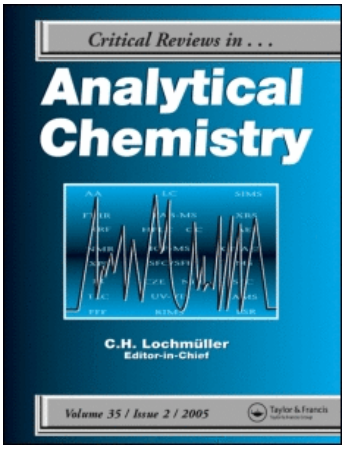

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State of the Art in Miniaturized Separation Techniques

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ABSTRACT: Miniaturized separation techniques have become very attractive recently because they offer a number of advantages over classic ones, for example, reduced chemicals consumption, separation improvement, and better sensitivity. It is also very important that they require minute samples, which is very often of primary importance in the environmental or biomedical sciences. In general, miniaturized systems that are being developed currently are divided into column and chip ones. For best performance of the separation microsystem, it must be properly designed in the terms of volumes and shapes of the parts it consists of. Theoretical considerations present this problem. As column systems are better known and understood the microcolumns are more frequently developed. Current works are focused on the preparation of unified columns that can be used in various techniques — for example, in micro-HPLC and CEC. Because of problems occurring in packed capillary column preparation and utilization, monolithic (rod) columns are developed to overcome the problems of inhomogeneity of packing or bubble formation under electroosmotic flow conditions. The requirements of minute sample analysis have been resolved by the construction of chip devices originating from total analysis system (TAS). Such systems are primarily designed for zone electrophoresis separations; however, micellar electrokinetic chromatography, gel chromatography, electrochromatography, or liquid chromatography can be performed using a chip device. In this article the most important trends in miniaturization of the separation systems are discussed, including theory, system preparation, and performance as well as exemplary applications.

I. INTRODUCTION

The process of development in chromatography has been observed since Tsvett introduced it in the beginning of XXth century;[1] however, the chromatography was not widely used before the 1930s. Its greatest development began in the middle of twentieth century, while the miniaturization of chromatography started in 1957 when Golay[2] introduced capillary columns into the gas chromatography. Since then capillary GC proved its usefulness; however, at present only a little further development of GC is observed. On the contrary — liquid chromatography is still being developed, especially new packing materials are prepared[3] and chromatographic systems are miniaturized. So, the term "miniaturized separation system" is presently used rather to describe liquid chromatography or electromigration techniques in their various modes (e.g., performed using a capillary or a chip device).

The articles published in last 2 decades showed an increasing concern over application of microcolumns and chips in separation science. Various problems of microtechniques have been described, for example, column packing, instrumentation, detection, multidimensional chromatography, unification of chromatographic techniques, and chip technology.[4-16] At present, two main trends of miniaturization of separation systems can be observed (Figure 1). First one is related to the miniaturization of column chromatographic systems. The development in this field was initiated in decreasing the column internal diameter in liquid chromatography; next step was the utilization of such columns in supercritical fluid chromatography (SFC), gas chromatography and finally in electromigration techniques, that is, in electrochromatography (CEC). Right branch in Figure 1 is related to the systems in which the separation is performed in a channel of a chip device. These two systems (columns and chips) are discussed separately.

From the historical point of view, Horvath e*t al.*[17,18] first introduced microcolumns into liquid chromatography in 1967. They used 1-mm internal diameter (I.D.) stainless steel packed column for the separation of ribonucleotides. After intro-

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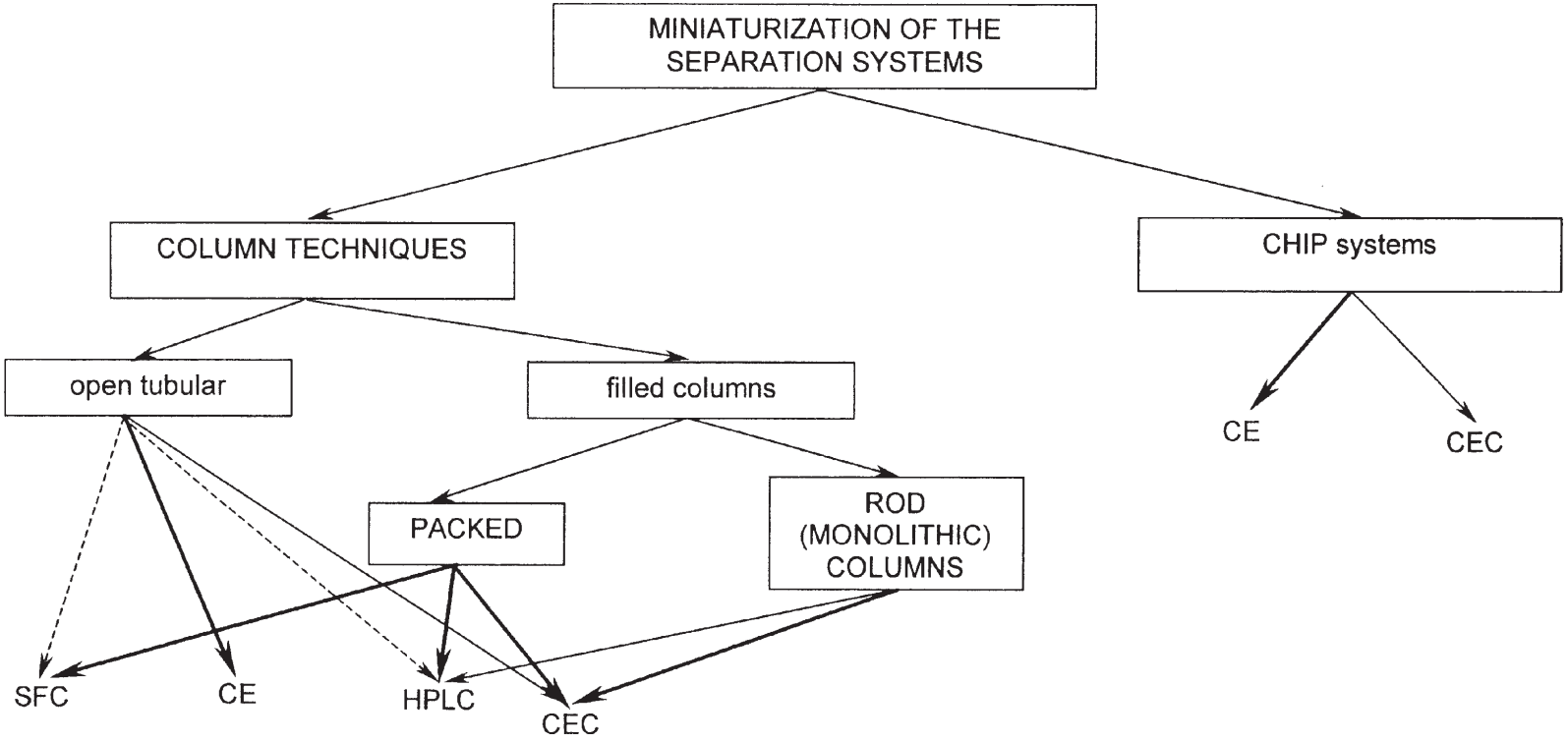


FIGURE 1. Trends in miniaturization of separation systems.

ducing capillary columns into the gas chromatography in late 1960s, the first papers by Giddings touched on the subject of possibility of usage of capillary open tubular columns in LC.[19,20]

Later, Ishii and co-workers[21] carried out first experiments employing LC columns with internal diameter smaller than 1 mm. At the same time Scott's group published the results of highly efficient separations obtained on 1-mm I.D. columns.[22,23] These articles as well as articles by Novotny et al.[24-26] and Yang[27] are regarded as key publications about microcolumn liquid chromatography.

The development of high-performance liquid chromatography toward the microcolumn technique is connected with several advantages over classic HPLC.[5] These are as follows:

- Large decrease in solvent and stationary phase consumption. It may be very important when expensive or dangerous phases are to be used;
- Very small samples can (and ought to) be used;
- Using micro-HPLC higher mass sensitivity is obtained;
- It is possible to pack very long columns using different techniques what results in a high resolution;
- Possibility of temperature programming;
- Convenience of selecting the operating conditions;
- Possibility of coupling with mass spectrometry as well as with various types of detectors commonly used in gas and liquid chromatography.
- Capillary columns may be the step toward unified chromatography where one capillary column is used in gas chromatography, liquid chromatography, and supercritical fluid chromatography. However, the concept of unified chromatography described by Young[6] was not widely discussed and at present there is a tendency rather to use the same column in LC and CEC or in LC and SFC.

II. TERMINOLOGY

It is very important to discuss briefly the terminology that has been used in the literature during last 2 decades. Several systems of the nomenclature have been proposed since the introduction of columns of smaller internal diameter:

Ishii[5] divided columns into groups accordingly to the internal diameter:

- 4.6 mm — conventional HPLC;
- 1.5 mm — semimicro HPLC;
- 0.46 mm — micro-HPLC;
- 0.15 mm — ultramicro-HPLC;
- 0.05 – 0.2 mm — (loosely) packed microcapillary column;
- 0.01 — 0.06 mm — open tubular (capillary) column.

Verzele and Dewaele[10] suggested the following system that takes into consideration various types of open-tubular columns as well:

- 5 mm and larger packed metal columns — preparative LC (Prep-LC);
- 3 to 5 mm packed metal columns — LC or conventional LC;
- 1 to 2 mm packed metal columns — microbore columns;
- 0.1 to 0.5 mm fused silica columns — Micro-LC;
- 25 to 100 μm columns obtained by drawing out glass or fused silica-packed tubes semi-packed open tubular capillaries — SPOT-LC
- 25 to 100 μm columns obtained by coating the wall with a porous layer or coated support but being open tubes — PLOT-LC and SCOT-LC.
- 1 to 50 μm wall coated open tubular columns — capillary-LC or WCOT-LC.

Approximately at the same time Barth and co-workers[28] used simplified nomenclature describing columns smaller than conventional ones:

- 0.5 to 2 mm — microbore columns;
- < 0.5 mm — micro LC columns.

Recently, Chervet et al. followed by Vissers[14,29] named LC techniques according to the flow rate range as it is presented in Table 1.

However, the term *capillary column* is used the most often and is usually related to the columns with internal diameter 50 to 300 μm.

TABLE 1
Terminology currently used in LC techniques. Adapted from Ref. 29.

Column i.d.	Flow rate	Name
3.2-4.6 mm	0.5-2.0 mL/min	conventional HPLC
1.5-3.2 mm	100-500 μL/min	microbore HPLC
0.5-1.5 mm	10-100 μL/min	micro-LC
150-500 μm	1-10 μL/min	capillary LC
10-150 μm	10-1000 nL/min	nano-LC (nanoscale-LC)

III. THEORETICAL ASPECTS OF MINIATURIZATION

The process of miniaturization of the separation systems can be considered in both economical and separation improvement categories. Since it would be an ideal situation to perform the fast and efficient separation at low cost, some theoretical aspects are noteworthy. A down-scale factor gives the information how much all volumes should be reduced in comparison to a conventional HPLC system:[29]

$$f = \frac{d_{conv}^2}{d_{micro}^2}, \quad (1)$$

where d_{conv} and d_{micro} are the internal diameters of conventional and microcolumns, respectively. It is easy to calculate that compared with classic HPLC, flow rates, injection and detection volumes, as well as connecting tubes should be approximately 207 times less for 320 μm i.d. capillary or 2116 times less for 100 μm capillary, assuming the same length.

As the flow rate in a column is given as:

$$F = \frac{u\pi d_c^2 \varepsilon}{4}, \quad (2)$$

the typical volumetric flow rates will be ca. 3.4 μL/min or 0.33 μL/min for the examples given above (assuming linear velocity u equals 1 mm/s, and total porosity $\varepsilon = 0.7$). Keeping in mind that typical flow rates in conventional HPLC range between 0.5 to 1.5 mL/min, the economical advantages of miniaturized techniques are obvious.

One of the most important advantages of the utilization of microcolumns is increased mass sensitivity. A compound injected onto a chromatographic column will be subjected to dilution during the chromatographic process. At the end of the column the chromatographic dilution (D) is expressed by the following equation:

$$D = \frac{c_0}{c_{max}} = \frac{\pi d_c^2 \varepsilon (1+k)\sqrt{2HL\pi}}{4V_{inj}}, \quad (3)$$

where c_0 is the initial (original) concentration of the solute in a sample, c_{max} is the concentration in the maximum of the peak (final), d_c is the column internal diameter, L and H are the column length and plate height, and V_{inj} is an injection volume. Chromatographic dilution increases with the square of the column diameter, which is the reason of proportionally lower dilution in the case of microcolumn when the same introduced mass is considered.

IV. INSTRUMENTAL REQUIREMENTS

It is known that a chromatographic system generally consists of a mobile phase source and delivery system, an injector, a separation column and a detector. All these parts are connected by the connecting tubes, unions, etc. When designing a liquid chromatographic system one should take into consideration peak broadening phenomena[4,5,17,22] that have a great influence on the system performance. Ideally, the recorded peak profile of the solute should depend only on the operating conditions of the column:

$$\sigma_t^2 = \sigma_{col}^2. \quad (4)$$

However, usually the conditions are not ideal and the obtained chromatographic band is broader than the column-dependent band. This is due to the dispersion and mixing effects taking place in the injector, connecting tubes, and detector cell. Additionally, the electronic system, that is, detector time constant, may have an influence on the band broadening. The contributions of these factors can be expressed as the sum of their variances:

$$\sigma_t^2 = \sigma_{col}^2 + \underbrace{\sigma_{inj}^2 + \sigma_{det}^2 + \sigma_{tub}^2 + \sigma_{tc}^2}_{\sigma_{ex}^2}, \quad (5)$$

where σ^2_t is the total peak variance (profile of the observed peak), σ^2_{col}, σ^2_{inj}, σ^2_{det}, σ^2_{tub} and σ^2_{tc} are the variances due to the column, injector volume and geometry, detector cell volume and geometry, connecting tubes, and time constant, respectively.

Peak volume (V_p) is the most important factor to be considered when designing a microchromatographic system.[5] For a column characterized by N theoretical plates and assuming a Gaussian concentration distribution of a solute, peak volume is proportional to the square of column internal diameter and inversely proportional to the square root of N:

$$V_p = 4\sigma = \frac{4V_R}{\sqrt{N}} = \frac{4V_0(1+k')}{\sqrt{N}} = \frac{\pi d_c^2 \varepsilon L(1+k')}{\sqrt{N}} \quad (6)$$

Therefore, the observed peak volume ($V_{p(obs)}$) will depend on the column contribution (V_p) as well as extracolumn (V_{ex}) peak volumes due to the contribution of the above mentioned parts of the system. Hence, the Eq. 5 can be rewritten as:

$$V_{p(obs)}^2 = V_p^2 + V_{ex}^2. \quad (7)$$

A. Injection

Using Eq. 7 it can be calculated that the injection volume that does not cause more than 5% of peak broadening is about 0.3 of the column peak volume, assuming the plug injection ($\sigma^2_{inj} = V^2_{inj}/K^2$, where K = 3.5 for perfect plug injection). Because such an injection cannot be performed using an actual injector, the volume should be less than above value. Various injection systems are used for introduction of a sample to microcolumn:

- stopped flow[21,26] — a portion of the sample was sucked into a sampling capillary, then the capillary was connected to the microcolumn and the analysis was performed; during the injection, the flow through the column is stopped. Kennedy and Jorgenson constructed a pneumatic microsyringe to inject the sample into an open-tubular column of 15 μm i.d. The main part was a micropippete drawn to 10 μm of outer diameter, which allowed to insert it into the column to inject the sample.[30] The K value was 5.56, while a split injector gave K = 4.43.
- split flow[25,27] — the most popular injection technique. The greatest disadvantage of this method is that most of a sample is lost as the split ratios are in the range 1:10 to 1:2000.
- heart-cut[31] — a method similar to the split injection, a very narrow band is "cut" from a large volume sample (e.g., 10 μL) at maximum concentration position using two cut-off valves and splitters. K value is approximately 4.
- utilization of miniaturized injection valves. The smallest commercially available valves have a volume of 10 to 20 nl. Because the maximum injection volume should be less than one-third of the V_p, the split-flow method or time-programmed injection should be used. It was also reported that diffusion and mixing that occur in the injectors of complicated flow path often causes peak tailing especially at low flow rates.[32] It is advantageous to turn the valve to the load position after some time (see Figure 2).

It must be mentioned here that the great influence of the sample solvent may be observed during the separation. If the solvent is stronger than the mobile phase, it can participate in elution resulting in shorter retention times or worse separation (see Figure 3). On the other hand, weaker solvent allows concentrating the solutes at the top

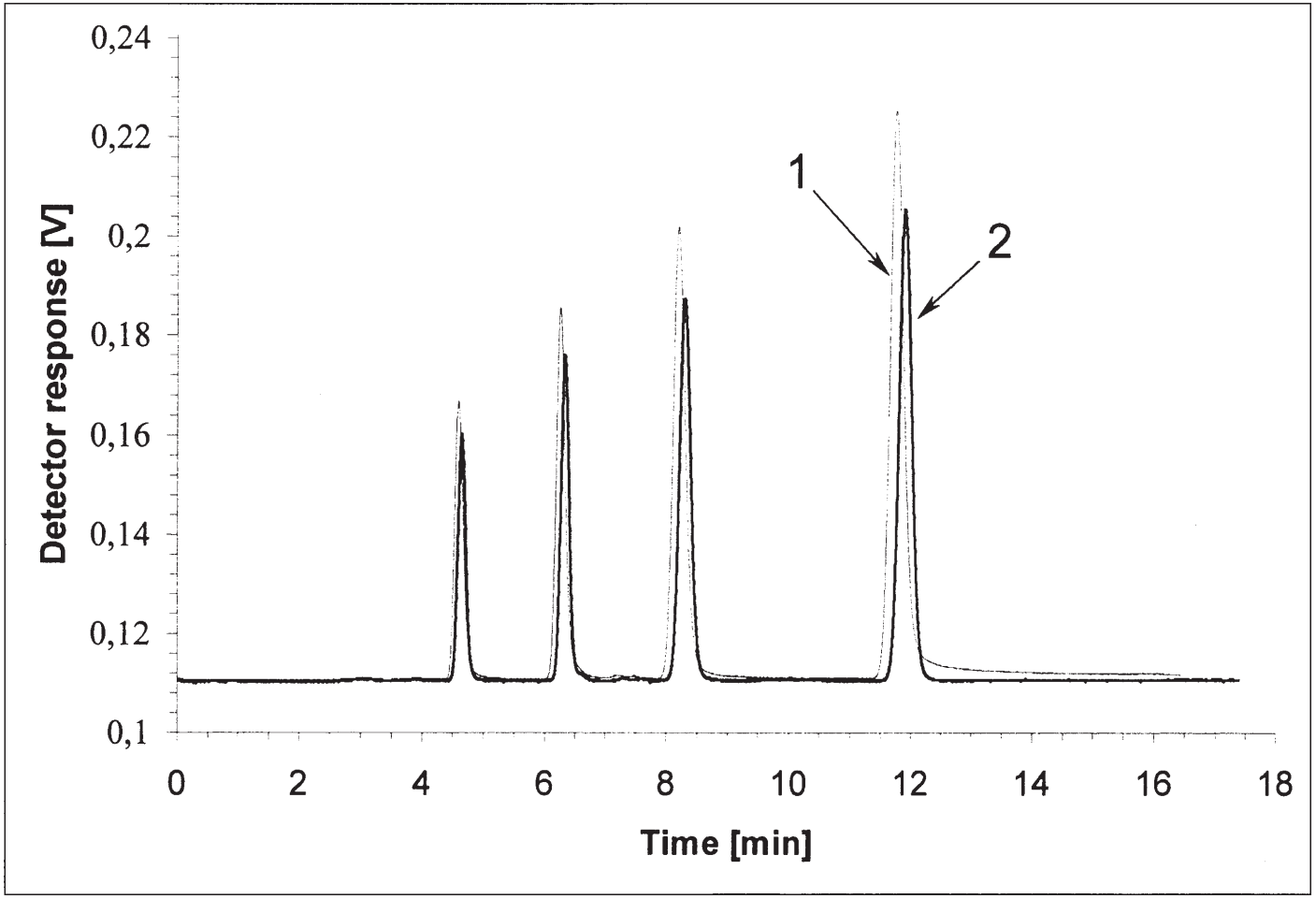


FIGURE 2. Effect of remaining the valve in INJECT position during the analysis (**1**) and turning it to the LOAD position after 2s (**2**) on peak symmetry. Column packed with Nucleosil 100-3 C-18, L = 270 mm, d_c = 161 μm. Detection performed "on column" at 254 nm. Compounds resolved: uracil, phenol, 1,1-diethyltoluamide, toluene.

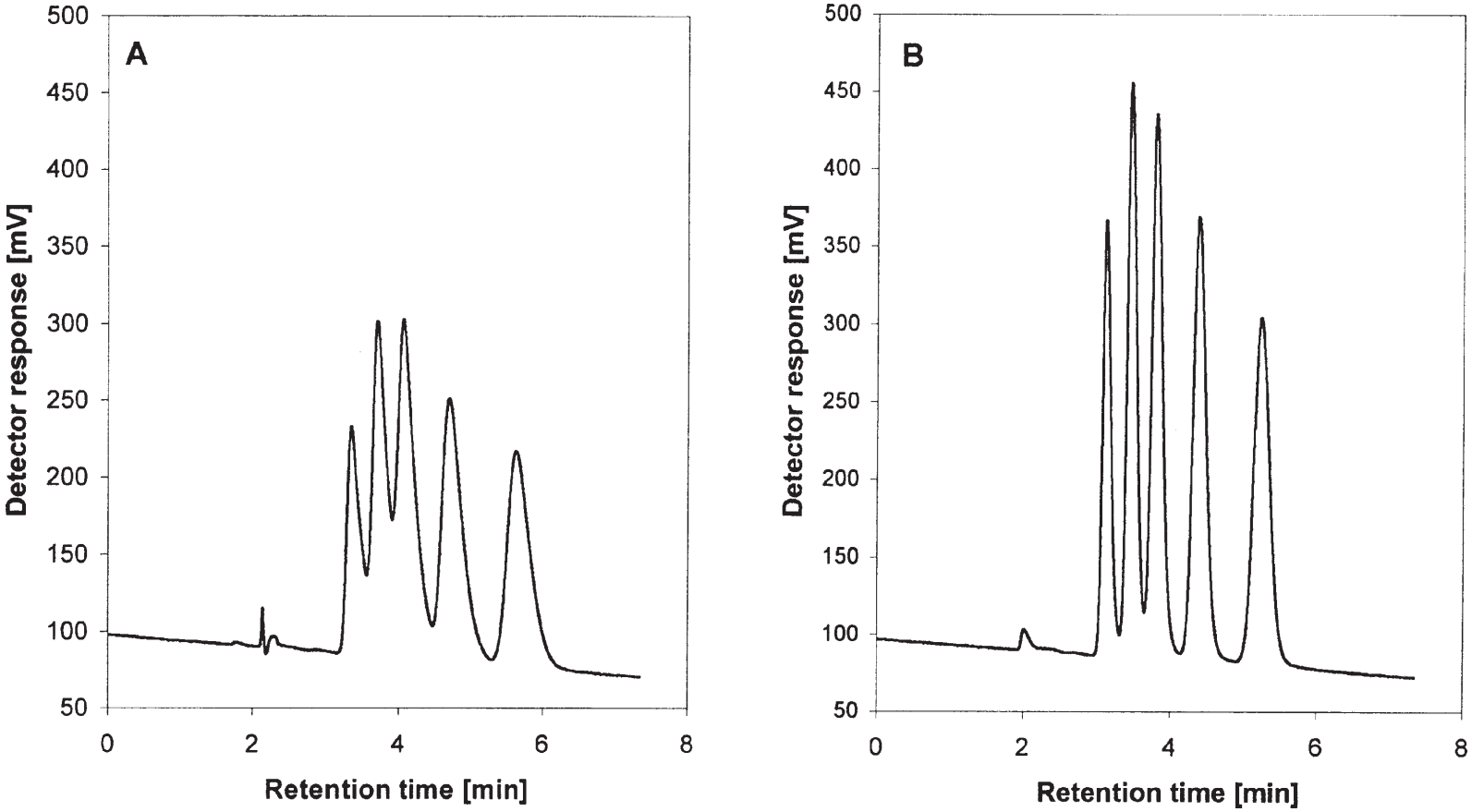


FIGURE 3. Separation of alkylbenzenes (C0-C4) on lauryl methacrylate monolithic column (L = 250mm, d_c = 100mm), using 75/25 acetonitrile/water mobile phase. Solutes (5 µL/mL each) dissolved in: **A** — acetonitrile, **B** — mobile phase. Split injection, detection performed "on-column" at 200 nm.

of the column before elution. In the first case the maximum allowable injection volume must not be exceeded, in the second one — it may be.

B. Detector Cell Volume and Shape

The peak eluting from the capillary column will be detected as a narrow band if the detector cell volume is sufficiently small. The contribution of the detector cell to the total variance is

$\sigma^2_{det} = V^2_{det}$ when the complete mixing occurs in the cell, or

$\sigma^2_{det} = V^2_{det}/12$ when the plug flow is considered.

As it was discussed by Martin et al.[33] and Kirkland et al.,[34] the actual cell shows intermediate effects. When its volume is up to 10% of the peak volume, the band broadening due to the detector cell can be neglected (see Table 2). Most of the currently used capillary columns are prepared to perform on-column spectrophotometric detection. In such cases the path length of the light beam usually equals the internal diameter of the column; therefore, the reduction of the column i.d. can reduce the sensitivity. To increase the sensitivity and keep small cell volume extended-path (longitudinal) cells were proposed.[35-38] Chervet and co-workers constructed and evaluated Z-shaped fused silica flow cell. For approx. 90 nL volume and approx. 2 cm (20.000 μm) of optical path length, the cell showed comparable to on-column detection (75 μm), extracolumn variance, and low detection limits for several hydrocarbons (e.g., 8 pg for fluoranthene).[35]

C. Connecting Tubing

All the above considerations suggest that any dead volume in the microcolumn system should be minimized to avoid extracolumn effects. The tubing, utilized in a chromatographic system to provide injector-column and column-detector connections, should also be downscaled as the column I.D. decreases. The contribution of the tubing to the peak variance can be expressed by the equation:

$$\sigma^2_{tub} = \frac{2\pi^3 D_M r^6_{tub} l_{tub}}{F} + \frac{\pi r^4_{tub} l_{tub} F}{24 D_M}, \qquad (8)$$

where D_M is a molecular diffusion, r_{tub} is a tubing radius, l_{tub} is a tubing length, and F is a flow rate[5].

Because molecular diffusion and tube radius are very small, the first term of the equation is negligible. Taking into account this fact and keeping in mind that the peak volume is four times standard deviation (Eq. 6) and $S_{tub} = \pi r^2_{tub}$, we obtain:

$$V^2_{tub} = \frac{2 S^2_{tub} l_{tub} F}{3\pi D_M}, \qquad (9)$$

where S_{tub} is a tubing cross-sectional area. For easy calculation it is advantageous to extract the $S^2_{tub} l_{tub}$ term and insert Eq. 2 with the above-mentioned assumptions (u = 0.1 cm/s, ε = 0.7, $D_M = 1 \times 10^{-5}$ cm²/s and allow 3% band broadening)[5]:

$$S^2_{tub} l_{tub} = 5.22 \times 10^{-5} \frac{V^2_p}{d^2_c}. \qquad (10)$$

The results of the calculations of connecting tubing lengths for different internal diameters are shown in Table 2.

Summarizing the above considerations, it is clear that for columns with I.D.<100 μm no connecting tubes should be used, and the column should be connected directly to the injector (using splitting device if necessary) and possess a detection window at the outlet frit.

V. PREPARATION OF THE COLUMNS

A. Column Material

Column blank material (a tube) should possess several properties that make the tube applicable in chromatography. The material for packed liquid chromatography, SFC, or CEC column should be characterized by:

TABLE 2
Peak Volumes and corresponding injection, detector cell volume and connecting tubing parameters for columns of various diameter (i.d.). Assumptions: L = 250 mm, N = 10.000, ε = 0.7, k′ = 0.

I.D. [mm]	V_p [µL]	V_{inj}	V_{det}	Connecting tubing length [cm] at various i.d. [mm]		
				0.25 mm	0.1 mm	0.05 mm
4.6	116	35 µL	11.6 µL	14	535	8568
1.0	5.5	1.65 µL	0.55 µL	0.65	25.4	408
0.3	0.49	0.15 µL	49 nL	0.057	2.2	36
0.1	0.055	16.5 nL	5.5 nL	0.0065	0.25	4.1
0.05	0.014	4.2 nL	1.4 nL	0.0017	0.066	1.1

- High mechanical strength because of very high pressure applied during packing process and utilization;
- Chemical inertness to used mobile phases (organic solvents, organic-aqueous mobile phases of various pH, supercritical mobile phases modified or not) and separated solutes;
- Smooth inner surface to achieve good packing results and avoid wall effects during utilization;
- Ease of use with various elements of apparatus (ferrules, tubings, unions etc.);
- To cause the electroosmotic flow (EOF) if required.

Classic HPLC columns are made of stainless steel with polished inner wall (to reduce wall effects) and are used successfully in most of applications.

Various materials have been used during microbore and capillary columns development. As it was reported by Horvath and co-workers,[17,18] stainless steel tubes with 1 mm i.d. were used first. Ishii and co-workers are the first to publish the results of the preparation and evaluation of packed micro-LC columns.[21] These first experiments were performed with PTFE (polytetrafluoroethylene) as column material packed with 5 to 30 μm particles. Takeuchi and Ishii[39] examined various column materials (PTFE, stainless steel, and Pyrex glass). They noticed that Pyrex glass gave good results because of smooth inner surface, but it was rather crispy and because of that — difficult to handle and operate.

Low resistance against the pressure usually restricts the use of polymeric materials such as PTFE or PEEK (polyetheretherketone); however, the best material that has been in use as far is fused silica. Takeuchi and Ishi first used it for packed capillary columns[40] and Yang for open tubular LC columns.[41]

Fused silica seems to be an ideal material for microcolumns. It offers such advantages as smooth inner surface, flexibility (due to the polyimide coating), good mechanical strength (fused silica can withstand pressures up to 80 MPa), and advantageous optical properties (UV transparency). This material is produced in a wide range of internal diameters and can be applied in gas chromatography, supercritical fluid chromatography (columns and restrictors), supercritical fluid extraction (restrictors), electroseparation techniques, as well as for capillary liquid chromatography. However, it should be mentioned here that several problems could occur during utilization of fused silica capillaries. Operating at high pressures (what is necessary during packing or SFC) can produce cracks and when capillary has a thin wall it may be destroyed. Capillary columns become dry very quickly when not used. As the drying may result in a loss of efficiency (formation of voids), the capillaries should be sealed or have ends dipped in vials containing liquid. It was reported that organic solvents such acetonitrile often damage polyimide coating that protects bare silica and from that reason packed columns are often kept in water when stored.[42]

B. Column Filling

Different techniques have been proposed for the preparation of packed capillary columns, including dry packing, slurry packing, supercritical fluid packing, or electrokinetic packing. The aim of the packing process is to fill the tube (column blank) with the adsorbent particles to obtain a homogeneous and stable bed of the stationary phase. The selection of the method depends on particle and column diameter, the mechanical strength of the material, and material type. Usually larger particles (d_p>20 μm) can be dry packed, while smaller ones, due to a high surface energy, are slurry packed. Knox[43] has discussed the conditions that must be fulfilled during the slurry packing; however, some of them are applicable in each method:

- The particles must not settle during the filling procedure,
- The particles must not agglomerate,
- The particles must hit the accumulating bed with a high-impact velocity,
- Each particle should have sufficient time to settle before it is buried by other particles,
- The slurry liquid must be easily washed out of the packing and should not react with it.

During the early period of packed microcolumn development there have been two major techniques

of column preparation. The group of Ishii[5] produced the columns by the slurry packing, while Novotny and co-workers[24-26,44-50] studied extensively "*drawn columns*" characterized by the length of several dozen meters. The preparation of such a column comprises of several steps including selection of the tube material and dimensions, filling the tube with the adsorbent, drawing and derivatization if necessary. As the drawing process (performed with a homemade or commercially available drawing machine) requires a high temperature, the tube should be made of soft glass characterized by low melting temperature; it allows the silica adsorbent particles to keep the shape during the process. The particles are introduced into the glass tube by means of gravity and are uniformly packed by the vibrator action. The uniformity of packing has a strong effect on column efficiency. The required internal diameter of the capillary can be easily controlled what allows to obtain the columns of required d_c/d_p ratio. For example, Tsuda and co-workers[51] studied the performance of capillary columns of d_c/d_p ratio between 3 and 12. The column performance, expressed by h, Φ, and E parameters,[24] was the best for the column characterized by d_c/d_p ratio 3 to 6. As the drawn columns are very long the analysis time could reach 2 to 3 h. Such type of columns are not developed further.

A dry filling technique was not widely used; however, several contributions have been reported.[52-55] When dry packing is performed, the static charge of the packing material should be reduced. It is usually performed by keeping the adsorbent in an atmosphere of, for example, ethanol vapors for several hours. The material prepared in such a way does not agglomerate and is discharged. The researchers obtained good results when such a technique was used. Crescentini et al.[52] compared the slurry and dry packing techniques and reported comparable results of the performance for 250 μm i.d. column packed with 5-μm particles. Yafeng reported high stability of dry-packed bed material, which could withstand at least 350 bar even outlet frit was removed, while slurry-packed bed was displaced at 80 bar. Moreover, the slurry-packed bed was completely damaged during rapid pressure drops that can occur when the system leaks.[55] However, Wilson and co-workers,[53] during the studies on d_c/d_p ratio, indicated worse results for smaller particles and suggested the slurry packing in such case. A kind of hybrid of wet and dry packing was reported by Capiello and co-workers,[56] who used 1% sodium lauryl sulfate solution to prepare the slurry of 5 μm C18 and C8 phases followed by applying the 100 bar pressure of nitrogen and continuous vibration. After the packing material was transferred into the PEEK capillary column, the nitrogen pressure was released and the column was flushed with water. The columns prepared by such "soap" packing showed reduced plate between 3 and 4 for anthracene.

Up to now, the slurry packing technique has been used most frequently because it bases on the experiences with the packing of the classic size columns. It has been widely described in the literature.[57-71] The concept of slurry packing relies on the preparation of packing material suspension in an appropriate solvent and introducing it into a column under pressure. Hence, the process can be regarded as filtration. Usually, the set-up similar to that presented in Figure 4 is used. The literature studies show clearly that there is no universal concept on how to efficiently pack the column, as each packing material can behave differently, however, the above–mentioned requirements should be fulfilled. Some theoretical approaches to the method have been presented by Vissers et al.,[71,72] Tong et al.,[73] and Shelly et al.[74,75] related to the coagulation properties of some materials or the filtration theory. A general tendency is to use low-viscosity slurry and packing liquids for faster transport of the particles from the slurry reservoir to the column (e.g., acetone, acetonitrile, hexane). The packing process itself can be difficult to perform.[76] For example, Kennedy and Jorgenson[64] reported that the adsorbent filled (15, 20, and 25 μm i.d.) column partially and stopped while the packing liquid was still flowing through the column. It was impossible to move the material even at a high pressure. The authors attributed this effect to: (1) imperfect sphericity and different sizes of the particles, (2) high slurry concentrations that caused agglomeration. Verzele et al.[65] used the term "bridging effect" to describe such phenomenon in small columns and described it as a one of the "wall effects" in liquid chromatography that particularly occur in microcolumns. In

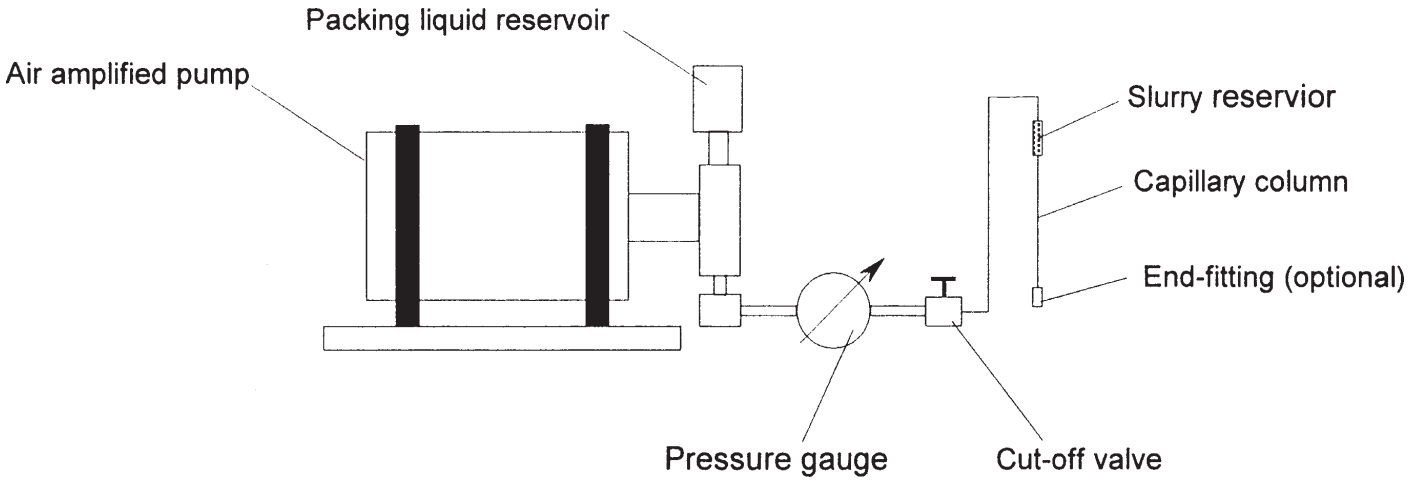


FIGURE 4. Slurry packing apparatus. (From Ref. 77.)

our laboratory we also faced similar problem, but we are not sure that all above explanations could be applicable. It was very difficult to pack some 5-μm materials into 200, 160, and 100 μm capillary even at a high pressure and using ultrasound probe. However, other 5 μm as well as 3 and 10 μm materials could be efficiently packed in the same capillaries using the same equipment. An example of slurry packing optimization results is shown in Figure 5.[77]

As it was stated by Poole and Poole,[82] irrespective of the method, the difficulty in obtaining uniformly packed bed increases as (1) the particle diameter decreases, (2) column diameter decreases, and (3) the column length increases. Keeping in mind Knox's rules,[43] it is obvious that column packed with liquids will have limited length due to a high viscosity of the liquids and limitations of the equipment that should provide a high velocity (provided by high inlet pressure) of the particles being packed.

Another column-filling technique that incorporates pressure to introduce the adsorbent into the capillary is packing with supercritical fluid (SF).[73,78-82] Owing to advantageous properties of the supercritical CO_2, that is, changeable density, low viscosity, and surface tension, it is possible to pack very long columns. For example, Malik and co-workers, using the apparatus shown in Figure 6, were able to pack columns (220 μm i.d.) up to 10 m in length with 5, 10, and 12 μm particles. The reduced plate heights obtained at SFC conditions were better for 12 μm particles (*h* ranged between 3.4 and 3.9) than for 10 μm (*h* = 4.4) and 5 μm (*h* = 5.4). They reported that the key factors that influence the obtaining of a good column are ultrasonic vibrations, pressure programming, proper restrictors, and decompression rate.[79] Tong et al. have also observed the positive effect of sonication and presented a mathematical model describing this process.[73] Because of a large number of factors affecting the column preparation by SF packing, Koivisto et al.[81] used a factorial design to find the most important of the parameters. Among the selected factors, the model obtained indicated high pressure, long restrictor, and the use of pressure ramp as primarily affecting the column quality. Surprisingly, the sonication was found to be necessary, but there was no significant effect when sonication power changed.

A method that requires no pressure drop is *electrokinetic packing* developed by Yan and coworkers.[84-88] There are several advantages of such a method:

- There is no high back pressure as it is generated in slurry packing because particles under a strong electric field are moved by electroosmotic flow in the column and move with their own electrophoretic mobility, if charged, hence very small particles can be packed,
- Several capillaries can be packed simultaneously what is more efficient and economical,
- to some extent, the electrokinetically packed column is more homogeneous in cross section because the particles move according to their charge-to-size ratio or move with the elecroosmotic flow, which exhibits almost no velocity differences over the cross-sectional area (conversely to parabolic profile of pumped liquid).

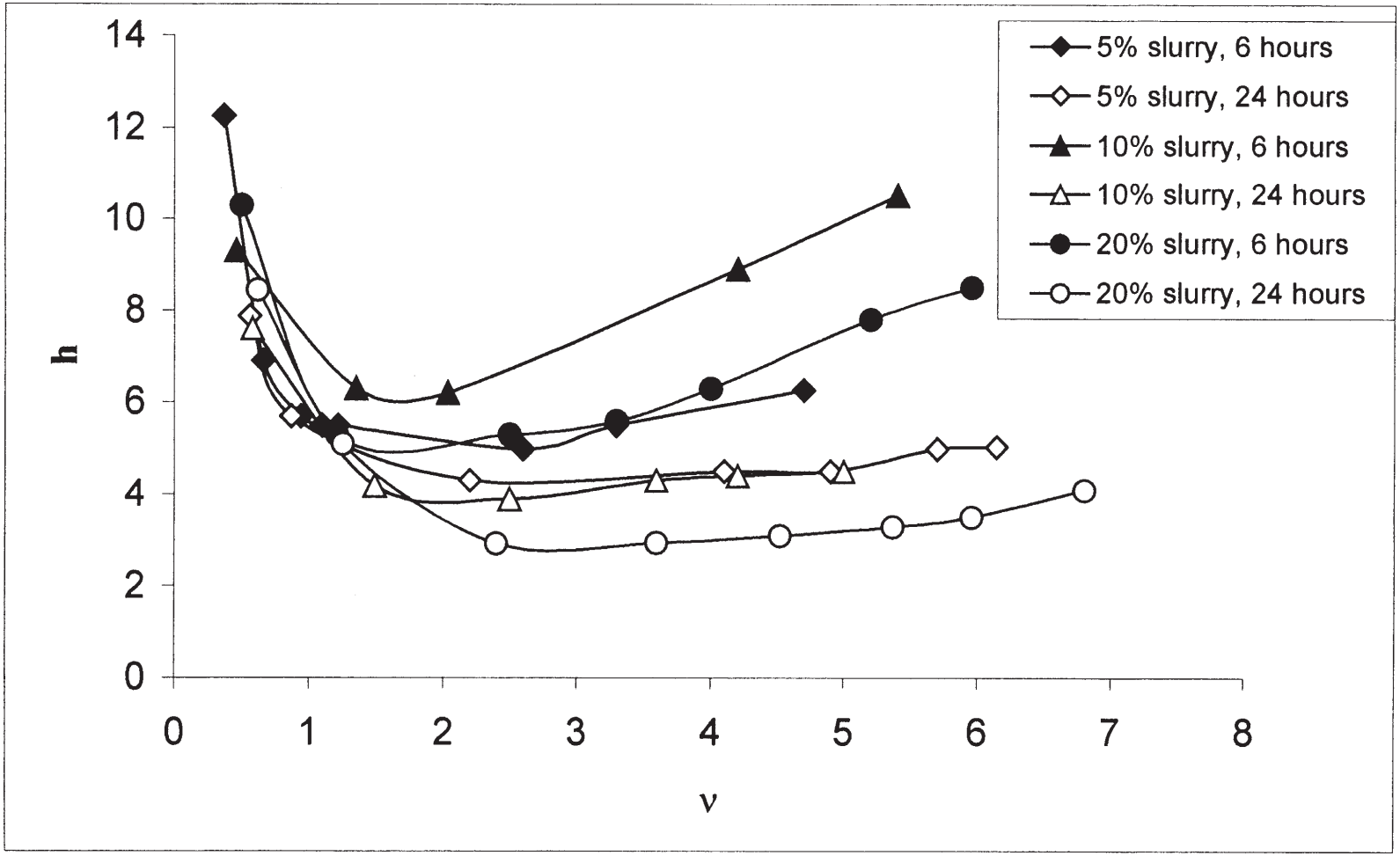
The scheme of the apparatus for electrokinetic packing is shown in Figure 7. The column prepared by electrokinetic packing of 3 μm ODS particles gave 102,000 theoretical plates per meter for naphthalene under CEC what was significantly better as for micro-HPLC (67,000 plates/m).[85] Dadoo et al.[88] efficiently packed 1.5-μm nonporous ODS and achieved a rapid separation of 16 EPA recommended PAHs obtaining >700,000 plates/m with in-column detection.

C. Column Form and Stability

The microcolumn stability and lifetime can be considered from two points of view. On one hand, the column performance is affected by the stability of the packed bed, on the other, by the stability of the retaining frits.

According to Knox et al.,[89,90] three regions can be distinguished in the packed classic column:

- Homogeneously packed central core;



A.

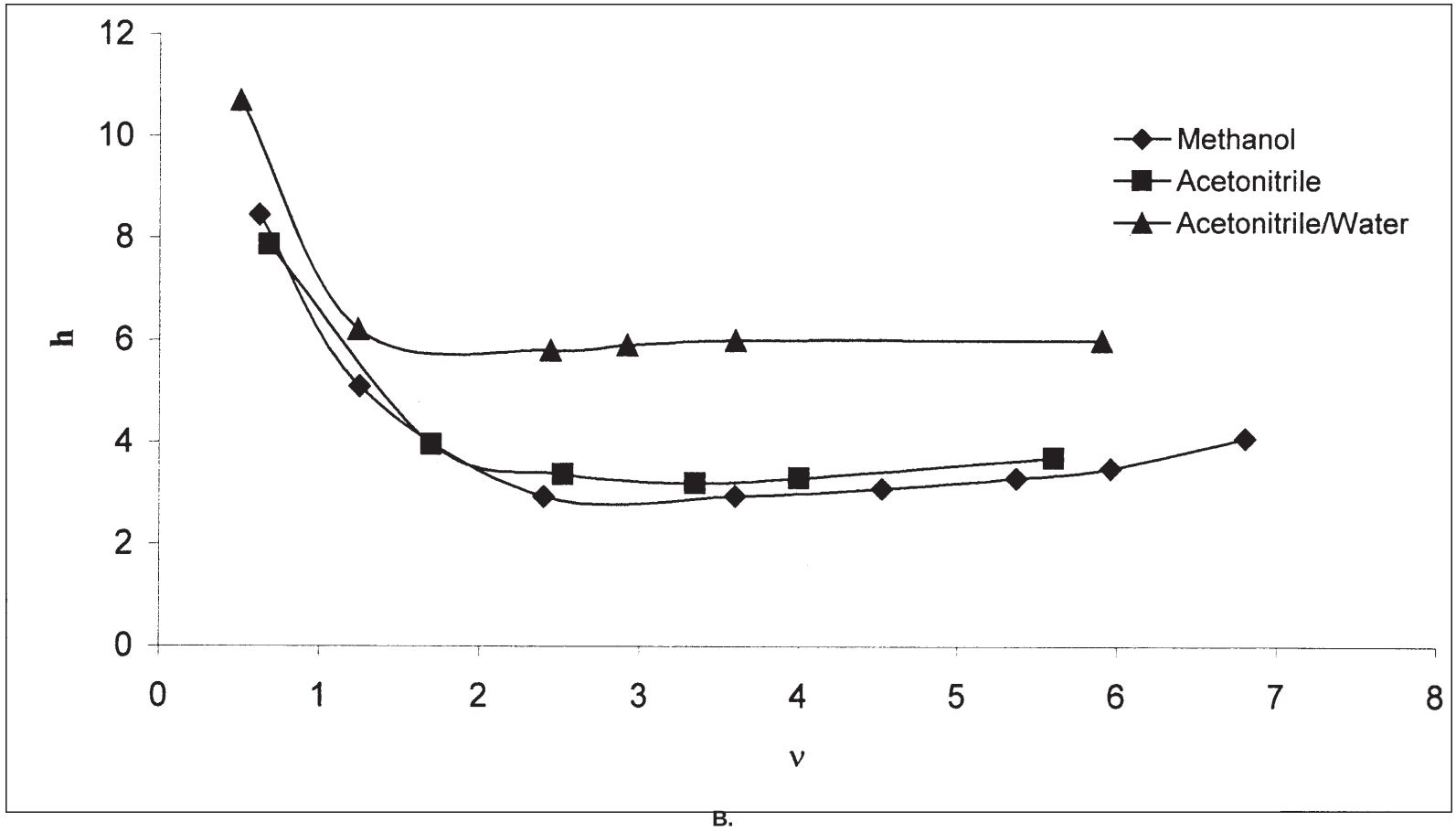


FIGURE 5. Optimization of slurry packing: **(a)** Nucleosil 100-3 C18 packed into 30 cm × 161 μm i.d. capillary. Slurry liquid: acetone, packing liquid: methanol. **(b)** Selection of packing liquid. (According to Ref. 77.)

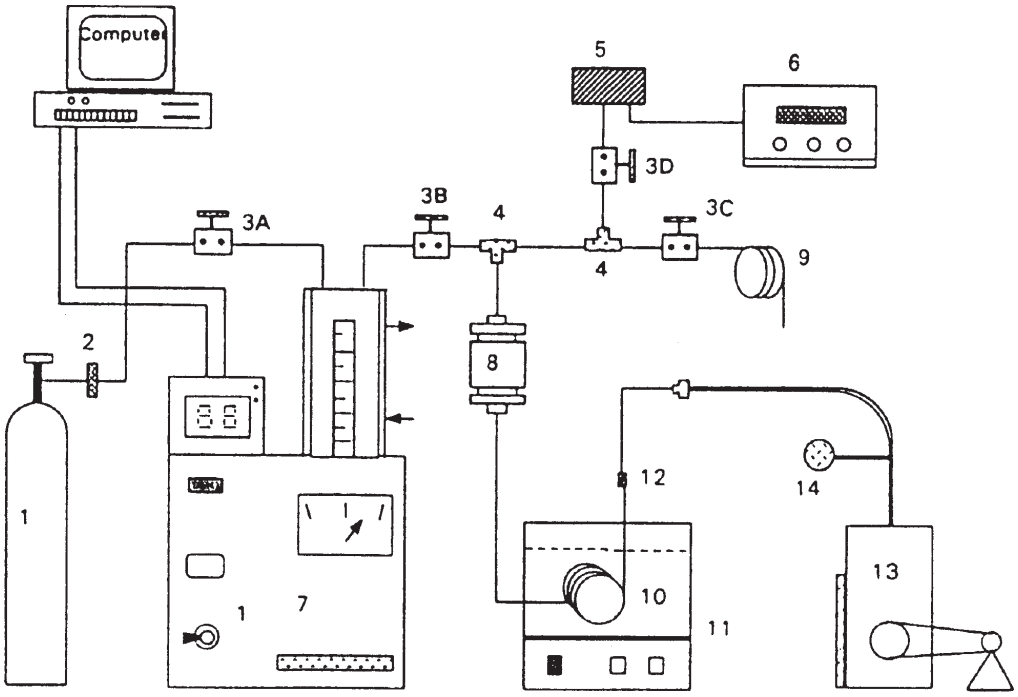


FIGURE 6. Apparatus for packing with supercritical fluid. (According to Ref. 79.)

- A layer of three particle diameters in which the structure is markedly different;
- The disturbed region of about 30 particle diameters between two former regions.

Due to dimensions the microcolumn packing consists of second and third regions or third region only (with respect to the d_c/d_p ratio), which means that microcolumns are less densely packed and are characterized by a higher permeability, and it may influence the efficiency. De Weerdt et al.[91] have studied the following factors influencing the microcolumn stability:

- Long-term stability under flow;
- Storage procedure;
- Solvent change over (gradient effects);
- High pressure pulses;
- Flexibility of the fused silica capillary.

They found 320 μm × 25cm column comparable or better than classic stainless steel column in spite of less dense packing structure. As the column top is the part where undesired effects start first (compacting of the bed), it is usually cut to restore column performance. Verzele et al.[65] reported that coating of the inner wall of the microcolumn with the polymer improved performance and lifetime of the column.

Like most of the LC columns capillary column consists of a tube (capillary), a stationary phase and the frits that retain the bed of stationary phase. As it was mentioned, to minimize extracolumn effects that contribute to band broadening the connection between the end of the column and the detector should posses the lowest possible volume dependently on the size of the column. Assuming that the column is attached directly to the injector (what is widely practiced) the only problem will be related to the outlet part of the column. Additionally, this part must be mechanically strong enough to withstand the packing and working pressure. Several forms are presented on Figure 8. Neglecting the utilization of typical commercial stainless steel end fitting, the simplest end fitting is presented on Figure 8a; it was applied, for example, by Takeuchi and Ishii[40,92] or Hoffmann.[62] Hoffmann reported that such connection could withstand up to 34 MPa during packing. A similar approach is presented on Figure 8b. The retaining frit is kept in place by a capillary with an outer diameter smaller than column's I.D. This capillary is fixed to the column using epoxy[71] or cyanacrylate glue.[68]

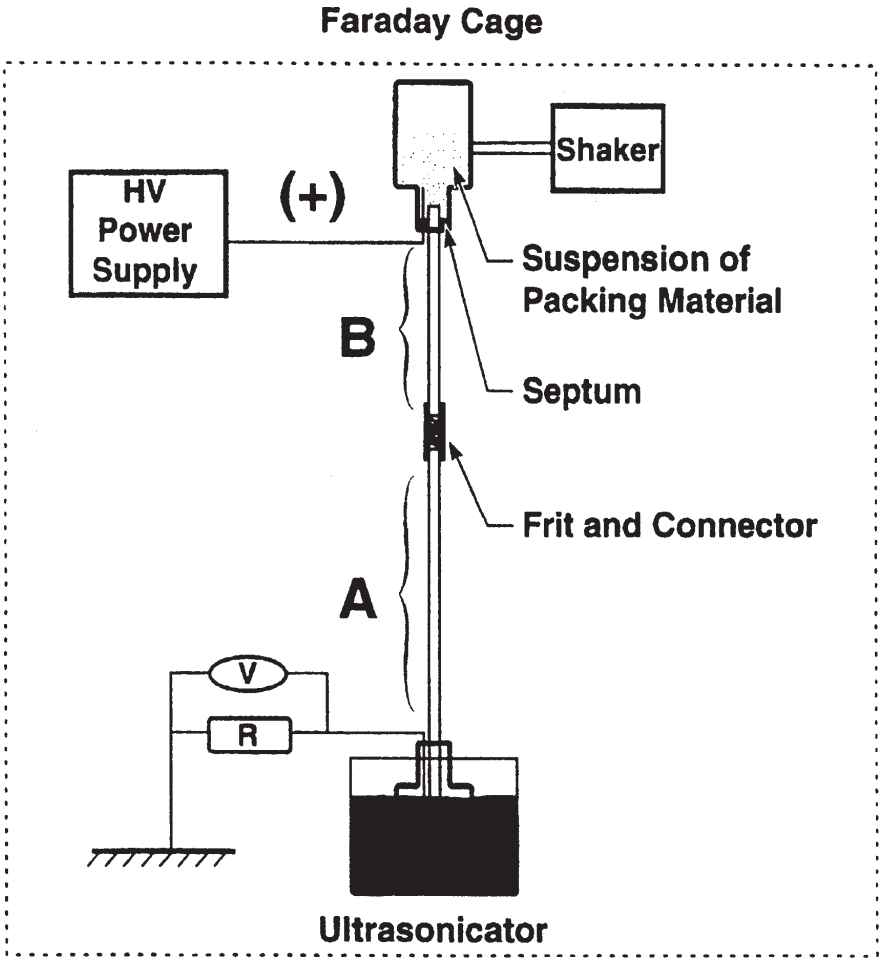


FIGURE 7. Electokinetic packing device. (According to Ref. 84.)

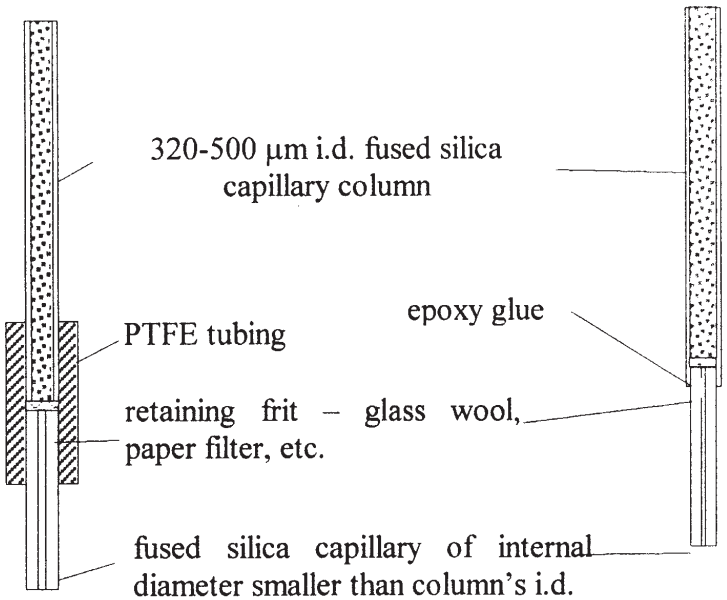
Bächmann et al. reported packing at 70 MPa[68] using such a system. Novotny used the same system (using epoxy glue) except the utilization of a 1-mm-long bed of coarser particles (25 to 75 μm) as an equivalent of a frit held in place by a smaller capillary.[63] The construction of a column shown on Figure 8a,b can be applied when the internal diameter of the column is not smaller than ca. 250 μm.

In order to avoid any extracolumn effects, the columns of smaller internal diameter are usually equipped with in-column frits while the detection takes place next to the outlet frit. Two solutions were proposed:

- Preparation of porous silica plug;
- Sintering of the adsorbent particles.

First method was proposed by Cortes et al.[93] and modified later by Trones et al.[94] It relies on the reaction of potassium silicate with formamide at 100 to 125°C. Such prepared frit could withstand at least 55 MPa.[94] More recently, Schmid et al. have prepared the in-column frits by fixing the packing bed ends with silica by a sol-gel reaction of a polydimethoxysiloxane (PDMOS) solution in a room temperature.[95] The frits were prepared after the capillary column were packed and, for successful preparation, the column ends should have been dried before wetting with PDMOS solution. Chirica and Remcho, using methacrylate polymers, immobilized the entire packing inside a capillary, obtaining in this way a fritless column. They reported excellent performance of the columns under HPLC (h = 2.2 to 2.5) and CEC (h = 1.1 to 1.5) conditions.[96]

Sintering of the adsorbent particles was applied, for example, by Kennedy and Jorgenson.[64] They tapped the end of the capillary onto the pile of the 5 μm silica particles, pushed them further



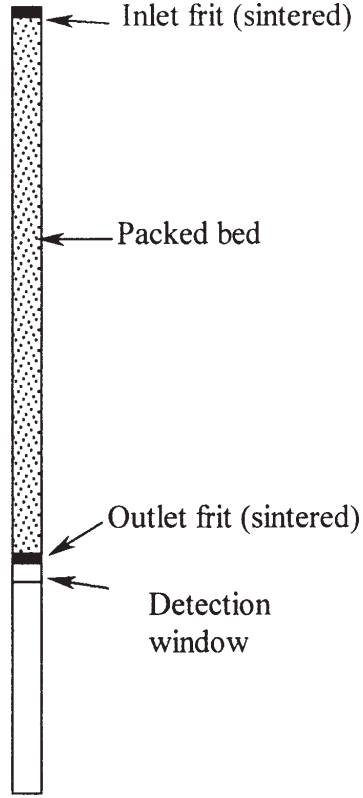


FIGURE 8. Capillary columns.

into the capillary and sintered by quick passing the end of the column through the flame of a microtorch. The free space between the frit and the column end was used for the mounting of the detector electrode. They reported that the frits were checked by applying the pressure of 55 to 69 bar. This method was later developed by wetting the silica particles with aqueous solution of potassium silicate or water[97] prior to sintering. Figure 8c shows the most frequently used form of a capillary column for HPLC or CEC. It consists of a single tube partially filled with the packing material and possessing the detection window at the outlet frit. The frits are prepared by sintering (using a heating filament) the packing material preferably flushed with water or water-containing solvent.[42,98,99] The sintering of the packing can be, of course, done when the column is flushed with a nonaqueous packing liquid (methanol, acetonitrile, etc.); however, the presence of water results in better permeability and homogeneity of the frit.

D. CEC and Problem of Bubble Formation

Capillary electrochromatography (CEC) has gained a great attention recently.[8,100-108] CEC is usually defined as a liquid chromatographic technique in which the mobile phase is not pumped but moves because of the presence of the electroosmotic flow so it is a hybrid of capillary liquid chromatography and capillary electrophoresis. In the early 1970s Pretorius proposed the utilization of the electroosmotic flow (EOF) instead of mobile phase pumping.[109] However, CEC was not widely used almost throughout the decade until Jorgenson et al. investigated electroosmosis in capillaries and Tsuda et al. performed electrochromatography in open tubular columns.[110,111] Later, Knox and Grant described some theoretical aspects of CEC.[112,113] Open tubular columns are not widely used in CEC because of their small internal diameters, which causes problems when optical detection methods are employed. Nevertheless, several groups successfully develop open-tubular electrochromatography focusing on various methods of wall modification.[114-121]

The majority of the CEC separations are performed on packed capillary columns prepared as described above. In CEC the role of the packing is not only limited to the separation itself but should participate in the electroosmotic flow generation as well. It is known that the EOF in a bare fused silica capillary filled with a buffer solution is connected with the presence of the double layer of cations (fixed layer and diffuse layer) at the capillary wall. Consequently, silica particles, also possessing surface silanol groups, exhibit the same effect. The linear velocity of the EOF (u_{EOF}) is given by the following equation:

$$u_{EOF} = \frac{\varepsilon_0 \varepsilon_L \zeta E}{\eta}, \qquad (11)$$

where ε_0 is a permittivity of a vacuum, ε_L is a permittivity of a liquid mobile phase, ζ is a zeta potential, E — electric field strength, and η is a mobile phase viscosity.

For a comparison, the mobile phase velocity under pressure-driven conditions depends on the particle diameter (d_p) and a pressure drop (ΔP) across the column:

$$u = \frac{d_p^2 \Delta P}{\Phi \eta L}, \qquad (12)$$

where Φ and L are column resistance factor and column length, respectively. Hence, the linear velocity of the EOF is independent of the mean channel diameter (no pressure drop term in the Eq. 11); this fact suggests that it is possible to use smaller packing particles than in HPLC. The utilization of, for example, 1.5 μm or smaller particles would be not possible under HPLC conditions because of a high-pressure drop, while the CEC separations using such packing materials were demonstrated.[42,88,98,122-125] Moreover, keeping in mind that the EOF is characterized by a flat flow profile, it is obvious that much higher efficiencies can be obtained.

One of the most important problems related to the utilization of packed column in CEC is bubble formation or drying out of the outlet part of the packing. The bubbles are generated below the outlet frit, in a nonpacked part of the capillary (see Figure 8) under electroosmotic flow conditions. These phenomena cause the current

breakdown and, of course, stop the mobile phase flow. Several theories were proposed to explain bubble formation. For example, Knox and Grant[113] suggested self-heating effect in the capillary as a major cause and recommended thermostating and operating at high pressure. The main drawback of this theory is the fact that under CEC conditions lower current is generated than in CE (lower buffer concentrations are used together with high content of organic modifier in the mobile phase), so the self-heating effect should not play the role. The second theory indicates the difference of the electroosmotic mobility between packed (lower mobility) and nonpacked (higher mobility) part of the capillary column. This difference causes the local pressure drop (effect of "suction") in the interface of these two parts.[126] The third theory is related to the frits that exhibit zeta potential different from the packing due to the absence of the chemically bonded phase thermally destroyed during sintering.[126] Moreover, the permeability,[127] length, and nature of the frit[128] also have the effect on the bubble formation. To minimize this problem, the mobile phase should be carefully degassed (sonication under vacuum or sparging with helium), the separation conducted under pressure or the frit surface be modified with octadecylsilane to reduce the above-mentioned zeta potentials differences. Seifar et al.[42] as well as Bailey and Yan[124] recommended the utilization of the surfactant (as an additive to the acetonitrile/buffer mobile phase) such as sodium dodecyl sulfate (SDS) below the critical micelle concentration to stabilize the EOF. Different utilization of a surfactant proposed Ye et al.,[129] who utilized cetyltrimethylammonium bromide (CTAB) added to the mobile phase for dynamic modification of a bare silica gel used as a stationary phase. After CTAB ions were adsorbed on the silica particles, their hydrophobic parts worked as a stationary phase for neutral compounds separation.

VI. MONOLITHIC COLUMNS

The problems of bubble formation in packed CEC columns can be reduced by the utilization of (1) packed fritless columns or (2) monolithic columns. In the first method tapers (external or internal) can be used in the outlet part of the capillary[130] to retain the bed particles without the need of sintering or the above-described methods of particle immobilization can be applied.[95,96]

The second approach relies on the utilization of a continuous, porous structure (a rod, monolith) as a stationary phase.[107,131-134] The concept of utilization of monoliths as separation media for capillary liquid chromatography was adapted from classic-size LC. Kubin et al. was probably the first to use the polymer monolith (poly(ethylene glycol methacrylate)) for separation (SEC of proteins) in 1967.[135] Later, in the early 1970s, several groups used polyurethane foams for gas[136-138] and liquid chromatography;[132,139] however, these materials did not exhibit the desired properties. Almost 2 decades later Hjertén et al. published the results of the separation of proteins performed on a compressed macroporous gel plug (copolymer of acrylic acid and *N,N′*-methylenebisacrylamide) 3 cm long and 6 mm in diameter.[140] Next, the extensive studies on preparation and utilization of monolithic columns have been performing since the beginning of the 1990s.

A. Polymeric Monolithic Columns

In general, the preparation of the polymeric monolithic column is very simple and follows the scheme presented on Figure 9. The empty capillary is filled to the desired length, with the polymerization mixture consisting of the monomers, initiator, and porogen solvent (see Figure 10). Then the polymerization is completed after thermal or photochemical initialization. Next, the column is flushed with the mobile phase using the pump or the electroosmotic flow.

1. Acrylamide-Based Monoliths

Acrylamide-based monoliths can be synthesized either in aqueous solution (or emulsion) or organic solvents. For example, Fujimoto polymerized acrylamide, *N,N′*-methylenebisacrylamide and 2-acrylamido-2-methyl-propanesulfonic acid in an aqueous solution containing ammonium persulfate

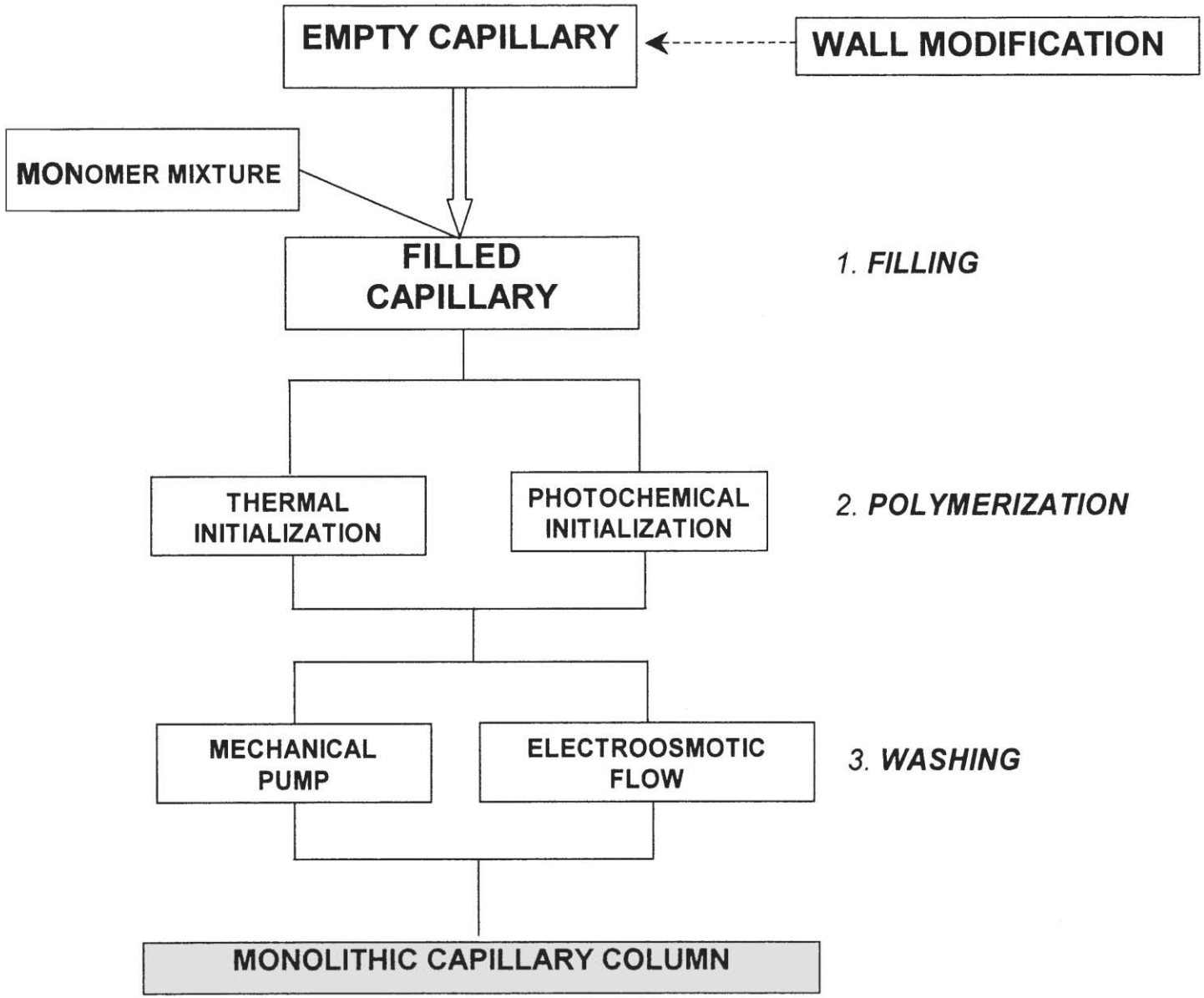


FIGURE 9. Preparation of polymeric monolithic capillary column. According to Ref. 133.)

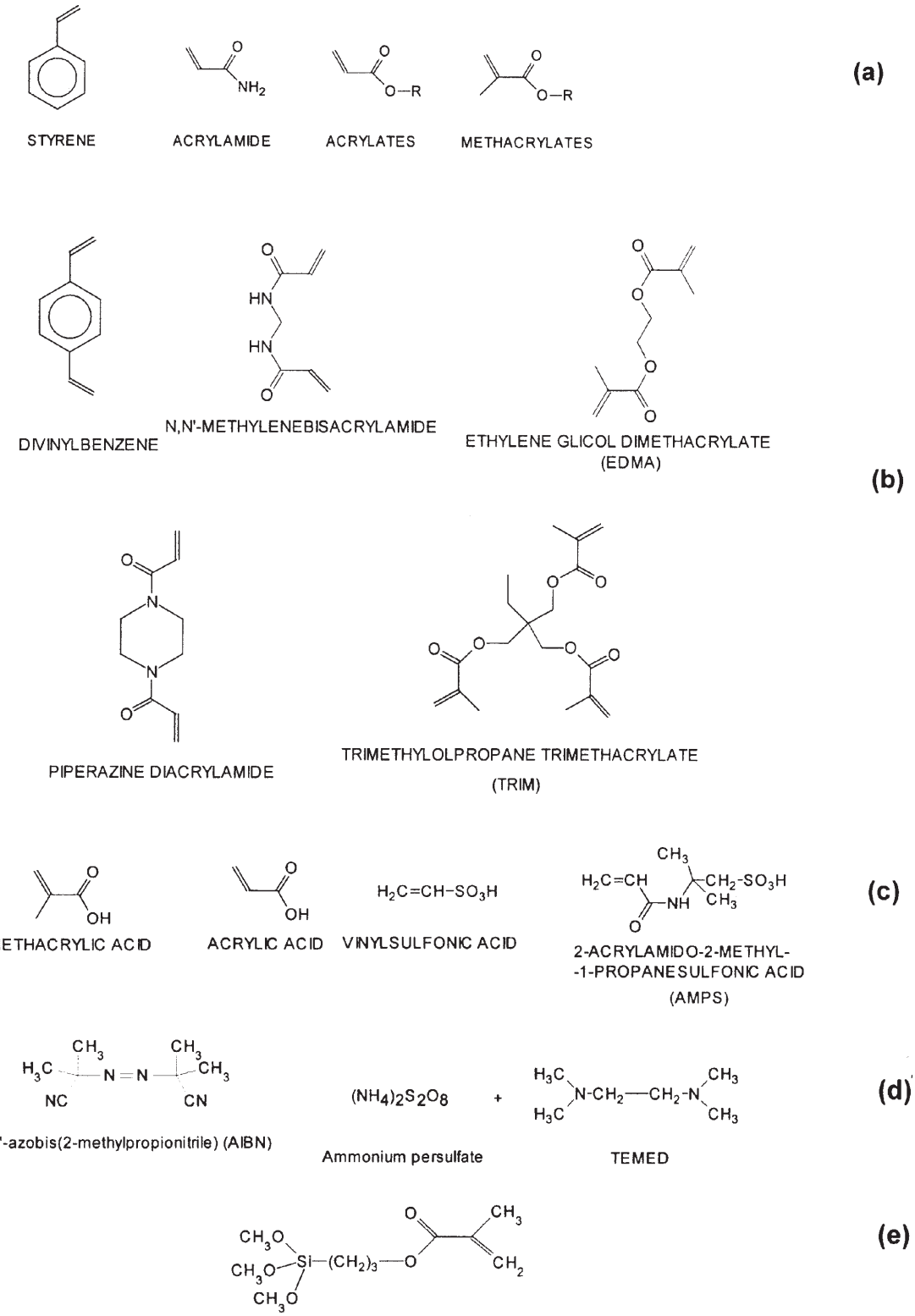


FIGURE 10. Reagents used for the preparation of the polymer monolithic columns: (a) monomers with functional groups, (b) crosslinking agents, (c) charged monomers responsible for the electroosmotic flow generation, (d) initiators, (e) silane for capillary wall modification.

and TEMED as an initializing system. The polymerization was performed in the capillary; the resulting charged gel was used for the separation of uncharged small molecules. The plate height of 6.9 μm was obtained for acetophenone; however, the retention times were rather long — 40 to 50 min. for the columns ca. 50 cm long. Fujimoto indicated that the mechanism of the separation on such a column was rather molecular sieving than solute-gel interactions.[141,142] The utilization of more hydrophobic monomer, *N*-isopropylacrylamide instead of acrylamide, allowed for the separation of steroids or polycyclic aromatic hydrocarbons (PAHs). Together with a quite high plate count for naphthalene (N = 107500/m), the retention times reproducibility was very good (% RSDs ranged from 2.71 to 3.56 for 7 days of operation). In this case the linear dependence of the retention factor k' on the mobile phase composition indicated a similarity to reversed phase chromatography.[143]

The preparation of highly cross-linked acrylamide-based monoliths was developed, for example, by Hjerten and co-workers.[144-146] For example, the polymerization mixture consisted of aqueous solution of acrylamide, piperazine diacrylamide, and vinylsulfonic acid. To control the hydrophobicity, butyl or stearyl methacrylate was also added. As these two compounds are not soluble in water surfactant was also added prior to emulsification by sonication. After initialization the emulsion was drawn into a capillary (75 or 100 μm) and left to complete the reaction. The properties of the columns were good; however, the separation of PAHs was greatly improved by: (1) changing the mobile phase composition or (2) addition of SDS to the mobile phase (see Figure 11).[145]

The process of sonication used for dispersion of water-insoluble monomers can be easily avoided if the proper solvent is used to dissolve all reagents (hydrophobic and hydrophilic). Palm and Novotny[147] used mixtures of aqueous buffer with *N*-methylformamide to obtain homogeneous polymerization solutions consisting of acrylamide, acrylic acid, methylene bisacrylamide, and alkyl acrylates (C4, C6, and C12). The buffer/*N*-methylformamide ratio varied from 50/50 for butyl acrylate to 5/95 for dodecyl acrylate. The addition of poly(oxyethylene) helped to obtain highly efficient columns. It is noteworthy that when changing the acrylamide/butyl acrylate ratio, the elution of the phenylketones changed (Figure 12).

2. Polystyrene-Based Monoliths

Wang et al. based on earlier experiences of polymeric beads (HPLC stationary phase) preparation and synthesized poly(styrene-*co*-divinylbenzene) rod in a large-diameter tube as far as in 1993.[148] More recently, the preparation of poly(styrene-*co*-divinylbenzene) monolithic capillary columns has been presented by Gusev et al.[149] and Xiong et al.[150]

Gusev et al. demonstrated the syntheses of rigid monoliths using mixtures of styrene and divinylbenzene as well as reactive vinylbenzyl chloride and divinylbenzene with a 0.1% addition of azobisisobutyronitrile (AIBN) as an initiator. The porous structure was provided by the porogenic solvents: methanol, ethanol, propanol, toluene, and formamide. The charged functional groups were introduced by reaction of surface chloromethyl groups with *N*,*N*-dimethyloctylamine. In such case positive-charged groups were introduced to provide the EOF. The columns were compared at *micro*-HPLC and CEC conditions and showed excellent efficiency (more than 200,000 plates/m), which was demonstrated on separations of peptides.

Great attention was paid to the capillary wall modification in order to provide the anchoring sites for the polymer, which resulted in a great mechanical strength under HPLC conditions. After treating the 75 μm i.d. capillary with NaOH at 120°C, it was washed with deionized water and acetone and dried with a nitrogen stream at 120°C. Then the capillary was filled with the solution of 50% (v/v) of γ-(trimethoxysilyl)propyl methacrylate in formamide containing 0.01% (w/v) of DPPH. DPPH was added as a inhibitor that prevents the polymerization via vinyl groups that could occur at elevated temperature. The reaction was performed at 120°C for 6 h, then the capillary was washed with acetone and dried. The necessity of using DPPH was documented with the SEM micrographs (Figure 13) that showed the quite large (comparing to the capillary dimensions) cleft between the polymer and capillary

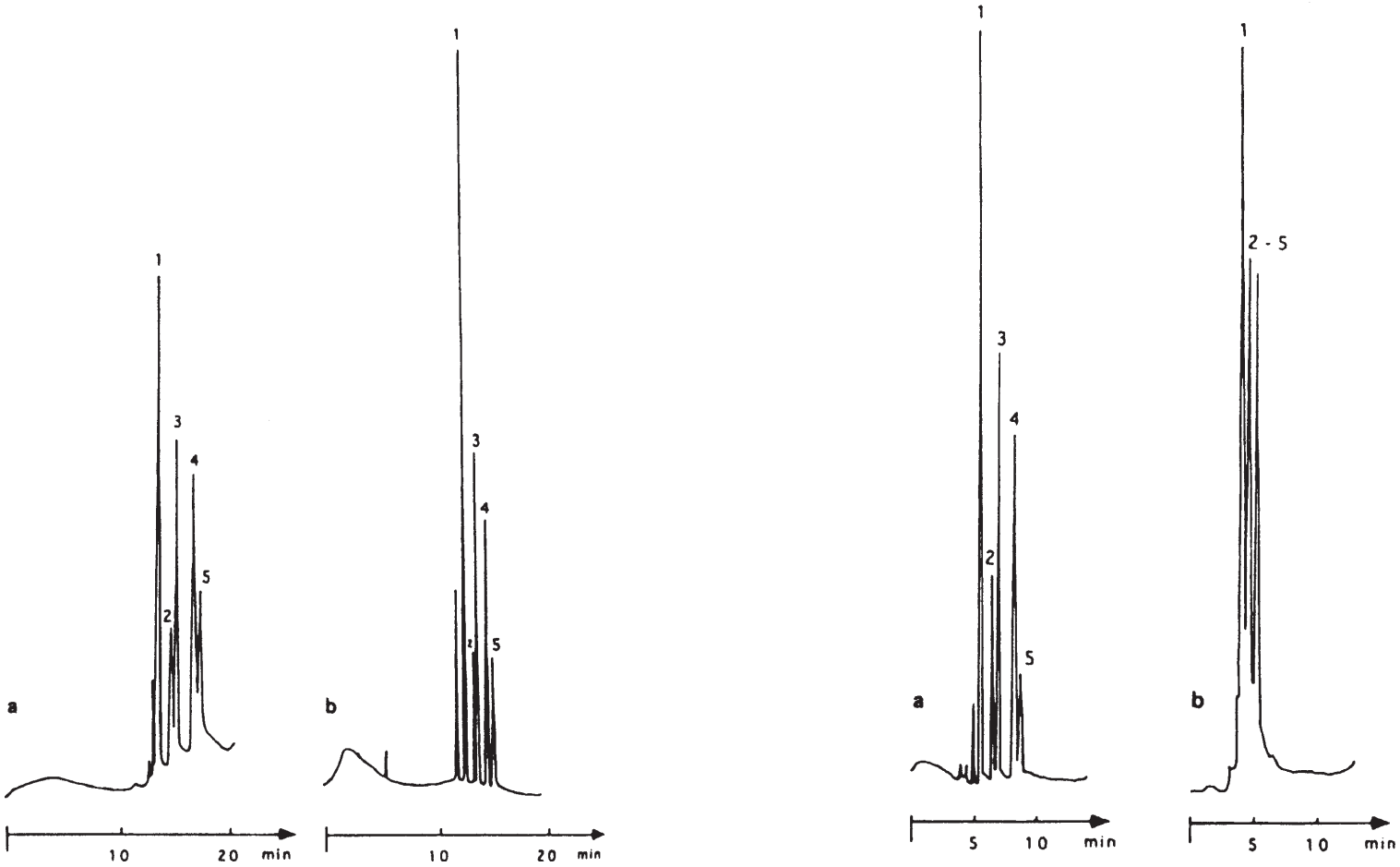
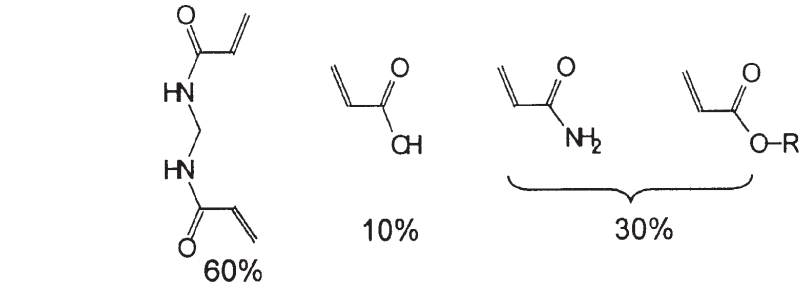


FIGURE 11. The effect of operating conditions on separation of PAHs: 1 — naphthalene, 2 — 2-methylnaphthalene, 3-fluorene, 4 — phenanthrene, 5 — anthracene. *Left chromatograms*: effect of changing mobile phase composition, column 16 cm of effective length, 75 μm i.d., voltage 3 kV, sample dissolved in 50/50 acetonitrile/buffer, (a) 60/40 isocratic separation, (b) separation started at 60/40 acetonitrile/buffer than changed to 70/30 acetonitrile buffer. *Right chromatograms*: effect of SDS addition, column 10 cm of effective length, 100 μm i.d., voltage 3 kV,: (a) mmol SDS added to the mobile phase, (b) without SDS. Mobile phase: 60/40 acetonitrile/4 mmol sodium phosphate pH=7.4. (According to Ref. 145.)



+ poly(oxyethylene) + (buffer+N-methylformamide) + (peroxidisulfate +TEMED)

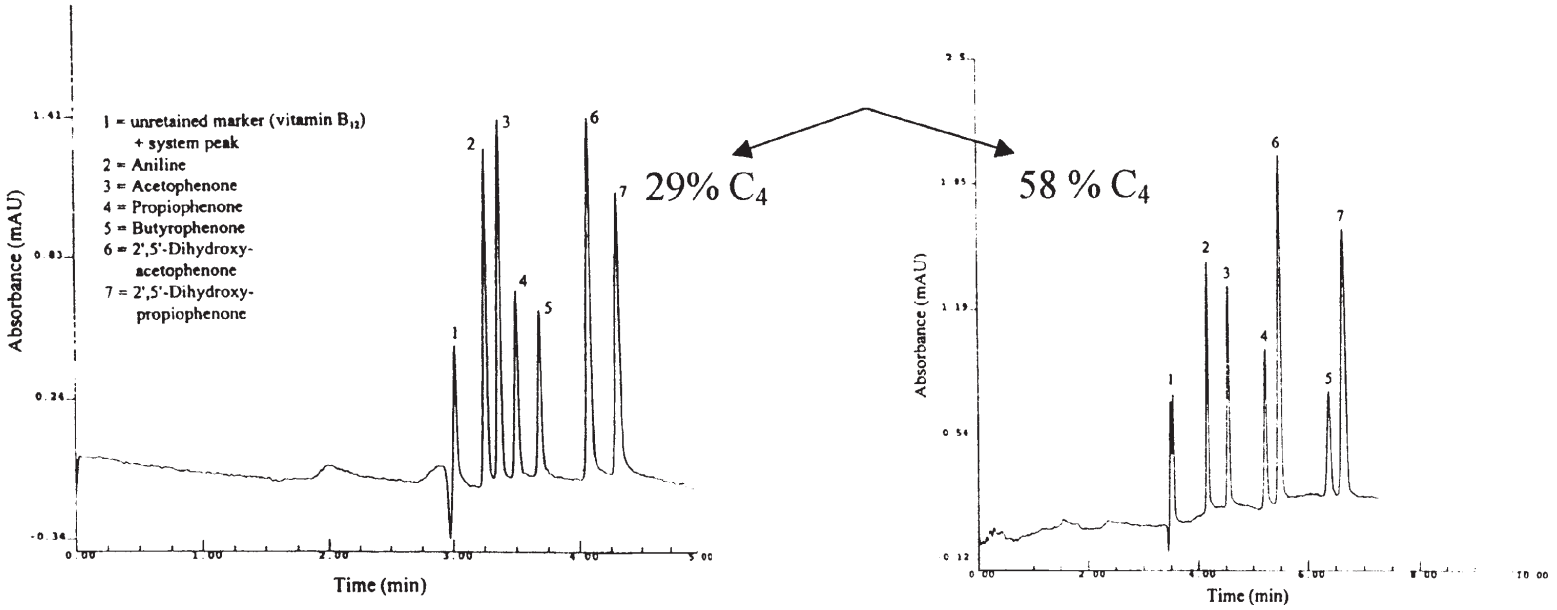


FIGURE 12. Effect of butyl acrylate content on retention of phenylketones separated on acrylamide-based polymer. (According to Ref. 147.)

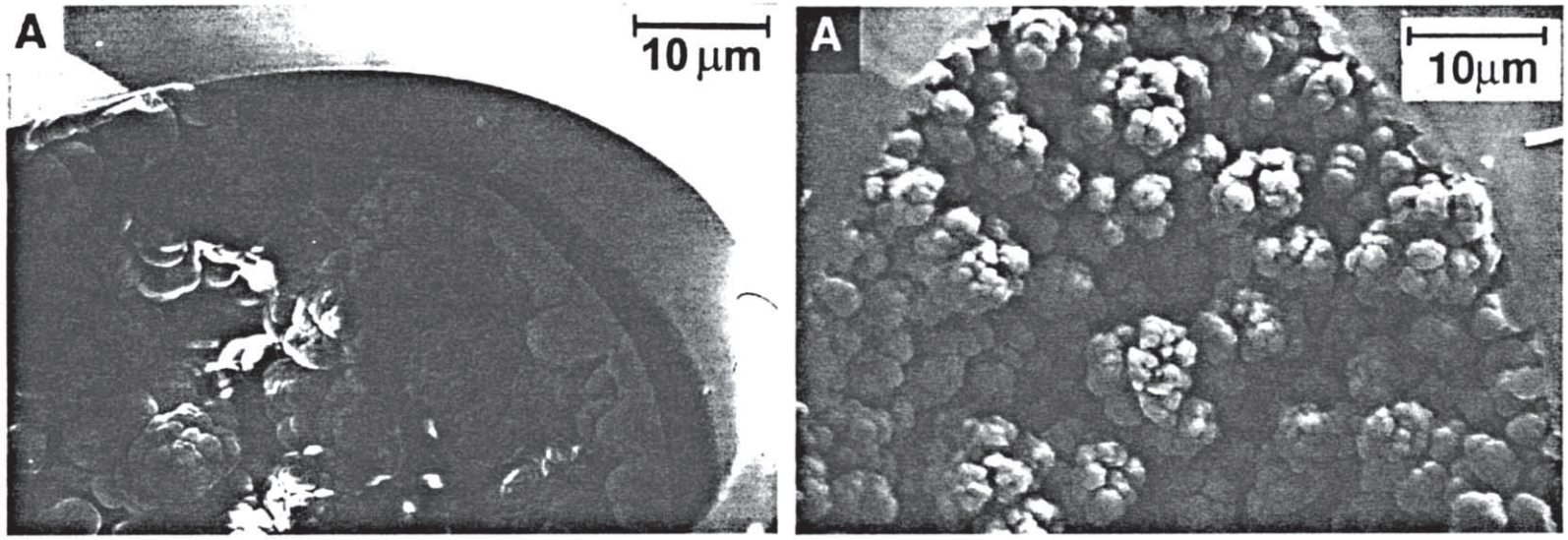


FIGURE 13. SEM micrographs of polystyrene based monoliths. Left: capillary wall modified without DPPH addition to the 50% (v/v) solution of γ-(trimethoxysilyl)propyl methacrylate in formamide. Right: picture: DPPH addition. (See text for details.) (According to Ref. 149.)

wall in the case when DPPH was not used (there were no free vinyl groups in that place). For a comparison Fujimoto,[142] following Hjerten,[151] modified the capillary using a mixture consisting of 40 μL of γ-(trimethoxysilyl)propyl methacrylate in 10 mL of 6 m*M* acetic acid and conducting the reaction at room temperature overnight.

Xiong et al. used 1:1 mixture of methanol with γ-(trimethoxysilyl)propyl methacrylate and also kept the capillary sealed at room temperature. No details on the wall-monolith connection were given. Poly(styrene-co-dininylbenzene) was synthesized using a mixture consisting of volume fractions of 5.0% styrene, 10% divinylbenzene, 5% methacrylic acid, and 80% toluene. Efficiencies ranged between 90,000 and 140,000 plates per meter; good separations of benzene derivatives were demonstrated.

3. Methacrylate-Based Monoliths

Very extensive studies on methacrylate-based monoliths were presented by Švec and co-workers.[131,133,134,152-157] The methacrylate monolithic columns were synthesized using R-methacrylate (R is a functional group, for example, butyl, glycidyl, *etc.*), ethylene glycol dimethacrylate (EDMA) as a crosslinking reagent, and charged 2-acrylamido-2methyl-1-propanesulfonic acid (AMPS). A ternary porogenic solution, consisting of water, 1-propanol, and 1,4 butanediol was specifically designed to obtain a homogeneous polymerization mixture of the above-mentioned hydrophobic liquid methacrylates and hydrophilic solid AMPS. Moreover, the porous properties of the monolith could be easily controlled by the percentage of 1-propanol (Figure 14).[155,156] As the electroosmotic flow depends on the number of charged functionalities, the increase of AMPS concentration increase the linear velocity of the mobile phase. However, it was found that keeping constant 1-propanol content in the porogenic solvent the pore size increased significantly with the AMPS concentration, which worsened the chromatographic properties of the monolithic column. Changing the 1-propanol content from 59.77 to 62.57% together with increasing content of AMPS (from 0.3 to 1.8%), it was possible to keep the mode pore diameter and pore volume on an almost constant level. It resulted in high separation speed (high AMPS concentration) and high efficiency (optimized pore size).

4. Molecularly Imprinted Monoliths

Chiral separations usually relies on interactions between enantiomers with a chiral selector. The selector that is a molecule possessing chiral atom or atoms can be a mobile phase component or can be chemically bonded to the stationary phase support. For separating a specific enantiomeric pair, many conditions have to be evaluated to obtain satisfactory results.[158] Molecular imprinting is a different approach for separation of chiral molecules and is a way toward artificial antibodies.[159-164] The scheme of the production of molecular imprinted stationary phase is shown in Figure 15.[165] One of the propranolol enantiomers used as a template is added to the polymerization mixture consisting of methacrylic acid, trimethylolpropane trimethacrylate (TRIM), and AIBN in toluene. Such a mixture was injected into the capillary and left to complete the reaction at -20°C under UV source (350 nm). After extraction, the imprints, possessing both a defined shape and arrangement of the functional groups, are formed. The eneatiomer used as a template is always more strongly retained as it fits to the cavity.

Lin et al.[166] have synthesized monolithic columns using L-phenylalanine anilide as a template and methacrylic acid and/or 2-vinylpirydine as the functional monomers and EDMA as a crosslinker. The molar ratio 5:1 of crosslinker to monomer was chosen as providing the best resolution during the separation of phenylalanine enantiomers. The polymer was bound to the fused silica capillary functionalized with the vinyl groups (reaction of silanols with thionyl chloride followed by Grignard reaction with vinyl magnesium bromide).

B. Silica Rods

Unlike the synthesis of the polymeric monolithic columns the preparation of silica rods is

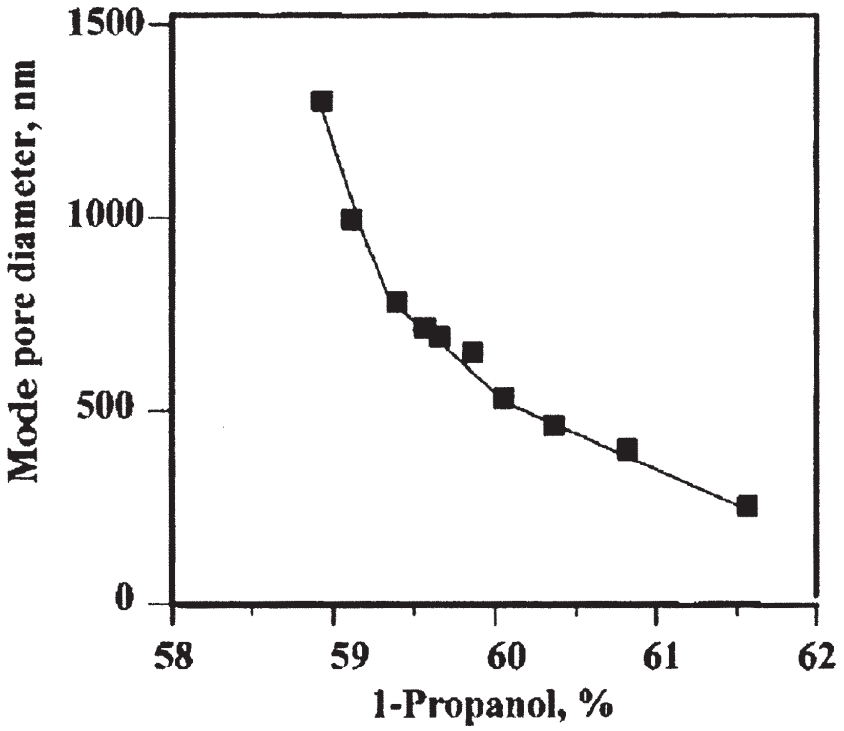


FIGURE 14. Effect of percentage of 1-propanol in the porogenic mixture on porous properties of monolithic polymers. Reaction conditions: polymerization mixture, ethylene dimethacrylate 16.00 wt%, butyl methacrylate 23.88 wt%, 2-acrylamido-2-methyl-1-propane-sulfonic acid 0.12 wt%, ternary porogen solvent 60.00 wt% (consisting of 10 wt % water and 90 wt% of mixtures of 1-propanol and 1,4-butanediol), azobisisobutyronitrile 1 wt% (with respect to monomers); polymerization time 20 h at 60°C. (According to Ref. 156.)

more complicated (see Figure 16); however, their properties make them very attractive.

Minakuchi and co-workers[167] reported the preparation of porous silica rods in 1996. The preparation relies on a hydrolythic polymerization of tetramethoxysilane accompanied by phase separation in a presence of water-soluble organic polymer. The resulting spongy structure typically consists of 0.3 to 5 μm silica skeletons, 0.5 to 0.8 μm through-pores and 2 to 20 nm mesopores in the skeleton. The mesopores size was controlled by the treatment with the aqueous ammonia as well as the temperature. For example, washing with 0.01 *M* ammonia produced silica rod with 14-nm mesopores, while 1.0 *M* ammonia gave 25 nm pores. In general, it was found that the chromatographically important size range of mesopores, between 5 and 25 nm median diameter, could be obtained by the utilization of 0.001 to 1 *M* ammonium hydroxide solution with the temperature up to 120°C.[168] A different procedure used urea as a hydrolyzable additive to the reaction mixture to provide high pH.[169] On the other hand, tetramethoxysilane/poly(ethylene oxide) ratio was found to have the effect on skeleton size and through-pores size.[168] In comparison to the chromatographic beds formed with silica particles, rod columns are characterized by a much lower pressure drop and a very good efficiency together with its low dependence on the mobile phase velocity.[167]

The great advantage of silica rod is possibility of derivatization using conventional methods developed for silica particles. For example, Ishizuka et al. derivatized silica rod prepared in a 25 cm × 100 μm i.d. fused silica capillary using octadecyldimethyl-*N*,*N*-diethylaminosilane at 60°C. The efficiency obtained was 5500 to 13,000 plates; however, small electroosmotic mobility was obtained due to the high surface coverage of the silica skeleton.[170] Fujimoto applied a slightly

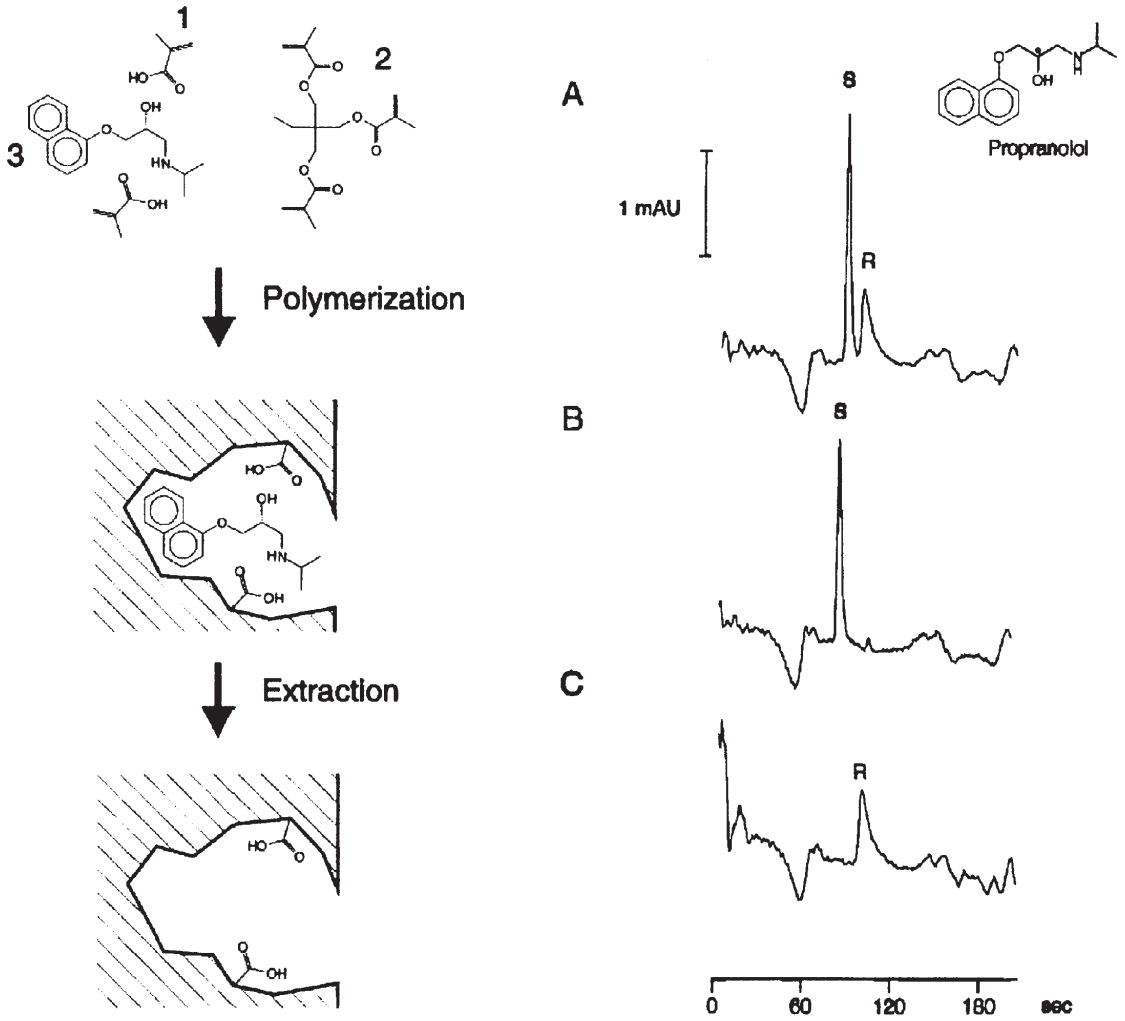


FIGURE 15. Examplary synthesis of monolithic molecularly imprinted polymer and the separation using a capillary column containing imprints of (R)-propranolol. The electrochromatograms show the separation of 100 μ*M* rac-propranolol (A), 50 μ*M* (S)-propranolol (B), and 50 mM (R)-propranolol (C). The samples were injected electrokinetically (5 kV, 3 s) and were separated at a constant voltage of 30 kV (857 V/cm). Acetonitrile/4 *M* acetate pH 3.0 (80/20 v/v) served as the electrolyte. UV detection was carried out at 214 nm. The capillary (75 mm i.d.) was thermostated to 60°C, and an overpressure of 7 bar was applied. (According Ref. 165.)

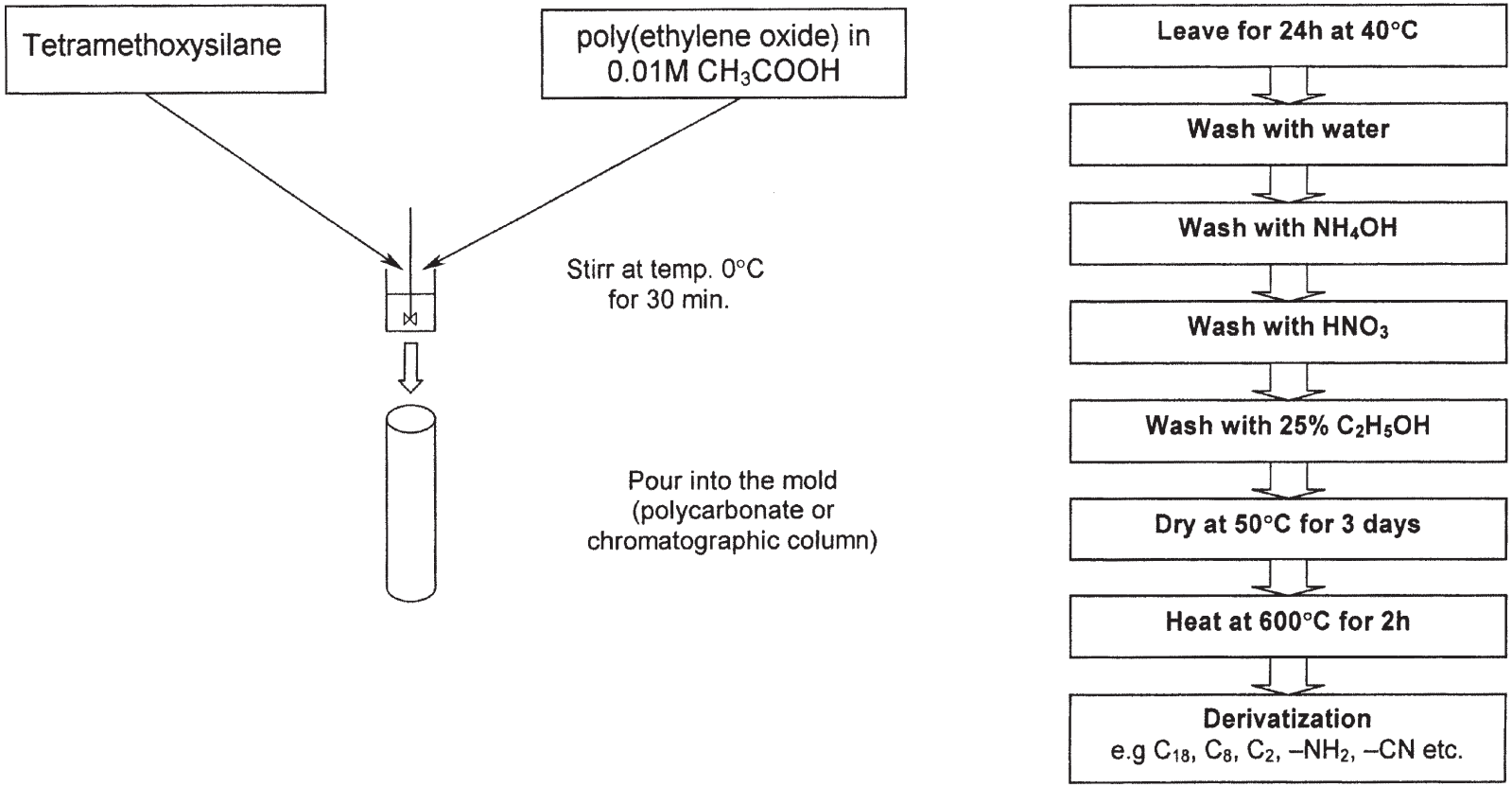


FIGURE 16. Exemplary scheme of fabrication of a silica rod. (According to Ref. 167.)

modified procedure of silica rod fabrication and obtained different rod morphology.[171] After modification with dimethyloctadecylchlorosilane good efficiencies 93,300 plates/m were obtained for valerophenone and 112,800 plates/m for methylparaben under CEC conditions. The EOF velocity was linearly dependent on the applied voltage and achieved approx. 1mm/s at 25 kV.

VII. COLUMN EVALUATION

Assuming that the separation system is well constructed in terms of dead volumes and shapes of the parts (see theoretical considerations) the obtained efficiency will depend only on the column quality. The evaluation of the separation column (i.e., "the part of the system where the separation takes place", e.g., the column or the channel of the chip) is usually performed when (1) new column is prepared — so it obvious to get to know whether it was well done, (2) evaluation of the column after some time of usage — to know whether the column parameters are kept, or (3) to compare different columns even if they are nominally the same, for example different C-18 phases.

So far, the evaluation of the columns, in the terms of its chromatographic performance, has been widely described for these packed with particles; however, some parameters can, of course, be used in the case of monolithic columns. Very useful compendia on this subject have been proposed, for example, by Bristow and Knox[172] or Buszewski et al.[173]

In general, in a pressure-driven system the quality of the column is evaluated from the point of view of resolution, analysis time, and pressure drop (ΔP) during the analysis. Such parameters as number of theoretical plates (N), height equivalent to the theoretical plate (H), and peak shape are taken into account. Different columns (packed with particles) can be compared using reduced parameters, that is, reduced plate h and reduced velocity ν:[172]

$$h = \frac{H}{d_p} \tag{13}$$

$$\nu = \frac{u d_p}{D_M} \tag{14}.$$

As the column efficiency (H) depends on a velocity of the mobile phase (u), very important data on the column quality can be obtained from $H = f(u)$ (or $h = f(\nu)$) plot. For a "good" column low H value arises slowly with the increase of the mobile phase flow. Basing on van Deemter equation:

$$H = A + B/u + Cu \tag{15}$$

which is usually applied for particle diameter $d_p >$ 25μm, for smaller particles Eq. (15) can be modified, and expressed as:

$$h = B/\nu + A\nu^{0.33} + C\nu \tag{16}$$

where A is related to the mobile phase flow between the particles and depends on the packing quality, B shows the contribution of the axial diffusion, and C is related to the mass transfer between stationary and mobile phases. These dimensionless parameters can be determined from $\log h = f(\log \nu)$ plot. Typical values are $A < 2$, $B < 4$, $C < 0.2$.

The column that is well packed and stable shows linear correlation between the pressure drop and the flow rate of the mobile phase.[173] Buszewski et al.[174] proposed a very effective and simple method for the evaluation of the column quality. It relies on rapid pulse changes of the direction of the mobile phase flow through the column. As the preferential sorption of one of the mobile phase component (methanol-benzene, THF-water, acetonitrile-water, methanol-water) changes with the pressure, the change of its concentration can be observed and detected using refractometric detector. As a result, the peaks similar to solvent peaks are obtained. If the peaks above and below the baseline are symmetric, the column is well packed. The main drawback of the method is that the rapid changes in flow direction break the column if it is not well packed.

A very effective parameter used for the evaluation of the column quality is a separation impedance E defined by Knox and Bristow as:

$$E = h^2\varphi = \frac{H^2}{K} = \frac{t_0 \Delta P}{N^2 \eta}, \quad (17)$$

where h is a reduced plate height and φ is a column resistance factor, and K is a chromatographic impedance (resistance). E is a very convenient parameter and it may be used for the characterization of packed and monolithic columns because K does not depend on particle size:

$$K = \frac{uL\eta}{\Delta P} = \frac{\eta L^2}{\Delta P t_0}. \quad (18)$$

According to Knox the column good columns exhibit $E < 2000$, that is, in the case of packed column $h = 2$ and $\varphi = 500$. In general — the higher efficiency and the lower pressure drop — the better is the column. Because the capillary columns are less densely packed than classic ones the column resistance factor may be lower than 500.[77] However, the pressure drop produced by a capillary column depends not only on the adsorbent bed but also on a quality of sintered frits (such effects do not play a role in classic column performance). There must be a compromise between their mechanical strength and permeability. The frits fabricated in a not optimal way (it must be experimentally determined for each stationary phase) could be too "weak" against the pressure or almost clog the capillary giving enormously high-pressure drop.

In the evaluation of the monolithic columns, the plate height values (H) are taken into account as well as pressure drops. For example, Minakuchi et al.[167] and Ishizuka et al.[170] compared silica rods with packed columns showing the plots of pressure drop vs. linear velocity and plate height vs. linear velocity (van Deemter plots). Moreover, E (separation impedance) values (Eq. 17) they calculated were as low as 400 at low flow rates.

As it was already mentioned, in CEC no pressure drop is observed — see Eq. 11. Hence, the evaluation of the column quality is based on limited number of parameters. Here, the plate height (H) or reduced plate height (h) can give some information on the column quality. In authors' opinion, if it is possible to perform, packed and monolithic capillary columns should be evaluated rather under HPLC conditions. In this way more data about the column quality can be obtained.

At present, the columns are often evaluated in the terms of the chromatographic characterization of the stationary phases, toward such characteristics as hydrophobicity, silanols activity, steric selectivity, or quantitative structure-retention relationships (QSRR). To do that it is very important to choose model compounds that will characterize the column. Various tests have been proposed in the literature, most of them have been compared, for example, by Claessens et al.[200] They are summarized in Table 4. The authors indicated that for compared tests good correlation between hydrophobic selectivity was observed; however, the silanol activities are not in agreement (except modified Engelhardt test and Tanaka test).

VIII. SEPARATIONS ON CHIPS

Continuous sample monitoring is an essential problem in a control of industrial or chemical production processes. This fact led to the development of automated analytical systems and induced the concept of *total analysis system* (TAS). The aim of the total analysis system is to automatically perform complete analysis, that is, sampling, transport, derivatization, and chromatographic or electrophoretic separation. Because of some disadvantages of these systems, such as poor separation speed and efficiency, slow sample transport and high chemical consumption, miniaturized TAS (μ-TAS) systems were developed.[186,187]

The application of micromachining techniques for fabrication of chip separation systems has been a trend for last decade. Unlike in column system the separation is conducted in microchannels etched on a small plate (Figure 17). The fabrication of such structures originates from microelectronic industry, where it has been used for integrated circuits production. In general, the process uses photolithographic and wet chemical etching schematically presented on Figure 18 followed by thermal bonding of a covering plate.[189-191] The materials that have been used are usually silicon, quartz, glass, or polymers; however, ma-

TABLE 3
Importants dates in monolithic column development.

Monolithic columns history

1967 – Kubin and coworkers - used highly swollen poly(2-hydroxymethyl methacrylate) for SEC of proteins

1970 – 1974 – several groups used polyurethane foams in GC and LC

1989 – Hjertén used compressed macroporous gel plug (copolymer of acrylic acid and N,N'-methylenebisacrylamide) for separation of proteins in cation exchange chromatography

1991 – Nakanishi and Soga prepared silica monolith using tetramethoxysilane

1992 – Švec i Frechet – monolithic polymeric column for HPLC

1995 – Hjertén used highly crosslinked acrylamide-based beds for CEC

1996 – monolithic silica columns

jority of such microsystems are fabricated in silicon or glass wafers.

The separation techniques that have been developed using chip systems comprise mainly electromigration techniques that are capillary elecrophoresis (CE),[190,199] capillary gel electrophoresis (CGE),[200] capillary isoelectric focusing (CIF),[201] micellar electrokinetic chromatography (MEKC),[202] capillary electrochromatography (CEC),[203,204] or HPLC.[204,205]

Capillary electrophoresis chip was first used in 1992.[190,192] Manz and Harrison have used the device presented on Figure 19. The set up consists of a base plate in which system of channels is etched. The separation is performed in channel 3 (30 μm wide and 10 μm deep), while the mobile phase is supplied by channel 1 (1 mm wide and 10 μm deep) from reservoir 1. The sample is injected by means of a syringe to fill channel 2, then the channels 1 and 3 are flushed with the buffer. A voltage applied between inlets 2 and 4 cause the EOF and draws the sample through the intersection point (ca. 9 pL). Subsequent voltage application between 1 and 3 introduces the sample to the separation channel 3. The number of the theoretical plates was quite high for separated dyes calcein (35,000) and fluorescein (19,000).

The utilization of quartz or glass provides the presence of the electroosmotic flow. However, in some cases it is advantageous to minimize this phenomenon. For example, Jacobson et al.[188] after the fabrication of a chip presented on Figure 17 covalently bonded acrylamide after modification of the wall with γ-(trimethoxysilyl)propyl methacrylate. Three metals, Zn, Cd, and Al, complexed with 8-hydroxy-quinoline-5-sulfonic acid (HQS), were then separated in less than 20 s. The utilization of laser-induced fluorescence detector (LIF) allowed for the detection of subfemtomole quantities of these metals.

Small channels of a chip can be very convenient tool for manipulation and processing of cells, permitting continuous observation due to the planar geometry. Li and Harrison observed transport of various cells through the micro device channels. By applying voltage in different reservoirs the direction of the transport could be changed as well as SDS could be applied causing lysis of the erythrocyte cells (see Figure 20).[206]

One of the advantages of chip devices is the possibility of performing almost complete analysis with, for example, derivatization. Wang et al. have used a simple pattern chip with precolumn derivatization channel (200 μm wide 3.6 mm long) that was wider than separation channel (50 μm wide and 74 mm long — Figure 21).[207] Efficient separation of OPA-derivatized amino acid could be performed during approx. 6 min.

TABLE 4
Tests for the evaluation of chromatographic columns.

Test	Testing solutes, mobile phase
Engelhardt (ref. 176, 177)	p-ethylaniline, aniline, phenol, ethylbenzoate, ethylbenzene, toluene, N,N-dimethylaniline, triphenylene, o-terphenyl Eluent: methanol/water 49/51 or 55/45 hydrophobicity = $k_{ethylbenzene}/k_{toluene}$, silanol activity= asymmetry of p-ethylaniline. Eluent: methanol/water 75/25 or 79/21 shape selectivity = $k_{triphenylene}/k_{o\text{-}terphenyl}$.
Galushko (ref. 178)	Uracil, aniline, phenol, benzene, toluene, Eluent methanol/water 60/40 hydrophobicity = $(k_{toluene}/k_{benzene})/2$; silanols activity = $1 + 3[(k_{aniline}/k_{phenol})-1]$ hydrophobic selectivity as well as size selectivity calculated from the phenol, toluene and benzene retention data retention expressed as a function of differences in free energies of solvation of the molecule and the mobile phase
Walters – silanol (ref. 179) Walters – hydrophobicity index	N,N-diethyltoluamide, anthracene; 100% acetonitrile Silanol activity = $k_{N,N\text{-}diethyltoluamide}/k_{anthracene}$. Benzene, toluene anthracene; Eluent: acetonitrile/water 65/35 hydrophobicity = $k_{anthracene}/k_{benzene}$.
Horvath (ref. 180)	N,N-dimethylaniline, triethylamine as silanol scavenger
Kaliszan (*e.g.* in ref. 181-183)	QSRR method – structure-retention relationship
Tanaka (ref. 184)	Uracil, thiourea, amylbenzene, butylbenzene, triphenylene, o-terphenyl, caffeine, phenol, benzylamine 1. methanol/water 80/20; hydrophobicity = $k_{amylbenzene}/k_{butylbenzene}$, amount of alkyl chains = $k_{amylbenzene}$, steric selectivity = $k_{triphenylene}/k_{o\text{-}terphenyl}$. 2. methanol/water 30/70; hydrogen bonding capacity = $k_{caffeine}/k_{phenol}$ 3. methanol/aqueous 0.02mM phosphate buffer pH=7.6 30/70; ion exchange capacity (IEC) at pH>7 =

	$k_{benzylamine}/k_{phenol}$. 4. methanol/aqueous 0.02mM phosphate buffer pH=2.7 30/70; ion exchange capacity (IEC) at pH<3 = $k_{benzylamine}/k_{phenol}$. Characterization in terms of surface coverage type of silanes, amount of silanols and ion exchange sites, hydrophobicity, steric selectivity, hydrogen bond capacity
Buszewski and Cendrowska (ref.185)	Uracil, bronopol, acetophenone, phenol, benzene, toluene Eluent: acetonitrile/water 80/20; classification of column quality measured as a selectivity with respect to bronopol, applied as an indicator of packing density, resolution and column lifetime.

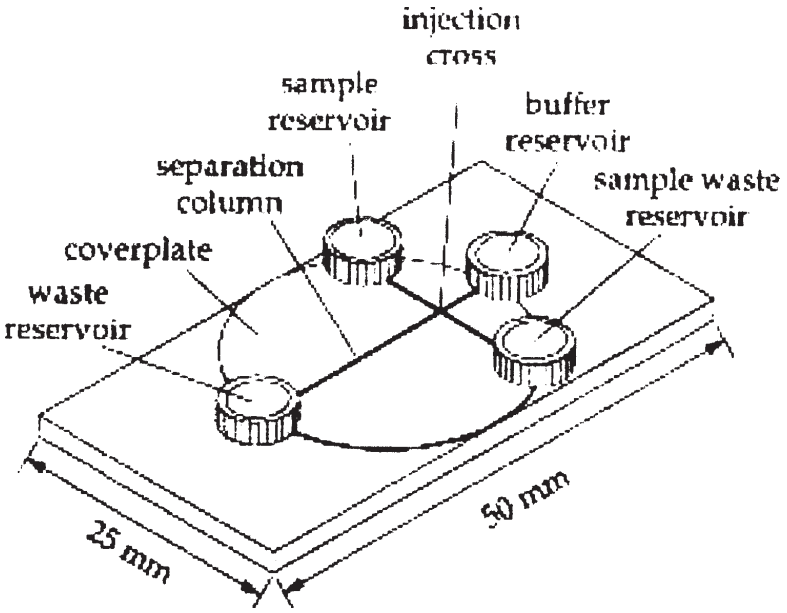


FIGURE 17. Schematic representation of a simple chip device. (According to Ref. 188.)

Despite all above-described separations have been performed on straight channel, the micromachining process allows for etching any pattern of the channels, for example, serpentine.[205,208] However, the length of the separation channel is usually constant and limited by the size of a wafer. A very interesting approach is the utilization of synchronized cyclic capillary electrophoresis. The separation can be performed in a cyclic device in which four separation channels are connected to form a square (see Figure 21). Applying voltages between appropriate buffer reservoirs allows for rotation of a sample around a channel, thus increasing separation column length. The plate heights obtained for Arg-FITC were 0.686 μm and 0.485 μm for MEKC and CGE respectively. The analysis time was much shorter than obtained on 75 μm × 50 cm capillary.[202]

Ericson et al. have recently demonstrated the electro- and pressure-driven chromatography performed on a chip with the channels filled with acrylamide-based monolithic bed (Figure 23).[204] About 300,000 plates per meter were obtained for acetone on a monolith derivatized with isopropyl and sulfonic (EOF generation) groups. The van Deemter plot is almost the same for capillary monolithic column and the chip, the shape of the curve is almost flat between linear velocities of ca 0.5 to 1.4 mm/s, thus providing fast and highly efficient separation. The monolithic support could be derivatized also with other functional groups, for example, ammonium, which allowed to anion-exchange chromatographic separation.

IX. SOME FINAL REMARKS

Miniaturized separation techniques have been developing toward capillary column and chip techniques. Both have their advantages and disadvantages, however, chip techniques provide more possibilities such as covering both liquid chromatographic and electromigration methods, performing pre- and post-column derivatization and exhibiting extremely low chemical consumption and easiness of simultaneous analyses of many samples.[209] Moreover, it seems that the creation of gradient in such devices is less complicated to perform than in capillary columns, it depends only on channel pattern and the way of application of the electric field between inlet vials and the outlet one.[210,211] From a practical point of view, the utilization of chip separation devices may be restricted by the number of the suitable detectors. Here, only electrochemical and LIF (laser-nduced fluorescence) detectors proved their usefulness.

ACKNOWLEDGMENTS

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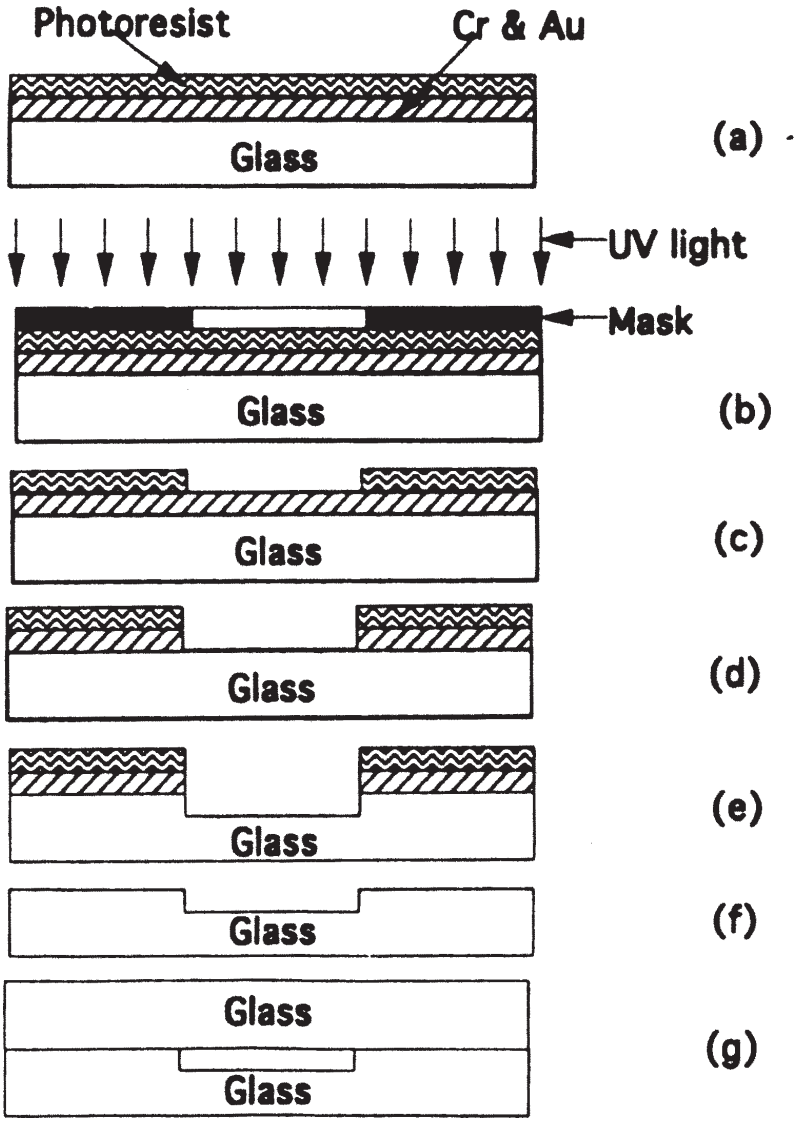

FIGURE 18. Photolitographic fabrication of chip devices: (a) Cr and Au masked glass plate coated with photoresist; (b) sample exposed to light through a master mask; (c) photoresist developed; (d) exposed metal mask etched; (e) exposed glass etched; (f) resist and metal stripped; (g) glass cover bonded to form capillary. (According to Ref. 191.)

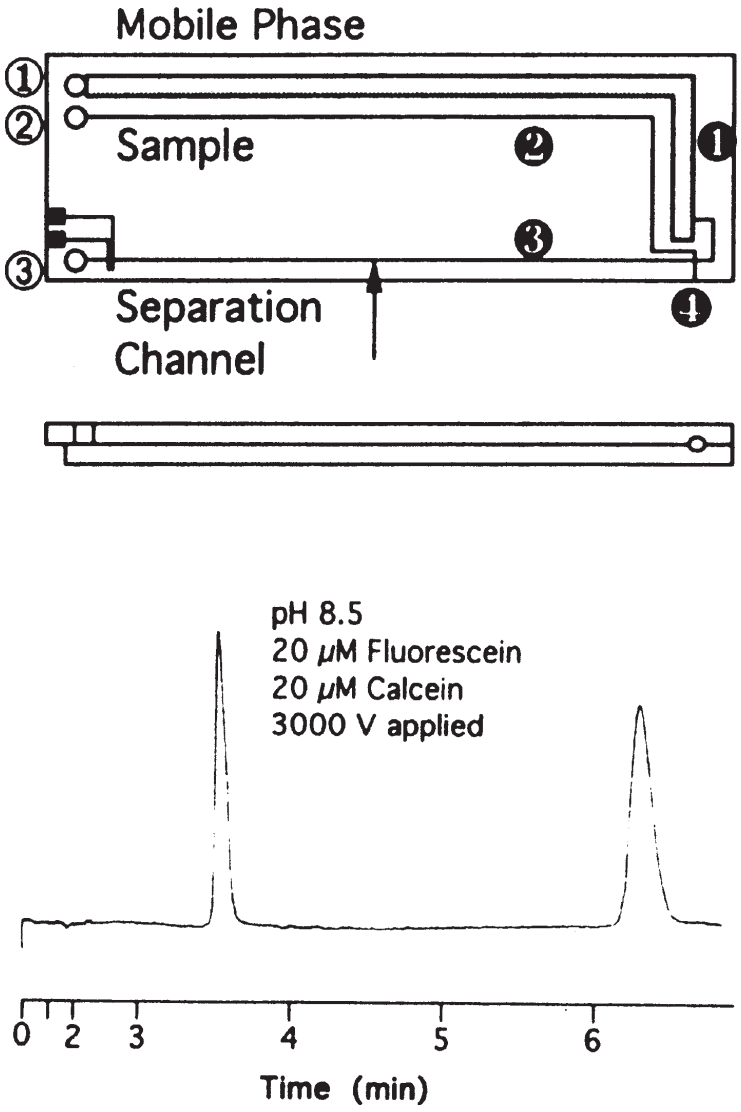

FIGURE 19. CE chip used by Harrison and Manz for the separation of dyes (calcein and fluorescein). Dimensions: $14.8 \times 3.9 \times 1$ cm. (According to Ref. 192.)

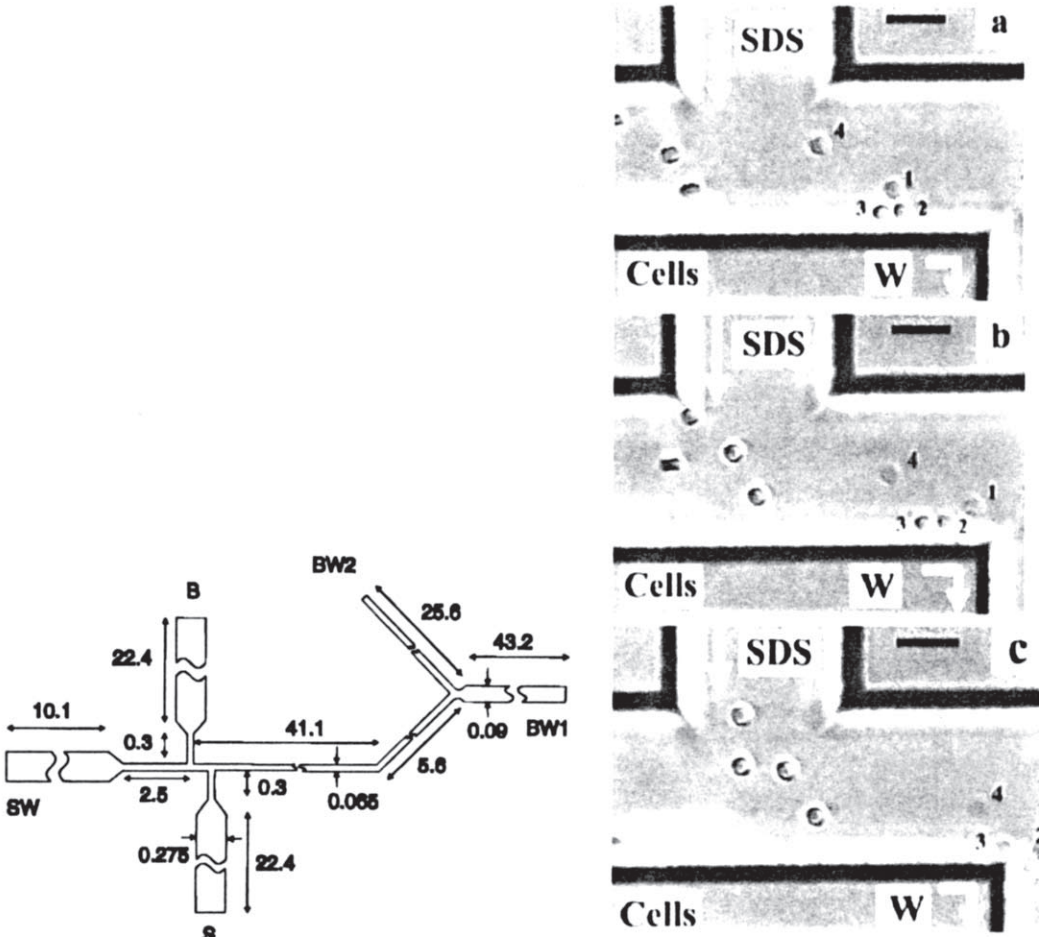

FIGURE 20. Photomicrographs of erythrocyte cell lysis observed in channels. Cells enter from the left and SDS from above. (According to Ref. 206.)

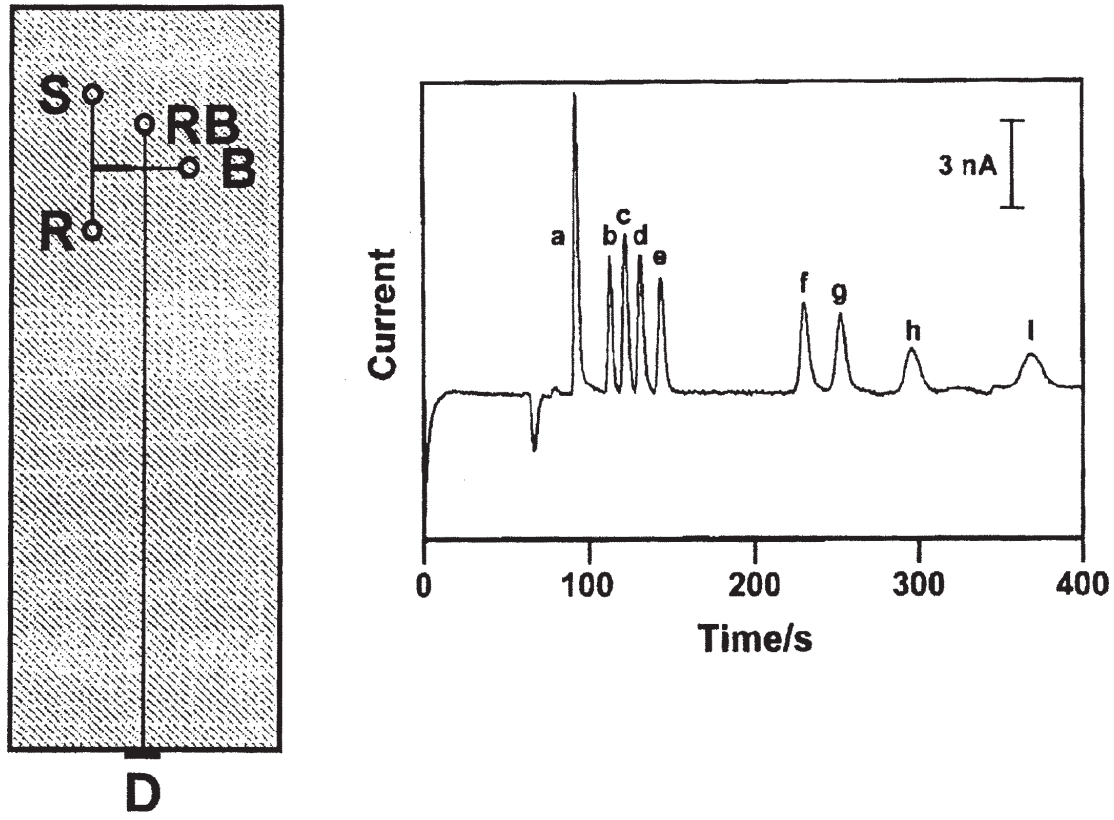

FIGURE 21. Simple chip for precolumn derivatization with electrochemical detection: S, R, RB and B are the reservoirs for sample, reagent, running buffer and buffer solutions. Electropherogram of the mixture containing 1.0×10^{-4} *M*: b — histidine, c- valine, d — isoleucine, e — leucine, 2.0×10^{-4} M: f — glutamic acid, g — aspartic acid, h — arginine, i — lysine. Buffer 20 mM borate + 30 mM SDS. Injection 1.5 kV, 3 s. Voltage 2 kV. Reagent solution: OPA/2ME (*o*-phtaldialdehyde + 2-mercaptoethanol). (According to Ref. 207.)

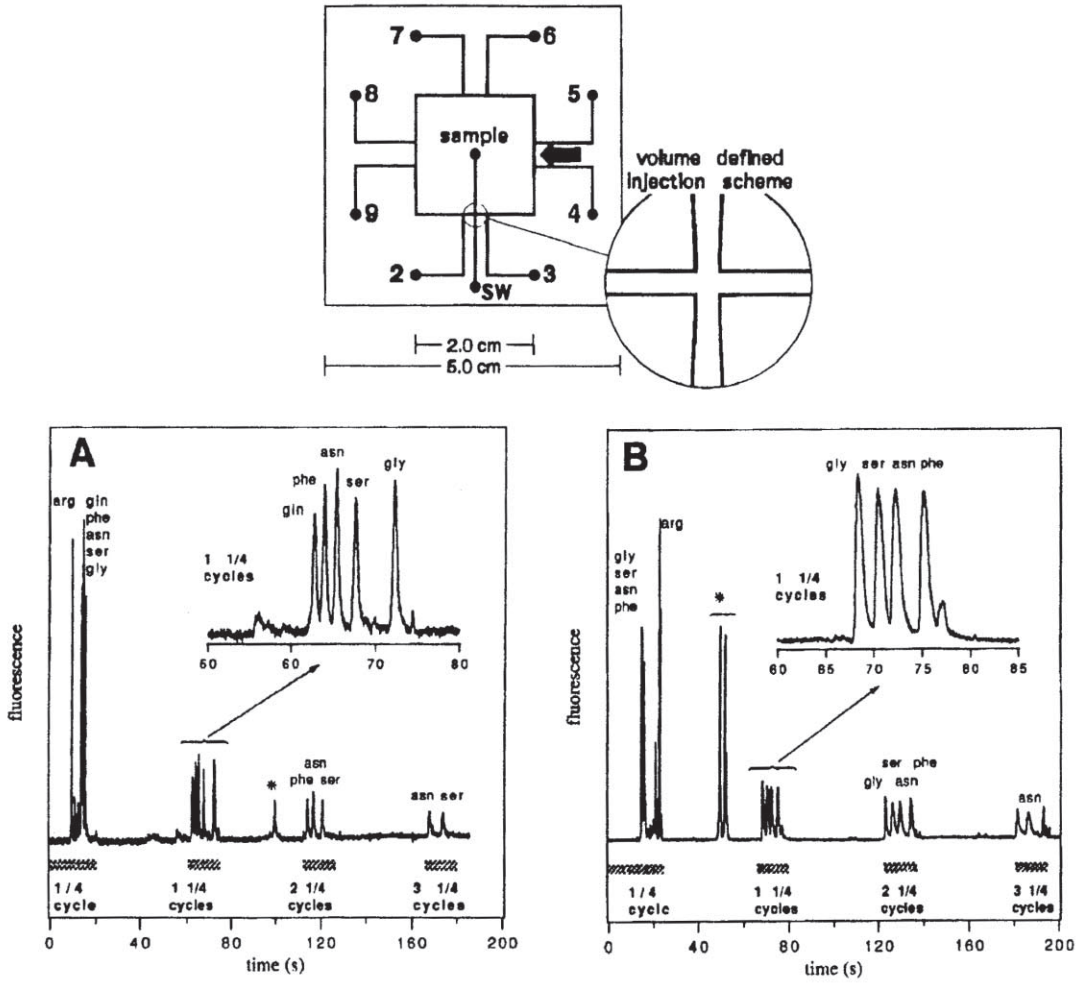

FIGURE 22. Schematic representation of a cyclic chip, with the channel width 40/20 μm top/bottom and 10 μm depth. Separation of FITC derivatives of aminoacids under MEKC (a) and CGE (B) is presented on bottom. (According to Ref. 202.)

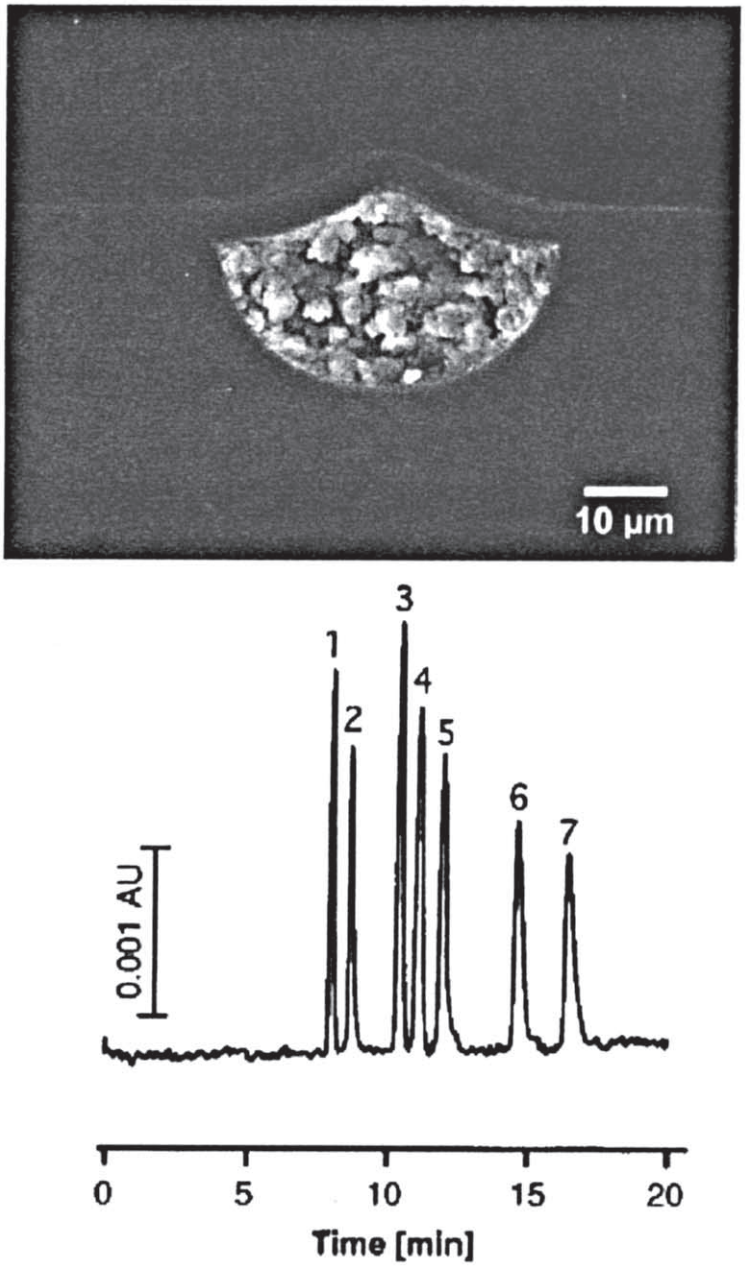

FIGURE 23. Channel filled with the monolithic support and electrochromatographic separation of alkyl phenones. Serpenine column geometry, 28.1 cm of effective length. Mobile phase: 30/70 ACN/5 m*M* sodium phosphate at pH 2.5. Voltage 16 kV, detection at 240 nm. Compounds resolved: 1—acetone, 2—aniline, 3—acetophenone, 4—propiophenone, 5—butyrophenone, 6—2,6-dihydroxyacetophenone, 7—2,5-dihydroxypropiophenone. (According to Ref. 204.)

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