LC and capillary electrophoresis – and nuclear magnetic resonance spectroscopy (NMR) [258–261]. The hyphenation of microcolumn LC with NMR has a number of advantages as compared to conventional high-performance liquid chromatography coupled with NMR spectroscopy. Due to the low solvent consumption fully deuterated solvents can be used. Suppression of the solvent signal is not necessary allowing the use of the complete chemical shift range for structural elucidation. 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, promoting the use of 50 nl volume continuous-flow NMR detection cells [259]. Shortcomings of continuous-flow NMR are poor sensitivity due to limited measuring time with each individual analyte, the flow-rate dependency of the NMR line width and the relatively large (high m*M* level) required analyte concentrations. Further, substantial bandbroadening and loss of chromatographic resolution is observed in NMR flow-trough cells. However, this is often compensated by the benefits of structural information detection NMR provides.

Continuous flow-detection allows for the use of proton NMR chemical shift values in the second dimension, as shown for the separation and identification of humulones, the main constituents of beer that are responsible for its bitter taste [261]. Two-dimensional NMR spectra are often recorded with stop-flow techniques. For example, a two-dimensional chromatogram of an isohumulone sample, including stereoisomers, homologues, isomers and impurities is shown in Fig. 8, where the aliphatic region of the NMR spectra is given in the second dimension. Solvent compression was not necessary in this instance due to the unrestricted use of deuterated solvents with capillary LC. However, no information about the chromatographic efficiency of the two-dimensional capillary LC–NMR system is available.

6. Applications

Microcolumn LC is applicable over a variety of different fields. Presently, its main application areas are bioanalysis, neuroscience and protein/peptide research. Sample availability and mass spectrometric compatibility are the main reasons behind the continued success. Other application areas are chiral separations and the analysis of industrial samples, such as polymers and additives [262]. The analysis of chemical warfare agents by capillary LC–ESI-MS [263] and the quantitative analysis of pesticides by solid-phase extraction-capillary LC [264] has been reported.

6.1. Protein/peptide research

The number of applications that can be found in literature that concern the analysis of biological

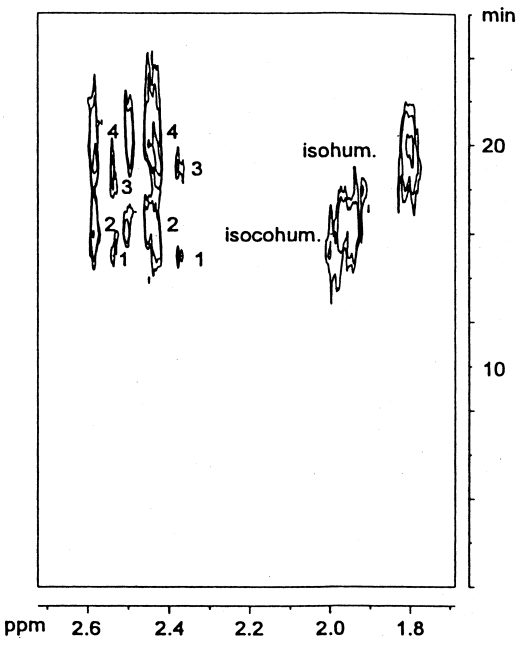

Fig. 8. Capillary LC–NMR chromatogram of the separation of an isohumulone mixture. The capillary LC system comprised a 20 cm×250 μm I.D. column packed with 5 μm C_{18} stationary phase. The mobile phase consisted of 0.05% phosphoric acid in D_2O/CD_3CN (40:60, v/v). A complete ^1H NMR spectrum was taken from 64 –128 consecutive scans (8000 point free induction decays over a 6024 Hz spectral window). The total acquisition time per row was 1 min 34 s for 64 scans and 3 min 8 s for 128 scans, respectively (adapted with permission from Ref. [258]. Copyright (1998) American Chemical Society).

compounds are numerous. Among the best examples in terms of microcolumn LC can be found in the protein/peptide research. For example, Henzel et al. [265–267] published a method, which isolated and identified proteins from two-dimensional gels by means of capillary LC and mass spectrometry analysis. Typically, a protein is characterized by means of an in-situ digestion after which the generated peptides are analyzed by means of MALDI-MS and capillary LC followed by ESI-MS or by protein sequencing. The combination of all these analysis results to the complete amino acid sequence of the protein. A typical example of a capillary LC separation from an in-situ digested, 2-dimensional gel-electrophoresis protein spot is given in Fig. 9. The most time-consuming steps in these type of protein characterization techniques are the manual sample preparations that are required for recovery of the protein from the gel. Nevertheless, this particular example shows that capillary LC can contribute significantly in the discovery and identification of proteins.

Other, similar examples have been published by Yates et al. [268,269]. 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.

Tempst et al. have reported the micro LC purification of peptides for combined chemical sequencing and MALDI-MS [270], and have described in detail on the microcolumn LC assembly and fraction collection, sample handling and peak selection for sequencing [271]. An experimentally detailed discussion on the description of the capillary LC system and miniaturized sample preparation has been published by Moritz et al. [272] on protein isolation and peptide mapping with capillary LC. Tryptic peptide maps derived from in-situ digested murine plasmacytoma were developed on a 0.2 mm I.D. capillary columns. Kassel et al. [273] evaluated packed capillary perfusion columns for rapid analysis and sequencing of enzymatic digest with ESI-MS–MS. 180, 320 and 1000 μm I.D. LC columns packed with small pore materials or perfusion particles were directly coupled to the ESI source of the mass spectrometer. With capillary perfusion columns retention times were reduced by 3–5 times with only a minor loss in chromatographic resolution. However, capillary perfusion LC–MS permitted for the identification of the same peptide fragments at the 25–50 pmol level. Kientz et al. [274] applied flow-injection capillary LC–ESI-MS to determine the molecular mass of a high-molecular-weight protein. Additional evidence of identification was obtained by detecting the presence of a disulfide bridge and by tryptic digestion of the protein. Collision induced dissocia-

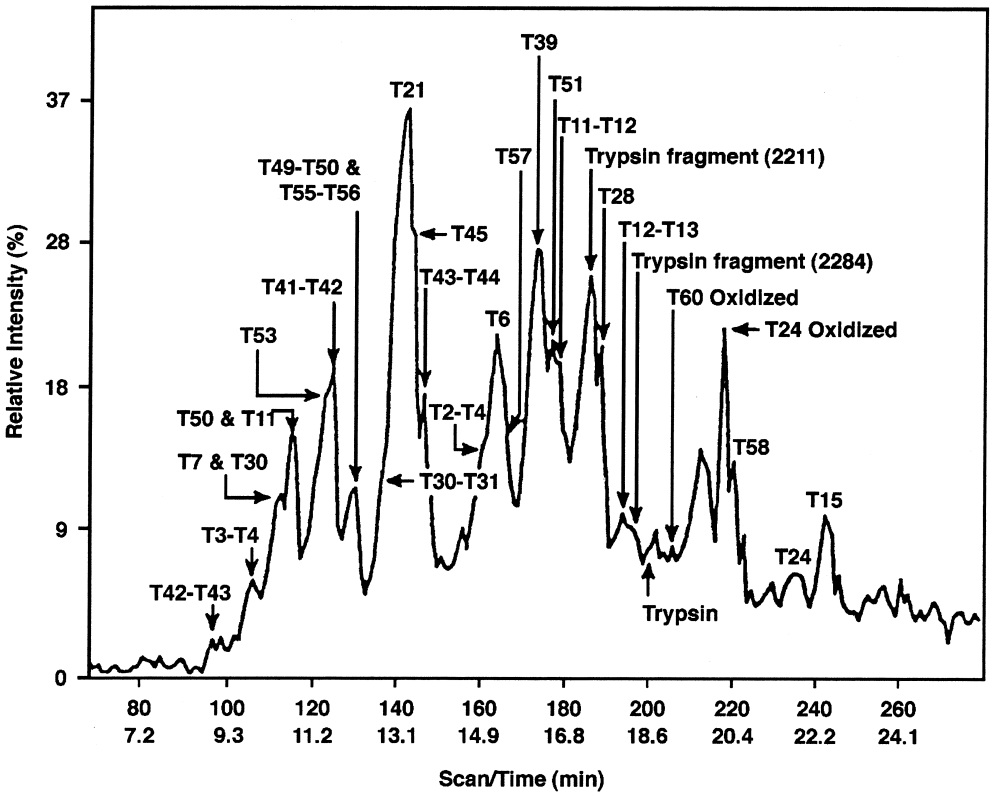

Fig. 9. Total ion current chromatogram obtained from an on-line capillary LC–ESI-MS analysis of an in-situ digestion of a protein spot from a two-dimensional gel. The amount of protein was~2 pmol. Peptide separation was conducted on a 180 μm I.D. capillary LC column (reprinted with permission from Ref. [267]).

tion (CID) spectra were recorded to determine the amino acid sequence of the major digest fragments.

All of the previously discussed examples refer to MS as the identification method for the peptide fragments. Battersby et al. [275] 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 by the 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).

6.2. Chiral separations

The chromatographic analysis of enantiomers is a rapidly growing research area, which has also been the subject of early microcolumn LC studies. Microcolumn LC is an applicable technique for enatiomeric separations since it is possible to apply new types of stationary phases that are normally very costly such as monoclonal antibodies and receptor proteins [276]. 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 [276–281], a chiral selector that is adsorbed on the packing material [276,282,283] or by using chiral mobile phase additives [276,284–287]. For instance, Takeuchi and co-workers [284–286] demonstrated the use of β- and γ-cyclodextrin as mobile phase additive for the separation of dansylated phenylalanine analogs [284] and phosphate enantiomers with ordinary octadecylsilica as the reversed-phase [285,286]. This, in contrast to the work of D'Aquarica et al. [287], who applied covalently bonded *N,N′*-3,5dinitrobenzoyl derivatives of trans-1,2-diaminocyclohexane as the chiral selector. The cost was significantly reduced for these smaller I.D. columns which allowed for the use of mobile phase additives and specially modified stationary phases.

6.3. Bioanalysis and neuroscience

On-line capillary LC – interfaced with either continuous-flow FAB-MS–MS or ESI-MS–MS was applied by Vouros et al. [288,289] 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 nature of the biochemical interaction [288]. 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 where heterocyclic aromatic amine DNA adducts of food derived carcinogenic compounds were detected [289]. The limit of detection of the target adduct was approximately 80 fmol, which was achieved by monitoring characteristic fragmentation patterns.

Vouros also reported on the analysis of vitamin D metabolites that may have a therapeutic effect in the treatment of leukemia [290]. However, overdosage of this steroidal hormone can cause adverse side effects. Capillary LC–tandem MS was employed to provide a high degree of 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 followed by continuous-flow FAB for characterization.

Femtogram detection limits for biogenic amines in rat brain tissues using micro LC with electrochemical detection was reported by Caliguri and Mefford [192]. The chromatographic separation of some indoleamine standards on a 25 cm×1.0 mm I.D. micro LC column is given in Fig. 10. The chromatogram represents the quantity of two compounds present in 0.84 μg of tissue. Under the reported conditions it would be feasible to quantitate the indoles in only 90 ng of tissue, which was in agreement with concentration values found in the literature.

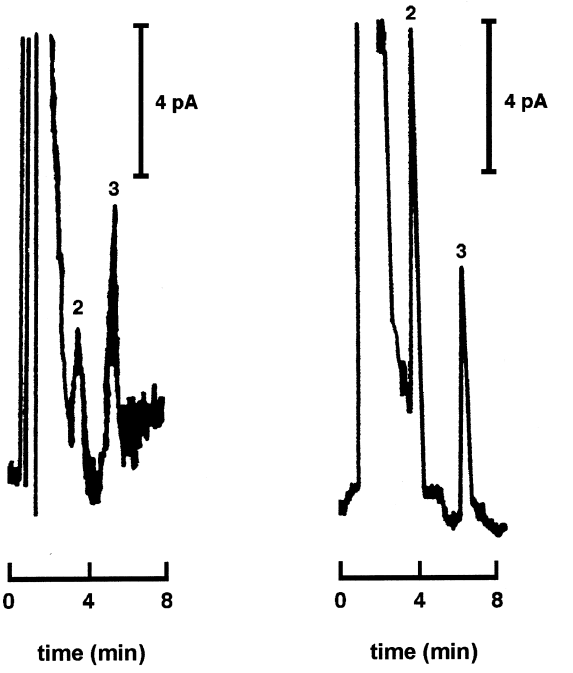

Fig. 10. Micro LC separation of an indole amine standard at the limit of detection level (left) and indoleamines in rat hypothalmus extract 0.94 μg tissue (right). Column: 25 cm×1.0 mm I.D. packed with 5 μm C_{18}; mobile phase: 0.1 *M* sodium acetate, 0.02 *M* acetate, 0.02 *M* citric acid, 50 mg l^{-1} EDTA, 100 mg l^{-1} sodium octyl sulphate and 4.5% acetonitrile (v/v); flow-rate: 40–50 μl min^{-1}; detection: +0.6 V vs. Ag/AgCl. Compound identification: (2) 5-hydroxy-tryptamine–HCl; (3) 5-hydroxy-indoleacetic acid (adapted from Brain. Res., 296, Caliguri and Mefford, Femtogram detection limits for biogenic amines using microbore HPLC with electrochemical detection, 156–159, Ref. [192], Copyright (1984) with kind permission from Elsevier Science).

Straub et al. [291] determined β-lactam residues in milk using perfusion capillary LC combined with ESI-MS. The separation detection of six key components was presented. β-lactam antibiotics are a widely used drug in veterinary medicine for the treatment of bacterial infections and are assigned as target high priority drugs. 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 critical for repetitive screening.

The determination of enzyme activity in single cells was presented by Hsieh and Jorgenson [292], who applied nanoscale LC for the separation of isotopically labeled catecholamines. A relationship between the type of cathecholamine and the enzyme activity of the cell was established. Eeckhoudt et al. [293] reported on the development of an assay for an anesthetic component compound which is a short-acting benzodiapenzine with hypnotic properties, and its main metabolite in human plasma. The limits of quantification were determined by means of micro LC with UV detection. The applicability of the method was demonstrated by studying the pharmokinetic in human volunteers. Hutton and Games [294] reported on the analysis of β-blockers by means of capillary LC–APCI-MS. Since the column flow was too low to ionize the drugs with a standard APCI source, a make up flow was added. Nevertheless, eight β-blockers were separated and identified in the full-scan-mode. Femtomol limits of detection could be achieved when the consecutive-reacting monitoring mode was selected.

Esmans and co-workers reported on the use of ion-pair capillary LC–ESI-MS for the analysis of cyclic nucleotides [295] and micro and capillary LC–MS–MS for the analysis of plant hormones [296]. The former study focused on the analytical conditions for conducting ion-pair chromatography in conjunction with ESI-MS. The low flow-rates and low ion-pair reagent concentration allowed the mass spectrometer to be coupled with ion-pair chromatography over a long period operation time. The later study focused on large volume injections using column-switching techniques, limits of detection and linearity of the technique for the analysis of plant hormones. A strictly qualitative method based on micro-LC–ESI-MS–MS to confirm the presence of anabolic hormone residues in bovine blood was presented by Draisci et al. [297]. Recovery studies with spiked samples were conducted to determine the accuracy of the developed method and its suitability for the detection of natural anabolic hormones in bovine serum.

Karlson [298] investigated post-column modification of the mobile phase in nanoscale and capillary LC–MS with alkali metal ions in order to form charged adduct ions. The target molecules investigated included modified cyclodextrins and oligosaccarides, bafilomycins and 18-crown-6. The optimum cation concentration was found to be independent of the type of cation and the relative sensitivity was found to increase slightly with the cation size. The signal-to-noise ratio could be improved by a factor of 20 for a cyclodextrin in the flow-injection-mode at 3 $\mu l\ min^{-1}$.

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 thorough overview of microcolumn LC and demonstrates the versatility of the technique by virtue of the many areas of application.

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