Advances in CHROMATOGRAPHY

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VOLUME 41
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ADVANCES IN
Chromatography

VOLUME 41
# Fundamentals of Capillary Electrochromatography

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I. INTRODUCTION

Capillary electrochromatography (CEC) can be considered as a hybrid of capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC). From another point of view, CEC is a liquid chromatographic method in which the mobile phase is electroosmotically driven through the chromatographic bed. The interest in CEC stems from the underlying idea that the unique properties of the electroosmotic flow (EOF) make it possible to realize highly efficient liquid chromatographic separation systems that can overcome the peak capacity limitations of current HPLC.

HPLC is a method with much lower chromatographic efficiency and peak capacity than open-tubular gas chromatography (OTGC), which can be regarded as the method of choice for those analytes that can be transferred into the gaseous phase. For those analytes that are not sufficiently volatile, however, OTGC is not an applicable alternative to HPLC. The need to improve the chromatographic efficiency and the peak capacity for nonvolatile analytes makes the development of alternatives to classical HPLC one of the major fields of activity in contemporary chromatographic research. The following techniques can be regarded as more efficient alternatives to HPLC: (1) supercritical fluid chromatography (SFC) [1], (2) capillary electrophoresis (CE), (3) micellar electrokinetic chromatography (MEKC) [2], and (4) capillary electrochromatography (CEC). Of these techniques, CEC is the closest to HPLC because the stationary and mobile phases can be identical in HPLC and CEC. In the case of ionic, acidic, or basic analytes, CEC offers (in analogy to MEKC) the possibility to combine chromatographic separation with separation due to electrophoresis, making it possible to separate strongly basic, moderately basic, weakly basic, neutral, weakly acidic, moderately acidic, and strongly acidic compounds in one run [3].

According to Colón et al. [4], the term electrochromatography was introduced by Berraz [5] to describe a form of paper electrophoresis. In 1952 Mould and Synge [6,7] demonstrated the use of electroosmotic flow in a thin-layer chromatographic system for the separation of polysaccharides in collodion membrane strips. Already in 1974, Pretorius et al. [8] realized the possible advantages of a liquid
chromatographic technique with an electroosmotically driven mobile phase. They introduced high-speed thin-layer chromatography (HSTLC) as a variant of thin-layer chromatography (TLC) with an electroosmotically driven mobile phase and much shorter run times than were obtained with traditional techniques. They also applied the principle of an electroosmotically driven mobile phase to liquid column chromatography with promising results.

In the course of the development of capillary electrophoresis [9,10], the idea of electroosmotic pumping of mobile phase through a packed bed was taken up again. In their landmark paper in 1981, Jørgenson and Lukacs [11] showed for the first time the possibility of performing reversed-phase chromatography in packed capillaries employing virtually the same apparatus as developed for CE. Since these pioneering works, development of CEC has started and several names have been coined for this type of capillary separation technique: liquid chromatography with electroosmotic flow [12], electroendosmotically driven liquid chromatography [13], electrically driven liquid chromatography [14], electroendosmotic capillary chromatography [15], electrokinetic chromatography with packed capillaries [16], and capillary electrochromatography [17,18].

The last term is now generally accepted and is the one most widely used referring to a separation technique in which the separation is achieved by retentive interaction of the solutes to be separated with an immobilized second phase (the stationary phase), while a liquid mobile phase is employed that is driven through the chromatographic bed predominantly via the effect of electroosmosis.

II. BASIC CONCEPTS

A. Instrumental Setup

CEC can be performed with identical experimental setup as used for CE and MEKC except for the need to use a capillary that contains an immobilized second phase which is responsible for the chromatographic separation. Figure 1 depicts a block diagram of an apparatus used for CEC. In most cases the mobile phase is a solution of buffering salts in a mixture of an organic modifier with water. With these very polar mobile phases, reversed-phase separations are achieved.

No pump is needed. Injection of the sample is performed directly into the high-voltage end of the separation capillary, mostly by electrokinetic injection. In this case, the vessel with mobile phase
is replaced by a vessel containing the sample and a programmed trapezium-shaped voltage ramp is applied for a few seconds. With electrokinetic injection the sample is dragged into the capillary by the effect of electroosmosis. In the case of neutral analytes, no discrimination of sample constituents takes place. The simplicity of electrokinetic injection compared to the difficulty of injecting a few nanoliters via a mechanical injection device into a stream of pressurized mobile phase constitutes one important advantage of CEC over micro-HPLC.

Various types of separation capillaries have been used so far. The preparation of separation capillaries by packing with reversed-phase silica gels (virtually exclusively octadecyl silica gel) has received the most attention in the early studies devoted to CEC. With packed capillaries, frits are necessary at both ends of the packed bed to keep the stationary phase migrating out of the capillary. However, the manufacture of inlet and outlet frits to retain the stationary phase is not a trivial task and is associated with many problems that will be discussed in a later section. Consequently, there is increased interest in the production of fritless separation capillaries. One approach to avoid the use of frits is open-tubular columns that are coated on the inner surface with a layer of stationary phase. Other approaches comprise the synthesis of soft gels anchored covalently
to the capillary walls or the synthesis of mechanically immobilized rigid gels.

In order to avoid intolerable extracolumn band broadening, detection has to be performed in a segment of the capillary, transforming this segment into the detection cell of the detector. Independent of the type of separation capillary used, the detection cell can be placed in an open section of the capillary behind the chromatographic bed (on-column detection), or detection is directly in a segment of the chromatographic bed itself (in-column detection). In Fig. 2 the differences between these two detection modes are illustrated taking a packed capillary as an example.

**B. Propulsion of Mobile Phase**

Ideally, in CEC the mobile phase is driven through the chromatographic bed exclusively by the effect of electroosmosis. Electroosmosis is an electrokinetic effect originating from the electrophoretic movement of the diffuse layer of the electric double layer that is formed at the interface between the liquid and the solid. In CE, electroosmosis is generated only at the electric double layer formed

![Diagram](attachment:image.png)

**Fig. 2** Difference between (a) in-column and (b) on-column detection in a packed capillary.
at the inner capillary wall. In CEC with packed or gel-filled capillaries, however, the inner surface generated by the porous plug inside the capillary is much larger than the inner surface of the capillary wall (see Fig. 3). Consequently, properties of the inner surface of the porous plug, and not properties of the inner surface of the capillary wall, determine the obtainable electroosmotic velocity. These considerations have been convincingly corroborated by experiments of Dittmann and Rozing [19], who have packed coated and noncoated capillaries with identical stationary phase and found virtually no dependence of the determined electroosmotic mobility on the existence or nonexistence of the coating, although in open-tubular capillaries the electroosmotic velocity was effectively suppressed by the coating (see Fig. 4).

The main driving force behind the interest of pioneering workers in CEC has been the fact that the electroosmotically driven flow in a porous plug (i.e., a capillary packed with particles) is virtually independent of the mean channel diameter. Smoluchowski has shown that Eq. (1) retains its validity if an open capillary is replaced by a porous plug [20].

![Diagram](capillary_wall.png)

**Fig. 3** Generation of electroosmotic flow in a packed capillary.
Fig. 4  Comparison of chromatograms obtained for the separation of neutral solutes with a packed (top trace) polyvinylalcohol-coated fused-silica capillary or a (mirror trace) bare fused-silica capillary. Capillary, 335 (250) mm × 100 μm; stationary phase, 3-μm porous octadecyl silica gel (CEC Hypersil C18); mobile phase, 80% acetonitril, 20% aqueous buffer (25 mmol/L TRIS-HCl, pH 8); temperature = 20°C; 1 = thiourea, 2 = ethyl paraben, 3 = propyl paraben, 4 = butyl paraben, 5 = pentyl paraben, 6 = naphthalene, 7 = hexyl paraben, 8 = fluorene, 9 = anthracene, 10 = anthracene, 11 = fluoranthene. (Reprinted with permission from Ref. 19.)

\[
\frac{V}{i} = \frac{\varepsilon_D \varepsilon_0 \zeta}{\eta \lambda_0}
\]  

(1)

where \( V \) = volume of liquid displaced per unit time, \( i \) = electric current strength, \( \varepsilon_D \) = dielectric constant of the bulk liquid, \( \varepsilon_0 \) = electric permittivity of vacuum, \( \zeta \) = electrokinetic potential (zeta potential), \( \eta \) = viscosity of the bulk liquid, and \( \lambda_0 \) = specific electric conductivity of the liquid.

Equation (1) can be used to describe the electroosmotic flow through a porous plug, provided that the flow is laminar, the local
radius of curvature of the particles and the size of the pores are large compared to the thickness of the double layer, and the effects of surface conductance are negligible. Porous plugs may be made of particles of quite irregular geometry, nearly spherical particles, fibers, bundles of capillaries, or porous rods [21]. However, in CEC the velocity of the mobile phase is generally not given as flow rate (volume/time) but as linear velocity (distance/time) determined via a non-retarded marker. By most workers, it is assumed that this linear velocity is identical or at least proportional to the electroosmotic velocity $v_{eo}$.

$$v_{eo} = \frac{\varepsilon D \varepsilon_0 \zeta}{\eta} \cdot E$$

(2)

where $E = $ local electric field strength.

Provided the size of the pores is large compared to the thickness of the electric double layer, the linear velocity is virtually independent of the mean channel diameter and directly proportional to the local electric field strength. This property of the electroosmotic flow makes it possible to employ separation columns in CEC that cannot be used (because of their high streaming resistance) in pressure-driven LC. Knox and Grant [13] examined the experimental conditions under which $v_{eo}$ is independent of the mean channel diameter. Assuming that the mean channel diameter must be larger than 10 times the thickness of the electric double layer $\delta$, they predicted for slurry-packed capillaries that the particle diameter $d_p$ has to be at least 40 times the thickness of the electric double layer $\delta$ and for drawn-packed capillaries $d_p \approx 20\delta$.

The thickness of the electric double layer $\delta$ is a function of the electric permittivity $\varepsilon$ of the liquid phase and of the ionic strength $I = \frac{1}{2} \sum z_i^2 c_i$ [13]:

$$\delta = \sqrt{\frac{\varepsilon kT}{1000 N_A e^2 \sum z_i^2 c_i}}$$

(3)

where $k = $ Boltzmann constant, $T = $ temperature (in kelvin), $N_A = $ Avogadro constant, $e = $ charge of electron, $z_i = $ valence number, and $c_i = $ molar concentration.

Employing Eq. (3), Knox and Grant [13] calculated that for a mobile phase (solution of a completely dissociated salt in water) con-
taining a 1:1 electrolyte at a concentration of 1 mmol/L, δ = 10 nm; and for a slurry-packed capillary the minimum particle diameter is 0.4 μm. In a later paper [18], they corroborated these predictions by experimental results employing capillaries packed with silica gels and octadecyl silica gels of particle diameters ranging from 1.5 to 50 μm. In their studies, the electroosmotic velocity was virtually independent of the particle diameter. With a stationary phase (beads of 0.5-μm diameter) synthesized by means of a modified Stöber process, Lüdtke et al. [22] proved the applicability of packings with such fine material in CEC. The electroosmotic mobility observed with these packings is not significantly reduced compared to packings with material of larger mean particle diameter.

Regarding the plate-height equation for a packed column (pressure-driven mobile phase) [23–25],

\[
H = 2\lambda_p d_p + \frac{2\gamma D_M}{v} + \frac{(1 + 6k + 11k^2)d_p^2}{24(1 + k)^2 D_m} v + \frac{8kd_p^2}{\pi^2 (1 + k)^2 D_S} v
\]  

(4)

where \( H \) = height of a theoretical plate, \( \lambda_p \) = packing factor, \( d_p \) = mean particle diameter, \( \gamma \) = labyrinth factor, \( v \) = linear velocity of mobile phase, \( D_M \) = diffusion coefficient in the mobile phase, \( k \) = retention factor of solute, \( d_f \) = effective film thickness of the stationary phase, and \( D_S \) = diffusion coefficient in the stationary phase, the tremendous impact of the mean particle diameter on the chromatographic efficiency obtainable with a given column is obvious. While the constraint for independence of the electroosmotic velocity of the mean channel diameter is fulfilled for the interparticle pores, in the case of porous packing materials with mean pore diameters of 6–10 nm it is assumed that there is virtually no electroosmotic flow in the intraparticle pores because of double-layer overlap.

Another important feature of the electroosmotic flow is its flat streaming profile (compared to the parabolic streaming profile generated by pressure difference-induced laminar flow) reducing band broadening due to mass transfer resistance in the mobile phase, and reducing the \( A \) term of the plate-height equation. The different streaming profiles generated in the electro-driven and the pressure-driven modes are depicted in Fig. 5. This figure also illustrates the reduction of the \( A \) term of the plate-height equation through independence of flow velocity of the channel diameter.
III. THEOREY

A. Migration Principles

In CEC the linear velocity of the mobile phase is determined via a “nonretarded marker.” No studies are available concerning the accuracy of those measurements. With reversed-phase packing material, thiourea is commonly used as “nonretarded marker.” Generally, the so-determined linear velocity is treated as an equivalent of the electroosmotic velocity $v_{eo}$. However, in the case of on-column detection (open section of the capillary behind the chromatographic bed, see Fig. 2), differences in the linear flow velocity in the chromatographic bed and in the open section have to be taken into account. In the chromatographic bed the mobile phase takes up only a fraction $\varphi_M$ of the inner capillary volume. Hence, the velocity of the mobile phase in the open section equals $v_{eo}$ in the chromatographic bed multiplied by $\varphi_M$. The corrected linear velocity in the chromatographic bed can be calculated using Eq. 5 [26].

$$v_{eo\text{(corrected)}} = \frac{L_b + L_o/\varphi_M}{t_0}$$

(5)

where $L_b$ = length of the chromatographic bed, $L_o$ = length of the open section between the end of the chromatographic bed and the
Fundamentals of Capillary Electrochromatography  /  11

detection window, and \( t_0 \) = migration time of the nonretarded marker.

In the case of noncharged analytes, the separation mechanism is identical to that in liquid chromatography. The chromatographic retention factor \( k \) for a retarded solute can be calculated from the migration time of a nonretarded marker and the migration time of the retarded solute. This retention factor is identical to \( K \cdot (V_s/V_m) \) (where \( K \) = concentration distribution coefficient, \( V_s/V_m \) = volume ratio in the chromatographic bed: volume of stationary phase/volume of mobile phase). In HPLC it is commonly assumed that the fraction of time spent outside the chromatographic bed with respect to the total migration time \( t_m \) can be neglected. In CEC with on-column detection, however, this approximation might not be valid, especially in the case of short packed sections of the separation capillary [22]. In this case, the retention factor must be corrected using Eq. (6) [26].

\[
k(\text{corrected}) = \frac{v_e \cdot (\text{corr.}) \cdot t_m}{L_b} - \frac{L_o}{L_b \cdot \varphi_M} - 1 \tag{6}
\]

where \( t_m \) = migration time of the retarded solute.

Euerby et al. [27] have shown that for noncharged solutes there is a negative linear relationship between the logarithm of the retention factor and the volume fraction of acetonitrile in the mobile phase (see Fig. 6). This relationship is known for liquid chromatographic separations. Taking into account that the velocity of the mobile phase is dependent on properties of the mobile phase [see Eq. (2)], optimization strategies in CEC for the separation of noncharged solutes can in part go back to those developed for HPLC.

In the case of ionized solutes, however, the situation is completely different. In this case, the separation is effected by both electrophoretic and chromatographic principles. Considering acids or bases as solutes and reversed-phase material as stationary phase, the following simplifying assumptions can be made:

Only the noncharged species contributes to the interaction of the solute with the nonpolar stationary phase.

The migration velocity in the mobile phase can be regarded as superposition of electroosmosis and electrophoresis, while the electric field strength is constant along the separation capillary.
If these assumptions are fulfilled, the migration velocity \( v_m \) of a solute zone in the chromatographic bed is given by [28]

\[
v_m = \frac{v_{eo} + v_{ep}}{(1 + k)}
\]

(7)

where \( v_{eo} \) = electrophoretic velocity, \( v_{ep} = \mu_{ep} \cdot F \) (\( \mu_{ep} \) = effective electrophoretic mobility, \( F \) = electric field strength).

According to Eq. (7), solutes can be separated by CEC that do differ not in \( k \) but in \( \mu_{ep} \). It is important to note that in case of ionized solutes the true retention factor cannot be determined in a single experiment from \( t_o \) and \( t_m \). Equation (6) is no longer valid. The effective electrophoretic mobility has to be determined in a separate experiment in an open-tubular capillary using the mobile phase of the chromatographic experiment as separation buffer.

Equation (7) implies that in the case of ionizable solutes, optimization strategies have to be completely different from those devel-
oped for HPLC or CE. However, the large number of influencing factors offers many parameters for selectivity tuning [3].

Rathore and Horváth [29] have highlighted that in the case of so-called duplex columns having an open segment between the end of the chromatographic bed and the detection window, the observed selectivity for ionized solutes depends on the length ratio (length of chromatographic bed/length of open section), as in the open section only electrophoresis contributes to the separation, while in the chromatographic bed both electrophoretic and chromatographic effects affect the separation of zones.

It has to be emphasized that the validity of Eq. (7) depends on the constraint that all simplifying assumptions listed above are met. This constraint is not fulfilled if the chromatographic retention mechanism is ion exchange (for example, separation of cations on a cation exchanger). Recently, Ståhlberg [30] has given a theory for zone migration in CEC for this generalized case. This theory is based on a solution of the mass balance equation. He showed that in the general case, the mixing of chromatographic and electrophoretic effects gives rise to strong nonlinear effects. Indeed, experiments with ion-exchange phases as stationary phases in CEC have brought unexpected results [31].

B. Efficiency

The first works in CEC done with capillaries packed with octadecyl silica gel concentrated on progress in efficiency compared to HPLC via the use of very fine packing material ($d_p \leq 3 \mu m$). These experiments have been made with neutral analytes in order to avoid electrophoretic effects. The maximum length of the packed bed is given not by the mechanical permeability of the packing but by the maximum voltage that can be applied between the ends of the capillary and by the electroosmotic mobility $\mu_{eo}$. In order to exploit the full potential of the separation system, a minimum linear velocity of the mobile phase has to be obtained. This minimum linear velocity corresponds to the optimum velocity of the plate-height curve. If the minimum linear velocity $v_{min}$ is 1.5 mm/s, the maximum voltage $U_{max} = 30$ kV and $\mu_{eo} = 0.25$ cm$^2$/s/kV, then the maximum total length $L_{tot,max}$ of the separation capillary is 500 mm with $v_{min} = \mu_{eo} (U_{max}/L_{tot,max})$.

Knox and Grant [13] postulated that in CEC with packed capillaries the reduced plate height can be significantly smaller than 2 because of the flat mobile-phase streaming profile. These theoretical
predictions have been corroborated by experimental results of Knox and Grant [18], Smith and Evans [32], and Robson et al. [33], who reported reduced plate heights \( h \) around 1 for retarded solutes. Some workers have demonstrated that with the same column (under identical conditions), a significantly better efficiency was obtained in the CEC mode than in the pressure-driven mode [18,34–37]. It can be concluded from these studies that with the so-called pseudo-electrochromatography [38] or pressurized-flow electrochromatography [35], a technique that uses a mixture of pressure-induced and electrokinetically induced flow, the chromatographic efficiency will be worse than with systems that use only electroosmosis for propulsion of the mobile phase.

Bruin et al. [39] have shown that the efficiency of electro-driven open-tubular liquid chromatography (ED-OTLC) is by a factor of ca. 2 better than that of pressure-driven open-tubular liquid chromatography (PD-OTLC). In the case of open-tubular columns the improvement in efficiency is due only to the impact of the mobile-phase flow profile on the \( C_m \) term of the plate-height equation.

The overall plate-height equation is valid in ED-OTLC and PD-OTLC [39]:

\[
H = \frac{2D_m}{v} + f(k)_m \cdot \frac{d^2_v}{D_m} + f(k)_s \cdot \frac{d^2_f}{D_s}
\]

where \( H \) = plate height, \( D_m \) or \( D_s \) = diffusion coefficient in the mobile or the stationary phase, respectively, \( v \) = linear velocity, \( d_i \) = inner diameter of the column, \( d_f \) = thickness of the stationary-phase layer, \( f(k)_m \) or \( f(k)_s \) = function of \( k \), and \( k \) = retention factor.

However, there is a difference in \( f(k)_m \) due to differences in the mobile-phase flow profile (see Fig. 5):

\[
\text{ED-OTLC:} \quad f(k)_{m}^{\text{ED}} = \frac{k^2}{16(1 + k)^2}
\]

\[
\text{PD-OTLC:} \quad f(k)_{m}^{\text{PD}} = \frac{1 + 6k + 11k^2}{96(1 + k)^2}
\]

Bruin et al. [39] highlight that in ED-OTLC, capillaries with wider inner diameter (up to 25 \( \mu m \)) can be used than in PD-OTLC without loss of efficiency, having advantages of detection, loadability, and column preparation. Figure 7 shows the influence of the column diameter and the type of mobile-phase propulsion on the plate
height for a given retention factor \( k = 1 \) according to Eq. (8) neglecting the resistance to mass transfer in the stationary phase [28].

With capillaries packed with octadecyl silica gel, acetonitrile–aqueous buffer mobile phases, and retarded noncharged analytes, extremely high efficiencies have been obtained in the isocratic elution mode at moderate holdup times. Seifar et al. [40] investigated the impact of the particle size on the performance. The mean particle diameter ranged from 1.5 to 50 \( \mu \)m. In accordance with theory, the plate height decreases with decreasing particle size. With porous particles the reduced plate height was about 2, but with the nonporous 1.5-\( \mu \)m particles, reduced plate heights of 1.3 were observed. On a 240-mm-long column, about 120,000 plates were generated (holdup time ca. 2.4 min, ca. 500,000 plates/m) [41]. The authors
state that in order to realize the observed linear velocity under pressure, about 1900 bar is required. With columns packed with 1.5-μm nonporous octadecyl silica gel, Bailey and Yan [42] were able to separate a series of 14 nitroaromatic and nitramine explosive compounds in under 7 min (see Fig. 8), featuring efficiencies of >500,000 plates/m. Dadoo et al. [43] used columns packed electrokinetically with 1.5-μm nonporous octadecyl silica gel to achieve rapid separations with high efficiencies. A sample containing 16 polycyclic aro-

Fig. 8 CEC separation of explosives. Capillary, 340 (210) mm × 75 μm; stationary phase, 1.5-μm nonporous octadecyl silica gel; mobile phase, 20% methanol, 80% aqueous buffer (10 mmol/L MES, 5 mmol/L SDS); HMX = octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine, RDX = hexahydro-1,3,5-trinitro-1,3,5-triazine, DNB = 1,3-dinitrobenzene, TNB = 1,3,5-trinitrobenzene, NB = nitrobenzene, TNT = 2,4,6-trinitrotoluene, 2,4-DNT = 2,4-dinitrotoluene, tetryl = methyl-2,4,6-trinitrophenylnitramine, 2,6-DNT = 2,6-dinitrotoluene, 2-Am-DNT = 2-amino-4,6-dinitrotoluene, 2-NT = 2-nitrotoluene, 4-NT = 4-nitrotoluene, 4-Am-DNT = 4-amino-2,6-dinitrotoluene, 3-NT = 3-nitrotoluene. (Reprinted with permission from Ref. 42.)
mestic hydrocarbons (PAHs) (classified as priority pollutants by the U.S. Environmental Protection Agency) was isocratically separated in under 10 min. With detection in a packed section of the capillary, >700,000 plates/m were obtained.

Lüdtke et al. [22] were the first to employ packings of submicrometer material in CEC. However, in their measurements the apparent reduced plate heights (not corrected for the nonpacked section of the capillary) are relatively high \( h(\text{minimum}) = 3.5–4 \). One possible explanation for this result is the fact that the \( B \) term of the plate-height equation [see Eq. 4] is not dependent on the particle diameter. Knox [44] has predicted that, using submicrometer material, all contributions from the \( A \) and \( C \) terms of the plate-height equation become insignificant compared to the band-broadening process, due to axial diffusion.

While the use of spherical nonporous material with mean particle diameter <1.8 \( \mu \)m with very narrow size distribution is one approach to obtain extremely high efficiency in CEC, the use of wide-pore material and pore flow effects is another interesting approach. Li and Remcho [45] report results obtained with octadecyl silica gels with mean pore diameters ranging from 6 to 400 nm. The authors assume that with large-pore material, perfusive transport of the mobile phase through the pores is possible, reducing significantly the plate height of the chromatographic system. The experimental results, indeed, support this theory. They also showed that the reduced plate height (mean particle diameter 7 \( \mu \)m) is dependent not only on the mean pore diameter but also on the ionic strength of the mobile phase, indicating "that the thickness of the electrochemical double layer obviously plays a significant role in perfusive transport through narrow channels." Recently, Stol et al. [46] and Santalla-Garcia et al. [47] reported, for columns packed with octadecyl silica gel (mean particle diameter 7 \( \mu \)m) with mean pore diameter of 400 nm, remarkably high efficiencies with mobile phases of moderate ionic strength. Up to 550,000 plates/m and reduced plate heights down to 0.26 were generated for retarded solutes. The column is acting "as if [one particle] is composed of several small particles." The utility of reduced plate heights as a measure for column quality in CEC on wide-pore stationary phases can be questioned. With wide-pore packing material of large particle size, it is possible to obtain efficiencies that can be obtained with nonporous material only with particles of considerably smaller size.
In 1995 Smith and Evans [31] observed extremely sharp zones (formally more than 8 million theoretical plates per meter) for basic antidepressants separated by CEC employing a strong cation exchanger as stationary phase and a mobile phase of pH = 3.5 (see Fig. 9). It is evident that a so-far-unknown zone-sharpening effect is involved. According to Stähler [30], this zone sharpening might be due to a combination of electric field and adsorption effects. However, it is known that the extremely sharp zones reported in [31] are concentration dependent and often not sufficiently reproducible for analytical purposes. It is interesting to note that abnormally high efficiencies have also been observed under reversed-phase conditions employing an octadecyl silica gel as stationary phase [48].

C. Comparison to Liquid Chromatography
Results presented by many workers [41–47] demonstrate that efficiencies between 500,000 and 700,000 plates per meter are attainable in isocratic CEC with packed capillaries. The number of plates

![Image](multichrom.png)

**Fig. 9** CEC separation of basic antidepressants. Capillary, 300 (200) mm × 100 μm; stationary phase, 3-μm sulfonic acid propyl silica gel; mobile phase, 70% acetonitrile, 30% aqueous buffer (50 mmol/L NaH₂PO₄, pH = 3.5); 1 = bendroflumethiazide, 2 = nortriptyline, 3 = clomipramine, 4 = methdilazine. (Reprinted with permission from Ref. 31.)
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in a real column, however, is significantly lower, due to limitations in the maximum applied voltage and the electroosmotic mobility limiting the maximum length of the packed capillary. In spite of these limitations, the attainable efficiency in CEC is about one order of magnitude higher than that in HPLC.

Assuming a column resistance factor of 750 and a pressure limit of 400 bar, Dittmann et al. [49] calculated the maximum plate number achievable at the optimum velocity under the constraints mentioned above (see Table 1). According to their calculations, 5–10 times higher plate numbers per column are achievable using CEC compared with those obtained using capillary HPLC. It has to be emphasized that increasing the electroosmotic mobility is an important parameter to increase the maximum plate number for a real column in CEC.

D. Extracolumn Band Broadening

In CEC the following extracolumn band-broadening contributions have to be taken into consideration: sample injection, detection, and data processing. With instrumentation designed for capillary electrophoresis, no significant band broadening due to detection and data processing is expected for CEC. Because of on-column injection and on-column (or in-column) detection, there is no band broadening due to transfer lines.

In 1966 Sternberg [50] investigated in detail extracolumn contributions to chromatographic band broadening. He showed that the second moment (variance) for the total distribution can be derived from the second moments for the partial distributions. He also discussed the second-moment distributions for various input functions.

Table 1 Achievable Efficiency in Capillary HPLC and CEC

<table>
<thead>
<tr>
<th>Particle size (µm)</th>
<th>Capillary HPLC</th>
<th>CEC</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Length (cm)</td>
<td>Plates per column</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>55,000</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>45,000</td>
</tr>
<tr>
<td>1.5</td>
<td>10</td>
<td>30,000</td>
</tr>
</tbody>
</table>

Source: Reprinted with permission from Ref. 49.
The input function is the distribution function of the analyte concentration in the sample zone due to the injection process.

Due to the use of injection valves with constant injected sample volume and pumps with constant volume flow, in HPLC the maximum sample volume given in volume units is of interest in dependence on the peak volume. These magnitudes are rather inconvenient in capillary electrochromatography. In CEC the sample is in most cases injected directly onto the column via the controlled application of a voltage (electrokinetic injection), and the velocity of the mobile phase is given not as flow rate (volume/time) but as linear velocity (distance/time) determined via a nonretarded marker.

Assuming that the contribution due to detection and data processing can be neglected, the tolerable injection plug length for a column of given length and efficiency can be easily calculated [51]. The partial second moment (variance, $\sigma_i^2$ for the injection plug (rectangular distribution) can be determined from the injection plug length $L_i$ [50].

$$\sigma_i^2 = \frac{L_i^2}{12} \tag{11}$$

The partial second moment (variance, $\sigma_C^2$) for the distribution due exclusively to the column is given by

$$\sigma_C^2 = \frac{L^2}{N} \tag{12}$$

where $L =$ length of the column to the detection window and $N =$ plate number.

The total second moment (variance of the recorded peak, $\sigma_{tot}^2$) is the sum of the partial second moments. It has to be emphasized that the variances are given in length units:

$$\sigma_{tot}^2 = \sigma_i^2 + \sigma_C^2 \tag{13}$$

With electrokinetic injection, $L_i$ can be determined employing Eq. (14), taking into account zone compression during the injection process due to enrichment of the solutes in the stationary phase:

$$L_i = t_i \mu_{eo} U_i L_{tot}^{-1}(1 + k_s)^{-1} \tag{14}$$

where $t_i =$ injection time, $\mu_{eo} =$ electroosmotic mobility, $U_i =$ injection voltage, $L_{tot} =$ total column length, and $k_s =$ retention factor for the solute of interest with the sample solvent as mobile phase.
According to Pyell et al. [51], in performing CEC with packed capillaries there is a bias between the theoretical and experimental data that can be understood as a deviation of the input function from the assumed rectangular distribution function. This deviation has to be taken into account by an experimental factor $F_I$. According to the data material presented in [51], $F_I$ equals 1.5.

Equation (15) gives the maximum tolerable injection plug length, $L_{\text{max}}$, for which the decision criterion (an increase of $w_h$ of about 5% due to extracolumn band broadening) is fulfilled:

$$L_{\text{max}} = 1.1 \cdot \frac{L}{\sqrt{N} \cdot F_I} = 0.7 \cdot \frac{L}{\sqrt{N}}$$  \hspace{1cm} (15)

With this set of equations tolerable injection parameters can be calculated for electrokinetic injection. For a fixed injection time Eq. (16) is valid, predicting the suitable injection voltage; while for a fixed injection voltage Eq. (17) is valid, predicting the suitable injection time.

$$U_{\text{max}} = 0.7 \cdot \frac{L \cdot L_{\text{tot}} \cdot (1 + k_s)}{\mu_e \cdot t_I \cdot \sqrt{N}}$$  \hspace{1cm} (16)

$$t_{\text{max}} = 0.7 \cdot \frac{L \cdot L_{\text{tot}} \cdot (1 + k_s)}{\mu_e \cdot U_I \cdot \sqrt{N}}$$  \hspace{1cm} (17)

In Table 2, optimum injection times are given for CEC with packed capillaries assuming that $k_s = 0$. The calculations show that with standard injection procedures (electrokinetic injection, $t_I = 5$ s, $U_I = 5$ kV) in CEC with packed capillaries of a total length ≤250 mm, the criterion for the tolerable extracolumn band broadening (an increase in $w_h$ by 5%) might not be fulfilled if the starting zone is not focused during the injection process.

In Fig. 10 the dependence of the peak width at half-height in length units on the injected plug length calculated according to Eq. (14) ($k_s = 0$) for alkyl- and arylbenzoates as test solutes is presented for a packed column [$N$(maximum) = 43,000–52,000, length to the detector = 258 mm]. The experimentally determined dependence of the peak width at half-height on the injected plug length corresponds to the curve predicted by theory. At low injected plug length ($L_I ≤ 1$ mm), $w_h$ is independent of $L_I$, while at high volume overload $w_h$ is linearly increased with $L_I$. 

---

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Table 2  Predicted Optimum Injection Time \( t_{\text{max}} \) Dependent on the Mean Particle Diameter \( d_p \) of the Packing with Electrokinetic Injection \( (U_i = 3 \text{ kV}) \) assuming following conditions: \( h = 2, L = 200 \text{ mm}, L_{\text{int}} = 250 \text{ mm}, \mu_{\text{so}} = 0.25 \text{ cm}^2/\text{s kV} \)

<table>
<thead>
<tr>
<th>( d_p (\mu\text{m}) )</th>
<th>( N )</th>
<th>( t_{\text{max}} (\text{s}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.0</td>
<td>10,000</td>
<td>4.7</td>
</tr>
<tr>
<td>5.0</td>
<td>20,000</td>
<td>3.3</td>
</tr>
<tr>
<td>3.0</td>
<td>33,000</td>
<td>2.6</td>
</tr>
<tr>
<td>2.0</td>
<td>50,000</td>
<td>2.1</td>
</tr>
<tr>
<td>1.5</td>
<td>67,000</td>
<td>1.8</td>
</tr>
<tr>
<td>1.0</td>
<td>100,000</td>
<td>1.5</td>
</tr>
<tr>
<td>0.5</td>
<td>200,000</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Source: Reprinted with permission from Ref. 51.

Fig. 10  Dependence of the peak width at half-height on the injected plug length (electrokinetic injection) for various alkyl benzoates. Capillary, 310 (258) mm \( \times \) 180 \( \mu \)m; stationary phase, 1.5-\( \mu \)m porous octadecyl silica gel; mobile phase, 80\% acetonitrile, 20\% aqueous buffer (2 mmol/L phosphate, pH 7.3); ■ = methyl benzoate, ● = ethyl benzoate, ▲ = benzyl benzoate, ▼ = p-tolyl benzoate, ◆ = iso-pentyl benzoate, □ = phenyl benzoate, ★ = butyl benzoate. (Reprinted with permission from Ref. 51.)
With in-column detection (see Fig. 2) and reversal of the mobile-phase flow by voltage switching, an analyte zone can be made pass several times through the detection area in the backward and forward directions. This principle has been used by Rebscher and Pyell [52] to estimate by experiment the extracolumn contribution to peak broadening in CEC with a packed capillary. By this method the length of the capillary column can be virtually increased by sections that are decided by the migration velocity of the analyte zone and the switching times. The variance of a peak $\sigma^2$ is proportional to the migrated distance. The migrated distance $x_m$ has to be calculated from the migration time and the migration velocity. A plot of $\sigma^2$ versus the migrated distance $x_m$ gives a straight line (see Fig. 11). The peak variance $\sigma_E^2$ due to extracolumn band-broadening processes corresponds to the $y$ intercept of the extrapolated straight line. The slope of the regression line permits one to calculate the true efficiency of the column [see Eq. 18], corresponding to the efficiency after elimination of instrumental band-broadening effects:

$$N = \frac{L}{m}$$

(18)

Fig. 11 Illustration of a method to determine experimentally the peak variance $\sigma_E^2$ due to extracolumn band-broadening effects by voltage switching and in-column detection. The peak variance $\sigma^2$ is plotted versus the migrated distance $x_m$. 
where $N = \text{plate number}$, $L = \text{length of the column to the detection window}$, and $m = \text{the slope of the regression line}$.

Rebscher and Pyell [52] found that with the packed capillary used in their study, the instrumental band-broadening contribution was excessive, largely reducing the usable efficiency of the chromatographic system. There is indication that final frits might be the main source of the observed excessive band-broadening effects. Some workers report higher achievable chromatographic efficiencies with in-column detection compared to on-column detection [18,26,53], observing that the internal frit at the end of the chromatographic bed can be a source of substantial dispersion of analyte zones as they migrate through the frit. With a separation capillary packed with nonporous 1.5-μm octadecyl silica gel, Dadoo et al. [43] obtained extremely high efficiencies only with in-column detection. They report a drop in efficiency from 600,000–750,000 plates/m for retarded solutes to 300,000–400,000 plates/m when shifting the detection area from just before the outlet frit to 0.5–1 mm after the outlet frit.

E. Thermal Effects

According to the considerations in Sections III.B and III.C, CEC has clear advantages over HPLC in terms of the obtainable efficiency for noncharged solutes. However, thermal effects have been neglected so far. Because of the electric propulsion of the mobile phase, Joule heat is generated in the separation capillary during the chromatographic run. The released power in the capillary corresponds to the applied voltage multiplied by the measured electric current strength. This generated heat is conducted first through the walls of the tube and then through the surrounding medium. As a result there will be a temperature difference between the capillary and the surrounding medium and there will be a temperature variation across the bore of the separation capillary. The temperature difference between the capillary and the surrounding medium does not lead to band broadening, but the temperature variation across the bore of the separation capillary does.

According to Knox [44], in CEC the parabolic temperature profile which exists across the capillary bore may cause variations in migration rates due to (1) changes in mobile-phase viscosity and (2) changes in the retention factors. In order to take this additional peak-broadening effect into account, an additional term has to be added to Eq. (4) or (8).
Grushka et al. [54] investigated in detail the effect of temperature gradients on the efficiency of capillary electrophoresis separations. They assumed that the actual velocity profile in the separation capillary is parabolic, due to the generated heat. Using the parabolic model, they developed an expression that relates the plate height to the capillary radius, the applied electric field strength, and the ionic strength of the mobile phase. This newly derived plate-height equation suggests that (1) at high electric field strength there is a significant peak broadening due to thermal effects, (2) the separation capillary should be thermostated, (3) thermostating at elevated temperature may have advantages, and (4) decreasing the concentration of the buffering salts in the mobile phase allows the use of wider capillaries. They state that "wider capillaries, in turn, would permit more convenient operation and large sample volumes." Wider capillaries allow one to improve detection limits in combination with on-column or in-column spectrometric detection. The upper limit of allowable capillary inner diameter is a function of the applied field strength, the specific conductance of the mobile phase, and the diffusion coefficients of the solutes.

Knox [44] calculated for capillary electroseparation methods the boundary conditions under which the plate-height contribution of the thermal effects is less than 10% the plate-height contribution from axial diffusion. It is interesting to note that, according to Knox, with parameters commonly given in CEC (concentration of buffering salt in mobile phase = 1 mmol/L, applied field strength = 100 kV/m), the maximum allowed capillary inner diameter is 150 μm.

Capillaries used in CEC usually have an inner diameter of 100 μm. For comparison, in CE and MEKC, capillaries mostly have inner diameters of 50–75 μm. Because of the high content of organic modifier and the low concentration of buffering salts in mobile phases employed for CEC, the electric current that is measured during a chromatographic run in CEC is one to two orders of magnitude lower than in other capillary electroseparative techniques. Wright et al. [55] even demonstrated for CEC the applicability of mobile phases that do not contain added buffer salts. A separation of 11 polycyclic aromatic hydrocarbons was performed in under 13 min by CEC with an acetonitrile/water mobile phase. Whitaker and Sepaniak [56] separated fullerenes by CEC with a packed column and acetonitrile/tetrahydrofuran or acetonitrile/methylene chloride as mobile phase. Working with mobile phases of extremely low specific electric con-

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ductivity might even make it possible to work in CEC with microbore columns and to perform highly efficient semipreparative separations.

Although the advantages of wider separation capillaries are known, this topic has rarely been addressed in experimental studies. Yan et al. [57] and Vissers et al. [58] demonstrated the possibility of performing CEC with capillaries of 320-μm inner diameter packed with 5-μm octadecyl silica gel.

While the possibility of using packed capillaries with an inner diameter ≫100 μm has been clearly demonstrated, very few data are available on the dependence (or independence) of the achievable chromatographic efficiency on the capillary inner diameter (I.D.). Rebscher and Pyell [59] estimated the influence of the capillary inner diameter on the achievable chromatographic efficiency independent of the influence of the quality of the frit and other randomly distributed parameters by packing several capillaries (inner diameters 75, 100, and 150 μm) with 3-μm octadecyl silica gel according to the same protocol. The comparison of the plate heights determined did not exhibit significantly lower plate heights for the capillaries with I.D. = 75 μm than for the capillaries with I.D. = 150 μm. Similar results have been obtained by Steiner et al. [60]. There might be, however, a significant drop in efficiency, if the inner diameter of the capillary is larger than 150 μm. Steiner et al. report a loss in efficiency of 50–60% when increasing the inner diameter from 100 to 180 μm.

Banholczer and Pyell [61] report that the retention factors for several noncharged solutes determined for a packed capillary (inner diameter = 100 μm) by CEC with thiourea as marker of the holdup time were independent of the concentration of buffering salts (NaH₂PO₄ + Na₂HPO₄) in the mobile phase (see Fig. 12). The phosphate concentration was varied in a range from 0.01 to 7 mmol/L. If there was a significant temperature rise inside the capillary due to Joule heating, a decrease in retention factors with increasing concentration of the buffering salts would be expected. The absence of any dependence of \( k \) on the ionic strength of the mobile phase verifies the assumption that in CEC with commonly given parameters the contribution of temperature gradients inside the capillary to the broadening of zones is negligible. Further investigations are needed to elucidate the influence of capillary dimensions, electric field strength, and specific electric conductivity of the chromatographic
Retention factors $k$ for several noncharged solutes versus buffer salt (phosphate) concentration in the mobile phase. Capillary, 422 (376) mm $\times$ 100 μm; stationary phase, 3-μm porous octadecyl silica gel (Nucleosil 100-3 ODS); mobile phase, 80% acetonitrile, 20% aqueous buffer (total phosphate concentration = 0.01–7 mmol/L, pH 7.2); solutes: ■ = methyl benzoate, ● = ethyl benzoate, ▲ = acenaphthylene, ▼ = acenaphthene, ◆ = pyrene. (Reprinted with permission from Ref. 61.)

bed (also, the stationary phase might contribute to the conductance of electric current) on retention data and efficiency.

F. Bubble Formation

In the early days of CEC, bubble formation during a chromatographic run was the most important practical problem that hampered the widespread use of this technique. In one of their pioneering works on CEC, Knox and Grant [18] have stated: “Drying out [of the packed capillaries] was particularly liable to occur with the wider capillaries and with the higher concentrations of electrolyte, indicating that self-heating was the primary cause.” They recommended thermostating of columns at temperatures close to ambient or op-
erating the whole column under pressure as the most effective preventive measures.

There are indications, however, that self-heating is not the primary cause of the formation of bubbles in the packing during a chromatographic run. The first indication is that the electric current strength that is measured during separation in CEC is one to two magnitudes lower than that is measured under routine CE or MEKC conditions. The results of Banholczer and Pyell [61] (see Fig. 12) strongly support the assumption that in CEC under standard conditions there is only a very small temperature rise inside the capillary.

Rebscher and Pyell [52] observed that the formation of bubbles invariably started with a semipacked column at the border between the packed and the unpacked sections of the capillary. They interpreted this observation as follows: “The following segment has a higher electroosmotic mobility than the preceding segment. Consequently, the second non-packed segment has the effect of a pump reducing the pressure in the packed section below the prevailing pressure.” This interpretation is corroborated in a theoretical study by Rathore and Horváth [62]. Rathore and Horváth studied the interface of the packed and open segments of a semipacked CEC column and discontinuities associated. They show that in order to satisfy the mass conservation law, in most cases a “flow-equalizing intersegmental pressure,” which is different from the pressure at the two ends of the column, develops at the interface of the packed and open segments.

A second reason for bubble formation is associated with frits. Rathore and Horváth [62] highlight that silica frits most likely have zeta potentials different from those of the bulk packings, and the discontinuity of zeta potential can lead to the development of flow-equalizing intersegmental pressure at such frits with concomitant bubble formation. Rebscher [63] observed that a completely packed capillary that invariably starts bubble formation at the inlet end during a chromatographic run can be used without problems, if the direction of the electroosmotic flow is reversed. Rebscher and Pyell [59] observed that if a prepared column has a frit with low (mechanical) streaming permeability, bubble formation during a chromatographic run is very likely to occur directly after the frit. This problem could be completely circumvented if the frit with low permeability was replaced by a second frit. These observations suggest that not only differences in the zeta potential between frit and bulk packing
but also an extremely low (mechanical) streaming permeability of the frit can result in bubble formation. Carney et al. [64] investigated factors affecting bubble formation. They concluded that bubble formation in CEC is a function of the length and nature of the frit. According to Carney et al., one possible solution of this problem is the recoating of octadecylsilane onto the silica frit, minimizing differences in the zeta potential between the frit and the bulk packing material.

The necessity to operate the whole separation capillary under pressure in order to avoid bubble formation is controversially discussed in the literature [33,55,65,66]. Van den Bosch et al. [67] emphasized that operating the whole column under pressure is not necessary with a robust separation capillary. Considerations above suggest that for the design of a robust separation capillary suitable for CEC at ambient pressure, it is important that the separation capillary be as homogeneous as possible over the full length in zeta potential and in the cross-sectional area of the inner volume of the capillary accessible to the mobile phase. It is important to note that these two constraints are ideally fulfilled with fritless capillaries filled completely with stationary phase. In this case, spectrometric detection has to be performed in the chromatographic bed (in-column detection).

IV. INSTRUMENTAL DEVELOPMENTS

A. Column Technology

Work in CEC has started with capillaries slurry-packed with octadecyl silica gel [11,13,15–17,32,35,38,52,68–70]. In order to keep the packing in place, frits had to be prepared at both ends of the packing. Only Knox and Grant [13] have also worked with drawn-packed capillaries that did not need frits to stabilize the packing. Tsuda et al. [71], Bruin et al. [39], and Pfeffer and Yeung [72] were the first to perform CEC with open-tubular capillaries. CEC is still an emerging technique. Improvement of instrumentation and column technology will be of prime importance for the further development of this technique and its use in validated methods. Neither the classical packed capillary with end frits nor open-tubular capillaries seem to be the ideal separation column for CEC. Consequently, column technology in CEC has become a vigorous area of research in the last years. Advances in column technology have been recently reviewed [4,65,66,73–76] and will be addressed only briefly.
With traditional packing procedures, frits are necessary to stabilize the chromatographic bed (see Fig. 2). Several methods have been reported for the production of frits in CEC: (1) reaction of sodium silicate solution with formamide to form a porous silica plug [77]; (2) sintering of a plug of native silica gel wetted with an aqueous solution of potassium silicate [78]; (3) sintering of a plug of native silica gel wetted with pure water [26]; or (4) sintering of a portion of the chromatographic packing itself with a heated filament after having flushed the column with water [32,68]. The slurry of the packing material is prepared either in an organic solvent or in supercritical carbon dioxide [33]. Mostly the packing material is transported into the column with the help of an external pump. Reynolds et al. [79] and Fermier and Colón [80] used columns packed by centripetal forces. Electrokinetic packing was described by Yan [81].

In CEC the stationary phase not only provides interaction sites for the solutes, it also plays the dominant role in the generation of the electroosmotic flow, hence the propagation of the mobile phase through the chromatographic bed. Consequently, the design of stationary phases suited for CEC not only has to keep in mind the retentive properties of this material but also the electrokinetic properties. Work in CEC started with stationary phases designed for HPLC. With these phases it might be possible to transfer directly a method that has been developed for HPLC onto CEC. The selectivity of the separation system may be altered by varying the stationary phase. Those stationary phases that have been used in the reversed-phase mode in HPLC should be applicable in CEC provided that their surface properties permit the generation of a sufficiently high electroosmotic velocity [82,83]. Some workers have tested successfully chiral stationary phases designed for HPLC in the CEC mode [84–90].

One important disadvantage of conventional silica-based materials, when used as stationary phases in CEC, is the dependence of the electroosmotic mobility on the pH of the mobile phase. However, some workers demonstrated the possibility of performing rapid analysis in CEC with capillaries packed with commercial octadecyl silica gel and mobile phases buffered at pH 2.5 [91]. Assuming that with a strong cation exchanger the electroosmotic velocity will be high even at low pH, Smith and Evans [31] tested the separation of basic drugs by CEC at low pH with a strong cation exchanger as stationary phase, with unexpected results (see Fig. 9). The ion exchanger was
made specifically for CEC, based on a specially prepared silica gel (\(d_p = 3 \, \mu\text{m}\)) onto which is bonded a propyl sulfonate group.

In order to make it possible to perform reversed-phase CEC with relatively high electroosmotic velocity quasi-independent of the pH, Zhang and El Rassi [92] introduced a novel silica-based multilayered stationary phase. This stationary phase comprises a relatively hydrophilic and charged sublayer attached covalently to the silica support and a retentative top layer of octadecyl groups bound chemically to the sublayer. Mixed-mode phases have also been employed [19,93,94].

Huang et al. [95] have chosen another approach to perform CEC with mobile phases of low pH. They have used a mixed-mode phase that contains octadecyl and dialkylamino groups (reversed-phase/anion-exchange phase). The amino groups determine at low pH the charge density at the surface and the direction of the electroosmotic flow is reversed with respect to the direction found with bare silica gel. Huang et al. have demonstrated that this phase is ideally suited for the separation of (positively charged) peptides.

Another approach is the synthesis of an octadecyl silica gel with light surface coverage of alkyl groups [96]. This stationary phase was designed to allow a relatively high electroosmotic velocity due to a high concentration of nonreacted silanol groups at the surface.

The properties of final frits keeping the stationary phase in place determine the mechanical stability of the packed columns and their liability to bubble formation. Frits are discussed as major sources of extracolumn band broadening. These problems make it very desirable to construct fritless capillary columns for CEC. The production of fritless capillary columns for CEC has been the object of very active research in the last years. Highly swollen cross-linked hydrophobic hydrogels or rigid monoliths have been prepared in fused-silica capillaries by in-situ synthesis, tapers have been used as an alternative to frits [97,98], and monoliths have been prepared by converting conventionally packed columns into ones with monolithic structure.

Fujimoto et al. [99] realized continuous beds by radical copolymerization of N-isopropylacrylamide and 2-acrylamido-2-methylpropanesulfonic acid with N,N'-methylenebisacrylamide as cross-linking agent in capillaries pretreated with (\(\gamma\)-methacryloxypropyl)-trimethoxysilane (inner diameter 50–75 \(\mu\text{m}\)). Other gel-based monoliths as stationary phases for CEC were synthesized by Liao
et al. [100], Ericson et al. [101], and Palm and Novotny [102]. Chiral separations have been achieved in CEC with continuous flow-through polymers [103–106].

Peters et al. [107–109] presented a technique for the one-step preparation of “molded” rigid polymer monoliths in nonpretreated fused-silica capillaries (inner diameter 100–150 μm). Mayer et al. [110] report the production of fritless capillaries (tapered end at the inlet side and neither frit nor taper at the outlet side); packed with 1.5-μm nonporous octadecyl silica gel. Asiaie et al. [111] succeeded in sintering packings of capillary columns. In the sintering process the compacted powder is converted into a monolith in which the particles are joined to each other by grain boundaries. Another possibility for the stabilization of packings and thus eliminating the need for frits is the embedding of packed particles in a rigid sol-gel or a silicate matrix [112–114].

From a technical point of view, open-tubular columns are the simplest approach to fritless and robust columns in CEC. Column technology for open-tubular CEC was reviewed in 1997 by Colón et al. [4] and Pesek and Matyska [75]. More recent developments are the fabrication of an organic–inorganic hybrid material by the sol-gel method as a thin porous film attached to the inner wall of fused-silica capillaries [115,116] and the preparation of polymer-coated 25-μm-I.D. capillaries by in-capillary copolymerization of N-tert-butylacrylamide with a charged monomer, after the pretreatment of the capillary inner surface with a bifunctional reagent [117].

Although open-tubular (OT) capillaries have some advantages over packed or filled capillaries, such as no liability to bubble formation and easy rinsing without the need to apply high pressure, OT capillaries are rarely employed in CEC. This might be due to the relatively small inner diameters of such capillaries having disadvantages in combination with optical detection methods. However, CEC with OT capillaries has a high miniaturization potential that has been used by Kutter et al. [118] for open-channel electrochromatography in combination with solvent programming using a microchip device.

B. Detection

Basically, in CEC the same detection techniques are applicable as in capillary electrophoresis: photometric detection, fluorimetric detection [119,120], or amperometric detection, for example. The cou-
pling of CEC with mass spectrometry has been successfully realized by several workers, mainly by employing electrospray ionization [34,38,95,98,116,121-127]. Also, the on-line coupling of CEC with nuclear magnetic resonance spectroscopy has been recently reported [128-130]. Guo et al. [131] coupled pressurized CEC with a condensation nucleation light-scattering detector using an electrospray interface. They demonstrated that condensation nucleation light-scattering detection is a sensitive and universal detection method for CEC. Qi et al. [132] highlighted the potential of thermo-optical absorbance detection as an attractive alternative to classical absorbance detection, interfacing a CEC system with an ultraviolet laser-based thermo-optical absorbance detector. High detection limits because of small detection volumes or low mass load can still be regarded as the Achilles’ heel of CEC, if not only the solution of a separation problem but also the achievement of low detection limits is needed to solve a specific analytical problem. It is obvious that further development of selective and sensitive detection techniques for CEC will be very important to broaden the application scope of this potentially highly efficient separation method.

There is one important difference between capillary electrophoresis and capillary electrochromatography in terms of optical detection. With both separation techniques, detection has to be performed in a section of the separation capillary in order to avoid excessive extracolumn band broadening (there are rare cases where capillaries are coupled [133]). Hence a section of the separation capillary has to be transformed into the detection cell. As in most cases, fused-silica capillaries are used, and the transparency of these capillaries in the VIS/UV region is given. However, the outer protecting polyimide layer has to be removed for a short section of the capillary to form the on-column detection cell, making this section of the capillary brittle and fragile.

The difference between CEC and CE in terms of optical detection is the fact that with CEC detection can be performed either in the chromatographic bed itself containing the stationary and the mobile phase (in-column detection, ICD) or in an open section of the separation capillary containing only the eluate (on-column detection, OCD). The terminology differing between these two detection modes was suggested in 1987 by Verzele and Dewaele [134]. In Fig. 2 the realization of these two detection modes is illustrated using a packed capillary as an example.
The inherent disadvantage of on-column detection is the appearance of a discontinuity at the interface between the filled and the open sections. Rathore and Horváth [62] have shown that these discontinuities may result in intersegmental pressure differences changing the nature of the mobile-phase flow from purely electroosmotically driven to partly pressure driven. In extreme cases even bubble formation may result. Typically, completely filled capillaries are more robust than partly filled capillaries. Analyte zones migrating through the interface between the filled and the open section can be substantially broadened (see Section III.D). In spite of these disadvantages, on-column detection is mostly applied.

In the case of in-column detection, the presence of two phases in the detection window results in diffuse scattering of light at the irregular interfaces between the two phases. Consequently, in the detection volume along the optical path of the detector there is (non-specific) intensity loss of the incident beam due to diffuse scattering, and also if the stationary phase does not absorb at the detection wavelength. The extent of intensity loss is dependent on the difference in diffraction index between the two phases. In the ideal case (match of diffraction indices of stationary and mobile phase) the detection window is transparent, becoming opaque or even nontransparent in the case of extreme differences in the diffraction index. Consequently, in-column photometric detection is possible only if the stationary phase does not absorb at the detection wavelength and if there are only moderate differences between the refraction indices of the two phases present in the detection volume. These constraints are not always fulfilled.

Banholczer and Pyell [135] compared in-column with on-column photometric (UV) detection performed with fused-silica capillaries of 180-µm inner diameter, packed with 3-µm octadecyl silica gel. They determined the influence of the detection mode on linearity of the calibration function, precision, and detection limit. In their experiments, the baseline noise for in-column detection (ICD) is about twice that for on-column detection (OCD). This negative impact on the detection limit is mitigated by a signal enhancement in the case of ICD. This signal enhancement is due to enrichment of the solute in the stationary phase and can be quantitatively described with Eq. (19):

\[ S_i/S_o = (1 + k) \varphi_M \]  (19)
where $S_I = \text{sensitivity for in-column detection}$, $S_O = \text{sensitivity for on-column detection}$, $k = \text{retention factor}$, and $\phi_M = \text{volume fraction of mobile phase in the chromatographic bed}$.

The validity of this equation was demonstrated experimentally [135]. Figure 13 compares chromatograms of the same sample obtained with ICD or OCD. All other experimental conditions except the location of the detection window were kept constant. The appara-

![Fig. 13 Comparison of chromatograms obtained with (a) in-column and (b) on-column photometric detection ($\lambda = 230 \text{ nm}$). Separation of alkyl benzoates (identical samples). (a) Capillary, 325 (270) mm × 180-μm I.D., (b) capillary, 400 (350) mm × 180-μm I.D.; stationary phase, 3-μm porous octadecyl silica gel; mobile phase, 80% acetonitrile, 20% aqueous buffer (2 mmol/L phosphate, pH 7.3) 1 = thiourea, 2 = methyl benzoate, 3 = ethyl benzoate, 4 = phenyl benzoate, 5 = benzyl benzoate, 6 = p-tolyl benzoate, 7 = butyl benzoate, 8 = iso-pentyl benzoate. (Reprinted with permission from Ref. 135.)
ent change in peak-height ratios is due to the dependence of the described enhancement effect on the retention factor.

Equation (19) was derived in analogy to the considerations of Guthrie and Jorgenson [136]. Rebscher and Pyell [26] have shown that Eq. (19) is also valid for fluorescence detection in packed capillaries, if variations in the fluorescence quantum yield due to environmental effects are neglected.

Taking Eq. (19) and the increase in baseline noise into account, the relative limit of detection (LOD) for a method employing ICD (in comparison to a method employing OCD) can be calculated as

\[
\text{LOD(ICD)} = \frac{\text{LOD(OCD)} \cdot F(\text{noise})}{(1 + k) \cdot \phi_M}
\]

(20)

where \( F(\text{noise}) \) = factor by which baseline noise in ICD is increased compared to baseline noise in OCD.

The validity of Eq. (20) has been verified experimentally for photometric detection [135]. With \( F(\text{noise}) = 2 \) and \( \phi_M = 0.7 \), the LOD with ICD is lower than with OCD provided \( k > 2 \). Consequently, there may be an improvement of the LOD for late-eluted solutes when employing ICD instead of OCD. Improvement of detection limits for late-eluted solutes by the use of detection in a packed section of the separation capillary [137] or by packed flow cells [138] was also reported for microcolumn liquid chromatography.

C. Gradient Elution

Gradient elution improves the peak capacity of a chromatographic system via zone compression and is indispensable for analyzing very complex samples. In CEC the following gradients may be applied: gradient of the composition of the mobile phase, temperature gradient, or voltage gradient.

Although in CEC temperature gradients (temperature between the melting and the boiling point of the mobile phase) can be easily realized because of the capillary dimensions of the separation column, this approach has not been reported so far, to the best of the author’s knowledge. The impact of a temperature gradient on retention factors of the solutes, however, will be much smaller than that of a gradient of the composition of the mobile phase.

In the case of using commercial automated CE equipment for CEC, the realization of stepwise gradients of the composition can be
performed easily and reproducibly by changing the (buffer) vial at the inlet side at a predefined time interval [139,140].

Linear gradients of the composition of the mobile phase have mostly been realized with help of a gradient-delivering HPLC pump. Behnke and Bayer [68] were the first to develop pressurized gradient CEC. If a pressure-induced additional flow is to be avoided, the head of the separation capillary “merely dips into the stream of mobile phase passing by.” Sample is injected via a normal type or microinjection valve [127,141–144]. Also, automatization via the use of an (HPLC) autosampler is reported [37,125,145]. In Fig. 14 the separa-

---

**Fig. 14** CEC separation of phenylthiohydantoin amino acids with gradient elution. Capillary, 207 (127) mm × 50 μm; stationary phase, 3.5-μm porous octadecyl silica gel (Zorbax ODS); mobile phase, acetonitrile, aqueous buffer (5 mmol/L phosphate, pH = 7.55) gradient; voltage 10 kV; photometric detection at 210 nm; solutes in order of elution: formamide, PTH-asparagine, PTH-glutamine, PTH-threonine, PTH-glycine, PTH-alanine, PTH-tyrosine, PTH-valine, PTH-proline, PTH-tryptophan, PTH-phenylalanine, PTH-isoleucine, PTH-leucine. (Reprinted with permission from Ref. 141.)
tion of phenylthiohydantoin amino acids achieved via gradient CEC (linear gradient formed by two HPLC pumps) is shown.

Yan et al [119] developed an experimental setup for gradient CEC with electrokinetic generation of the gradient of the composition of the mobile phase. Two high-voltage power supplies are used to generate two electroosmotic flows in two channels (connected to two mobile-phase reservoirs) that are merged in front of the column head. The voltage of the two high-voltage power supplies is controlled by a computer. The ratio of the electroosmotic flow rate between the two channels delivering the mobile phase is gradually changed, thus generating a gradient of the composition at the mixing tee.

Other workers used a miniaturized titration device for gradient CEC [116,146]. Also, voltage programming is reported as an alternative to mobile-phase composition gradient programming [147]. It has to be emphasized, however, that this technique is of limited applicability, as no variation in the retention factor is achieved by voltage gradients.

V. MOBILE-PHASE CONSIDERATIONS

Not only surface properties of the stationary phase but also bulk properties of the mobile phase determine the electroosmotic mobility in the chromatographic bed. Hence, in CEC, optimization of the composition of the mobile phase must consider not only retention of solutes and selectivity of the chromatographic system (as in HPLC) but also observed electroosmotic mobility and achieved chromatographic efficiency. It is evident that strategies for the optimization of the mobile phase in CEC must differ from those that have been developed so far for HPLC, although the underlying retention mechanism for noncharged solutes is basically the same.

Parameters determining the electroosmotic velocity in packings of porous particles are not completely understood [148]. Currently, users of CEC must rely on phenomenological recordings of dependencies of chromatographic parameters such as velocity of the mobile phase or efficiency on properties of the mobile phase.

Regarding Eq. (2), the tentative assumption can be made that in case of a given stationary phase, constant electric field strength, and constant ionic strength in the mobile phase, the observable
linear velocity of the mobile phase is proportional to the ratio of the dielectric constant $\varepsilon_d$ to viscosity $\eta$ of the bulk liquid. Banholczer and Pyell [61] determined the linear mobile phase velocity with one packed capillary for various organic solvent–aqueous buffer mixtures (keeping the ionic strength constant) and correlated this magnitude to $\varepsilon_d/\eta$ of the corresponding organic solvent–water mixture (see Fig. 15). Although there is a trend, a direct correlation of $\varepsilon_d/\eta$ to the observed mobile phase velocity is not possible. The deviation from the regression line indicates possibly that the electrokinetic potential $\zeta$ cannot be regarded as independent of the mobile-phase composition, although the ionic strength has been kept constant.

Highest electroosmotic mobilities can be obtained in CEC with acetonitrile–aqueous buffer mixtures, making this mixture currently the mobile phase of choice in CEC. Similar results have

![Graph](image-url)

**Fig. 15** Electrophoretic mobility $\mu_{eo}$ plotted versus ratio dielectric constant $\varepsilon_d$ to viscosity $\eta$ of the corresponding solvent–water mixture. Capillary, 422 (376) mm × 100 μm; stationary phase, 3-μm porous octadecyl silica gel (Nucleosil 100-3 ODS); mobile phase, organic constituent/phosphate buffer (c(phosphate) in mobile phase = 1 mmol/L, pH = 7.2). (Reprinted with permission from Ref. 61.)
been obtained by Dittmann and Rozing [70] when changing from acetonitrile to methanol or THF as organic modifier in the mobile phase.

In an open capillary the electroosmotic mobility $\mu_{eo}$ is related to the thickness $\delta$ of the double layer adjacent to the wall surface [149]:

$$\mu_{eo} = \frac{\sigma \cdot \delta}{\eta}$$  \hspace{1cm} (21)

where $\sigma$ = surface charge density and $\eta$ = viscosity of the bulk liquid.

According to Eq. (3), $\delta$ is a function of the ionic strength $I = \frac{1}{2} \sum_i z_i^2 c_i$. Hence, in open-tubular capillaries for a given electrolyte (only the concentration of the buffer component is varied), the electroosmotic mobility $\mu_{eo}$ is inversely proportional to the square root of the ionic strength. The same relationship should be expected for capillaries filled with a porous plug. Indeed, Choudhary and Horváth [150] found for a packed capillary a linear relationship between $\mu_{eo}$ and the square root of the concentration of added NaCl ($c = 20$–$60$ mmol/L). Also, Dittmann and Rozing [19] found for a packed capillary a decrease of $\mu_{eo}$ with increasing buffer concentration ($c = 2$–$20$ mmol/L).

In CEC, however, the concentration of the buffer component in the mobile phase is often restricted to low concentrations ($c \leq 1$ mmol/L) because of the limited solubility of the buffer component in the solvent mixtures employed as mobile phases. Wan [151] determined $\mu_{eo}$ dependent on the concentration of sodium phosphate ($c = 10^{-3}$–$10^{-5}$ mol/L) in the mobile phase with a capillary packed with octadecylsilica gel. The author obtained maximum $\mu_{eo}$ at an intermediate concentration of sodium phosphate. Similar results have been obtained by Knox and Grant [18], who found maximum $\mu_{eo}$ at an intermediate concentration of the mobile-phase component NaH$_2$PO$_4$ at $10^{-3}$ mol/L.

In order to measure electroosmotic mobilities that are not distorted by the influence of the open section of the separation capillary, Banholczer and Pyell [61] determined $\mu_{eo}$ in capillaries completely packed with octadecylsilica gel employing acetonitrile/aqueous phosphate buffer as mobile phase, varying the phosphate concentration [$c$(NaH$_2$PO$_4$) + $c$(Na$_2$HPO$_4$)] in a range from 0.01 to 7 mmol/L.
The experiment was repeated under identical conditions with a second column in order to give a rough estimate of the column-to-column variability. In Fig. 16 the observed electroosmotic mobility is plotted against the logarithm of the phosphate concentration in the mobile phase. The results obtained for the two capillaries are depicted in the same figure. For the two capillaries, $\mu_o$ passes through a maximum at a phosphate concentration of 0.4–4 mmol/L. These studies show that for porous plugs there is a deviation from what is described for OT capillaries.

The results obtained for packed capillaries suggest that in CEC the ionic strength in the mobile phase is an important parameter. In order to obtain maximum velocity of the mobile phase, a sum concentration of buffering salts of ca. 1 mmol/L is appropriate.

![Graph showing electroosmotic mobility versus buffer salt concentration](image)

**Fig. 16** Electroosmotic mobility versus buffer salt (phosphate) concentration: ■ = column 1, $L = 442$ (390) mm × 100-μm I.D., mean of three measurements; ◆ = column 2, $L = 422$ (376) mm × 100-μm I.D., mean of five measurements, error bars = standard deviation; stationary phase, 3-μm porous octadecyl silica gel (Nucleosil 100-3 ODS); mobile phase, 80% acetonitrile, 20% aqueous buffer (total phosphate concentration = 0.01–7 mmol/L, pH 7.2); separation voltage 25 kV, electrokinetic injection 5 s at 5 kV, photometric in-column detection, $\lambda = 230$ nm, marker of holdup time thiourea. (Reprinted with permission from Ref. 61.)
VI. APPLICATIONS

Initially CEC was applied mainly to the analysis of samples of pharmaceutical interest Dulay et al. [152] demonstrated the routine application of CEC with a commercial CE instrument, separating a mixture of neutral compounds with a packed capillary. Hundreds of consecutive runs were performed over a period of weeks. They concluded that “CEC separations can be achieved in a reliable and routine manner.”

The potential to apply CEC in pharmaceutical analysis has been investigated by Euerby et al. [27] in the analysis of a wide range of structurally diverse pharmaceutical compounds. They state that “The repeatability of retention of CEC using reverse-phase materials is excellent and is a distinct advantage over CE using bare capillaries. The repeatability of peak area and height is surprisingly good compared to conventional CE and is comparable to many commercially available HPLC systems.”

Applications of CEC (including chiral analyses) were reviewed in 1998 in an excellent paper by Cikalo et al. [66]. Recently, various workers have been able to show that CEC can be used successfully to analyze compounds in various biological matrices, e.g., urine or plasma [145,153]. Sandra et al. [154] reported the analysis of triglycerides in vegetable oils. Dermaux et al. [155] employed CEC to analyze free fatty acids and fatty acid phenacyl esters originating from vegetable oils and margarine. They compared data obtained with CEC and micro-HPLC. CEC was found to be much superior in terms of efficiency and speed of analysis.

Saeed et al. [91] report the application of CEC to the quantitative analysis of individual mono- and dihydroxy phenols in tobacco smoke. According to Saeed et al., the method presented provides the analysis of real samples in significantly shorter times than achieved by current GC and HPLC methods. Dadoo et al. [43] presented the isocratic separation of 16 polycyclic aromatic hydrocarbons (classified as priority pollutants by the U.S. Environmental Protection Agency) by CEC with a packed capillary in under 10 min. Bailey and Yan [42] realized the complete separation of a series of 14 nitroaromatic and nitramine explosive compounds under isocratic conditions in under 7 min (see Fig. 8).

Currently, there is much interest in the evaluation of the potential of CEC for the analysis of biomolecules. CEC has good comapti-
bility with mass spectrometry. In addition, electrospray ionization in mass spectrometry has evolved into a powerful tool for the analysis of biomolecules. CEC/MS might fill the place of HPLC/MS when high separation efficiency is needed. So far, published applications of CEC/electrospray ionization mass spectrometry, CEC/ion trap storage/reflectron time-of-flight mass spectrometry, CEC/electrospray ionization time-of-flight mass spectrometry, or CEC with photometric (UV) detection in the field of bioanalysis include: separation of phenylthiohydantoin amino acids [98,111,141,156] (see Fig. 14), separation of peptide and protein mixtures [94,95,116,157], analysis of peptide and protein digests [124,126,127,158], separation of isomeric polycyclic aromatic hydrocarbon–deoxyribonucleoside adduct mixtures [140], separation of derivatized mono- and oligosaccharides [96], separation of purine and pyrimidine bases and their nucleosides [159], and separation of oligonucleotides [68].

VII. CONCLUDING REMARKS

Capillary electrophromatography is currently a field of very active research. This hybrid of liquid chromatography and capillary electrophoresis has proven to be superior over HPLC in terms of efficiency and offers in the case of ionizable solutes selectivities that are different from HPLC and CE.

Not all phenomena that have been observed by those applying CEC are fully understood, e.g., band shapes found for ionic compounds separated on an ion exchanger. However, practical problems that hampered rapid development of this method in the early days of CEC seem to have been overcome. If theoretical predictions that the inner diameter of separation capillaries in CEC can be considerably larger than in CE without loss in efficiency prove to be well founded, detection limits of methods using CEC will be largely improved, and CEC would gain an additional advantage over CE. In order to construct robust, highly efficient, and reliable separation columns for CEC, fritless capillaries are desired. From a theoretical point of view, capillaries filled homogeneously with a bed of stationary phase are best suited to meet the needs of CEC.

Encouraging results obtained with CEC are in accordance with predictions based on separation science theory. These results justify current efforts made in the synthesis of new stationary phases, in instrumental developments, in the design of coupling devices for the
coupling of CEC with powerful spectrometric techniques (e.g., mass spectrometry or nuclear magnetic resonance), and in the development of new detection techniques meeting better the requirements for detection in a section of the capillary than those techniques that have been developed for HPLC. A sound theoretical understanding of parameters influencing a separation achieved by CEC will be very important in order to exploit the full potential of this new separation technique.

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2 Membrane Extraction Techniques for Sample Preparation

I. SAMPLE PREPARATION TECHNIQUES

In the field of chromatographic analysis, sample preparation is a somewhat neglected aspect, even if it has received increased attention during the last years. It is frequently necessary to separate the analytes from a matrix containing various high-molecular disturbing materials, usually insufficiently characterized. This task is usually called cleanup, and it is one of the main objectives of sample preparation. Well-known examples where cleanup is mandatory are drug analysis in biological liquids such as blood or urine, and trace organic environmental analysis, where humic substances influence the detection of the analytes. Another aspect of sample preparation is enrichment of the analyte—i.e., to increase its concentration over the matrix background in order to decrease the detection limits. Obviously, these objectives are intimately connected. A third objective, which is becoming increasingly important, is the possibility for automation of the entire analytical process. Apart from the higher sample throughput possible, automation permits the use of
closed analytical systems, leading to better control of contamination in trace analysis. According to a survey study [1], more than 60% of the total time spent on chemical analysis is used for sample preparation, while only 7% is used for the final analysis per se, using various chromatographic or spectroscopic techniques. It is obvious that there is a great potential for saving by automating the sample preparation process. The most commonly used techniques for sample preparation in organic analysis are liquid-liquid extraction (LLE) and solid-phase extraction (SPE). LLE is the classical technique for sample preparation, and it has been described in several recent reviews [2,3]. This technique was the dominating one a few years ago [4] and is still so. For example, in the U.S. Environmental Protection Agency (EPA) protocols for environmental analysis, most methods still prescribe LLE. This technique offers large potential for tuning the extraction by chemical means, such as incorporating different specific reagents. LLE also has a high tolerance for interfering compounds,
and it provides physical separation of the extracted analyte from the sample. With LLE, it is possible in many applications to achieve efficiently both cleanup and enrichment. However, there are also some serious drawbacks, such as large consumption of solvents, difficulty of automation and on-line connection to analytical instruments, as well as often troublesome formations of emulsions. SPE is the more modern of these techniques, and it is amply described in several recent books [5,6]. By means of a solid sorbent, available in many chemical and physical forms, analytes are extracted from the sample phase (normally aqueous) and subsequently eluted with an organic solvent. There are a number of ways to automate SPE in high-performance liquid chromatography (HPLC), either by using dedicated instruments, so-called SPE workstations, or by precolumn techniques [6]. The related technique of solid-phase microextraction (SPME) [7,8] is easily automated, especially in connection with gas chromatography. Some drawbacks with the SPE techniques are insufficient retention of very polar compounds, limited selectivity, and high costs associated with disposable sorbent
materials. In addition, even if the amount of solvent needed for SPE is decreased compared to LLE, it might still be significant. Some aspects of SPE and LLE are easily compared in the EPA sample preparation protocols. For example, in a generic EPA SPE procedure (Method 3535) [9], 85 mL of organic solvent is needed for extraction of 1 L of water sample (30 mL for elution, 55 mL for washings and conditioning). Method 3510, which is the corresponding LLE method, is applicable to many more analytes. It requires 180 mL of organic solvent, which is more, but not dramatically so. In both these methods, the volume of the extract is reduced to 1-10 mL before analysis. As alternatives to the SPE and LLE techniques, different membrane techniques have been suggested, as recently reviewed by van de Merbel [10]. A simple division is between porous and nonporous membranes. Porous membranes are used in dialysis to separate low molecular analytes from high-molecular matrix components, and cleanup is achieved in that way. There is no discrimination between different small molecules. There is no enrichment possible, rather a
dilution. Variations of porous membrane techniques are microdia

lysis [11,12], which is used extensively in neuroscience research for

in-vivo sampling, and electrodialysis [10], in which an electric field is

employed to selectively transport charged compounds over a dialysis

membrane. In addition, a number of micro- and nanofiltration tech

iques belong to the field of porous membrane techniques. By a com

bination of dialysis with SPE, both cleanup and enrichment can be

performed. This is the ASTED process [13,14], the basis for a com

mercial automated instrument (Gilson S.A., Villeiers-le-Bel, France). Typical applications involve drug analysis in blood plasma.

This somewhat neglected technique was recently reviewed [10]. Porous membrane techniques are strictly not extraction tech

iques, and they are not further considered here. In this review, the

focus is on membrane extraction techniques utilizing nonporous

membranes. A nonporous membrane is a liquid or a solid (e.g., poly

meric) phase that is placed between two other phases, usually liquid

but sometimes gaseous. One of these phases is the sample to be pro

cessed, the donor (or feed) phase. On the other side of the membra


is the acceptor (or strip) phase, in which the extracted analytes are collected and transferred to the analytical instrument. With this arrangement, the versatile chemistry of LLE can be employed (and extended) in a format that is amenable to automation. This can provide unsurpassed cleanup efficiency and enrichment factors, in most cases with insignificant or no use of organic solvents. The following description will be focused on these techniques, their connection to chromatography and to capillary electrophoresis, as well as their applications to biological and environmental analysis.

II. MEMBRANE EXTRACTION TECHNIQUES

A number of nonporous membrane techniques have been described for sample preparation in analytical chemistry. The main versions are summarized in Table 1 and described in the following sections. In Fig. 1, typical units for membrane extraction are shown. Usually they are constructed of two blocks of inert material with a machined groove in each. When the blocks are clamped together with a membrane between, a flow-through channel is formed on each side of the membrane; the upper one is the donor channel, and the lower
Table 1 Types of Membrane Extraction

<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviation</th>
<th>Phases</th>
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<tbody>
<tr>
<td>Supported liquid membrane extraction</td>
<td>SLM</td>
<td>Aq/org/aq</td>
</tr>
<tr>
<td>Microporous membrane liquid-liquid</td>
<td>MMLLE</td>
<td>Aq/org/org</td>
</tr>
<tr>
<td>Polymeric membrane extraction</td>
<td>PME</td>
<td>Aq/polymer/aq or org/polymer/aq or aq/polymer/org</td>
</tr>
</tbody>
</table>

Refs. a [15,16] [16,17] [18] [19,8] [19,9] [19,10]

First ref. for analytical sample preparation, recent review

one is the acceptor channel. For sample preparation, channel volumes are in the range 10-1000 μL. An extraction unit based on a hollow fiber membrane is also shown in the figure. Here the acceptor phase is inside the fiber lumen and the donor channel is the annular volume between the outside of the fiber and the inside of a surrounding tube or cylindrical hole. This type of unit can be made with
channel volumes as small as 1 |LIL [20,21]. With proper modification,

the membrane units shown in Fig. 1 are in principle applicable to

all versions of membrane extraction.

A. Supported Liquid Membrane Extraction (SLM)

Supported liquid membrane extraction has been used for some time

for industrial separations, mainly for extraction of metal ions from,

e.g., wastewater [24,25]. The use of SLM for sample preparation in

analytical chemistry was proposed by Audunsson [15], and the field

has been the subject of several earlier reviews [10,16,22,26-28]. In SLM, the membrane consists of an organic solvent, which

is held by capillary forces in the pores of a hydrophobic porous

membrane. Typical solvents are long-chain hydrocarbons such as n

undekane or kerosene and more polar compounds such as di-hexyl

ether, tri-octyl phosphate, and others. Various additives can in

crease the efficiency of extraction considerably, as described below.

Fig. 1 (a) Membrane unit with 1-mL channel volume. From Ref. 22 with

permission from Elsevier Science. (A = blocks of inert material, B = mem

brane), (b) Membrane unit with 10-|LIL channel volume. From Ref. 23 with
Hollow fiber membrane unit with 1.3-JJL acceptor channel (lumen) volume. From Ref. 21 with permission. Copyright 1997 American Chemical Society.

(1 = O-rings, 2 = polypropylene hollow fiber, 3 = fused silica capillaries, 4 = male nuts). In Fig. 2, the basic principle for SLM extraction of basic compounds, e.g., amines, is presented. The sample pH is adjusted to a high enough value for the amines to be uncharged, and the acceptor channel on the other side of the membrane is filled with a stagnant acidic buffer. When the sample is pumped through the donor channel, the uncharged amines (B) are extracted into the organic membrane phase. After having diffused through the membrane, an amine molecule will be immediately protonated at the membrane-acceptor interface and therefore prevented from reentering the membrane. This is referred to as trapping, and it results in a transport of amine molecules from the donor to the acceptor phase, which after the extraction is transferred to an analytical instrument, either manually.

Fig. 2 Schematic description of the SLM principle. For details, see text.
or on-line by a flow system. As the extract is aqueous, the technique is best compatible with reversed-phase liquid chromatography or ion chromatography. If the trapping is virtually complete, practically all analyte in the acceptor will be in the form of ammonium ions. Therefore, the concentration gradient (which controls the mass transfer rate) of the diffusing species (the free amine) will be practically unaffected by the total concentration of amine in the acceptor phase. This leads to a potentially high degree of concentration enrichment (several hundred or thousand times, depending on volumes and time), when more and more sample is pumped through the donor channel and collected in the acceptor channel. It is obvious that acidic compounds (HA) will be completely excluded from the membrane as they are already charged in the alkaline donor phase. This also holds for permanently charged compounds. Neutral compounds (N) may be extracted, but not enriched, as the concentration in the acceptor phase will never exceed that in the donor. Macromolecules, such as proteins, will typically be charged and therefore rejected. Finally, the extraction rate of un
charged macromolecules will be very low due to their low diffusion coefficients. Thus, under the conditions mentioned, the SLM extraction will be highly selective for small, basic compounds. There are many possibilities to tune the chemistry in the three phases to enrich different classes of compounds. Acidic compounds may be extracted in a similar way as amines by reversing the pH conditions in Fig. 2. Adding reagents, e.g., ion-pairing or chelating, to the donor phase permits the design of SLM extraction systems for various permanently charged compounds and metal ions. Selectivity and mass transfer can be further enhanced by incorporating various carrier molecules or ion complexes in the membrane phase. Various trapping reagents in the acceptor phase can be used to prevent analytes being extracted back into the membrane. It is also possible to add soluble antibodies to the acceptor phase, so analytes can be selectively trapped as antigen-antibody complexes, leading to ultimate selectivity [29]. This principle can also be used for enrichment of permanently neutral species. Summarizing the principles of SLM extraction: neutral, extract
able species should be formed in the donor phase (or at the donor membrane interface); these species should be transported through the membrane and in the acceptor phase become transformed to an other, nonextractable species. Chemically this is similar to liquid liquid extraction into an organic solvent, followed by a back extraction into a second aqueous phase.

A thorough treatment of the theory and principles of the SLM process has been presented [30], and some highlights are the following. The extraction efficiency E (fraction of analyte molecules that are recovered in the acceptor) is a function of many parameters. These include the magnitude of the partition coefficient of the analyte between the aqueous phases and the organic (membrane) phase, trapping conditions in the acceptor, flow rate of the donor, character istics of the membrane, and dimensions of the membrane. The influence of the partition coefficient K is somewhat complex.

At low values of K, E is small, as the analyte is insufficiently ex tracted into the organic membrane and the mass transfer is limited by the diffusion transfer through the membrane. At intermediate
values, the mass transfer is limited by the transport properties in
the flowing donor phase, and in this region the most efficient extrac
tion is obtained. At very high values of \( K \), i.e., for very hydrophobic
compounds, the stripping of analyte into the acceptor phase becomes
the limiting factor, and the observed extraction efficiency will de
crease, as relatively large amounts of analyte will be left in the mem
brane. In a recent study [31], it was found that the most efficient
extraction is obtained when the octanol-water partition coefficient
as a rough estimate is around \( 10^3 \). The trapping conditions in the acceptor are also important for
the extraction efficiency. If the trapping is not complete, the extrac
tion efficiency will decrease with time, leading to less precise quanti
tation. This was detailed in a recent paper [32]. The extraction efficiency is highest for very low donor flow rates,
and decreases as the flow rate increases. On the other hand, increas
ing the donor flow rate also increases the amount of analyte that is
introduced into the extraction system, and the net result often is an
increase in the amount of accumulated analyte in the acceptor dur
ing a given time. Thus, given enough sample volume, high
flow rates lead to lower detection limits. With very small sample volumes, a low flow rate might be needed in order to squeeze as much analyte as possible from the sample. Thus, it is obvious that it is not necessary to strive for the maximum value of E, and that this parameter should not be confused with recovery. For good quantitative performance, the important issue is to find conditions that lead to reproducible values of E, and the value of this parameter will be included in the calibration. This is analogous to, e.g., mass spectrometry, where the ionization efficiency usually will be far below 100%, still providing satisfactory quantitation.

B. Microporous Membrane Liquid-Liquid Extraction (MMLLE)

As mentioned above, the SLM technique will not work well for highly hydrophobic compounds, especially if they cannot be trapped efficiently. Examples of such compounds are hydrocarbons, PCBs, etc., which can easily be extracted from water into an organic solvent, but cannot be extracted out into an aqueous acceptor as required by the SLM principle. For such applications, the technique of MMLLE
is more suitable. Here the acceptor phase is an organic solvent and
the same solvent forms the liquid membrane by filling the pores in
the porous hydrophobic membrane [16]. In Fig. 3, the principle of
MMLLE is sketched. Chemically, this is analogous to conventional
liquid-liquid extraction, but performed in a flow system, easily auto-
mated and interfaced to analytical instruments. As the extract ends
up in an organic phase, the technique is most easily interfaced to
gas chromatography (GC) or to normal-phase liquid chromatography
(NP-HPLC). In principle, the membrane could also be hydrophilic,
which would lead to aqueous phase in the membrane pores. As far
as we know, this has not yet been tried for analytical purposes.

Fig. 3 Schematic description of MMLLE. For details, see
text.

LLE in a flow system (in the form of flow injection analysis) has
been described many times, as reviewed by Valcarcel [33], but then
the organic and aqueous phases are mixed in the same flow channel
and later separated. The practical problems with the phase separa-
tion seem to have prevented this technique being widely
used. In MMLLE, the phases are never mixed and all mass transfer between the phases takes place at the membrane surface.

The extraction efficiency is limited by the partition coefficient. If it is very high, it is possible to work with a stagnant acceptor and still get considerable enrichment into a very small extract volume. With smaller partition coefficients, it might be necessary to arrange a slow flow of the acceptor phase to remove the extracted analyte and maintain the diffusion through the membrane.

C. Polymeric Membrane Extraction (PME)

One of the drawbacks of SLM extraction is the possible instability of the liquid membrane, even if this problem usually seems to be overrated, at least for analytical laboratory applications, where a membrane lifetime of several days to weeks is sufficient. This is discussed further in Section IV.C. In any case, by exchanging the SLM with a polymeric membrane, usually a silicon rubber membrane, the membrane lifetime is considerably increased. However, with a fixed composition of the membrane, the possibilities for chemical tuning
(e.g., the application of carriers) of the separation process is greatly reduced. This is probably most serious for relatively polar analytes, where the hydrophobicity of the membrane has to be reduced. Also, as diffusion coefficients in polymers are lower than in liquids, the mass transfer is slower, leading to slower extractions. On the other hand, as the membrane is virtually insoluble, any combination of aqueous and organic liquids can be used. Melcher [18,34] has developed this principle, both with a trapping acceptor (as described above for SLM), and with a solvent in the acceptor channel, leading to a situation similar to MMLLE.

D. Membrane Extraction with a Sorbent Interface (MESI)

The above techniques are all characterized by liquid donor and acceptor phases. For easy interfacing with gas chromatography a gaseous acceptor phase is the most convenient. The MESI technique realizes this principle [8,19]. It can be applied to either gaseous or aqueous samples, and the equipment consists of a membrane module with a (usually) silicone rubber hollow fiber, into which the analytes are extracted from the surrounding liquid or gaseous
sample. The technique works best for volatile and relatively nonpolar compounds. Inside the fiber flows a gas, which transports the analyte molecules away from the membrane into a cooled sorbent tube where they are trapped. By heating the sorbent trap, the analytes are desorbed and transferred to GC analysis. Incidentally, the MESI principle can be seen as a gas-phase analogy to the ASTED principle for liquid chromatography; see above. In Fig. 4, a typical MESI setup is shown. All components are connected in-line so that the carrier gas for the GC passes through the membrane fiber and the sorbent trap, resulting in a completely integrated instrument setup. Sampling can also be made off-line with the extraction module and sorbent trap in, e.g., field sampling, and the sorbent trap can later be connected to the GC and desorbed in a separate step. Matz [35] recently presented and compared this and a few other variants.

Fig. 4 Components of the MESI system coupled to a gas chromatograph.

(From Ref. 8, with permission.) Membrane Extraction for Sample Preparation I 65

III. INTERFACING MEMBRANE EXTRACTION AND SEPARATION
Some of the membrane techniques can be performed in a manual and off-line way, not connected directly to a chromatographic or electrophoresis analysis instrument. A good example is a manual version of SLM extraction (named LLLME) [36] utilizing disposable units for extraction of amphetamine from blood plasma samples and subsequent off-line analysis by capillary electrophoresis. For environmental applications [37,38], the extracts from large SLM units are sometimes collected manually and injected into the HPLC using an autosampler. This permits sampling outside the lab and duplicate chromatographic analyses. In addition, connections to flow-injection analysis systems (FIA) can be mentioned, where the uses of gas-diffusion membranes (aq/gas/aq), as well as dialysis cells, are widespread [33]. Early realizations of MMLLE [39] and PME [40] also involved FIA systems. More recent examples are an SLM-FIA setup for Cr(VI) [41] and an MMLLE-FIA application involving a chemometric evaluation of diode array spectra after an aq/org extraction was recently presented [42]. However, the membrane extraction techniques are well suited
for automatic connection to chromatographic instruments, and this principle leads to many advantages, so it will now be discussed in some detail.

A. Interfacing with Liquid Chromatography

Several of the membrane extraction techniques, such as SLM and PME, typically lead to an aqueous extract. Such extracts can most straightforwardly be analyzed with reverse-phase liquid chromatography (RP-HPLC). For this purpose, flow systems can easily be built up around peristaltic pumps and pneumatic valves, controlled by electronic timers, integrators, or computer systems. A typical such system is shown in Fig. 5. It was originally set up for SLM extraction of chlorinated phenols from natural waters [43], but was used also for other environmental applications [44,45]. The sample is acidified and pumped with a peristaltic pump (1) through the donor channel. During the extraction, the alkaline acceptor is kept stagnant. By switching a valve (5) after the extraction, it is transported further on, neutralized, and moved to a...
(9), where the analytes are adsorbed and focused. After switching the injection valve (8), the analytes are transferred to the analytical column. Reagent water for washing the donor channel between the samples can be introduced by means of a valve (2). There are also provisions to rinse the precolumn with acid between runs. This type of system has been used mainly in environmental applications for extraction of relatively large amounts of natural water with large membrane units (channel volumes 1 mL). The precolumn ascertains that all extracted analytes in one extract are analyzed in one chromatographic run. Alternatively, a smaller membrane unit can be used, so that the entire extract (or a major part of it) can be contained in the injection loop and thus injected directly into the liquid chromatograph without a precolumn [46,47]. The same approach was used by Melcher [18] and by researchers from Salamanca, Spain, in a series of papers dealing with MMLLE in org/aq or org/org configurations [48-50]. For biological samples of about 1 mL, the peristaltic pump/solenoid valve approach is not suitable. More accurate
instrumentation can be constructed using an autosampler and syringe pumps.

As an example, a fully automatic system is shown in Fig. 6. It was built around an "intelligent sample processor" Model 231 (Gilson).

Fig. 6 Experimental setup for SLM-HPLC determination of biomolecules in blood plasma or urine. (Adapted from Ref. 23 with permission. Copyright 1994 American Chemical Society.)

and originally constructed for the determination of basic drugs in blood plasma [23]. The same principle was used also in other studies [47,51,52], and this is probably the most versatile setup for automated SLM-HPLC applications. The plasma samples are held in vials in an autosampler rack.

Immediately before extraction, the alkaline donor buffer is added by means of the syringe pump and robotic needle. The alkalized sample is pressed through the donor channel in the membrane unit. After the extraction is completed, the contents (10 μL) of the acceptor channel are transferred by means of a second syringe pump into the injection loop and subsequently injected into the chromatographic column. The operations of the sample preparation system are
synchronized with the chromatographic computer system, so one sample is extracted during the chromatographic run of the previous sample. Thus, the sample throughout is determined by the length of the chromatogram, typically ca. 15 min. A miniaturized SLM extraction cell, comprising a hollow fiber membrane and having 1.3 JLL acceptor volume inside the fiber lumen, was developed and satisfactorily connected to packed-column capillary liquid chromatography by using a miniaturized loop injector [20]. Often the extract after MMLLE and PME is organic, and in those cases, interfacing to NP-HPLC is more suitable. This can be realized with an autosampler in essentially the same way as described above [53].

B. Interfacing with Gas Chromatography

The interfacing between a membrane technique and capillary GC is an integral feature in MESI for volatile compounds. The extract is in the form of a gas and the analytes are trapped on a sorbent column and thermally desorbed directly into the GC column; see Fig. 4.

Slightly different technical realizations of this idea have been pre
sented [8,35,54,55], differing mainly in the type of heating and the physical arrangement of the membrane. For liquid extracts, GC with capillary columns poses quite stringent demands on the sample: it should be essentially water-free, and the volume is quite restricted. This means that SLM and other techniques leading to aqueous extracts are less suitable for on-line connection to GC. With packed columns, however, which can tolerate injection of aqueous samples, automated connections were early realized [56-58]. By exchanging the aqueous solvent for hexane in a solvent-exchange interface, as described by Vreuls and co-workers [59], it was possible to interface an SLM extraction system to capillary GC [51]. The resulting system was quite complex, although completely automated extraction and analysis of local anesthetics in blood plasma was achieved with good performance. Membrane extraction techniques such as MMLLE and PME with organic acceptors can be interfaced to GC relatively easily. This can be realized by means of large-volume injection methodology, a topic that recently was thoroughly reviewed [60]. As a simplification of the above-mentioned SLM-GC system [51], an MMLLE-GC sys
tem for the same application was constructed, resulting in a system that was considerably more easily operated and more rugged [17].

This system is shown in Fig. 7. The extraction part is similar to that in Fig. 6: the donor phase is pumped with the syringe pump (1), the samples in the autosampler (2) are alkalized one by one and passed through the donor side (3) of the membrane unit. With the solvent pump (5), acceptor solvent (hexane) is slowly pumped through the acceptor channel (4) and subsequently to a 400-uL loop (7) connected to an injection valve (8).

 Provision for nitrogen purging (5) was needed to decrease carryover effects. After extraction, the contents of the loop were transferred to the GC, equipped with a retention gap (10), a retaining precolumn (11), a solvent vapor exit (14), and a capillary column (12). The GC system was set up as described by Grob and Stoll [61]. By miniaturization, the connection MMLLE-GC can be further simplified. A device called Extraction Syringe (ESy) has
recently been described [62]. See Fig. 8. The main part is a single hydrophobic hollow fiber (1), mounted in the center of a Kel-F piece (A; 3) with a drilled hole. A stainless steel needle (B, 2) is extruding from the end of the fiber. The lumen of the fiber contains the organic acceptor phase, with a volume of a few microliters (5). The sample to be extracted is pumped (6) around the fiber and the analytes are partitioned into the organic solvent. The instrument is placed directly on top of a gas chromatograph for automated extraction and injection onto the GC by means of a pneumatic piston (C), so the operation mimics the operation of an autosampler injection. With a few microliters of injected volume, no special arrangements have to be made with the gas chromatograph; a conventional splitless injection is appropriate. Although this type of MMLLE-GC connection is yet little tested, it seems that this principle has significant advantages over
other principles for connection of LLE to gas chromatography, and

as such it should have a large application potential.

C. Interfacing with Capillary Electrophoresis

As the sample volume in capillary electrophoresis (CE) has to be

very small, typically in the nanoliter region, on-line connection of

sample pretreatment devices is difficult. By means of various so

called stacking procedures, several microliters can be introduced

and the analytes compressed in the beginning of the separation capillary. This simplifies the task of in-line connection, but additional

aspects such as high voltages hazards still make the topic difficult

and inconvenient. Two recent reviews [63,64] describe this in detail.

There are not many examples of direct connection of nonporous

membrane techniques on-line with CE, but a number of dialysis applications have been presented [10]. Bao and Dasgupta [65] con

nected a short piece of hollow fiber membrane in-line with the capillary. This was made with both porous membranes for dialysis and

gas-phase transfer and with a polymeric (silicone rubber) mem

brane. In the latter case, acceptor trapping of phenols in
an alkaline buffer was accomplished in the same way as described for SLM above, and significant enrichment was obtained. As the membrane was short in comparison with the separation capillary, no stacking was required and the plate number of the separation was not appreciably influenced. There seems not to be any follow-up of this work, which seems rather promising, although it is easy to predict a number of practical problems. Another principle for membrane-CE interfacing is to utilize a separate micromembrane cell (Fig. 1c), equipped with a hollow fiber membrane with a small volume [21]. This approach provides a more stable system and necessitates a stacking procedure, giving an increased overall concentration enrichment. The setup was not entirely automated, as it required a manual connection of the capillary to the outlet of the membrane unit, although this in principle could be handled with a suitable valve.

IV. WHAT CAN BE ACHIEVED BY MEMBRANE EXTRACTION?

In this section, the main advantages with membrane extraction in sample preparation for chromatography and related techniques will
be discussed. Compared to other techniques, the membrane extraction techniques provide advantages in various ways. Membrane extraction probably offers the highest degree of selectivity and cleanup from complicated matrixes of all known techniques, and it is possible to achieve very high enrichment factors with preservation of the selectivity. Automation and on-line connection to instruments for chromatographic final analysis can be readily made and, compared to most other techniques for sample preparation, the use of organic solvent is much reduced, in most cases essentially to zero. This is true for a large number of compound classes, in various matrices and concentration ranges.

A. Cleanup and Selectivity

All types of nonporous membrane extraction procedures will in principle lead to a high degree of cleanup, especially between small and large molecules. The analytes to be analyzed must dissolve into the membrane, pass through it, and redissolve in the acceptor phase. In many cases, the conditions of extraction can be set so that this chain of events is possible only for a strictly limited range of
compounds.

The possibilities to achieve this tuning are best for the SLM technique, where selective reactions in all three phases can be utilized for this purpose, but also for the other membrane extraction techniques, there are a number of such possibilities. A simple example is the already-mentioned principle for specific extraction of basic compounds from an alkaline sample (donor) to an acidic acceptor.

To increase the selectivity further, the pH difference between the donor and acceptor can be optimized, so only amines of a limited range of pK_a are extracted. As an example, if the pH is 11 and 2 in the donor and acceptor, respectively, both aliphatic and aromatic amines with pK_a in the approximate range 4-11 will be extracted and enriched (5-10 with optimal efficiency). On the other hand, with an acceptor pH of 5, only amines with pK_a in the range 7-11, i.e., preferentially aliphatic amines, will be enriched, as the more weakly basic aromatic amines will not be trapped efficiently. By selecting the polarity of the membrane liquid to match the polarity of the ana
lytes, the selectivity can be further increased. With certain additives in the membrane phase, the extraction properties can be changed radically. An example of this is shown in Fig. 9, where the extraction of carboxylic acids of different polarities is strongly influenced by the contents of TOPO (tri-octyl phosphine oxide) in the membrane [66]. The most polar acid, lactic acid, was not extracted at all without TOPO, but the extraction was significantly improved by the additive. Butanoic acid, however, was well extracted without TOPO, and essentially unaffected by its concentration. There are a number of other possible additives that have been used in SLM extraction for enhancing the extraction efficiency of different classes of compounds, such as chelating or complexing reagents, crown ethers, ion-pair formers, artificial receptors, etc. Some of them are mentioned in the applications section below.

Fig. 9 Influence of TOPO content in di-n-hexyl ether on extraction efficiency E. (Adapted from Ref. 66, with permission from Elsevier Science.) Both in biomedical analysis and in environmental analysis, an important objective for sample preparation is to remove high molecular-weight material. In the case of biomedical analysis, such
material is usually proteins, and in environmental applications it is mainly humic substances. The membrane extraction techniques are all very efficient in this respect; such high-molecular-weight compounds are often charged and therefore not extracted into organic liquids. Even if they are noncharged, the transport is so slow that their extraction is negligible. Therefore, membrane extraction leads to very clean extracts in different applications. In Fig. 10 [23] it is shown how some basic drugs, extracted from blood plasma with the instrumentation shown in Fig. 6, gave HPLC chromatograms that can hardly be distinguished from extractions from pure aqueous buffer solution. However, the response in blood plasma was significantly lower. That effect was traced to binding of the drug to plasma proteins, and preliminary studies of the possibility to evaluate protein binding effects including their kinetics have been started. Another example (Fig. 11) [52] concerns the extraction of polar drug metabolites from urine, where the chromatograms again are virtually indistinguishable from water solutions. Here, however, the...
Fig. 10 (a) Chromatograms of Amperezide (I), its metabolite (II) and homolog (III) with the subsequent blank after enrichment from blood plasma.

(b) Corresponding chromatograms after enrichment from an aqueous buffer solution. Concentrations 4μg/mL of I and II, 8 μg/mL of III. (From Ref. 23, with permission. Copyright 1994 American Chemical Society.)

Fig. 11 Chromatograms of a water solution (a) and a urine sample (b), both spiked with 3-OH-PPX (1; 1.0 \mM), 4-OH-Ropivacain (2; 0.80 μM), 3-OH Ropivacain (3; 0.83 μM) 9 PPX (4; 1.0 \mM) 9 Iso-PPX (5; 0.84 μM), and Ropivacaine (6; 0.90 μM). (From Ref. 52, with permission of Elsevier Science.)

response is the same in urine and water, as there is no protein binding involved. Figure 12 compares the cleanup possibility of SLM extraction and SPE for triazine herbicides in spiked natural water [67]. The SPE chromatogram (a) shows a characteristic "humic hump," with the analytes influenced by matrix peaks of nearly the same order of magnitude. The chromatogram after SLM extraction (b), with analyte concentrations that are twice lower, shows neither the "hump" nor any disturbing matrix peaks, thereby demonstrating a higher
degree of cleanup resulting in lower detection limits. The above examples all refer to SLM extraction. Although there are fewer degrees of freedom in PME and MMLLE, here also good selectivity can be obtained. In Fig. 13 [50] is shown chromatograms of sunflower oils, unspiked and spiked with triazine herbicides, after an org/aq MMLLE extraction into an acidic acceptor. The membrane device was on-line connected to RP-HPLC as described above.

B. Enrichment

The different membrane extraction techniques behave differently when it comes to concentration enrichment factors. In aq/org types of extraction (MMLLE and PME), the maximum concentration enrichment factor is limited to the value of the partition coefficient between the donor and the acceptor phases. In those techniques, appreciable extraction factors are possible only when the partition coefficient is large; the same situation as for ordinary LLE. This does not preclude that considerable enrichment factors can be obtained when the conditions are favorable. Extraction enrichment factors of about 250 times were obtained in MMLLE extraction of cationic surfactants in natural water [53]. Also, in an aq/org PME-GC determi
nation of chlorinated hydrocarbons and other compounds [34], ex-
traction factors up to 200 times were obtained. In SLM, on the other hand, the enrichment factors are not lim-
ited by the partition coefficient, but from the trapping conditions in
the acceptor phase, as was recently detailed [32]. With some simpli-
fications, the maximum enrichment factor $e(\text{max})$ for SLM extraction
of a basic compound as described in Fig. 2 and the accompanying
text, depends on the acceptor pH (pH $A$) by

Fig. 12 Chromatograms (LC-UV) of methoxy-s-triazine herbicides: (a) SPE
extraction of spiked river water (1.0 $\mu$g/L of each analyte); (b) SLM extrac-
tion of spiked river water (0.5 $\mu$g/L of each analyte). Peak designation: 1, Simetone; 2, Atratone; 3, Secbumetone; 4, Terbumetone. (Reproduced from Ref. 67 with permission from Elsevier Science.)

Fig. 13 Chromatograms of sunflower oil (a) before and (b) after spiking with
1.0 ppm of each triazine: (1) atrazine, (2) ametryne, (3) prometryne, (4) ter-
butryne. (From Ref. 50, with permission of the authors and Elsevier Science.)

Fig. 14 Enrichment factors of aniline (1), 3-chloro-4-methylaniline (2), 3,5
dichloroaniline (3), and 3-methyl-5-nitroaniline (4), all
0.1 mg/L. Acceptor: 0.1 M sulfuric acid (pH ~ 1). (From Ref. 32, with permission. Copyright 1998 American Chemical Society.)

The dissociation constant of the analyte is pK a. Thus, with reason ably strong bases, it is easy to achieve large values for the maximum enrichment factor. On the other hand, as detailed below, to really obtain high enrichment factors, a high sample/extract volume rate is necessary. This is illustrated in Fig. 14, showing the attainment of the max imum enrichment factor for four aniline derivatives with pH A = 1.

For aniline itself (1), pK a = 4.6 leads to a maximum enrichment fac tor of about 4000 times. This is apparently not reached until after long extraction times (the experiment was ended after 25 h of extrac tion and 6 L of sample, giving a final enrichment factor of about 2000 times and still increasing). On the other hand, for the weakly basic 3,5-dichloroaniline (3), pK a = 2.5, giving the maximum enrichment factor of only about 32 times, which is quickly reached after a short time of extraction. The situation can be improved by increasing the acid concentration in the acceptor, as shown in the cited
work. Seen in another way, the enrichment factor obtained is given by

the following relation:

where \( V_s \) is the volume of the extracted sample and \( V_A \) is the volume

of the extract, in SLM the volume of the acceptor channel. From this

equation, it is seen that even if \( J? \) approaches 1, the enrichment fac

tor is never larger than the volume ratio. The strength of SLM in this

context is that it can provide relatively high extraction efficiencies at

the same time as the extract volume is kept small. For nontrapped

techniques such as MMLLE, it might not be possible to achieve a

large \( E \) with a stagnant acceptor (if the partition coefficient is not

enough large) and therefore the acceptor must be pumped, leading

to larger \( V_A \) and smaller \( E_e \). The same, to a higher degree, is true

for dialysis. This limitation in MMLLE is overcome by introducing

a secondary focusing step. For capillary GC applications this can be

a retention gap [17], and for LC the solvent strength can be selected

so a column focusing effect is obtained [53]. The same limitation is overcome in MESI (and in the ASTED
dialysis approach) using a solid-phase column where the analytes
are trapped. For these cases, the \( V_A \) in the equation above could be considered as the desorption volume of the solid-phase trap. Obviously, selectivity is a prerequisite for enrichment; it is pointless to enrich disturbing compounds also. In Fig. 15 is shown a result from a combined SLM-HPLC-CE application [68], where Bambuterol, a basic drug, was extracted from blood plasma using SLM. Further, it was introduced into a micro-HPLC column, from where a heart cut was transferred to the CE and finally analyzed using the double-stacking procedure and enantiomeric separation. The overall concentration enrichment factor is here about 40,000 times and the detection limit in blood plasma is ca. 0.15 nM for each of the Bambuterol enantiomers with simple UV detection in CE. The main origin of the high enrichment factor is here not the SLM step, but in analyte focusing on the HPLC column and in the stacking, but it would be impossible without the high degree of selectivity provided by the SLM step.

C. Automation and Unattended Operation

As the membrane extraction process can be most conveniently performed in flow systems, it is easy to devise arrangements
employing pumps, autosamplers, solenoid and rotary valves, etc., with computer control that can provide more or less automated operation. In Section III and Fig. 4, a number of examples of such systems are shown.

Fig. 15 Electropherograms showing plasma containing 10 pM physiostigmine as a protease inhibitor (a) and plasma additionally containing 0.5 nM of each Bambuterol enantionmer. Peaks labeled A are the Bambuterol enantionmers and the peak labeled B is the physiostigmin. (Adapted from Ref. 68, with permission of ICS Technical Publications, Inc.)

For unattended operation of automated systems, the high selectivity and cleanup possible with membrane extraction is an advance, as it prolongs the usable lifetime of columns, etc. On the other hand, membrane stability could be a limiting factor. For the PME, MMLLE, and MELSI techniques, this is not a big problem, as the membranes used are polymeric and durable. Sometimes, fouling by dirty samples can be seen, but it is possible to devise washing schemes in automated membrane extraction to diminish the prob
lem. In any case, a smooth membrane surface is less amenable to fouling than, e.g., an SPE column, and it is easier to wash. It was noted [20] that the pore size of the membrane could have an important impact on the membrane fouling in MMLLE of blood plasma samples. For SLM extraction, the membrane stability is less obvious, and the issue is often raised. Here, an organic solvent is held in the pores of a hydrophobic porous membrane placed between two aqueous flowing streams. Obviously, this demands that the solvent used is nonsoluble in water and that the capillary forces that hold the liquid in the pores are enough strong to withstand inevitable pressure differences over the membrane. Practically, these potential problems are not crucial. No serious problems with pressure differences have been ever seen with SLM setups, and simple calculations show that pressures of several bar are necessary to "blow" out the organic phase from the pores in typical cases. The solubility of nonpolar solvents in water is very small, and a solvent such as n-undecane, which has been used extensively, forms membranes that are stable for months. Some problems may be encountered when more polar
membranes are needed. A medium-polar solvent that has been used extensively is di-rc-hexyl ether, which is stable in SLM membranes nearly as well as Tz-undecane. The inclusion of various additives might compromise the stability, and the matter calls for careful attention. Additives as hydrophobic as possible (maybe modified with alkyl chains) are advantageous. The hydrogen-binding additive TOPO, as mentioned above, can be readily used. Membrane preparations with 10% TOPO in di-^i-hexyl ether are stable for weeks [52].

The material of the liquid membrane support seems to influence the stability somewhat, the most commonly used PTFE membranes being slightly better than polypropylene membranes. The regeneration of the SLM is made in a few minutes by simply soaking the membrane support in the desired liquid, wiping, and reinstalling the SLM in the membrane holder. For hollow fiber membranes, in-situ regeneration has been shown to work well [20].

D. Solvent Consumption

Compared to alternative sample preparation techniques, membrane extraction demands very little solvent. This is a significant advan
tage, as the cost of high-purity solvent is high, both for purchasing and for destruction. Even more important, the environmental implications of these solvents are considerable, both for the laboratory workers and for the outer environment. This is especially true for chlorinated solvents, where different types of restrictions and bans are discussed and partly already implemented in certain countries.

MESI and PME with aqueous acceptor do not require any solvent, and SLM extraction requires only negligible amounts of high-boiling organic liquid in the membrane. Only for MMLLE and PME with an organic acceptor are small amounts of conventional organic solvents needed. For the MMLLE described above for analysis of local anesthetics in blood plasma [17], less than 1 mL of hexane was used for each sample and for the ESy operation only ca. 20 (iL was needed [62]. Thus, the membrane extraction techniques compare favorably with the alternative techniques in terms of solvent consumption.

V. FIELDS OF APPLICATION OF MEMBRANE EXTRACTION

The membrane extraction techniques are applicable to a variety of
sumed to be relatively complete for the analytical use of nonporous membrane extraction techniques in various applications.

A. Analytes

With SLM extraction, acidic or basic compounds can be extracted with simple procedures involving a pH difference between donor and acceptor (see Section II.A). There are many applications with acidic compounds, mainly in environmental applications, for example, phenoxy acids [69-71], sulfonyl urea herbicides [44,72], phenolic compounds [43,73,74], salicylic acid, and carboxylic acids [46,47,66,75-77]. Also, a number of basic compounds have been extracted with SLM extraction, such as aliphatic amines [15,56-58,78], triazine herbicides [32,37,38,67,79], aniline derivatives [80], as well as various basic drugs and drug metabolites [20,21,23,36,51,52,68,81-83].

For compounds other than simple acids or bases, alternative chemical extraction schemes have been suggested involving ion pairing, chelation, complex formation, and immunological recognition. This has been applied to amino acids [84-89], metal ions [90-93], and anionic surfactants [45]. There are also a few
applications of polymeric (silicon rubber)

membrane extraction of phenols [18,49,94,95] and salicylic acid [96]

using an aqueous trapping basic acceptor, a principle that is very

similar to SLM extraction with MMLLE and PME, leading to an organic extract, other

types of analytes can be extracted. This applies mainly to nonionizable compounds such as toluene, chlorobenzenes, and naphthalene

[18,34,97], but also to vitamin E [48], triazine herbicides [42], cat

ionic surfactants [53], and organotin compounds [93]

Finally, with MESI, where the extract is gaseous, only more or less volatile compounds are extracted. This technique has been applied to solvents such as benzene, toluene, ethylbenzene, chlorobenzene, xylene, xylenes, and similar compounds [35,54,55,98-100].

B. Matrices

As the membrane extraction techniques are intrinsically very selective, the main application areas for these techniques are relatively complicated matrices. In the biological field, a number of applications of blood plasma analysis have been shown. In this case, it is in many cases possible to achieve in one step a very high degree of cleanup, so the analytes of interest are more or less
completely separated from the matrix [17,20,21,23,36,51,57,68,74,81,82,96].

Other matrices of biological origin were urine [36,52,56,83,101] and manure [75]. Another important field of application for membrane extraction is environmental analysis. A large number of extractions from natural water (lake or river water) as well as wastewater have been presented [37,38,42-45,53-55,67,69,72,79,80,91,92,102]. In a few cases, a field version of SLM extraction has been used to perform on-site time-integrating sampling of herbicides in a river [70,71]. Some studies of trace organics in nutrient solutions for hydroponic cultivation of tomatoes [73] and in soil solutions [47,76] were performed.

Both outdoor [46,58,78] and indoor [77] air have been sampled using impinger and washbottle techniques, and the extracts were extracted and enriched by membrane extraction. Air, both ambient and in headspace over a liquid or solid sample, has also been extracted with the MESI technique [54,98,99]. Finally, a number of industrial and food matrices were extracted, such as process water [18,34,97], oils [49,50,94,95], and butter [48].
VI. CONCLUSIONS

The selection of a membrane extraction technique for sample preparation, as well as selection among different such techniques, depends mainly on the properties of the analytes and the matrix, and also on the requirements for detection limits, automation, etc. Generally, as described above, the membrane techniques offer in many cases superior cleanup and enrichment while allowing good possibilities for automation and in-line connection to the final analytical instruments if required. For suitable analytes, mainly acidic and basic compounds, but also other ionizable compounds, the SLM extraction provides the highest selectivity and enrichment possibilities. With uncharged compounds and very nonpolar compounds, MMLLE (or ESy) would be a better choice. The use of PME would be motivated for cases where exceptional membrane stability is required, and this approach can be used for all combinations of aqueous and organic phases, and therefore utilize both SLM chemistry with a trapping acceptor and MMLLE where a conventional LLE chemistry is applied. For volatile analytes and direct connection to gas
chromatography, MESI is the preferred alternative. It can be applied to both gaseous and aqueous samples as well as to the headspace over a liquid or solid. In a near future, the ESy technique may become an important competitor where the need for an enrichment column is eliminated.

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3 Design of Rapid Gradient Methods for the Analysis of Combinatorial Chemistry Libraries and the Preparation of Pure Compounds

to the simple sample mixtures of combinatorial chemistry, where

one desires to separate the desired compound from raw materials and byproducts. Thus we can use the same tools demonstrated until

now to analyze the performance of columns and gradient conditions

for the rapid analyses required in combinatorial chemistry. How

ever, the question posed by the needs of combinatorial chemistry extend beyond what has been discussed so far. One would like to know what are the chromatographic conditions that result in the best resolving power if the constraint is the total run time of the analysis. In the following, we will address this question first for a single column. In the subsequent paragraph, we will then examine different columns and particle sizes. The column selected for the starting discussion is a column of 5 cm length packed with 5-μm particles. We can vary freely the flow rate (or linear velocity) and the gradient duration, and we are interested in the resolving power of the column as a function of both parameters. For the complete analysis, Eq. (10) needs to be expanded to incorporate the dependence of the column plate count on the gradient running conditions: (17) where \( L \) is the column length, \( H \) is the height equivalent to a theoretical plate, \( d_p \) is the particle size, and \( a, b, \) and \( c \) are constants. \( D_M \) is the diffusion coefficient of the sample, and an appropriate typical value for the samples of interest needs to be selected; \( u \) is the linear velocity, which can be expressed as \( L/L_0 \). We can incorporate this definition of the plate count into the resolution equation: (18) This equation contains all the parameters necessary for a complete analysis of the optimal gradient conditions as a function of the column choice. The gradient parameter \( B \) and the diffusion coefficient \( D_M \) depend on the type of analyte. Typical values can be selected for small molecules, peptides, or even large-molecular-weight analytes. The column length \( L \), the particle size \( d_p \), the column dead time \( t_0 \), and the gradient slope (or simply the gradient time \( t_g \)) are all parameters whose influence on the resolving
power of the gradient separation we would like to explore. In the discussions in the next paragraph, we will examine the influence of particle size and column length in greater detail. As a tool, we will use a three-dimensional plot of resolution versus column dead time and gradient duration. Figure 5 contains such a plot for the 5-cm, 5-μm column selected as our first example. The column dead time is depicted in seconds on the x axis, the gradient duration, also in seconds is the y axis, and the resolution is shown in arbitrary units on the z axis. As expected, the resolution increases with increasing gradient time. For every gradient time, the resolution exhibits a maximum as a function of the flow rate. For very slow gradients, this maximum coincides with the minimum of the plate height-versus-velocity curve. For short gradients, the maximum resolution occurs at a higher flow rate. This is due to the interplay between the slope of the gradient, which decreases as $t_0$ decreases at constant gradient time $t_g$ and the loss in plate count at higher velocities. Near maximum resolution is reached with a gradient of nearly 3 h (10,800 s), but quite good resolution is obtained already at a gradient run time of 10 min (600 s), at a column dead time of about 25 s. For extremely short gradients, with a gradient duration Fig. 5 Gradient resolution as a function of gradient duration and column dead time for a 5-cm, 5-μm column. This graph is very suitable to examine column capabilities under gradient conditions. of 2 min or less, the best results are obtained with a column dead time of only about 7 s. Of course, the overall resolving power of such a short gradient is significantly inferior to that of the longer gradients. A few additional comments are necessary. On any diagonal line from the front to the back of the graph the ratio of the gradient duration to the column dead time remains constant. A constant gradient profile means that the elution pattern of the separation remains constant as well. This is the single-column equivalent of Fig. 4: only the height equivalent to a theoretical plate (HETP) and the column plate count change under these circumstances. Of course, the resolution increases as the gradient duration increases, until the minimum of the plate height-versus-velocity curve is reached. An example of this is shown in Fig. 6. Fig. 6 Comparison of gradient chromatograms for a simple mixture of small-molecular-weight analytes as a function of the gradient time, but under conditions where the gradient volume remains constant. The elution pattern does not change, but the resolution increases as the gradient duration increases. B. Gradient Resolution as a Function of Column Length and Particle Size In this section we
will further pursue the thought process started at the end of the last paragraph. We will explore the resolving capabilities of columns of different length and of different particle sizes. This discussion will include the constraints on speed imposed by the column back-pressure. Finally, we will explore the simultaneous scaling of column length and particle size. Experience from isocratic chromatography teaches us that under these circumstances the maximum resolving power as well as the fastest speed of analysis remain constant [12]. Let us first explore the effect of different column length on gradient resolution, keeping the particle size constant. Figure 7 shows a diagram of resolution versus column dead time and gradient duration for a 15-cm, 5-nm column. For simplicity of visual comparison, the length of the resolution axis and the gradient duration axis reFig. 7 Gradient resolution as a function of gradient duration and column dead time for a 15-cm, 5-nm column. For comparison with Fig. 5, main the same as in Fig. 5, which shows the same plot for a 5-cm, 5-nm column. However, the length and the scale of the column dead time axis are shifted. This is due to the fact that we imposed an upper pressure limit of about 40 MPa on the column operating conditions in both graphs. The consequence of this is a ninefold shift in the shortest column dead time possible when the column length at constant particle diameter changes by a factor of 3. Due to the pressure constraint, the 15-cm column also does not reach the maximum resolution at very short run times. Only for gradient run times over 5 min (300 s) can a maximum in the resolution at constant gradient duration be reached. On the other hand, for very slow gradients the 15-cm column reaches a higher resolution than the 15-cm column. Under these conditions, the performance of both columns approaches what we know about isocratic chromatography. The difference in the performance capability of the 5-cm and the 15-cm columns is shown in Fig. 8. Here we plot the maximum resolution obtained at every gradient duration versus the gradient duration. Such a plot allows a direct comparison of the maximum capability of each column for a given expenditure in analysis time. For very fast gradients, the resolution obtained with the short column is similar to the resolution obtained with the longer column. The advantage of the higher resolution obtainable with the longer column becomes significant only for slower gradients with a gradient duration longer than about 1000 s or 15 min. On the other hand, the column backpressure of the shorter column is lower, which improves the column stability. Consequently,
the shorter column is a better choice for very fast separations. Let us next explore the influence of particle size for columns of equal length. The first parameter to consider is the column backpressure. At constant column length, the back-pressure increases inversely proportionally to the square of the particle size. This means that a column with a smaller particle size will reach the backpressure limit of the instrumentation at a lower linear velocity than a column with a larger particle size. However, the column with the smaller particle size is expected to exhibit the higher plate count. This improvement in plate count should also be discernible in gradient chromatography. A comparison of 4.6 mm X 50 mm columns packed with two different particle sizes is shown in Fig. 9. The upper graph shows the performance of the 5-jm column, the lower graph the performance of the 3.5-jm column. Only the data for steep gradients are shown, with a maximum gradient duration of 1200 s or 20 min. As expected, the maximum resolution is obtained with the slowest gradient, at a column dead time of about 30 s for the 5-jm column and about 27 s for the 3.5-jm column. The resolution maximum obtained with the 5-jm column is slightly less than 80% of the resolution maximum of the 3.5-jm column. We have imposed a pressure limit of about 25 MPa for both graphs. This means that the 5-jm column can be used at a shorter column dead time than the 3.5-jm column, as indicated in the last paragraph. However, even for the 3.5-jm column, the performance maximum at a given gradient time occurs at higher pressures only for gradient times below 150 s. On the other hand, the maximum resolution for a 75-s gradient is obtained with the 5-jm column well within the pressure limits and is identical to the performance of the 3.5-jm column at the pressure limit. Consequently, for target analysis times of about 5 min, the 3.5-jm column is preferred over the 5-jm column, but for very short analysis times under 2.5 min the pressure limitation of the HPLC instrument needs to be taken into account. However, there is another approach to shorten analysis time with less impact of the pressure limitation of the instrument: we can change the column length and the particle size simultaneously. Fast Gradients for 5 cm 5 micron Column Fast Gradients for 5 cm 3.5 micron Column Fig. 9 Comparison of the resolving power for fast gradients of two columns of equal length but different particle sizes. Column length 5 cm, particle sizes 5 um (upper graph) and 3.5 um (lower graph). Under these circumstances, the pressure required to reach a particular \( t_0 \) is the same for all columns [12]. Conversely, the shortest analysis time achievable at a given pressure
limitation is independent of the choice of the particle size. This is shown in Fig. 10. We have plotted the maximum performance at each gradient duration for a 10-cm, 10-\textmu m column, a 5-cm, 5-\textmu m column, a 3-cm, 3-\textmu m column, a 2-cm, 2-\textmu m column, and a 1-cm, 1-\textmu m column. The shortest run time imposed by the pressure limit is 75 s. If one waits long enough, the maximum resolving power of all columns reaches the same value. However, for realistic gradient run times the shorter columns packed with the smaller particles always result in a higher performance than the longer columns with the larger particles. For example, for a 100-s gradient the resolution values are 3.0 for the 10-cm, 10-\textmu m column, 6.0 for the 3-cm, 3-\textmu m column, and 13.2 for the

Fig. 10 Comparison of the maximum resolution capabilities of several

4

Molecularly Imprinted Extraction Materials

for Highly Selective Sample Cleanup and

Analyte Enrichment Francesca Lanza and Börje Sellergren
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Table 2 Sample Application, Washing and Elution Conditions in Previous

MISPE Protocols

Table 2 Continued

SD = selective desorption.
SA = selective adsorption.
CZE = capillary zone electrophoresis.
TFA = trifluoracetic acid.
TEA = triethylamine.

ELISA = enzyme-linked sorbent assay. strongly bound analytes such as stronger nitrogen Brønsted bases, efficient elution has been achieved using eluents of the same base solvent but with the addition of small amounts of acids (e.g., acetic acid, trifluoroacetic acid) or base
Sample Cleanup of Biological Fluids

The first MISPE protocol reported in the literature [24] involved the use of an imprinted dispersion polymer in a column format, for the selective enrichment of pentamidine (1) in urine. In this case the high selectivity of the polymer allowed the analyte to be detected directly in the eluate without the need for any further chromatographic separation (entry A in Tables 2 and 3). In view of the high selectivity of MIPs, this is a viable approach which brings the benefits of shorter analysis times and simpler instrumentation. In most cases, however, MISPE has been used before a chromatographic separation step. The MIP has been applied in a batchwise extraction [29] or in columns or cartridges [26-28,30-32,39]. The former format was used by Andersson et al. for the analysis of the analgesic drug sameridine (3) in human plasma (entry C in Tables 2 and 3) [29]. Quantification limits below nanomolar concentrations were required. In order to circumvent problems related to bleeding of nonextracted template (see below), this group used a compound (14) that is structurally related to the analyte as template. The structural similarity is unlikely to seriously impair the binding of the analyte and thus the detection limit can be significantly lowered. The accuracy and intra-assay precision of the complete MISPE protocol based on the batchwise preconcentration of sameridine from human plasma were at least as good as those of the routine liquid-liquid extraction method. The advantage of the MISPE protocol was the high selectivity, resulting in less interference in the subsequent GC analysis and potentially higher sensitivities by applying larger sample volumes. The evaluation of a MIP for the solid-phase extraction of propranolol (9) (entry G in Tables 2 and 3) from biological fluids was addressed by Martin et al. [33,39,40], who compared the cumulative recovery curves obtained with different solvents of increasing eluotropic strength containing various modifiers. It appeared that the conditions chosen for the elution of the retained propranolol were extremely important to ensure a selective extraction. In fact, the imprinted material nonspecifically adsorbed a range of molecules structurally related to the target analyte. 

Fig. 6 Reversed-phase and normal-phase modes of operation in the off-line application of MIPs in SPE. served for the routine liquid-liquid extraction method. The advantage of the MISPE protocol was the high selectivity, resulting in less interference in the subsequent GC analysis and potentially higher sensitivities by applying larger sample volumes. 

Fig. 7 RAM or C18-MIP coupled column system for prefractionation based on size and polarity and transfer of analyte to a solvent for enhanced recognition of the target analyte.
Results for the Protocols in Table 2 Polymers were prepared using MAA (AK) or 4 vinylpyridine (4VPY)/MAA (1/1) (L) as functional monomers and EDMA as crosslinking monomer in the presence of the template and various solvents (porogens) as shown schematically in Fig. 4. EtOAc = ethyl acetate
etate, THF = tetrahydrofuran, RT = room temperature. a Polymerization technique. Thermochemical initiation at elevated temperatures or UV photochemical initiation at low temperature. b Weight of dry MIP packed in the SPE cartridge or column. c Volume and type of sample added to the MIP phase. d Typical concentration range used in the extraction tests. e Exact or approximate recoveries reported. f Polymer obtained as dispersible agglomerates of micrometer sized particles. g Polymer prepared in water suspension. h Column dimensions: 80 x 4 mm. i Column dimensions: 150 x 4.6 mm. j Recovery by loading and washing in pH 5 buffer. In parentheses the corresponding value after loading and washing in pH 7 buffer. k Recovery obtained by HPLC quantification. In parentheses is the corresponding value when quantification was done by ELISA. Usually similar and dissimilar to propranolol. However, a careful choice of eluent allowed the nonspecifically bound analytes to be washed off first and propranolol to be recovered quantitatively in a second step. Interestingly, comparing the strong modifiers trifluoroacetic acid (TFA) and triethylamine (TEA), only the latter resulted in selective cleanup. Also, matching the wash solvent with the porogenic solvent seemed to result in higher selectivity and recovery. Stevenson et al. compared MISPE with immunoaffinity extraction for the quantification of tamoxifen (11) in urine and plasma (entry I in Tables 2 and 3) [31, 41]. This group reported considerable problems with template bleeding, i.e., continuous release of small amounts of nonextracted template, particularly upon solvent changes. A number of different wash procedures were tested, but none gave satisfactory results that would allow reliable quantification of low levels of the drug. Although the materials could be used for the selective extraction at higher concentration levels and the manufacturing of the materials was simple, the immunoaffinity technique was favored since these phases allowed the drug to be selectively extracted from biological fluids, at low concentration levels and with high accuracy. Similar problems were reported by Venn et al. in the application of MISPE in the quantification of the developmental drug darifenacin (10) in blood (entry H in Tables 2 and 3).
A comparison of MIPs using different functional monomers and porogens with respect to chromatographic selectivity for the drug was first performed. Using MAA as the functional monomer, the resulting MIP could discriminate efficiently between the drug enantiomers and was therefore employed in an off-line SPE procedure. The blood was deproteinized by addition of acetonitrile (50%) and the sample applied directly to the MISPE cartridge. By washing the cartridge with acetonitrile, the drug was efficiently retained on the MIP, whereas early breakthrough was seen on the nonimprinted control cartridge. The selectivity was further investigated by comparing the extraction of a number of structurally related analogs and substructures. Thus the materials performed well in terms of selectivity, capacity, and robustness. Nevertheless, applications for trace- or ultra-trace-level analysis was again precluded due to the excessive template bleeding. Extraction of Analytes of Environmental Concern Triazines belong to a group of widespread herbicides that have become major pollutants of soil and ground waters [43,44]. Therefore analytical methods are needed to allow them to be monitored at concentrations below micrograms per liter. The triazines are available in large numbers with small structural differences and with different known basicities and hydrophobicities (Table 4). This class of compounds is therefore well suited as a model system in molecular imprinting [45]. Previous results indicate that highly selective MIPs can be prepared against triazines [45-48]. The strongest interactions are expected to be a cooperative hydrogen bond between the nitrogen para to the chlorine (or thiomethyl) substituent and an exocyclic amino group of the triazine and the carboxylic acid group of MAA [49]. This interaction is also accompanied by secondary interactions which can be the association of a second acid group to the triazine ring system or shape complementarity arising from the polymer backbone. Thus the polymers effectively discriminate between chloro- and S-triazines. For the chlorotriazines, highest selectivity is seen using MAA as monomer; whereas for the S-triazines, trifluoromethyl acrylic acid is the monomer of choice [50]. The use of MISPE for the cleanup of beef liver extracts for the quantification of atrazine (5) by HPLC-UV or ELISA was evaluated by Muldoon and Stanker (entry E in Tables 2 and 3) [30]. In both cases the application of MISPE resulted in improvements in analyte quantification. In particular, for the HPLC method the use of MISPE

Table 4 Structures of Triazines with Various Brønsted Basicities and
Hydrophobicities improved the accuracy and precision and lowered the limit of detection. In the case of ELISA, better accuracy was achieved in the determination, although the precision was similar. With the analyte present at parts-per-billion levels, the reliability of either determination method would have been marginal without the MISPE step. The application of MIPs prepared using triazines as templates to the solid-phase extraction of water samples requires drying the cartridge after the sample application in order to remove water traces which would disrupt the interactions between the analytes and the sorbent. In the protocol described by Matsui et al. [25], the aqueous sample was applied to the MIP cartridge, which was then carefully dried prior to a selective dichloromethane wash (entry D in Tables 2 and 3). HPLC-UV analysis of wastes and extracts showed that all the impurities were washed off without significant elution of simazine (4) and that the analyte was recovered quantitatively in the eluate. Recently, the group of Barcelo performed a detailed investigation on the off-line application of chlorotriazine-imprinted MIPs in the analysis of real environmental water samples [36]. Two imprinted polymers were synthesized using either dichloromethane or toluene as porogen and terbutylazine as template and were implemented to the solid-phase extraction of six chlorotriazines in natural water and sediment samples. All extracted samples were analyzed by liquid chromatography/diode array detection (LC/DAD). Several washing solvents, as well as different volumes, were tested to remove the matrix components adsorbed nonspecifically on the sorbents. This cleanup step was shown to be of prime importance to the successful extraction of the pesticides from the aqueous samples. Interestingly, the wash solvent giving the highest level of cleanup and recovery was the same as that used as porogen. Optimal analytical conditions were obtained using the MIP imprinted with dichloromethane as porogen, with 2 mL of dichloromethane used in the washing step and the preconcentrated analytes eluted with 8 mL of methanol. The recoveries were higher than 80% for all the chlorotriazines except propazine (53%) when 50- or 100-mL groundwater samples, spiked at the 1-ug/L level, were analyzed (Fig. 8). The limits of detection varied from 0.05 to 0.2 |ug/L when preconcentrating a volume of 100 mL of groundwater sample. Natural sediment samples from the Ebre Delta area (Tarragona, Spain) containing atrazine and deethylatrazine were Soxhlet extracted and analyzed by Fig. 8 LC/DAD chromatogram at 220 nm obtained after preconcentration of 100 mL of groundwater sample spiked at 1 ug/L through (a) a blank cartridge prepared using a
nonimprinted control polymer and (b) a cartridge prepared using a polymer imprinted with terbutylazine. Peaks: 1 = deisopropylatrazine, 2 = deethylatrazine, 3 = simazine, 4 = atrazine, 5 = propazine, 6 = terbutylazine, and I.S. (internal standard) = diuron. The methodology developed in this work. No significant interference from the sample matrix was noticed, thus indicating good selectivity of the sorbents used. The above studies show that the imprinted sorbents can be used in two modes: in the reversed-phase mode (sample application) and in the affinity mode (dichloromethane washing). Otherwise there are only few reports on the use of MIPs for the pretreatment of samples of environmental concern. Recently an MIP imprinted using the herbicide bentazone as template was synthesized and evaluated by Baggiani et al. [27] in frontal liquid chromatography (entry L in Tables 2 and 3). The material was then used as sorbent for the solid-phase extraction and a preliminary procedure for the enrichment of the analyte from water samples was tested. Good recoveries (91-96%) and concentration factors of 3.2-15.2 were found. B. On-line Protocols In the development of a cleanup step for nicotine-containing chewing gum, an on-line MISPE procedure was developed (Fig. 9) and the resulting method compared with an SPE run on a nonimprinted blank column (entry F in Tables 2 and 3) [28]. The sample pretreatment would in this case be particularly suited for MISPE, since the analytes together were present in a nonpolar solvent, used to dissolve the chewing-gum matrix. Nicotine MIPs were prepared by the standard protocol using MAA as the functional monomer. The resulting polymers were crushed, sieved, and packed into standard HPLC columns for evaluation. Using acetonitrile as wash solvent, nicotine could be eluted as a sharp peak from the imprinted sorbent by adding 0.2% TFA. This led to quantitative recoveries in a large concentration interval. The elution conditions were then optimized on-line. The chewing-gum extract was added to the column and the column was washed with acetonitrile, followed by elution of the analytes. As seen, there is a pronounced difference between the MIP and the blank column in terms of breakthrough volumes and recovery of the analytes in the elution step. Whereas three of the four analytes broke through prior to the elution step on the blank column, only the most hydrophobic analyte—p-nicotyrine—broke through on the MIP (Fig. 10). Transferring the procedure to an off-line mode, quantification by reversed-phase HPLC showed that all analytes except p-nicotyrine were recovered.
Due to the enhanced stability of the nicotine MIP, achieved by thermal annealing, and the reusability of the MIP, the procedure may be suited for automation. MISPE with Pulsed Elution A method for the analysis of theophylline in blood serum based on molecularly imprinted solid-phase extraction with pulsed elution (MISPE-PE) was developed by Mullett and Lai (entry J in Tables 2 and 3) [34]. The method made use of an MIP column for the on-line enrichment of theophylline and injection of a small plug of methanol to produce a rapid pulsed desorption of the analyte. The sample was applied in chloroform, since in this solvent a complete retention of the analyte was observed. The matrix constituents (e.g., lipids) and the potential interferences (e.g., other drugs) which were not recognized by the binding sites were rapidly eluted with chloroform. Then a rapid and quantitative recovery of theophylline was accomplished in a pulsed format through injection of 20 nL of methanol. In this way the eluting analyte could be detected directly by UV. The pulsed elution approach was further applied to micro-SPE columns (0.8 mm I.D., 80 mm long), which offer the advantage of lower consumption of solvents and faster analysis times [51]. With the application of 20 nL solvent pulses it was demonstrated that the nonspecifically bound analytes could be removed and nicotine could be recovered Fig. 10 Adsorption and elution of 100 nL of a placebo chewing-gum extract spiked with nicotine (0.5 mg/mL) and (3-nicotyrine, cotinine, and myosmine (0.05 mg/mL). The step elution was as follows: MeCN, 0-8 min; MeCN + 0.2% TFA + 2.5% H2O, 8-12 min; MeCN, 12-15 min. The peak identity was established by separate injections and spectral analysis using a diode array detector. Rest refers to still-not-eluted 3-nicotyrine, cotinine, and myosmine. In MISPE, only 3-nicotyrine could be detected in the adsorption and wash steps. quantitatively over a linear dynamic range of 1-1000 ng/L and with a detection limit of 1.8 ng/mL. On-line Coupled Column Approaches

The drying step required in many off-line procedures is not compatible with on-line operations. Moreover, if the MIP columns are to be reused, fouling caused by macromolecular matrix components precludes direct injection of biological fluids. In these cases an attractive alternative is to use a precolumn designed to transfer low-molecularweight analytes into an organic solvent while simultaneously excluding biological macromolecules. This is possible using a coupled column system where the analytes are first adsorbed nonspecifically on a multipurpose SPE column, e.g., a hydrophobic sorbent (C18 reversed-phase or restricted-access sorbent), and then transferred to a dedicated MIP sorbent in an organic medium. The approach is
suitable for on-line applications, as recently demonstrated by Boos et al. [37] and Bjarnason et al. [38], and has the advantage of allowing the direct injection of biological samples (Fig. 7). Furthermore, the column switching allows the pretreatment step to be separated from the analysis so that one sample can be pretreated while another is being analyzed, leading to a higher sample throughput.

VI. THE DEVELOPMENT OF NEW MISPE PROTOCOLS

Four different steps are involved in the development of a new solidphase extraction protocol based on molecular imprint: synthesis of the material, assessment of its recognition properties, development of an extraction protocol, and final validation of the protocol. First, a suitable combination of monomers and a porogenic solvent have to be found on which the template can be successfully imprinted [17]. The thermodynamic stability of the monomer-template assemblies is the key factor in this step. One or more functionalized vinyl monomers are sought which are capable of forming strong noncovalent interactions with the functional groups of the template. The monomer-template assemblies need to be stable enough for them to be transformed to specific binding sites during the polymerization. The structure and morphology of the polymer matrix are strongly influenced by the type and amount of cross-linker and porogen [52]. These factors affect in turn the sorption-desorption kinetics and the stability of the material and therefore require careful optimization as well. Once a suitable material has been found, a chromatographic characterization step is required in which the recognition properties of the sorbent are evaluated in different mobile phases. In order to predict suitable extraction protocols, the sorbents should thus be characterized in terms of affinity, selectivity, and sample load capacity (breakthrough volumes) for the analytes. Except for establishing recognition based on the retention times of the template and analogs at one or two different concentrations, frontal analysis or batch rebinding at several concentrations yields thermodynamic information in terms of binding energy and mass transfer kinetics [22,53,54]. The assessment can also be done by an off-line SPE procedure in which the cumulative recovery curves of the sorbents are evaluated in different solvents and pHs. A suitability test for propranolol-imprinted sorbents, to be used in SPE of plasma and urine samples, was developed by Olsen et al. [40]. This was based on the solvent composition required to give a recovery of either 20% or 50% of the extracted material (ES 20 or ES 50 values) on the blank and the imprinted cartridge. The method was developed in order to allow a rapid estimate of the specific and nonspecific contributions to overall binding.
Finally, a validation step for the overall MISPE procedure is mandatory to allow the use of the method itself in place of the regulatory methods. The issues to be considered here are the accuracy and precision of the method based on the MISPE protocol, its limit of quantitation, selectivity, and ruggedness. In particular, the interand intra-assay precision need to be checked with real samples and with certified reference materials and methods [29,30]. VII. POLYMER SYNTHESIS-RELATED FACTORS

Each step in the imprinting procedure (from the synthesis to the validation) needs to be thoroughly investigated and optimized when a new MIP is synthesized against a given template. In particular, the selection of appropriate functional monomers capable of forming stable complexes with the template is critical for the success of any MISPE protocol. The type and number of functional groups present in the template provide some guidelines for the choice of the functional monomers. Ionic or hydrogen-bonding interactions are usually the first interactions to be exploited in targeting the template. Thus nitrogen bases have been successfully imprinted by using carboxylic acids such as MAA as functional monomers (see Table 3). Here the strength of binding, disregarding cyclic hydrogen-bonded structures, increases with the acidity of the functional monomer and the basicity of the template [55]. Likewise, basic functional monomers (2- and 4-vinyl pyridine, diethylaminoethyl methacrylate, aminomethyl methacrylate, allylamine, N-vinyl pyrrolidinone, 4-(5)-vinylimidazole, polymerizable amidines) have been used to imprint acidic templates and acidic functional monomers (methacrylic acid, trifluoromethacrylic acid, itaconic acid, p-vinylbenzoic acid) have been used to imprint basic templates [17]. The selection of the possible functional monomers can further be based on the extensive literature from the area of host-guest chemistry [56], the binding mode to biological receptors [2], affinity chromatography [57], or crystal engineering [58]. Reports can be found describing stability and stochiometry of complexes between structural analogs of the template and functional monomers, and these reports can thus serve as initial guidelines in the monomer selection. In a number of cases, combinations of two or more functional monomers giving terpolymers or higher polymers have resulted in polymers showing better recognition properties than the corresponding co-polymers [59,60]. However, these systems are complicated by monomer-monomer association which competes strongly with the template-monomer association unless one of the monomers has a particular preference for the template. So far the success of any imprinting protocol has depended more on chemical intuition than on a rational approach.
However, for general use of the imprinting technique, rational synthesis and evaluation protocols need to be established. A key step in this development process is the rapid identification and optimization of the main factors affecting the recognition properties and the morphology of the polymers. These factors are the type and concentration of functional monomers, cross-linking monomer, free-radical initiator, and porogen. Further factors related to the polymerization conditions are the pressure and temperature of polymerization. Owing to the timeconsuming procedures generally adopted for the preparation of MIPs, the assessment of the recognition properties of large numbers of MIPs so prepared is not realistic on a practical time scale. Instead, a system allowing parallel synthesis of large groups of MIPs and in situ screening of the recognition properties is desirable. One solution consists of scaling down the batch size, allowing the materials to be synthesized in vials or wells suited for direct analysis of the recognition properties. VIII. METHODS FOR SYNTHESIS AND SCREENING OF LARGE GROUPS OF MIPs A scaled-down version of the established monolith procedure was recently developed by Takeuchi et al. [50] and by Lanza and Sellergren [61], in which molecularly imprinted polymers were prepared on the bottom surfaces of chromatographic autosampling vials and assessed directly by batch rebinding tests. In both cases, triazines were used as model templates and the functional monomers were used as screening parameters. In the work of Takeuchi et al., the relative amounts of functional monomers (MAA and TFM) were varied to optimize the recognition properties of MIPs prepared using ametryn and atrazine as templates, whereas six different functional monomers were screened by Lanza and Sellergren using terbutylazine (TER) as template. In both cases the polymers were prepared by photoinitiation following previous protocols with ethyldimethacrylate as a cross-linking monomer and dichloromethane as solvent. The standard batch size was scaled down ca. 100 times to a total volume of 100 µL. As seen in Fig. 11, the screening was performed in two steps. The primary assessment is based on quantitative HPLC or UV-absorbance analysis of the amount of template released from the polymer in a given solvent. Among the six functional monomers tested, methyl methacrylate, 4-vinylpyridine, and N-vinyl-2-pyrrolidone led to rapid and quantitative extraction, whereas methacrylic acid and trifluoro
methylacrylic acid led to polymers that retained the template the most. A modest increase in the recovery was obtained after additional washing in dichloromethane but, as expected, only after adding strongly competing solvents such as acetic acid were high overall recoveries of the template obtained. The selection criteria in the first screen were based on the amount of released template in a given solvent, i.e., acetonitrile or dichloromethane, the latter being the same solvent as the one used during polymerization. As discussed above, this solvent is commonly the solvent in which the polymers exhibit the most pronounced recognition [35]. Thus, in a particular batch, if a quantitative release is seen in this step the resulting polymer cannot be expected to rebind a significant amount of the template and may thus be discarded. In this step the polymers prepared using MAA and TFM are the only polymers that can be expected to show enhanced rebinding of the template in this solvent. After having established useful functional monomers, a secondary screening for selectivity was performed. In this screening, the rebinding of the
template to the MIPs was investigated in parallel to the rebinding to the corresponding blank, nonimprinted polymers [61]. Alternatively, an internal standard, structurally related to the template, may be added and the differential binding investigated [50]. Supporting the validity of the suggested screening approach, the MAA and TFM MIPs exhibited enhanced rebinding and selectivity. A scaled-up version of the MAA polymer was assessed as stationary phase in chromatography. As seen in Table 5, a number of structur
5 Biomembrane Chromatography: Application to Purification and Biomolecule-Membrane Interactions

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2.


Transformation of Analytes for

Electrochemical Detection: A Review of

Chemical and Physical Approaches

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I.

II.

III. ABBREVIATIONS INTRODUCTION A. Microseparation Techniques B. Detection Challenges in Microseparation Techniques C. Electrochemical Detection and Microseparation Techniques TRANSFORMATION METHODS AND SUMMARY TABLES A. Primary Amines B. Secondary Amines C. Peptides D. Thiols E. Alcohols 204 206 206 206 207 208 209 217 220 221 225

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1. ABBREVIATIONS AP APM ASC BBQ BDF BR/Cu(II) CA Ce(IV) OX CLOD DEDC DFB/HPLChv-EC DHBH DM-PITC DMA/EDC DMPM DNPHD DPBE DNPT DTC EX FA FBA FCAC 4-Aminoantipyrine N-(4-Anilinophenyl) maleimide o-Acetylsalicyloyl chloride 3,5-di-ter"Butyl-1,2-benzoquinone
3-Bromo-1,1'-dimethyl-ferrocene Biuret reagent
[Cu(II)/tartrate] 2-Cyanoacetamide Ce(IV) oxidation
Concentration limit of detection Diethylthiocarbamate
2,4-Dinitrofluorobenzene and HPLChv-EC
2,5-Diiodoxybenzohydrazide p-N,
AT-Dimethylaninophenylisothiocyanate 2,4-Dimethoxyaniline
HC1 and 1-ethyl3(3-dimethylaminopropyl)carbodiimide
A'/-(4-Dimethylaninophenyl)maleimide
2,4-Dinitrophenylhydrazine
1-(2,5-Dihydroxyphenyl)-2bromoethanone 3,6-Dinitrophthalic
anhydride Dithiocarbamate Ethyl xanthate 3-Ferrocenyl
acyl azide Ferroceneboronic acid Ferrocenyl acid chloride

FEA/EDC
FEITC
FITC
FM
FSC
HEDC
HPLC-hvEC
IEMN/Pt
Me/CDS
MLOD
MP
NBD-C1
N-HSP
N-SEP
NDA/CN
NPHD
NQS
OPA
OPA/BT
SAC 1-Ferrocenylethylamine and
1-ethyl3(3-dimethylaminopropyl)carbodiimide
2-Ferrocenylethylisothiocyanate Ferrocenylisothiocyanate
AT-(Ferrocenyl) maleimide Ferrocenesulfonylchloride
doos(2-Hydroxyethyl)dithiocarbamate HPLC and postcolumn UV
irradiation with electrochemical detection 2-
[2-((Isocyanate)ethyl] -3-methyl-1,4naphthoquinone and
platinum reduction Metal/carbon disulfide Mass limit of
detection 2-Methoxyphenol
7-Chloro-4-nitrobenzo-2-oxa-1,3diazole
AT-Hydroxysuccinimide phenol AT-Succinimidyl
3-ferrocnylepropionate Naphthalene-2,3-dicarboxaldehde/
CN' 4-Nitrophenylhydrazine P-Naphthoquinone-4-sulfonate
o-Phthaldehyde (OPA) o-Phthaldehyde(OPA)/ter¿-butylthiol
o-Phthaldehyde/(3-mercaptopoethanol o-Phthaldehyde
(OPA)/sulfite p-Aminophenol p-Aminophenol and
2-bromo-1-methylpyridinium iodide Pyrrolidinecarbodithioate
Phenylhydrazine Phenylethoxycyanate
1-(2-Pyridylazo)-2-naphthol-6-sulfonic acid 8-Quinolinol
Salicylaldehyde Salicylic acid chloride TD-hv Titanium
dioxide postcolumn photolysis TNBSA
2,4,6-Trinitrobenzene-sulfonic acid TTAA
(R,R)-0,0'-di-p-toluoyl tartaric acid anhydride Zn/HEDC
Zinc bis [(2-hydroxyethyl)dithiocarbamate]

II. INTRODUCTION

A. Microseparation Techniques
The miniaturization of separation systems has received much attention in the scientific literature over the past 10-15 years. Progress has been made in providing reliable commercial microbore HPLC equipment and capillary electrophoresis (CE) instrumentation, and novel technology is currently being developed for microchip electrophoresis [1-11] and capillary electrochromatography (CEC) in packed columns [12-16]. An obvious advantage of using miniaturized separation technology is its small sample requirement, which has made possible studies such as the separation of the contents of single cells [17-21] or the on-line separation of in-vivo microdialysis samples [22]. Physical attributes such as efficient heat dissipation and flow dynamics are advantages of miniaturization and make it possible to perform efficient separations in the high electric fields encountered in CE and CEC.

B. Detection Challenges in Microseparation Techniques

Over the past decade, there has been a continued trend in the miniaturization of various analytical techniques, especially in the area of liquid-phase separations; however, advances in the separation com
ponent of an instrument place serve demands on the associated de
tection module. The challenge comes from the fact that detector
dimensions in miniaturized systems need to be proportionally down
sized. For optically based detectors this results in a shortened path
length and diminished sensitivity. When fluorescence detection is a
viable option, a partial answer to this issue lies in the use of lasers
as the excitation source. In general, laser-induced fluorescence (LIF)
detection has provided for high-sensitivity detection of analytes in
small-volume detection cells and has found application on numerous
occasions in CE [19,23-28]. Despite such successes laser-based de
tectors are still limited by a lack of generality due to the few choices
of available excitation wavelengths and the limited number of com
pounds that can be excited at these wavelengths. For home-made
LIF detectors, other limitations often are related to operator train
ing, and the general tediousness of setting up and optimally op
erating a laser-based detector on a daily basis. Many of these aspects
are not of issue with commercially available systems that
have recently become available. Unfortunately, from a practical standpoint,

there is still a high initial start-up cost and limited lifetime for many

of the lasers. This situation may change dramatically in the next
decade as more solid-state lasers are developed that may provide

for excitation at wavelengths less than 400 nm. Turning to another

approach, mass spectroscopy (MS) clearly offers another solution to

high-sensitivity detection in small volumes. Significant improve
ments in commercial instrumentation has occurred over the past de
cade, and now it can be stated that MS has become a reliable quanti

tative tool for the bioanalytical chemist. As present, perhaps the

greatest limitation of MS is the prohibitively large start-up costs and

the high maintenance costs.

C. Electrochemical Detection and Microseparation Techniques

Another viable solution for trace determination in small volumes is
electrochemical (EC) detection. Electrochemical detection has been

applied successfully to microcolumn separations, including its use

in both microbore HPLC, microbore open-tubular liquid chromatog
raphy, and capillary electrophoresis, and these applications have been reviewed [29]. The application of electrochemical detection in the low-volume flow cells of miniturized separation systems is potentially very large, due to the inherently sensitive nature of EC detection and the fact that the technique actually benefits from miniaturization. Generally, signal-to-noise ratios become more favorable with miniaturization in electrochemical detection [30], whereas these ratios become less favorable when using optical detection methods. Two detrimental aspects to the use of electrochemical detection are negative interactions of the analyte and matrix with the electrode surface, and, as is the case for fluorescence, the relatively limited percentage of analytes possessing electrochemical activity. The former manifests itself in electrode fouling and decreased detector response, which can be alleviated by the use of internal standards and/or mild detection potentials, where electrode surface fouling is negligible. The lack of reasonable electrochemical detectability can be addressed by transformation of the molecule to
an electrochemically active form through chemical
derivatization or

physical means. Previously reported transformations of this
type

were reviewed in 1985 [31]. The present review includes an
overview

of those approaches, plus additional techniques that have been de

veloped and described in the past decade.

III. TRANSFORMATION METHODS AND SUMMARY TABLES

The included tables represent a comprehensive collection of the re
ported chemical and physical methods used for the transformation of

poorly detectable compounds to electrochemically detected analytes.

The individual tables and associated discussion are
categorized by

the type of compound transformed and include precolumn and post
column methods. Details included in the tables are the reagent or

method name, specific analyte(s) determined, reported limits of de

tection (S/N = 3), reaction conditions required to obtain this detec

rounded to one significant figure and given as "concentration limits

of detection" (CLOD) rather than the sometimes utilized "mass lim

its of detection" (MLOD). The application of microbore HPLC, capil

lary electrophoresis, and capillary electrophoresography,
with

their inherently low mass loading capabilities, has made it useful to

quote limits of detection in terms of concentration injected onto the

column rather than mass injected. MLOD can be extremely small

in the case of the three aforementioned techniques and not fairly

reflect the transformation methods utility in allowing for detection

at trace levels. In some cases during this review, a conversion to

CLOD requiring the injection volume was made from a MLOD re

ported by the author. When no injection volume was specified in the

reported experimental procedure, it was assumed to be 20 fiL when

an analytical HPLC column (4.6-mm I.D.) was used. For reports con

taining data for multiple compounds, the CLOD listed is for the com

pound having the greatest sensitivity. The notation "$" indicates

that the quoted detection limit was obtained on a pure synthetic

standard of the derivative or a sample transformed at high concen

tration and diluted, both situations which might not be representa

tive of detection limits in the transformation of real samples. Reac

tion conditions include time and temperature as reported by
the author. The notation "§" in reaction conditions indicates that an ex
traction, precipitation, or evaporation was performed as part of the
derivatization procedure either to remove excess derivatization re
agent or to concentrate the derivatized analyte, steps which may
negatively influence precision and limit accessibility to small
volume samples. Finally, the potentials listed in the tables are ver
sus a Ag/AgCl reference electrode or have been converted to allow
this comparison.

A. Primary Amines

The greatest proportion of functional group transformations for elec
trochemical detection has been the derivatization of primary
amines. This category includes primary alkylamines, amino
acids,
and peptides. Due to their unique structure, the derivatization of
peptides is covered in a separate section, but as most peptides pos
sess one or more primary amine functionalities, they can usually be
derivatized with the reagents listed in this category.

The most commonly applied reagent system for primary amines
is o-phthaldehyde (OPA), which was reported as a fluorogenic deri
vatization reagent for primary amines in 1971 [85]. This compound was later discovered to additionally possess electrogenic characteristics [35,51,86]. The condensation reaction of the 1,2-dialdehyde of OPA with a primary amine to form the electroactive 1,2-disubstituted isoindole (Reaction 1) is unique among derivatization reagents in that OPA is hydrolytically stable in water, undergoes a rapid reaction at room temperature, and is specific for the primary amine functionality. The drawback in utilizing OPA is the limited stability of Reaction 1 the isoindole condensation products. The instability of the isoindole formed in this reaction has been linked to the steric nature of the amine being derivatized [87], the type of thiol, and the excess of OPA in the reaction mixture [88]. It was determined using radiolabeled phenylalanine and tyrosine that only 50% of the total derivatized product injected onto a reverse-phase high-performance liquid chromatography (HPLC) column actually reaches the detector intact [34], although such results must be carefully interpreted because isoindole product stability is highly dependent on the
various reaction conditions and parameters. The substitution of sulfite for the traditional thiol co-reagent was observed to enhance isoindole stability, but in the case of electrochemical detection, optimum detection potentials were raised approximately +200 mV for these more negatively charged isoindoles [59]. Most reactions of primary amines with OPA are complete within 1-2 min at room temperature, and the derivatized analytes, because of their instability, are normally analyzed immediately upon formation. CLODs in the low-nanomolar range can be obtained using oxidative detection at +500 to +850 mV (see Table 1). More recently, OPA has shown great utility in the determination of amino acids in biological samples of interest to neuroscientists.

These studies, including those obtained by microdialysis, generate samples in the microliter size range, which often require special handling techniques, automated sample transfer, and the use of microbore chromatography. In one report, a comparison was made of five different OPA/MCE systems for the determination of aspartate and glutamate in microdialysis samples (5-10 \(\mu\)L) [38]. The best method
in the report, which included the use of microbore chromatography

and on-line microdialysis-derivatization-HPLC, allowed nanomolar CLOD ranges using a detection potential of + 700 mV. Other investigators have determined amino acids in rat brain tissue [55] and amino acid neurotransmitters in the cat visual cortex [37], with reported detection limits in the high and middle nanomolar range, respectively, when using OPA/BT. Optimized OPA/BT derivatization conditions, when used in conjunction with microbore chromatography, enabled other investigators to obtain a high-picomolar CLOD range for y-aminobutyric acid with detection at +800 mV [53]. Part of the optimization aspects included the use of glycine and iodoacetamide as scavengers for excess OPA and tert-butylthiol, respectively. A similar reagent for the electrogenic derivatization of primary Ta b l e

1 Pr

i m
Amino Acids
- Glutamate
- Aspartate
- Aminobutyric acid (GABA)
- Aminolevulinic acid
- Thyroxine
- Spermine
- Spermidine
- Putrescine
- Cadaverine
- Taurine
- Acetylated homotaurine
- Histamine
- Histamine
- Tryptamine
- Tyramine
- Phenylethylamine
- Putrescine
- 6Diaminohexane
- Tryptophan
- Carboxymethylcytochrome metabolites

E (mV) + 600 + 750 + 700 + 700 + 450 + 730 + 650 + 700 + 500 + 1200 + 500

CL0D to 503 nM 40 nM 55

M10 nM 30 nM 2 nM to 100 nM 6 uM Reaction conditions 425°C, 13 min 4°C, 1 min r.t., 12.5 min r.t., 1 min r.t., 30 s r.t., 210 min 0°C, 6 min r.t., 20 s 1 min r.t., 5 min N/A r.t., 2 min

References [3237] [38] [3941] [42] [43] [44] [45] [46] [47] [48] [49] [50]

Table

Continued

ed Reagent Analyte

E (mV) CL0D Reaction conditions ReFS. OPA / MCE OPA / BA

TOPA / BTOPA / SulOPAN D / CNAmphe	a
m i n e H e p t a m i n o l N o r e p h e d r i n e P h e n e t h y l a m i n e 2 H e p t y l a m i n e y A m i n o b u t y r i c i A m i n o a c i d s B a c l o f e n y A m i n o b u t y r i c i A l k y l a m i n e s A l a n i n e A r g i n i n e G l u t a m i c a c i d S a r i n e T y r o s i n e A m i n o a c i d s G l u t a t h i o n e B i s p h o s p h o n a t e a c i d ( G A B A ) a c i d ( G A B A ) a l e n d r o n a t e + 8 5 0 + 6 0 0 + 8 0 0 + 7 0 0 + 7 5 0 + 7 0 0 + 8 5 0 + 6 0 0 + 8 5 0 0 + 9 6 0 * + 8 5 0 + 7 5 0 + 8 0 0 + 9 0 0 + 6 5 0 2 0 0 n M 4 0 n M 3 0 0 p M 6 n M 1 0 n M 2 0 0 n M 3 0 0 n M 5 0 n M / A t o 1 0 n M 1 0 0 p M 1 0 n M 2 u M 5 n M f 1 0 n M r . t . , 3 0 m i n r . t . , 3 m i n r . t . , 2 m i n r . t . , 2 m i n r . t . , 2 m i n r . t . , 1 m i n 2 5 ° C , 2 5 m i n 3 7 ° C , 1 5 m i n r . t . , 1 m i n 2 0 ° C , 0 2 5 h r . t . , 1 0 m i n r . t . , 1 m i n r . t . , p o s t c o l u m n r . t . , 3 0 m i n r . t . , 1 5 m i n [ 5 1 ] [ 5 2 ] [ 5 3 ] [ 5 4 ] [ 5 7 ] [ 5 5 ] [ 5 6 ] [ 5 8 ] [ 5 9 ] [ 6 0 ] [ 6 1 ] [ 6 2 ] [ 6 3 ] [ 6 4 ] [ 6 5 ]


Transformation of Analytes for Electrochemical Detection I


32. L. Canevari, R. Vieira, M. Aldegunde, and F. Dagani,


Transformation of Analytes for Electrochemical Detection I

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I. INTRODUCTION

Trace metal determination and speciation have received particular attention in the last years, mainly with respect to environmental samples and industrial products analysis. Liquid chromatography (LC) has become one of the main powerful analytical tools for the analysis of complex matrices (e.g., foods, new materials, pharmaceutical and environmental samples) and speciation studies, in the field of metal analysis. LC has stimulated studies on new more selective materials, enabling difficult separations, and for the improvement of detection sensitivity and selectivity. Spectrophotometric or electrochemical detection has been coupled with postcolumn reactions, but a new approach, hyphenation, concerning the coupling of unconventional detectors with LC, has become the emerging field of research. Some general reviews or comments with particular regard to ion chromatography, complexation ion chromatography, as well as advances in detection techniques summarize LC potentialities.
and will be introduced hereafter. In this chapter, with
respect to the new philosophy of simple
removal of interferents and/or analyte preconcentration
before separation and specific detections of metal ions, a brief
introduction to
sample handling is presented. Classical and more recent
applications of liquid chromatography for the analysis of metal
species are
presented, and the different approaches are discussed with
particular reference to ion-exchange, ion-pair, and chelation
separation
mechanisms.

II. SAMPLE HANDLING

The main practical problems in real sample analysis are
related to
collection, dissolution, cleanup,
trace enrichment, and matrix elimination. Sampling and
storage will not be considered here, but a few words must
be devoted
to sample treatments, due to the need for matrix removal, sample
preconcentration, and/or derivatization. Sample cleanup can
be performed off-line, prior to chromatographic analysis, or
on-line, incor
porated into the chromatographic hardware. Among the cleanup
procedures, those using ion-exchange mechanisms can be used
to
reduce the alkalinity or the acidity of a sample [1] by using a high
capacity cation-exchange resin in the H⁺ form or a high-capacity
anion-exchange resin in the OH⁻ form, respectively. Alternatively,
electrochemical devices can be used [2,3]. Similar procedures can be
designed to suit different sample types, by varying the form of the
resin used to achieve an alternative chemical modification of the
sample, e.g., reversed phase (see below) for removing nonpolar com
pounds. The cleanup resins are often commercially available as dis
posable cartridges, offering a rapid and versatile sample pretreat
ment.

A. Matrix Removal and Analyte Preconcentration

Different approaches can be followed [4] in order to remove the ma
trix and preconcentrate the analytes: separations can be static or
dynamic. The chemical techniques used in preconcentration must
provide analyte isolation as well as enrichment through minimal
sample manipulation in order to avoid contamination and to obtain
low sample blanks. Such procedures are usually defined off-line or
on-line, involving sample treatments which are performed indepen
dently of or in direct connection with the analytical
instrumentation.

Among the main methods—evaporation, precipitation,
co-precipita
tion, flotation, extraction (homogeneous or heterogeneous),
sorption
(adsorption, chelation on functionalized supports,
ion-exchange),
electrochemical methods, and special techniques (e.g.,
hydride gen
eration)—particular attention has been devoted to the
broadly
named liquid-liquid or liquid-solid extractions
(solid-phase extrac
tion, SPE), probably the most effective of the techniques
employed
nowadays. From a practical point of view, the liquid-liquid
extraction can be performed following two procedures: ionizable
com
pounds can be extracted into organic solvents as "neutral"
ion pairs
in the presence of an appropriate ion-pairing agent; or
hydrophylc
compounds, which are difficult to remove from the aqueous
phase,
can be extracted by forming a hydrophobic complex with an
appro
priate complexing agent and the reaction—may be used for a
selective
isolation. The most widely employed extractants for metal
ions form
neutral chelates which have greater affinity for organic solvents than for the aqueous phase; the metal determination following this approach can be performed by direct analysis of the organic phase.

The liquid-liquid extraction methods may be used for selective or group separation of trace elements, collected in the extract, or for matrix removal. These methods have the advantage of simplicity and rapidity, but the low concentration factors achieved are one of their major drawbacks. The extraction of compounds from a solution onto a solid phase (SPE) using silica, alumina, celite, charcoal, polymeric, or ion-exchange resins has long been practiced. Silica gels or polymers have been bound with a wide variety of functional groups (e.g., alkyl, phenyl, amino, ciano, diol, alkylsulfonate, and quaternary ammonium groups), and more recently with chelating functional groups (e.g., carboxylic, quinolines, iminodiacetic) to provide a specific interaction with analytes. No attempts are made hereafter to define foams, resins, or sorbents of inorganic or organic nature.

It must be mentioned that if silica-based supports are used, the re
residual silanol sites provide, for the analyte, a second type of binding sites, so it is advisable to use as supporting material in the column either a silica C18 end-capped type or a polymeric-based C18 derivatized support. For SPE the acting mechanisms, for the retention of metal ions and their species, coupled with the different kinds of materials, are: Adsorption of neutral species as such or obtained via interaction with hydrophobic groups formed by adding a proper ligand able to originate neutral complexes with the analytes or adding an ion-interaction reagent able to originate ion pairs, with hydrophobic properties, with metal ions or their charged complexes Chelation obtained by reaction between metal ions and coordinating groups grafted on the solid Ion-exchange performed in two ways: cation exchange, which involves a direct exchange of the metal ion with the counterion of the resin and its retention; or anion exchange, which requires the preliminary addition of a ligand (usually sulfonated or phosphonated) able to form a negatively charged complex with the analyte From a practical point of view, liquid-solid extraction is commonly performed by driving the sample through a column (microcolumn, cartridge, microtube, or other kind of container) packed with the proper stationary phase, namely, a preconcentrator. When complexation is required in on-line systems, the sample of interest is injected into the manifold and merged with the chelating agent at a confluence point downstream. After passing through a mixing coil, which allows enough time for the chelate to form, the metal chelate
is preconcentrated on the column. This procedure is superior to the
direct addition of the chelating agent to the sample, since in this
case a purification cartridge can be added before mixing sample and
ligand. To overcome the problems arising from off-line complexation
and preconcentration (e.g., sample poisoning, extraction proce
dures), great effort has been devoted to develop on-line preconcentration
procedures and, for on-line high-performance liquid chromatography (HPLC) applications the preconcentrator could be inserted
instead of the injection loop. Obviously, in consideration of the mechanism and materials selected, the parameters to be kept under control will be proper pH for complex formation (precipitation must be
avoided); ligand or ion-interaction reagent concentration and solubility (sufficient to originate complex or ion pair but not so high as
to compete for resin sites); ionic strength (mainly for ion-exchange
competition); and, from a practical point of view, elution velocity of
the sample must be the maximum allowed by absorption, chelation,
or ion-exchange kinetics utilized for metal-ion retention.
In all cases, attention must be paid to ensuring the compatibility of the preconcentration step (the strength of the retention of analytes onto the preconcentrator) with the composition of the eluent used for the subsequent recovery of the analytes and their separation. Strictly related to the above-mentioned procedures, based on SPE, multidimensional liquid chromatographic techniques prove to be successful in matrix removal and trace separation and involve the use of two or more columns. The multidimensional technique is also known as "heart cut" column switching. The term "column switching" includes all techniques by which the direction of the flow of the mobile phase is changed by valves so that the effluent (or a portion of it) from the primary column is passed to a secondary column for a defined period of time. The column-switching technique has also been used in ion chromatography (IC) to solve the column overloading problems encountered when difficult samples have to be analyzed. In this case, matrix elimination is effected mechanically [5] rather than chemically via sample pretreatment. The technique
can employ two four-way valves inserted before and after a precolumn (e.g., guard column, separator column, or an entirely different type of column from the separator column, such as a reversed-phase column). By configuring the valves in such a way that the bulk of the matrix is diverted to waste and only a heart cut of the analyte of interest is transferred to the separator column, the system is effective in either eliminating or at least simplifying sample preparation.

Fig. 1 Schematic diagram of column switching. Initial valve position: the sample flows through the column CS12A and then into the CS10. Valve switched: the eluent goes into CS10 and then into CS12A. [Reprinted from M. A. Rey, J. M. Riviello, and C. A. Pohl, Column switching for difficult cation separations, J. Chromatogr. A, 789: 149-155 (1997), with permission from Elsevier Science.]

tive in either eliminating or at least simplifying sample preparation.

[6] (see Fig. 1). It must be emphasized that a great number of papers, referring to column switching or multidimensional liquid chromatography, concern methods that do not actually use two chromatographic columns, but rather use simply a short column (preconcentrator) cou
B. Derivatization

Derivatization is an alternative approach to solve the mentioned problems. Derivatization is a chemical reaction able to modify the nature of the analytes or of the matrix, and can be usefully employed both pre- and postcolumn. In the first case the metal species are usually reacted, e.g., with a chelating agent that enables their separation by means of the selected mode. In the latter case the reaction is used for enabling analyte detection or lowering the detection limits; that is, e.g., for metal ions detectable with poor sensitivity by conductometric detection, a reaction with metallochromic ligands may enable their UV-visible detection with higher sensitivity. In this case a postcolumn reactor (PCR) "reaction coil," where the reaction takes place, is inserted between the analytical column and the detector and reached by the eluent and the reagent solution. The main requirements are (1) reduced dead volumes due to tubes, connections, and reactor; and (2) the reaction of derivatization must be very
fast, in order to reduce the time spent inside the reactor and to avoid peak diffusion. A particular case of "derivatization" is represented by the suppressed conductometric detection in IC. To enhance the detection sensitivity, the contribution to the conducibility due to the eluent is "suppressed" through a chemical reaction (the main components of the eluent are transformed into water, weak acids, or weak bases). To define a border for the term "derivatization," some other more specific approaches, e.g., hydride generation or ion generation, followed in the new techniques involving the coupling (hyphenation) of HPLC with "unconventional" detectors (e.g., atomic spectroscopy, mass spectrometry, inductively coupled plasma-mass spectrometry), will be discussed hereafter.

III. CHROMATOGRAPHIC MODES

Several modes of HPLC can be performed for the analysis of metal species, including normal-phase chromatography (NPC), reversed phase chromatography (RPC), ion-pair or ion-interaction chromatography (IPC, IIC), ion chromatography (IC) (cation and anion exchange, ion exclusion), chelation ion chromatography (CIC),
their couplings—multidimensional and multimode chromatography. The basic principles, retention mechanisms (processes involving solute interactions in both the mobile and stationary phases), materials, and applications are summarized hereafter.

A. Normal and Reversed-Phase Chromatography

Both these two modes of HPLC are used for the separation of metal chelates and organometallies, so each will be discussed below. Normal-phase chromatography (NPC) is characterized by the

use of an inorganic adsorbent or chemically bonded stationary phase

with polar functional groups (e.g., silica, cyanoalkyl-silica) and a nonaqueous mobile phase (one or more polar solvents diluted to the desired eluting power). The mechanism acting in the NPC is a liquid-liquid partition, and both retention and selectivity are dramatically influenced by the presence of polar additives (water) in the mobile phase. Reversed-phase chromatography (RPC) employs nonpolar solids of high surface area (usually alkyl-bonded silica packing, e.g., C8 or C18 groups grafted to the silica surface) as stationary phase and an aqueous-organic solvent mixture as mobile phase. Retention in RPC

and
occurs by nonspecific hydrophobic interactions of the solute with the stationary phase. Secondary chemical equilibria (see below) optimize separation selectivity by varying the mobile-phase composition. The solute retention is attributed to both adsorption and partition phenomena, while neutral and ionic solutes can be separated simultaneously. The current approach for normal-phase chromatography (NPC) is based on the formation of metal chelates (e.g., diacetylbisthiobenzhydrazones, dithizone, diethyldithiocarbamate), their extraction from the sample, their injection and elution, performed with organic solvent mixtures of n-heptane/benzene, toluene, diethyl ether/acetonitrile or similar. A typical reaction for heavy-metal ions is with ammonium tetramethylenedithiocarbamate (ATDC) [7], which gives neutral complexes. These kinds of chelates can be separated in the same manner as neutral compounds on normal-phase silica columns (e.g., 1% propanol in hexane). The main problems encountered in NPC are connected with the thermodynamic stability of the metal chelates and solubility, the need for extraction, and the risk of pollu
tion of the sample when traces must be detected. Among the
mentioned mechanisms, normal-phase chromatography is not at present the most widely used. RPC has been widely used for the separation of neutral or weakly charged metal complexes, but the more extensive applications are based on ion-pairing mechanism, so related procedures will be detailed hereafter. Analysis for trace metals is carried out by the formation of metal chelates with separation by RPC on C18 columns and the use of organic-based mobile phases. Dithiocarbamates are the most frequently reported complexing agents, due to the strong chelating ability of their sulfur groups and their ability to form nearly water-insoluble metal salts with all metals except sodium and other alkali and alkaline earth metals. Dilli et al. [8] completed a comprehensive study on reversed-phase HPLC behavior of diethyl dithiocarbamate (DEDTC) complexes of Cu, Co, Cr, Ni, and Hg with a variety of columns and mobile phases. In this case DEDTC complexes were preformed off-column (60°C, 15 min), extracted into chloroform, and finally dissolved in CH3OH and injected
for the separation onto a C18-column (juBondapak, Waters). The study showed that the ligand must also be present in the mobile phase for low concentration of chelates, to avoid their dissociation. In addition, the better suitability of a water-methanol eluent with respect to a methanol-acetonitrile-water mixture has been demonstrated. In this way one avoids interference due to the involvement of acetonitrile in ternary complexes formation.

A similar procedure was developed for Co and Ni DEDTC-chelate separation on dimethyloctadecylsilyl-bonded silica. The preconcentration of chelates by a double extraction (diethyl ether followed by methanol) coupled with UV detection allowed the attainment of 5 and 50 ng/L detection limits for Co and Ni, respectively [9]. More recently, a detailed study of the RP-LC behavior of metals (Zn, As, Fe, Cd, Pb, Ni, Cu, Hg, Co, and Cr) complexed by reaction with a homologous series of five dialkyldithiocarbamate ligands, of general formula R₂NCS₂⁻, has been reported [10]. A single C18-column (juBondapak, Waters) and either methanol-water or methanol-acetonitrile-water mobile phase proved suitable for the separation of metals with
the exception of As, Fe, and Zn. The ternary system was superior for higher homologs. Studies to overcome the main drawback of an extraction step, due to the low solubility of DEDTC complexes, were also made, and a thiosemicarbazone was synthesized that was able to form soluble complexes [11].

2-Acetylpyridine-4-ethyl-3-thiosemicarbazone complexes of Co, Cd, Fe, Ga, Ni, In, and Zn were separated on a PS-DVB column and the mechanism was found to be a mixed-mode one (RP and IIC) since Co, Fe, and In complexes are positively charged and the best separation was achieved by adding NaClO₄ to the CH₃OH mixture. The problem arising from the presence of more than one coordination form for the corresponding central ion for nitrogen-ox ygen coordinated complexes (which produces a general peak broadening) has been tentatively solved by Ming et al. [12]. Their work uses a precolumn and on-column derivatization for the separation of V(V), Co, Fe, and Ni through the formation of binary and ternary peroxo complexes with 4-(2-pyridylazo)-resorcinol (PAR) and H₂O₂.

The study showed that, with methanol-water eluent and a RP
col

umn, V(V)-PAR binary complexes yield two peaks which are con
verted into a V-PAR-peroxo complex single peak if H 2 0 2
is added
to the sample within PAR before elution.
2-(5-bromopyridylazo)-5
diethylaminophenol (5-Br-PADAP) is another 2-pyridylazo
complex
ing reagent that is highly sensitive and selective. Studies for the
separation and determination of metal ions [Cu, Co, Fe, Ni,
V(V),
Pd] as 5-Br-PADAP chelates by RP-HPLC showed that only the
retention of Co(III)-5-Br-PADAP complex is affected by
varying the
concentration of surfactant added to the eluent [13]. In
addition, a
stronger interaction resulted for tetrabutylammonium (TBA)
with
respect to cetyltrimethylammonium (CTA) and cetylpyridinium
(CP)
cations. The alkyl group, such as that of CTA or TBA, may
interact
with the C18 chain on the stationary phase by molecular
interaction
so that the charged part of the surfactant is exposed on
the surface,
increasing its polarity so that the Co chelate is eluted
earlier. Follow
ing this approach, a selective preconcentration method with
a cation
exchange resin for RP-HPLC of the Co-5-Br-PADAP complex was
recently developed [14]. Co complex, in aqueous solution, is readily oxidized to the Co(III)-5-Br-PADAP inert cationic complex, which is retained on a sulfonated XAD-4 resin. Co is detected spectrophotometrically (588 nm) after elution onto a C18 analytical column (Capcell SG-120) with a methanol-water eluent added of ethylenediaminetetraacetic acid (EDTA) and TBA and without 5-Br-PADAP. The absence of 5-Br-PADAP favors the dissociation e.g., of Cu and Zn chelates, and other metal ions eluted later, such as Fe and Ni, do not interfere. The detection limit for Co in water samples is reported to be 5.9 ng/L. Various azo dyes have also been considered for the chromatographic separation of metal chelates on a reversed-phase RP-18 column, and the study focused on the separation and determination of V(V) at trace levels in natural waters [15]. The originality of this investigation is due to the optimization of the RP column selectivity by introducing a tetraalkylammonium salt into the system. The metal chalates considered are neutral or cationic and ion-paired complexes are not involved, whereas other metal ions (e.g., Fe, Al)
do not interfere in the determination. 8-Quinolinol (HQ) is another extensively used ligand for the separation of metal ions by HPLC. For this ligand the methods are also based on metal-ion complexation, usually by heating the sample in the presence of HQ, one or two extraction steps with an eluent-compatible solvent, and injection of complexes into the chromatographic system. As an example, the simultaneous determination of Mo(VI), V(V), Cu(II), and Fe(III) at the parts-per-billion level in sea water can be mentioned [16]. To overcome the problems arising from off-line complexation and preconcentration (e.g., sample poisoning, time consuming) a column-switching technique has been proposed [17]. In the mentioned study two compatible eluents of different eluotropic strength were selected, one (CH$_3$CN/H$_2$O) to concentrate the metal HQ complexes onto a precolumn (Nucleosil C18) and the second (CH$_3$CN/H$_2$O/HQ) to elute the analytes from the precolumn onto the analytical column (C18). The linear dynamic range is from 5 ppb to 10 ppm for Al and from 40 ppb to 5 ppm for Cu and Fe. LC has been shown to be a powerful technique for the determination of trace noble metals, and among the different ligands em
ployed, thiazolylazo reagents play a remarkable role in RP-HPLC.

Studies on the retention of Pd, Pt, Rh, and Ru chelates of 1-(2-thiazolylazo)-2-naphthol (TAN) enabled the determination of Pd and Ru [18]. Basova et al. [19] separated the 4-(2-thiazolylazo)resorcinol (TAR) complexes of Rh, Ru, Cu, and Co on an ODS column with acetonitrile. Separation and determination of Rh, Ru, and Os chelates with TAR was also reported [20]. Saraswati et al. [21] used TAR as a chelating reagent in the reversed-phase HPLC separation of transition metals from rare earth elements in low-alloy steels by increasing the concentration of the eluent (octane-1-sulfonate-tar taric acid). For noble metals more satisfactory results have been ob tained using a new thiazolylazo reagent, 2-(6-methyl-2-benzothiazolylazo)-5-diethylaminophenol (MBTAE) [22]. MBTAE complexes (Pt, Os, Ni, Co and Rh, Pd or Ir, Ru) were separated and determined by RP-HPLC using both C18 and C8 columns with methanol-n-butanol-water or methanol-water eluents, respectively [23,24]. Several papers focusing on the analysis of the isomers of metal complexes have been published, and recent advances in HPLC have
made available columns in which the active solid phase is chiral,

for the separation of enantiomers. Akama et al. [25] achieved the

identification of two geometric isomers of the chromium(III) complex

with 1-phenyl-3-methyl-4-benzoyl-5-pyrazolone, through HPLC con

tinuous-flow, fast-atom bombardment, mass spectrometry. Aceto

nitrile-water and rc-hexane-tetrahydrofuran as the mobile phase

for an ODS and a silica gel column were used. Carreri et al. [26]

have shown the NP and RP HPLC potentiality in a comprehensive

study on seven iron-carbonyl compounds substituted with alkynes,

R 2 C 2 . Several stationary phases, including silica, CN, C18, and phe

nyl columns, coupled with positive-ion and negative-ion chemical

ionization mass spectra of the compounds obtained with particle

beam mass spectrometry (MS), were considered for the separation

and the structural elucidation of organometallic compounds. Finally,

a comprehensive review has recently been published [27] on normal

phase (NP) and reversed-phase (RP) HPLC modes for the analysis

of organometallic compounds and metal coordination
compounds, in
the synthesis and reaction of metal complexes, in studies of the ki
netics and mechanism of the complexation of the metal complexes,
and in bioinorganic chemistry.

B. Ion Chromatography

The term ion chromatography (IC) referred in the past to ion exchange chromatography only. Ion-exchange chromatography is basically a simple process based on a reversible interchange of ions between a solution and a solid, inorganic or polymeric insoluble material, containing fixed ions and exchangeable counterions. Analytes are separated on the basis of their different relative affinities for the ionic centers of the stationary phase. With respect to the use of the conductometric detection, two ion chromatographic modes are defined: suppressed and nonsuppressed. In the first mode, before detection, the eluate is driven through the suppressor unit, where the background conductivity is greatly reduced, so that the sensitivity with which sample ions can be detected is increased. Nonsuppressed ion chromatography is performed without the use of a suppressor unit, with ion-exchange resins of low exchange capacity and
very
dilute eluents, so that the background conductivity is quite low. In the cation-exchange technique the metal ions are normally reacted with an anion of a weak acid to reduce their charge density
in the eluent solution before entering the separation column, where they are separated owing to their respective affinities toward the active sites of the separating resin. Ligands are also required to avoid precipitation when an acidic eluent is not suitable for the selected columns. Ion chromatographic separation by anionic exchange of metal ions involves their presence as negatively charged complexes, which can be obtained in two ways. The first way, off-line, is through their formation before the chromatographic separation (precolumn complexation—complexes must be stable enough to avoid decomposition during separation or ligand must be added to the eluent). The second way, on-line, is based on the complexation in the chromatographic column itself, by adding the proper ligand to the eluent. Ion chromatographic separation of metal ions based on anionic exchange offers the potentiality of different selectivity, reduced problems for metal-ion hydrolysis, and application
plex sample matrices. Therefore, ion-exchange chromatography is based on electrostatic interactions between the ions to be exchanged, but other reactions may occur, e.g., hydrophobic reactions between the sample and nonionic regions of the stationary phase, or additional reagents are intentionally introduced in order to achieve or optimize the separation. This means that in IC, in addition to the ion-exchange reaction, the secondary chemical equilibria (SCE) play a relevant role [28].

Figure 2 shows a schematic illustration of the equilibria existing on a cation- or an anion-exchanger between a solute cation ($M^{2+}$), an added ligand ($H_2L$), and an eluent cation ($enH^+$). In the first case, see Fig. 2a, the complexation due to the deprotonated ligand moderates the cation exchange through a pulling effect. Figure 2b represents the equilibria involved for the retention of an anionic complex, where the excess of the ligand itself (deprotonated) or another anion added to the eluent, competes for the fixed sites. Finally, Fig. 2c shows the pushing and pulling coupled effects of the ligand and of
the competing cation, where complexation reduces the availability of the free M\(^{2+}\) for the exchange. It must be underlined that in all cases the eluent pH is a determining parameter, since it is acting both on the ligand dissociation and on the cation protonation. Metal ions can be retained on silica-based ion-exchange columns and silica itself can act as both anion and cation exchanger. Ion ex changers produced by chemically bonding ion-exchange groups to a silica backbone were the main type of early column packings, pellicular ion exchangers formed by coating a silica core with a polymeric ion-exchange material. Polymer-coated silica cation exchangers for IC can be synthesized by depositing varying film thicknesses of a prepolymer onto porous silica; immobilization is achieved by in-situ cross-linking reactions using radical starters or radiation. Poly(butadiene-maleic acid) (PDMA) is the preferred polymer. An advantage of silica-based materials is the low probability of secondary interactions between solute ions and the silica substrate and higher column capacities over synthetic materials. On the other hand, serious Fig. 2 Secondary chemical equilibria in cation- (a, c) or
anion- (b) exchange

chromatography. Metal ion M$^{2+}$ as such or as an anion complex: (a) cation exchange, the ligand (L$^2$) exerts the pulling effect on the cation analyte (M$^{2+}$) through the complexation; (b) anion exchange, the deprotonated ligand (L$^2$) competes (pushing effect) with the anion complex (ML$^{2+}$); (c) cation exchange, pulling and pushing effects of the ligand and of a competing cation on the retention of M$^{2+}$.

drawbacks exist with silica-based materials. Both low and high pH values must be avoided: below pH 2, covalent bonds linking the ion exchange functionality become unstable and the functional groups are cleaved; while above pH 8, silica matrix may be dissolved. When IC was first introduced [29], column packing for cation determinations consisted of surface-sulfonated 25-Lim polystyrene beads cross-linked with 2% divinylbenzene, and they were used with packed-bed suppressors. Due to the large differences in selectivity of alkaline earths toward alkali cations, HCl was needed to elute the monovalent cations, while stronger divalent eluent components (e.g., m-phenylenediamine) were required for the elution of divalent ca
tions. Due to the long column equilibration time between the two eluent systems, it has proven difficult to provide simple isocratic elution to allow the separation of both classes of cations in a reasonable period of time, so that two different columns were dedicated for the two classes of cations. Nevertheless, chromatograms were characterized by quite long total run times and by poor peak efficiency. Heavy metals have also been separated on these kinds of materials with diluted strong acids and/or organic complexing agents. The development of new methods of synthesis for ion chromatography (latex-coated columns, IonPac CS3, Dionex Co.), together with the replacement of ra-phenylenediamine by the zwitterion 2,3-diamino propionic acid monochloride [30], made it possible to simultaneously analyze both the alkali and alkaline earth elements, in the presence of ammonium, in one column, and to improve peak efficiencies, although analysis still required a long time and baseline resolution was not completely achieved. A different kind of column containing carboxylate functionalities, instead of the traditional sulfonate ones
with a low selectivity for the hydronium ion, the so-called Schomburg column, was introduced by Kolla et al. [31]. This stationary phase, based on a poly(butadiene-maleic acid) (PBMA) co-polymer silica gel coated, coupled with eluents containing slightly acidic complexing agents (e.g., tartaric acid), was used to separate Li\(^+\), Na\(^+\), NH\(_4\)\(^+\), K\(^+\), Mg\(^{2+}\), Ca\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\) in nonsuppressed IC. The unique selectivity of the carboxylic groups, and the competition of the chelating agent in the eluent with the cation-exchange sites for divalent cations only, provided good separation and short analysis times, the only limitation being the operative pH range imposed by the silica material. The performance of PBMA silica columns for separation of alkali, alkaline earth, and heavy- or transition-metal ions has been studied in the presence of different organic complexing agents (oc-hydroxyisobutyric, tartaric, citric, oxalic, pyridine-2,6 dicarboxylic acid, and EDTA) and applied to determine cations in water, food, ore, sole brine, and other real samples [32,33]. Common reversed stationary phases, permanently coated with suitable hy
drophobic agents such as alkylsulfonates or alkylsulfates, have also
been used for the separation of transition and heavy metals
[34,35]. A great improvement in metal-ion chromatography was ob-
tained with new synthetic packing materials basically characterized
by an ethylvinylbenzene substrate cross-linked with 55% divinyl
benzene surrounded by solvent compatible functional groups (sul-
fonic, carboxylic/phosphonic, carboxylic/phosphonic/crown ether),
(IonPac Columns, Dionex Corporation, Sunnyvale, CA, USA).
The above-mentioned polymers in some cases (e.g., IonPac
CS5 and CS5A) bear both quaternary ammonium and sulfonate func-
tional groups, in a pellicular layer located on the core of the beads.
The presence of both cation- and anion-exchange groups enables
more sophisticated separations: (1) Sun et al. [36] speculated on this
property and separated Ge and Sn, by pure ion exchange, as cation
and anion, respectively. Metals were determined simultaneously on
a IonPac CS5 by working at the proper pH (<1.5), ensuring the pres-
ence of HGeO\textsuperscript{3} and Sn\textsuperscript{2+} species; (2) Motellier et al.
[37] developed a liquid chromatographic method with an on-line preconcentration
step (IDA chelating column) using pyridine-2,6-dicarboxylic acid (PDCA), which forms negatively charged complexes with metals (Co, Cu, Ni, Mn, Zn), and a CS5 analytical column. They showed that the exchange mechanism for Ca on the analytical column is much different from that of transition metals (pure ion exchange of positively charged complexes). The method proved to be suitable for waters of low ionic strength. This kind of columns (IonPac CS 5A) allowed the simultaneous determination of heavy and transition metals with a gradient program (based on oxalic acid and sodium chloride eluents) coupled with: (1) spectrophotometric detection after postcolumn reaction with 4-(2-pyridylazo)resorcinol PAR, (2) 2-[(5-bromo-2-pyridylazo)-5-diethyl-aminophenol. This procedure was used for biochemical samples (technique validated by five standard biochemical references) [38] and nitrate/phosphate fertilizer solutions (detection limits 1-30 ng) [39], respectively. The main difficulty which had to be overcome was the greatly different selectivity of the ion-exchange resins, normally used to separate monovalent and divalent cations, respectively. These resins
were characterized by a much larger selectivity for divalent cations than for monovalent cations, thus divalent cations have greater retention times than monovalent ones. As an alternative way to simultaneously determine mono and divalent cations, a column-switching (dual-column) technique was reported [40]. Samples are eluted through two separate columns, each optimized for the chromatography of one group of cations. Separation is accomplished by switching the eluent flow paths from one column to the other. This method has several advantages compared to gradient techniques (e.g., shorter run times, no equilibration period between runs). Nevertheless, this technique requires sophisticated instrumentation, and sensitivity for divalent cations is poor.

Column-switching techniques between latex-based IonPac CG3 and CS10 columns have been used by Betti et al. [41] to determine alkali and alkaline earth elements in sea water. Transition and heavy metals have been determined in sea-water samples by multidimensional liquid chromatography [42]. The separation was achieved by coupling a dynamically coated sorbent column, a
preconcentrator, and

a chelating column. The metal concentrations (Cu, Ni, Co, Mg, Ca,

Sr, Fe and Pb, Zn, Mn, Cd) in sea-water samples were

5.0-50 |ng/L. As discussed in depth, samples containing alkali metals, alka

de earth cations, and the ammonium ion are difficult to analyze.

Environmental samples, at low levels of ammonium in matrices with

a high concentration of sodium, are a typical case. This is due mainly
to the similar selectivities of ammonium and sodium ions for the

common stationary phases containing sulfonate or carboxylate cat

ion-exchange functional groups. This problem has been solved by a

column-switching technique which enables the determination of

trace concentrations of the common inorganic cations (Li, Na, K, Mg,

Ca) and ammonium in the presence of large concentrations of either

sodium or ammonium [43]. Figure 3 shows, step by step, the isocratic

elution (eluent: methanesulfonic acid) of monovalent and divalent

cations during a column switching between a carboxylated (IonPAc

CS 12A) and a sulfonated column at decreased cation-exchange site

density (IonPAc CS 10), which provides the needed selectivity. Considering the experimental data available
for the separation
of these cations, the best separations were achieved
through the use
of polymer-based columns containing carboxylic (IonPac CS12,

Fig. 3 Step-by-step isocratic elution of monovalent and
divalent cations by
column switching. After elution of monovalent cation from
CS12A to CS1D
column, the valves are switched and the order of columns is
reversed. [Re
printed from M. A. Rey, J. M. Riviello, and C. A. Pohl,
Column switching
for difficult cation separations, J. Chromatogr., 789:
149-155 (1997), with
permission from Elsevier Science.]

CS14) [44,45] or carboxylic and phosphonic (IonPac CS12A)
[46,47],
or carboxylic/phosphonic/crown ether (IonPac CS15) [48]
functional
ities. Figure 4 clearly shows the different selectivity
achieved with
IonPac CS 12A (a) and IonPac CS 15 (b), due to the presence
of an
18-crown-6 ether group (permanently attached on the
macroporous
substrate beads of the later column). Since the
development of new materials for mono- and divalent
cations, expert systems for the planning of appropriate
dilutions,
suitable detector output ranges, and standard additions
were devel
oped for alkali and alkaline earth metals in mineral waters
[49].
Moreover, applications of the optimized systems (CS12 and methanesulfonic eluents) for the determination of Na\(^+\), K\(^+\), Mg\(^{2+}\), and Ca\(^{2+}\) in melted-snow samples from alpine sites [50] and for the determination of elements in Antarctic samples [51], have been shown. The high capacity which characterizes some of these stationary phases provided the means to develop a method based on high-volume direct injection for trace-level determination [52]. Satisfactory results for the determination of Na\(^+\), K\(^+\), Mg\(^{2+}\), and Ca\(^{2+}\).

Fig. 4 Comparison of selectivities achieved by carboxylic/phosphonic groups (IonPac CS 12A) and carboxylic/phosphonic/crown ether groups (IonPac CS15). (a) Column: IonPac CG 12A and CS 12A; eluent, 15 mM sulfuric acid; flow rate, 1.0 mL/min; peaks, 1 = Li\(^+\), 2 = Na\(^+\), 3 = NH\(^+\), 4 = K\(^+\), 5 = Mg\(^{2+}\), 6 = Ca\(^{2+}\). (b) Column, IonPac CG 15 and CS 15; eluent, 15 mM methanesulfonic acid + 7.5 mM hydroxylamine + 5% acetonitrile (40\(^\circ\)C); flow rate, 1.2 mL/min; peaks, 1 = Li\(^+\), 2 = Na\(^+\), 3 = NH\(^+\), 4 = Mg\(^{2+}\), 5 = Ca\(^{2+}\), 6 = K\(^+\). [Reprinted from M. A. Rey, C. A. Pohl, J. J. Jagodzinski, E. Q. Kaiser, and J. M. Riviello, A new approach to dealing with high-to low concentration ratios of sodium and ammonium ions in ion
Ca²⁺ in well water samples were also obtained using an IC Pak CM/D (Waters) column with an eluent containing citric acid and pyridine-2,6-dicarboxylic acid (PDCA) [53]. Tartaric and dipicolinic acids have also been used for mono- and divalent cations [54]. Ohta et al. [55] studied the cation-exchange properties of a commercially available unmodified silica gel (Develosil 30-5) in the acidic region. They obtained the simultaneous separation and determination of Na⁺, NH₄⁺, K⁺, Mg²⁺, and Ca²⁺ in environmental water samples by coupling nitric acid with a selective complexation (PDCA) of divalent cations. They also demonstrate the main cause of cation exchange attributed to aluminum present as an impurity in the silica gel [56]. More recently, the modification of a silica gel by a coating method with Al enabled the cation-exchange separation of mono- and divalent cations [57]. A synthesized weak cation exchanger (co-polymerization of vinyl groups covalently bound onto silica surfaces with acrylic acid) has

chromatography, J. Chromatogr., 804: 201-209 (1998), with permission from Elsevier
been used as stationary phase and the pure ion-exchange
mechanism is modified by adding 18-crown-6 ether and
acetonitrile to the
mobile phase [58] for alkali and alkali earth cation
separation.

Highly sensitive and excellent separation for these
cations, in various natural samples, was achieved (run time 15 min) using
an unmodified silica gel and 1 m ¥ oxalic acid/3 mM 18-crown-6
eluent [59]. It seems of interest to mention that Dumont and
Fritz [60]
showed that increase in the separation factors and change
in the
elution order of alkali metal cations could be obtained using
nonaqueous solvents with macroporous cation-exchange resins
(low-capacity PS-DVB sulfonated resin). This behavior is due to
the change
in solvated ionic radii for Li and Na, so that higher
resolution is
obtained in the separation of ions that usually elute close
together
(Li/Na, K/NH 4 + ). The use of lithium ion and neutral pH
or methanol
and lithium chloride as the eluent gave very low detection
sensitivities and poor separation of mono- and divalent cations on
porous
silica [61-63]. More recently, the separation of
eight-inorganic mono
valent cations was achieved on a porous silica gel microcolumn with a 30% (v/v) acetonitrile and benzyltrimethylammonium (BTMA) chloride in the mobile phase [64]. This result could be explained through a reduction of the hydrophobic interaction between cations and BTMA introduced onto the surface of silica through cation exchange and hydrophobic interactions. The advantage of the method is that BTMA cation both competes with analytes for the exchange sites and is used for their indirect UV detection. Cation exchange performed on silica using weakly or neutral eluents gives very good sensitivities for cations. Ion chromatographic separation of metal ions based on anionic exchange offers the potential of different selectivities, reduced problems for metal-ion hydrolysis, and can be applied to complex sample matrices. Notwithstanding the fact that many organic acids (from mono-, di-, tricarboxylic acids to chelating agents such as oc-hydroxyisobutyric acid, tartaric, citric, oxalic, pyridine-2,6-dicarboxylic acid, 1,2-diaminocyclohexanetetraacetic acid, diethylenetriaminopentaacetic acid) have been used for the simultaneous ion chromatography
of anions, alkali, alkaline earth, and heavy metals [65-67], ethylene diamminetetraacetic acid (EDTA) plays a fundamental role. Since EDTA forms, at the proper pH, negatively charged complexes with divalent or trivalent metal ions, the possibility of simultaneous separation of anions from metal ions is also feasible, as well as the speciation of metal ions. In these procedures, such complexes can be obtained both off-line and on-line. Some examples of applications of an EDTA eluent have been reported for the determination of anions and divalent cations in natural and pharmaceutical samples [68]. Both UV and conductivity detection were used, working at pH values of 6-8. Experiments were also performed with binary eluent systems comprising EDTA as complexing agent. An UV absorption reagent was used to enhance detection limits for Ca^{2+} and Mg^{2+} [69,70]. For sea-water samples [71], silica-based anion-exchange analytical columns enhanced sensitivity and enabled detection limits from 20 μg/L for Mg^{2+} to 0.4 mg/L for Ca^{2+} with UV and conductivity detection and eluent pH at 4.8. Komarova et al. [72] studied the ion chromatographic behavior of anionic EDTA complexes of vanadium (V) and (IV). As mentioned above, the
ion chromatographic determination of metal-EDTA complexes can be performed with anion-exchange columns [68,71,73-76]. In this way, anions and metals can be separated as anionic complexes in a single run. In addition, this approach enables specific determinations; e.g., Se(VI) was determined (detection limit 4.8 ng/L) in real samples by suppressed anion chromatography in the presence of anions and heavy-metal ions [74]. Alternatively, the separation of the metal-EDTA complexes can be carried out with a cation-exchange column [66]. In this case the retention mechanism of analytes involves the cation exchange of free metal ions (e.g., Cu, Fe, Zn, Ni, Pb, Mn, alkali, and alkaline earth metals) which are present at low pH values. Detailed studies on the behavior of metal-EDTA complexes in cation chromatography as a function, e.g., of pH and ionic strength, provides data that can be used to develop selective models for understanding the contributions of different charged or neutral species on the retention mechanism involved.

A selective method for preconcentrating and determining Pb at trace
levels, 0.5 (ig/L detection limit, was developed based on these consid
erations [77]. Recently, the simultaneous determination of common
inorganic anions, magnesium and calcium ions, in various environ
mental waters has been achieved by indirect UV-photometric detec
tion ion chromatography coupling trimellitic acid-EDTA eluent
with an anion-exchange column (TSKguardgel QAE-SW) [78]. Theoretical approaches to the retention of anionic metal com
plexes have been developed for anion exchange [75,76,79] and for
cation exchange [77,80]. Ion chromatography, as well as reversed-phase liquid chroma
tography, is used increasingly for the separation of lanthanide met
als, but classical ion-exchange techniques do not always provide sat
isfactory separation. An approach to rare earth separation has been proposed by
Strelow et al. [81], who evaluated the distribution coefficients for
yttrium and some lanthanides between AG 50W-X4 resin and hy
droxyethylenediaminetriacetate in monochloroacetate buffer solu
tion, the results have been applied to the quantitative separation of
yttrium and neodimium from samarium and heavier lanthanides.
In these conditions, the control of pH is essential because the peak
positions are very sensitive to change in this parameter. Silica gel based sulfonated-form cation-exchange gel and sulfonated polystyrene gel columns were used with lactic (LA) and oc-hydroxyisobutyric (HIBA) acids by Kawabata et al. [82]. A gradient elution with HIBA or LA enabled them to overcome interferences due to polyatomic ions and isobars in ICP-MS detection of rare earths; e.g., GdO+ and GdOH+ overlap all the isotopes of Yb and Lu, and LaH+ hinders the free isotope of Ce. The method gave a complete separation of all rare earths within 1-5 ng/L detection limits, and the method has been successfully applied to the analysis of Tm, Yb, and Lu impurities in a Gd matrix and Ce in pure La 2O3 [82]. The Dowex 50 X 8 cation-exchange resin has been used by Fariñas et al. [83] as a tool to separate rare earth elements, as a group, in geological materials from the matrix elements, using in increasing HCl concentrations (from 2 to 6 M). The determination of lanthanides is finally performed by ICP spectrometry. The separation of each rare earth element can be performed either with a cation- or anion-exchange mechanism, according to the
eluent composition and the properties of the stationary phase. Cat
ion exchange of lanthanides has been performed in pellicular, latex agglomerated columns (IonPac CS3 [84] and CS10 [85]) in the presence of appropriate chelating agents such as HIBA. Lanthanides form complexes with HIBA that lower the affinity of the lanthanide for the cation-exchange resin. The elements are eluted according to the stability of the complexes formed, with Lu (the most stable complex with HIBA) eluting first and La (the weaker complex with HIBA) eluting later. In the anion-exchange mechanism, lanthanides have been predominantly separated using a mixed-bed column (IonPac CS5 and, more recently, CS5A [86-89]) containing both anion- and cation-exchange sites. Different complexing agents can be used in a gradient mode, as single components or in a mixture, to obtain a baseline resolution among each rare earth element. The first nine elements (La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, and Dy) have been determined as impurities in a YbF₃ matrix, used in the production of optical fibers, by coupling oxalate and diglycolate as ligands in the eluent with a
mixed-mode ion-exchange packing (IonPac CS5) [88]. Through the
optimization of the method [89] the co-elution of Ho and Er
was avoided, enabling the determination of 11 elements in the
presence of excess of Yb in the matrix (see Fig. 5). Detail was also
given on the different contributions to the retention mechanism (anion
or cation exchange) when such a mixed-bed column is used, showing the
second chemical equilibria involved [89] (see Fig. 6). Finally, an example of a very limited number of papers dealing
with ion-exclusion chromatography applications to metal determina
tions is given. Tanaka et al. [90] have developed an anion-exclusion
method in nonsuppressed IC of mono- and divalent cations, estab
lishing a simple technique to be applied in acid rain, under
ground, lake, and forest soil waters. The simultaneous separation of
Na⁺, NH₄⁺, K⁺, and Mg²⁺, Ca²⁺ has been obtained with an anion
exclusion chromatographic column packed with polymethacrylate
based weakly acidic cation-exchange resin in the H⁺ form
(TSKgel

Fig. 5 Chromatogram of a YbF₃ sample spiked with lanthanides, 5 mg/L
each. Stationary phase, IonPac CS5: Eluent gradient: \( t = 0 \) min, 80 mM oxalic acid; \( t = 15 \) min, 26 mM oxalic acid, 23 mM diglycolic acid; \( t = 22 \) min, 80 mM oxalic acid. Detection at 520 nm after postcolumn derivatization with PAR. (From Ref. 89.)

OA-PAK-A 300 X 7.8 mm). An eluent containing 0.75 mM sulfuric acid, 2 mM tartaric acid, and 7.5% methanol allowed the separation of mono- and divalent cations in about 25 min.

C Ion-Interaction Chromatography

Ion-interaction chromatography (IIC), also called soap chromatography, ion-pair chromatography, and dynamic ion-exchange chromatography, is a typical example of a chromatographic process based on secondary equilibria. Two approaches are usually followed to perform an IIC separation of metal ions: 1. Eluents containing an ion-interaction reagent (IIR) (e.g., alkylammonium salts, alkyl sulfates, or alkylsulfonates) are used and stationary phases (conventional RP or polymers) are dynamically modified into low-capacity ion exchangers.

Fig. 6 Equilibria involved in lantanides (M\(^{3+}\)) separation in the presence of ligands (L\(^{2-}\)) in a mixed-bed column (IonPac CS5). (From Ref. 89.) Elution of cations is achieved by their complexation and ion pairing of the negatively or positively charged complexes with IIR. During the separation, the retention of neutral analytes, analytes having the same or opposite charge with respect to the IIR, will be unaffected, decreased, or increased. Organic modifiers, and in some cases the complexing agent, are
also added to the eluent. 2. Common reversed-phase stationary phases are permanently coated with suitable hydrophobic agents such as alkylsulfonates or alkylsulfates with a sufficiently long alkyl group. In this case stationary phases are preloaded with the proper, very lipophilic, IIR. The mechanism of elution is governed by the mobile phase in two ways: (a) eluents containing a strong driving cation and a small amount of complexing agent ("push-pull" method, e.g., mobile phase containing ethylenediammonium cation and tartaric acid); (b) eluents containing a very weak driving cation and higher concentrations of complexing agent ("pure pull" mechanism). Since pH can alter the stoichiometry of the complexes and their overall charge, resolution of chelates is greatly dependent on eluent acidity. Therefore, research in this field is devoted to evaluating the nature and concentration of proper ligands and IIR as well as organic and ionic strength modifiers and eluent pH. The wide number of parameters involved and governing retention reflect the great versatility of the system to manipulate selectivity and to obtain good separations even when complicated matrices have to be separated. Nevertheless, the number of variables involved turns out to be a drawback when the effect of each mobile-phase parameter has to be considered during the modeling of the ion-interaction mechanism, which indeed has been controversial to be interpreted since the introduction of ion-interaction chromatography in the early 1980s.
The ion-pair formation interaction and the dynamic ion-exchange interaction modes are illustrated schematically in Fig. 7. Wide work on IIC ion-paired metal complexes of nitroso-naphthol sulfonates, with liquid-liquid extraction and on-line derivatization, has been done by Siren [91]. A modification of the proposed methods consists of an on-line derivatization of metal ions; in this procedure the metals are injected into a methanol-water eluent containing a quaternary ammonium bromide (e.g., cetyltrimethylammonium, CTA) and, after the column, they are mixed with a ligand solution (1-nitroso-2-naphthol-6-sulfonate) [92]. This procedure makes the metal-ion separation governed by the kinetics of formation of complexes and ion pairs and retention onto the postcolumn mixer-reactor system. On-column DEDTC chelates formation-preconcentration and RP-HPLC separation has been used for the determination of Cd, Cu, and Ni in sea-water samples [93]. A C2-bonded silica microcolumn, loaded with a dithiocarbamate-cetyltrimethylammonium ion pair, enabled the retention of metal ions and their complexation. Elution on ODS analytical columns was optimized by adding
cetyltrimethyl ammonium (CTA) bromide to a CH$_3$CN-H$_2$O mixture, owing to the relative instability of Cd-DEDTC anionic complex which was eluted as a neutral ion pair. Azo ligands have also proven suitable for trace-metal ion separation and determinations. The chromatographic behavior of 3-(5-chloro(Plasmocorinth B) and its metal-ion complexes has been studied in ion-pairing reactions for metal preconcentration and separation by Fig. 7 Schematic illustration of (a) ion-pair formation interaction and (b) dynamic ion-exchange interaction for an anionic solute (anion metal complex, ML") and a free metal ion (M + ). HPLC [94] in the presence of tetrabutylammonium ion. The separation of analytes, optimized with a flow-gradient elution, was successfully applied to river-water samples, enabling analyte metals to be separated from alkaline and alkaline earth elements. A detailed study on the effects of the mobile-phase composition (IIR, counter ion, organic modifier, pH) on IIC separation and electrochemical detection of metal complexes with SPADNS, Acid Alizarin Violet N,
and Plasmocorinth B was made [95]. The optimized procedure enabled the determination of Cu, Co, Fe, Ni, and V at microgram-per liter levels in natural waters. The suitability of a ligand for metal-ion determinations in an ion-interaction chromatographic mechanism depends on the selectivity of the ligand for the metal cations considered. An approach to select the appropriate ligand has been followed by Sarzanini et al. [96]. In this approach, the determination of the thermodynamic and conditional stability constants of Cu 2+, Ni 2+, Fe 3+, and Al 3+ with cyclo-tris-7-(1-azo-8-hydroxynaphthalene-3,6 disulfonic acid), calcion, or calcichrome (see Fig. 8), allowed selection of the proper experimental conditions for further application in the IIC separation. In this case pH was the key parameter for controlling selectivity and thus avoiding interferences by certain metal ions. The separation became specific for Al/Fe or Ni/Cu coupled cations by working at 4.5 or 7.5 pH values, respectively (Fig. 9). Metal ions have also been separated as anionic chelates with pyridylazosulfoaminophenol derivatives [97,98]. The retention behavior of these metal chelates in IIC has been elucidated.
as a function of eluent composition [99] with respect to the significant differences found in methanol-water and acetonitrile-water systems as a function of the volume fraction of water [100]. More recently, 2-(5-nitro-2-pyridylazo)-5-[(A^-n-propyl-A^-O-sulfopropylamino)]-phenol (nitro-PAPS) has been used as a pre-column chelating reagent for the simultaneous determination of V and Co, in steel samples, by IIC with an ODS column and an acetonitrile-water eluent containing TBA (IIR) and EDTA [101]. The method enabled detection limits of 17 ppt for V and 55 ppt for Co without preconcentration and with a spectrophotometric detection. Octadecyl-bonded silica permanently coated with sodium dodecylsulfate in the presence of complexing agents was considered for the separation of transition metals [102]. In that work an ion-exchange mechanism similar to that of fixed-site exchangers is shown. Fig. 8 Molecular structure of the ligand Calcion. (From Ref. 96.) Fig. 9 Determination of Al 3+ and Fe 3+ at pH 4.5 (left) and Ni 2+ and Cu 2+ at pH 7.5 (right) in a tap-water sample. Eluent 53-47% (v/v) methanol in
water containing 40 mM buffer, 25 mM TBA, 25 mM NaCl, and
5.0 \times 10^{-7} 
M Calcium. Tap water as such (dotted line) and spiked with
1 ppm of each
metal ions (solid line). (From Ref. 96.)

to occur, and both the pushing effect of the eluting cation
and the
pulling effect of the complexing anion are taking place but
the latter
plays a dominant role in the process of elution. A
significant exam
ple of the mentioned approach is the detailed study of
Cassidy and
Sun [103]. They compared the performance of an anion
separation
with a cation separation, each based on an ion-interaction
system
that used cetylpyridinium chloride or ra-octanesulphonate
to modify
a reversed stationary phase. In the first case, transition
metals (Mn,
Co, Ni, Cu, and Zn) were eluted with an oxalate eluent. The
anion
exchange system provided column efficiencies comparable to
that for
the cation system. This approach may be attractive for
solving ana
lytical problems taking into account the considerably
different order
of separation obtained by the two systems. A recent study
on chro
matographic behavior of metal ions [Fe(III), Cu, Pb, Zn,
Ni, Co, and
Mn] in IIC when the stationary phase is modified with various alkanesulphonates (1-10 carbon atoms in the alkyl chain) must be mentioned [104]. Reversed-phase ion-pair procedures involving EDTA have also been considered in optimizing separation and detection of metal species. Different techniques such as precolumn derivatization without complexing agent in the eluent or on-column derivatization may be less efficient and give rise to peak broadening. Ion-pair reversed phase high-performance chromatography has been investigated by coupling EDTA with tetraethylammonium (TEA) [105], tetrapropylammonium (TPA) [106], and tetrabutylammonium (TBA) [105,107,108] bromide ion-pairing agents. TBA resulted the best suitable ion pairing agent in all cases, and the use of EDTA in the eluent [105,108], together with high complexation constants, shifted equilibrium in favor of chelate formation reaching lower detection limits. The data obtained [105] clarify some aspects of the separation mechanism of ion-interaction chromatography for metal ions in different oxidation states and confirm that the retention of divalent and trivalent...
lent metal ions complexed with EDTA takes place through an ion exchange mechanism in which the ion exchanger is dynamically generated by the retention of the counterion in the stationary phase [108]. Studies on the separation and determination of metal ions [Cu, Co, Fe, Ni, V(V), Pd] as Br-PADAP chelates 2-(5-Br-2-pyridylazo-5-diethylaminophenol) by RP-HPLC showed that only the retention of the Co(III)-5-Br-PADAP complex is affected by variation of the concentration of the surfactant added to the eluent [109]. Stronger interactions are shown to result for tetraalkylammonium (TBA) with respect to cetyltrimethylammonium (CTA) and cetylpyridinium. An alkyl group, such as that of CTA or TBA, may interact with the C18 chain on the stationary phase by molecular interaction so that the charged part of the surfactant is exposed on the surface, increasing its polarity so that the Co chelate is eluted earlier. Vachirapatama et al. [110] developed a method based on precolumn ternary complex formation of Nb and Ta with Br-PADAP and citrate.

The selectivity of the separation of metal complexes,
ion-paired with TBA, was optimized by the concentration of methanol and citrate in the mobile phase. The method, applied to geological materials, is characterized by detection limits of 30 and 410 ppt for Nb and Ta, respectively. Dynamic ion exchange allows the use of bonded microparticulate alkyl silicas as nonpolar stationary phases with aqueous buffers containing low concentrations of hydrophobic ions for the separation of rare earth elements. Lanthanide separation was pioneered by Casidy [111] using gradient elution with sodium octanesulfonate as an ion-interaction reagent, which provides virtual ion-exchange sites by adsorption on a nonpolar Cis stationary phase with oc-hydroxyisobutyric acid as complexing eluting component. Quantitation is achieved by postcolumn reaction with Arsenazo III and optical absorbance detection at 658 nm, with typical detection limits below 1 ng for each element. Alternatively, 2-(4-pyridylazo)resorcinol (PAR) can be used as postcolumn reagent. In most cases lanthanides need to be precomplexed with proper ligands and then separated. The most widely used ligands are lactic
glycolic [113,114], HIBA [115-118], and nitrilotriacetic [119] acids. In certain cases, the achievement of complete resolution among all the rare earths is difficult. Kuroda et al. [113,114] showed that glycolato complexes are eluted within 20-30 min, but their method suffers from incomplete resolution between Ho and Sm, Eu, Gd, Tb, and Dy, which co-elute. The same authors showed that better resolution and separation is achieved using lactate and laurylsulfate as hydrophobic ion in the eluent [112]. Dodecylsulfate [115,120] and 1-octanesulfonate [121] have been employed in the determination of some lanthanide elements in apatite, bastnesite, and monazite samples. A coupled column chromographic procedure based on a semipreparative reversed-phase column and a dynamic cation-exchange column has been proposed by Lucy et al. [122]. This configuration has been applied to the determination of lanthanides in uranium matrix, due to the problem of degradation of neutron economy of nuclear reactors by impurities of rare earths. A C 18 column and HIBA eluent has been used to remove uranium, while a Cis column loaded with C 2 OSO 4 and a HIBA gradient
enabled lanthanide determination after postcolumn reaction with arszenazo III. Several examples of coupling both isocratic and gradient reversed-phase separations with ICP [123-125] and ICP-MS [126] detections are available. Theoretical approaches to the retention of anionic metal complexes have also been developed for IIC or dynamic ion exchange [127,128], and a comparison of prediction power between theoretical and neural-network models in ion-interaction chromatography of metal complexes, having single or double charges, has also been published [129].

D. Chelation Ion Chromatography

Trace-metal analysis with ion chromatography procedures becomes very difficult with samples of high ionic strength such as concentrated brines or sea waters, as the ion-exchange sites can become "swamped" with salt ions or, in the case of ion-pair chromatography, the pairing mechanism is modified. For these kinds of samples the selectivity of the chromatographic separation can be enhanced by the use of chelation ion chromatography. Chelation chromatography
involves both the formation of coordinate bonds and an ion-exchange process, due to free or protonated chelating groups which act as ion exchange sites, in the stationary phase and, in some cases, complexation in the eluent. Chelation ion chromatography or, more correctly, high-performance chelation ion chromatography (HPCIC), is based on the use of high-performance substrates for trace-metal separation and determination. Two main approaches can be followed to obtain proper stationary phases: (1) chemical bonding of the chelating group to the substrate; or (2) coating of a substrate with a ligand which is permanently trapped onto the substrate. Kantipuly et al. [130] illustrated the extensive range of chemically bonded chelating resins available for the separation and concentration of trace metals, among many combinations of chelating ligands and supporting materials. The different kind of stationary phases, available on the market or laboratory made, have recently been reviewed [131] and they are silica- or polymer-based materials with chelating agents grafted onto the surface. Alternatively, chelating ligands can be im
mobilized by adsorption onto a styrene-divinylbenzene co-polymer, silica gel, or other synthetic polymer. For the separation of metal ions, these precoated columns use an aqueous mobile phase at a relatively high concentration of an inorganic salt, e.g., 0.5-1.0 M KNO₃. The most widely used resin to preconcentrate and to separate elements and groups of elements in sea water, applied for the first time almost three decades ago [132], is an iminodiacetate resin (i.e., Chelex-100). Since this resin was successfully used in batch analysis for the determination of many metals in complex matrices, it was packed in a column form and eluent forced through at 1-2 mL/min.

Experimental results showed that only partial recoveries of some metal ions could be obtained under these conditions. Despite attempts to improve metal retention on Chelex-100 resin, it was finally concluded that the low recovery of metal ions was due to a physical degradation of the resin under pressure because of the low degree of cross-linking of the polystyrene-divinylbenzene supporting polymer. Among many combinations of chelating ligands and supporting
materials, Bonn et al. [133,134] successfully characterized and used stationary phases with silica-bound iminodiacetic acid (IDA) functions. The chromatographic behavior of some elements (Mg, Fe, Co, Cd, and Zn) is optimized under a mixed-mode mechanism, ion exchange and chelate formation with eluents (e.g., citric or tartaric acid), but other ions (e.g., Cu, Ni, and Pb) are irreversibly retained and strong complexing agents such as pyridine-2,6-dicarboxylic acid (PDCA) are required for the complete elution [135]. A more highly cross-linked macroporous PS-DVB has been developed [136] with iminodiacetate functional groups that allow operations at high pressure without physical degradation (MetPac CC-1 column, Dionex).

At pH 5.2-5.6, polyvalent metal ions are selectively concentrated into MetPac CC-1, alkali metals and anions are not retained, and a selective elution of alkaline earth metals can be achieved using ammonium acetate. Concentrated metals and lanthanides are eluted, with the exception of chromium, with acid into a cation-exchange column, acting as interface before the analytical column, from which they
are successively driven to the ion chromatographic separation with a PDCA or an oxalate complexing eluent and detection is obtained after postcolumn derivatization with PAR. Detection limits range from 0.2 to 1 ug/L (Fe, Cu, Ni, Zn, Co, Mn, Cd, Pb) [136,137]. The procedure was successfully used for metal-ion determination in sea water from the Venice lagoon, and with 60-mL sample preconcentration the detection limits for Cu, Ni, Zn, Co, and Mn were lowered to 0.05-0.1 ug/L [138]. A modification of the above-mentioned procedure allowed metal recoveries from chelating resin with the same eluent used for the ion chromatographic separation (75 mM H2SO4 / 100 mM HCl/100 mM KCl) and with a cation-exchange column with higher capacity. In this way detection limits of 10 and 30 ng/L were achieved for Cd and Pb, respectively, in sea-water samples (200 mL) [139]. Voloschik et al. [140] used a silica gel-based sorbent with chemically bound amidoxime functional groups for the selective determination of metals (Mn, Pb, Cd, Co, Ni, Zn, Cu, and Hg) in waters, since this kind of resin showed a weak affinity for Mg and
Ca and
this eliminated their interference in the determination. The examples described above are in some cases mentioned as chelating chromatography but actually refer more than to an actual chelating ion chromatography, to application of chelating materials to preliminary separation and/or preconcentration of metals. The development of CIC, based on ligands chemically bound to silica or polymer phases, refers to a technique applied for a long time in metal-ion preconcentration and matrix removal before spectroscopic determination based on permanent loading of sorbents by chelating agents [141-144]. Jones and co-workers widely investigated this field in order to improve detectability of both alkaline earth and heavy metals. They showed that the chelating ability of Xylenol Or ange and Chrome Azurol S coated onto polystyrene-divinylbenzene neutral hydrophobic substrates, changes markedly with different substrates [145]. Studies devoted to transition-metal determination [146-148] allowed the optimization of an interesting procedure based on 10-juim-particle-size PS-DVB resin, 100 A pore size, impreg
nated with Xylenol Orange, stable from pH 0.5 to 11.5 [149]. The stationary phase enables the removal of Ca and Mg during the sample on-column preconcentration at pH 6, and the separation and determination of Zn, Pb, Ni, and Cu with a step-gradient pH elution.

The only drawback of the method is the coelution of Cd-Mn, but satisfactory results are obtained for sea-water samples. This is a good example of how small variations in chelating ability between dyes can be very useful for specific separations. Using HPCIC the retention order of metal ions, including earth metals, is reversed with respect to IIC, and so barium is eluted first as a sharp peak, followed by Sr, Mg, and Ca. The barium separation and determination was successfully optimized in oil-well brines, at milligram-per liter concentrations in 1600-mg/L Ca samples [150]. A neutral hydrophobic resin (PS-DVB), first impregnated with methylthymol blue (S^-'-o/sfN^-'-dicarboxymethyDaminomethylthymol-sulfonephthalein) was coupled with an acid elution (0.5 M KNO_3 + 0.5 M lactic acid) and UV detection (PCR: PAR + ZnEDTA). Detection limits of
1 mg/L for Ba and 8 mg/L for Sr, respectively, were reached. Also, this Sr and Ba separation was optimized in milk powder analysis with a column impregnated with phthalein purple (o-cresolphthal ein-3"-o-s-methyleneiminodiacetic acid) [151]. A recent paper de tails an investigation into the parameters involved in the production of a range of dye-impregnated chelating columns (10 chelating dyes, mainly on triphenylmethane- or azo-based dyes) for preconcentration and separation of alkaline earth, transition metals, and heavy metals at trace levels within an application devoted to Al determination in sea water [152]. More recently, a porous graphitic carbon reversed-phase column has been used with a mobile phase containing a selective metallochromic ligand for the separation of alkaline earth metals (o-cresolphthalaein complexone) [153]. To extend the range of metals separated by dynamically coated columns, a polystyrene divinylbenzene reversed-phase column was precoated with methyl thymol blue (MTB), a metallochromic ligand which complexes metal ions through two iminodiacetic acid functional groups.
kind of column proved suitable for samples at ionic strengths up to 1.0 M NaCl and was highly selective for U0 2 2+.

Large-volume injection (2.0 mL) and an eluent step-gradient procedure enabled the determination of U0 2 2+ without interference, at microgram-per-liter concentration in a saline lake sample spiked with 1 mg/L Mn, Cd, Zn, Pb, Ni, and Cu. Finally, Paull et al. [155], using a mobile phase containing MTB, developed dynamic chelating chromatography, in which the metallochromic ligand is included within the mobile phase. The new system, compared to MTB-precoated chelating columns [154], allows the separation of transition and heavy metals and shows different selectivity and improved peak shapes.

IV. METAL SPECIATION

Metal speciation refers to the identification and quantitation of the different forms of a particular element, such as organometallic, chelated, or free forms, or different oxidation states in a particular sample. Since variations in the chemical form define the toxicity or essentiality of the analyte, this information is particularly crucial in
environmental and toxicological investigations. Robarts et al. [156],

about 10 years ago, reviewed more than 440 papers on metal speciation by liquid chromatography, but the growing interest in this field depends on the possibility of coupling the separative power of LC with more sensitive and selective detection techniques. Detection and quantification of the separated species at trace levels was generally performed by atomic spectroscopic techniques.

When these are coupled with a separation system, information on speciation instead of the total concentration or amount of element(s) of interest is provided. Atomic absorption spectrometry can be used as a sensitive detector after chromatographic separation [157].

Among atomic emission techniques [158,159], the majority of applications have been based on inductively coupled plasma (ICP) emission spectroscopy [160,161]. This source is more suitable for LC, since the chromatographic flow rate is compatible with the conventional (i.e., pneumatic chamber) ICP interface. When used as a detector for HPLC, ICP offers good sensitivity, a dynamic range of over
five orders of magnitude, and multielement detection capabilities.

However, conventional direct connection HPLC-ICP coupling can suffer from poor transport efficiency, particularly when pneumatic nebulizers are used. Such coupling also demonstrates low tolerance for many of the organic solvents commonly employed in HPLC eluents. Investigations have therefore been performed to characterize the effect of mobile-phase composition and flow rate on HPLC ICP methods such as new kinds of nebulizers, e.g., a microflow ultra sonic nebulizer or direct-injection nebulization (DIN), increasing transport efficiencies to the ICP interface. Finally, ICP has been used as a source for mass spectrometry (MS), and ICP-MS has become one of the most powerful techniques for speciation analysis when coupled with separation procedures [162-165]. As mentioned, inductively coupled plasma atomic emission spectrometry (ICP-AES) and inductively coupled plasma mass spectrometry are two different and sensitive methods used in trace-metal speciation. The lower limits of detection (subnanogram to subpicogram levels), the wide linear ranges and isotope analysis capability, and
the high precisions (0.1-5.0% RSD) associated with ICP-MS make it more advantageous than ICP-AES. In addition, the capability of ICP-MS for isotope ratio determinations allows isotope dilution analysis to be used. More recently, a perspective on the current technology and applications of LC-MS has been published [166,167]. Some examples on speciation methods and advances for As, Se, Pb, Hg, and Cr will be detailed hereafter.

A. Arsenic

The molecular forms of arsenic subject to speciation analysis are anions, e.g., arsenite As(III), arsenate As(V), monomethylarsonate (MMA), and dimethylarsinate (DMA); cations, e.g., arsenobetaine (AsB), arsenocholine (AsC), and tetramethylarsonium ion (TMAs); or uncharged compounds at neutral pH, e.g., arsenous acid. There is no general rule or trend that relates toxicity with oxidation state or chemical form and number of substituents linked to the central atom, but As(III) is more toxic than As(V). Recently, arsenic speciation in humans and food products has been reviewed [168].

A detailed study on AsB, AsC, and TMAs cations was done by Biais et al. [169]. An evaluation of direct, ion-pairing, reversed
phase, and cation-exchange chromatographic procedures involving
different stationary phases and eluents has been made in order to
optimize atomic absorption detection through a new on-line thermo
chemical hydride-generation interface (THG). Strong cation-ex
change chromatography was incompatible with the THG interface,
solving the problem of AsC-TMAs co-elution on C18 column. The
best choice was a normal-phase HPLC approach: cyanopropyl sta
tionary phase and a methanolic eluent containing a silanol masking
agent. The optimized procedure gave detection limits of 13.3, 14.5,
and 7.6 ng for AsB, AsC, and TMAs cations, respectively. A simple method [170] has been developed for As(III) and As(V)
speciation. An extractive chromatography has been performed by
eluting samples (pH 2.5) through a stationary phase, silanized diato
mite, modified with dioctyltin dichloride (C 8 H 17 ) 2 SnCl2 [171]. As(V)
was selectively retained and As(III) passed through the column,
As(V) recoveries (85-115%) and column regeneration were obtained
with 2 M HCl. The arsenic contents in the eluates were determined
either by flame AAS or graphite furnace AAS; detection
limits were not given, but the linearity of the method was 0.01-0.20 \( \text{ag/mL} \). Arsenic speciation was evaluated by separation on an anion- and a CASI ion-exchange column and off-line hydride-generation electrothermal atomic absorption spectroscopy, for As(III), As(V), AsB, MMA, DMA, p-aminophenylarsenate (p-APA), and AsC [172]. In this study, As(III) co-eluted with AsB. A thermal decomposition procedure was introduced to convert organoarsonicals into As(V) before reduction by sodium borohydride, and detection limits resulted comparable to HPLC-HG-AAS [173] and HPLC-ICP-MS [174] procedures. Ion chromatographic methods for elemental speciation (As, Se, and Cr) using microbore columns with direct-injection nebulization by ICPAES have been described by Gjerde et al. [175]. An anion HPLC procedure with a phosphate eluent was developed for arsenite As(III), arsenate As(V), monomethylarsonate, dimethylarsinate, arsenobetaine, and arsenocholine separation [176]. After optimization, five peaks only were well resolved, due to the overlap of As(III) and AsB. The analytes were determined by hydride-generation atomic absorption spectrometry (HG-AAS). The arsenic content of the unresolved peak was assigned to
As(III) + AsB or As(III), with or without a microwave-assisted oxidation step

[177,178] before HG-AAS determination on two consecutive injections. The method applied to urine samples gave, with a cleanup procedure, detection limits within 0.15 μg/L and 2.5-5.3% standard deviations. Similar studies were also done for DMA, AsB, and AsC in deionized water, urine, and cleanup dry residue from urine samples [179]. The stability of arsenic species, in relation to food treatment procedures (seafoods and mushrooms), has been investigated with an HPLC-UV-HG-AFS (AFS, atomic fluorescence spectrometry) method [180]. A separation with a strong anion-exchange resin (BAX-10) and a gradient eluent concentration (K2SO4) at 60°C has also been proposed for As(III), As(V), MMA, DMA, and AsB [181], but in this case a complete separation was not achieved due to the co-elution of inorganic species. Rauret et al. [182] developed an IC-HPLC procedure for As(III), As(V), MMA, and DMA separation and ICP-AES determination by coupling the systems with the hydride-generation sample introduction technique. The procedure was improved [183] by checking two
different columns (Nucleosil-5SB and Hamilton PRP X-100) and by comparing isocratic and gradient elution. The peak profile was improved by filtering the data corresponding to low concentrations with a Fourier transform. With such a procedure, detection limits between 2.7 As(III) and 11.4 As(V) µg/L were obtained. Several reports have appeared on LC coupled with ICP-MS for metal speciation determination As [174,184-187]. A direct-injection nebulizer interface work [188,189] demonstrated that reversed phase microbore columns and eluents containing ion-pairing agents could be coupled with mass spectrometric (MS) detection for arsenic speciation. Good efficiency was also obtained using interfaces based on hydride-generation manifolds. Hydride generation was used to increase the efficiency of conventional pneumatic nebulizers in LC ICP-MS studies of arsenic speciation [190,191] and to avoid interference from the polyatomic species which has the same m/z value as 75 As isotope. Even by using a membrane gas separator [192], the determination was subject to the interference of the molecular ion $\text{ArCl}^+$. The removal of 40 Ar 35 Cl + interference was
by coupling ion chromatography and ICP-MS detectors, and lowered detection limits resulted for As(III), As(V), DMA, and MMA with the use of an He-Ar gas mixture as ionization source (0.032 and 0.080 ng for DMA and MMA, respectively). A separation of As(III), As(V), MMA, and DMA was performed with reversed-phase ion-pair liquid chromatography (IIR: TBA phosphate) [194]. The eluate was delivered to the hydride-genera tion system after a prereduction with L-cysteine at 95°C in diluted nitric acid. In this way ArCl + was removed and conventional detection limits were reduced to 11-51 ng/L values. Seven molecular forms of arsenic [As(III), As(V), MMA, DMA, AsB, AsC, and tetramethyarsonium, TMAs] were separated, by anion- and cation-exchange HPLC, and detected by on-line flame atomic absorption spectrometry (AAS). The potential interference due to phosphorus on the 193.7-nm arsenic line is avoided because it is separated by an anion-exchange HPLC procedure [187]. Silica based and polymeric cation-exchange columns were examined, and complete separation of cationic species and DMA was achieved by isocratic elution (pyridine eluent) on a bare silica column (— Si — OH.
groups act as cation-exchange sites. The retention of DMA at low pH was attributed to the presence of DMA + species. In a further development of the method, Larsen et al. [195] studied the determination of eight arsenic compounds in urine with anion- and cation exchange separations by coupled HPLC-ICP-MS. The sample is silica C18 cleaned and introduced into the system; four anionic [As(III), As(V), MMA, and DMA] and four cationic [AsB, AsC, TMAs, and trimethylarsine oxide (TMAO)] arsenic compounds were detected at m/z 75, since chloride is separated chromatographically and does not interfere. In the cation-exchange chromatographic system the chloride eluted with the void volume, and in the anion-exchange procedure is eluted more than 100 s later than the last analyte peak, well separated from the arsenic species. TMAO, not included in the previous work, eluted in the cation-exchange system due to protonation of the As = 0 bond in the acidic mobile phase. A pH value of 10.3 in anion separation enabled the retention of arsenous acid, which is eluted with the void volume in other anion-exchange chro
matographic systems [196]. The procedure [195] has been applied
to investigate the speciation of arsenic in seafood samples and to
elucidate the biosynthetic pathway involved in marine metabolism
[197]. In this field, Harrington et al. [198] described
extractions and cleanup procedures to isolate water-soluble arsenic species in marine brown algae for their determination by both GF AAS and HPLC ICP-MS. More recently [199], a speciation of these compounds was obtained by micellar liquid chromatography coupled with ICP-MS detection. The method, based on a micellar mobile phase (CTAB, propanol, and borate buffer), and on a PRP-1 separation column coupled with the ICP-MS system, allowed linear dynamic ranges of three orders of magnitude and detection limits in the picogram range (90-300) and overcame the problems of chloride since it is not coeluted with any of the four arsenic species. A detailed study on the suitability of ion spray (IS) technique for arsenic speciation analysis in biological samples must be mentioned. A cation-exchange HPLC has been coupled to IS-MS(-MS) detection for analysis of organoarsenic species. Elemental
and molecular, dual-mode analysis is presented using standard mixtures.

Although detection limits are not low as those obtained by HPLC ICP-MS, the results indicate IS-MS-MS as a complementary technique to ICP-MS for speciation analysis [200]. Papers published since 1980 on As and Se speciation in environmental matrices have recently been reviewed by Guerin et al. [201].

B. Selenium

The thermochemical hydride-generation (THG) interface mentioned for arsenic speciation has also been used for HPLC-AAS determination of selenoniocholine (SeC) and trimethylselenonium cations (TMSe) [202]. The cation-exchange chromatography that resulted was unsuitable for the THG interface, and cations were separated on a cyanopropyl stationary phase with a methanolic phase containing a silanol-masking agent. The calculated detection limits were 43.9 and 31.3 ng for SeC and TMSe, respectively. An improvement of chromatographic behavior and reduced detection limits were observed by adding trimethylsulfonium iodide to the mobile phase. Houck et
al. [203] obtained, for inorganic selenium species, Se(IV) and Se(VI),
detection limits of 10-20 ng/mL with ion-pairing reversed-phase
(silica C18) microscale-liquid chromatography (eluent: methanol
water-TBA, flow rate 50 |iL/min) and a direct-injection nebulizer
(DIN) coupled with ICP-MS. More recently, Yiang et al.
[204] coupled a similar ion-pair chromatographic separation with an ICP-MS
detector using an ultrasonic nebulizer. By this approach the detection limits for TMSe, Se(IV), and Se(VI) were 0.17, 0.76, and 0.53 ng/mL, respectively. Se(IV) and Se(VI) species were also separated by Shum et al. [205] on an anion-exchange microcolumn (eluent:
sodium carbonate-bicarbonate, flow rate 100 |iL/min) with a DIN ICP-MS detection system. Isotope ratio measurements on chromatographically separated species of Se gave detection limits of 7-8 ng/mL for both of the species (based on 78 Se and peak-area measurements). A Nucleosil 100-SB anion-exchanger column was used with an eluent ammonium citrate for HPLC fraction collection and electrothermal atomic absorption spectrometry of Se(IV),
Se(VI), and TMSe speciation [206]; the detection limits ranged from 11 to 32 ng/mL in water and urine matrices. Inorganic selenium species in aqueous samples have also been separated using an anion-exchange column with a two-step eluent-switching procedure [207]. The best separation was obtained with 25 mM K_2 SO_4 eluent, switching to 200 mM after 200 s at a flow rate of 2.0 mL/min. After the separation by on-line acidification, microwave reduction, and hydride generation, the species were detected with an atomic fluorescence detector. The method provided detection limits of 0.2 and 0.3 ng/mL within 1.5% and 2.0% RSD for selenite and selenate, respectively. Speciation of eight selenium compounds has also been obtained with a strong cation-exchange column by interfacing the chromatographic system with an ICP-MS by a high-pressure hydraulic nebulizer [208]. Speciation of some organic selenium compounds has been considered in a recent review [209]. The speciation of organic selenium compounds (SeCy, SeMet, and TMSe ion) by HPLC-ICP-MS in natural samples (enriched yeast, human serum, and urine) has also been
performed on a reversed-phase analytical column (Hamilton RP1)

[210]. By optimizing the eluent polarity and ion-pairing agent (pentane sulfonate) with regard to TMSe cationic species, a satisfactory separation is achieved and inorganic species are eluted in the void volume. Detection was performed by ICP-MS using $^{82}$Se for quantification, and enabled to detect 0.20, 0.60, and 0.20 ng/mL for SeCy, SeMet, and TMSe, respectively, within a 0- to 500-ng/mL analytical dynamic range. Quijano et al. [211] reported the use of a mixed column for the speciation of selenocystine (SeCy), selenomethionine (SeMet), selenite, and selenate with ICP-MS. The method was improved by including two additional selenium species (TMSe and selenoethionine) using a Spherosorb 50DS/A microcolumn and phosphate buffer eluents at pH 2.8 and 6.0, respectively. The method enabled detection limits in urine matrix of 0.5 ng/mL and precision better than 5% for the six selenium compounds [212]. A detailed study on inorganic selenium and selenoaminoacids speciation has been made by on-line coupling a high-performance liquid chromatographic-microwave-digestion system (HPLC-MW)
with an AA, an ICP, or an ICP-MS spectrometer [213]. The system proposed allows complete separation of selenoaminoacids in urine samples, but the inorganic selenium peaks are overlapped; their speciation is obtained, however, by carrying out a second injection and detection with the microwave turned off. The ICP-MS detector provided the lowest detection limits, 0.16, 0.59, 0.66, and 0.19 ng/mL for total inorganic Se, selenomethionine, selenoethionine, and Se(IV), respectively. From previous experience [213, 214], a detailed comparison of quadrupole ICP-MS and double-focusing sector field ICP-MS for the speciation of selenium in urine was made, also comparing different HPLC-ICP-MS interfaces (DIN, MW-HG) [215].

C. Lead

Organolead compounds are present in the environment by biomethylation of inorganic lead and as a result of the use for a long time of tetraalkylead compounds as antiknock additives in gasoline. Röbeck et al. [216] evaluated the HPLC behavior of tetramethyllead (TTML) and tetraethyllead (TTEL) on a LiChrospher 60 column with different eluent mixtures. Both acetonitrile-LiClO4 and metha
nol-LiClO₄ eluents showed that a 10% aqueous solution 0.1 M LiClO₄ is sufficient to achieve separation of TTML and TTEL, but a ternary solvent (methanol-chloroform-LiClO₄) optimizes separation and reduces analyzing time (4 min). The method, applied to natural waters, gave, with normal pulse amperometric detection at a glassy carbon electrode, detection limits of 15.5 and 17 ng for TTML and TTEL, respectively. Shum et al. [188] achieved the separation of two alkyllead (TTML, TTEL) and three organomercury species (methyl-, ethyl-, and phenylmercury) using ion-pair chromatography and ICP-MS detection. The separation was made by a PEEK microcolumn (5 cm X 1.6 mm I.D.) packed with C18 material and with an acetonitrile-water eluent containing ammonium pentanesulfate at 100 μL/min flow rate. Detection limit was 0.2 pg and the method was validated with a freeze-dried urine reference material. The separation of TTML, TTEL, and triphenyl-lead (TPhL) by ion-pair HPLC and ICP-MS detection was also obtained with a methanol-water eluent containing 4 mM sodium pentanesulfate [217]. With this
the main problem encountered was the separation of TTEL from inorganic lead. In an attempt to optimize this separation, a gradient elution was applied [210]. During the optimization of chromatographic parameters, only inorganic and methyllead resulted, strongly affected by a change of the ion-pairing agent concentration, and 8.0 mM gave the best choice for resolution and for lowering the influence of salt concentration on the nebulizer tip and sampling orifice of the mass spectrometer. Inorganic, triethyl-, triphenyl-, and tetraethyl-lead were well resolved with a gradient methanol concentration, from 40% to 90%, over 10 min. The high concentration of organic solvent gave a loss of sensitivity only for TTEL, which was the last to elute, and the relative detection limits were 2.8, 3.5, 77.5, and 7.4 ng/ml for TEL, TPhL, TTEL, and inorganic lead, respectively. The method has been successfully applied to the quality control of the water supply of a U.S. Environmental Protection Agency (EPA) laboratory (trace metal contents) (Cincinnati, OH, USA). More recently, Brown et al. [219], on the basis of work of Al-Rashdan et al. [217,218], optimized
the ion-pair HPLC separation of trimethyllead from Pb 2+, by a gradient program from 10:90 to 30:70 ratio of methanol to buffer (HAc/NaAc) between 4 and 7 min. Coupling the chromatographic system and ICP-MS by a single-pass spray chamber with a concentric glass nebulizer, a 0.40-ng Pb/g detection limit was obtained for trimethyl lead ions. On-column derivatization procedures have also been developed for simultaneous organic ionic lead and mercury species separation.

Cammann et al. [220] developed an on-line enrichment on a RP-18 pre-column by adding methyl thioglicolate to the sample. Trimethyl-, triethyl- dimethyl-, and diethyl-lead, methyl- and ethyl-mercury were separated on an Hypersil ODS column with a mixture of methanol and citric acid buffer. Detection limits in the range 270-800 ng/L were obtained by spectrophotometric detection (UV, 235 nm).

D. Mercury

A method has been developed based on the formation and separation of methyl, ethyl, phenyl, and inorganic mercury complexes with ammonium tetramethylenedithicarbamate [221]. An ODS RP-18 col
umn was used and the elution was performed with an acetonitrile-water-APDC-buffered eluent. Detection was achieved by interfacing a glass flow cell between the chromatographic system and a cold-vapor atomic absorption spectrometer (CVAAS). The on-column procedure, in comparison with precomplexation, showed reduced detection sensitivity only for inorganic mercury, which is very high with respect to the other species. The on-line procedure was chosen and, after eluent optimization, detection limits between 0.5 and 0.015 ng/mL were achieved by coupling a sample (100 ml) preconcentration onto a C18 microcolumn. Diethyldithiocarbamate (DDC), hexamethylenammonium (HMA)-hexamethylenedithiocarbamate (HMDC), and pyrrolidinedithiocarbamate (PDC) were tested for enrichment and separation of methyl, ethyl, methoxyethyl, ethoxyethyl, phenyl, and inorganic Hg complexes [222]. A RP C18 column was used and the best results were obtained with PDC complexes coupled with an acetonitrile-water-buffered eluent. Analytes were determined by ultraviolet, postcolumn oxidation, cold-vapor atomic absorption spectrometry (UV-PCO-CVAAS) [223]. The UV-PCO
was introduced to destroy the complexes, after elution, to increase
the yield of the following reduction step for CVAAS determination.

Methyl- and ethoxyethylmercury co-eluted but, since HC1 pretreatment of samples decomposes the latter, it was possible to evaluate
both species by analyzing treated and untreated samples. Detection
limits of 5.0 jig/L, obtained with the HPLC-UV-PCO-AAS system,
were lowered to 0.5 ng/L with a preconcentration (300-mL samples)
of mercury chelates on a microcolumn (Hypersil-ODS RP C18).
ICP-MS detection associated with ultrasonic nebulization was
used for methyl-, ethyl-, and inorganic mercury after separation on
a C18 reversed-phase column with a methanol-acetonitrile-2-mercaptoethanol eluent containing ammonium acetate [224]. Detection
limit values (0.4-0.8 ppb) that were 10 times lower than those with
LC-ICP-MS and a conventional nebulizer, and comparable to those
for LC-ICP-MS with cold vapor generation, were obtained. Munaf et al. [225] proposed a preconcentration and liquid chromatographic separation of methyl, ethyl, and inorganic mercury
based on microcolumns (Develosil-ODS and STR-ODS-H) with a
cysteine-acetic acid eluent. The cysteine concentration was the limiting factor because low concentrations are insufficient to elute mercury species and too large concentrations hindered the mercury-cysteine complex decomposition before the cold-vapor detection. The sensitivity of the method was enhanced by coupling a preoxidation step (with potassium peroxodisulfate and Cu catalyst) to the reaction for mercury-vapor generation; in this way a 0.1-ng Hg detection limit was obtained. An ion chromatographic separation of methyl, ethyl, and inorganic mercury as cysteine complexes was developed [226]. The eluent composition (acetic acid, sodium perchlorate, and cysteine) was optimized with respect to the separation procedure and to the reductive reaction (NaBH₄) which permits the detection of mercury with CVAAS. On-line preconcentration procedures were also investigated using both C18 and an ion-exchange microcolumn. The detection limits, for 100-mL samples, were 2, 10, and 4 ng for Hg, CH₃Hg, and C₂H₅Hg, respectively. Tetra-n-alkylammonium bromide ion-pairing agents and so
dium halides in methanol-water mixture were investigated as mobile phases for the separation of inorganic mercury and organomercury species (methyl-, ethyl-, benzyl-, phenyl-) [227]. The effect of tetrathylammonium (TMA), tetraethylammonium (TEA), and tetrabutylammonium (TBA) ions on the capacity factors of the species investigated was examined. The retention of Hg\(^{2+}\) was greatly dependent on the concentration of ion-pair reagent and its molecular size, and the capacity factor for Hg\(^{2+}\) increased with an increase of both parameters. In contrast to this behavior, organic mercury species showed lower capacity-factor changes. The TBA ion-pairing agent was efficient for the separation of all the species and the addition of sodium chloride to the mobile phase gave better peak shapes and lower retention times. When UV and DCP detection was compared, the sensitivities resulted in opposite behavior and the detection limits ranged from 0.2 to 8.0 ng with UV and from 255 to 175 ng with DCP for benzylmercury and methylmercury species. Relative high detection limits for DCP may be attributed to the high carbon content (organic solvent and ion-pair reagent).
enhancing the background and low atomization efficiency due to larger ion-pair species difficult to penetrate into the DCP.

E. Tin

At the beginning of 1990, cyano phases (cyanopropyl-bonded silica gels) were used in normal-phase mode (eluent: hexane-acetonitrile tetrahydrofuran) and tetraalkyl-tin compounds were separated according to their polarity under the following elution order: tetrabutyl-, tetraethyl-, tributyl chloride, tetraphenyl-, triethyl chloride, diphenyl dichloride, and diethyl dichloride [228]. The peak shapes and resolution were improved by an iodine chloride on-column pretreatment. UV detection (220 nm, time constant 50 ms) showed a complete separation in about 90 s with 6 mL/min mobile-phase flow rate.

No details were given on detection limits and analytical dynamic range of detectable concentrations. Astruc et al. [229,230] did a theoretical and experimental study on on-line discontinuous detection in liquid chromatography by graphite furnace atomic absorption spectrometry (GFAAS) and its application to butyltin compounds at trace
Separations of butyltin moieties have been obtained using a Nucleosil column with a 0.001% tropolone solution in toluene. The chromatographic procedure has been applied to tetrabutyl,tributyl-, dibutyl-, and monobutyltin speciation in water. The first two analytes are co-eluted and the last strongly retained onto the column.

By coupling on-line detection by GF AAS, the detection limit for dibutyltin was 10 ng/L. A highly fluorogenic reaction between triphenyltin (TPhT) and 3-hydroxyflavone in a micellar medium (Triton X-100) has been coupled with an ion-exchange chromatographic method (column, cation exchanger Partisil10 SCX; eluent, methanol-water, 0.15 M ammonium acetate) enabled reaching a detection limit of 0.02 ng for 200-µL sample injection [231]. The method was applied to the determination of TPhT in bottom sea water after enrichment on a C18 cartridge and gave satisfactory results at the nanogram-per-liter level [232]. The cation-exchange chromatography of tributyltin and triphenyltin, usually performed with ammonium acetate eluents, has been optimized by the addition of benzyltrimethylammonium chloride.
(BTMA), which also allows the indirect detection of trialkyltins [233]. The method has been applied to trimethyltin and triethyltin speciation in addition to the above-mentioned compounds. The detection limits obtained (0.15-2.5 mg/L) were not so low, due to the type of detector used, but the chromatographic approach is attractive. Various types of couplings have been developed that utilize both inductively coupled plasma atomic emission spectrometry (ICP AES) and direct-current plasma atomic emission spectrometry (DCP-AES) [234] or ICP-MS [235] for detection. A reversed-phase liquid chromatography (column, C8, 3 Jm, 30 X 3 mm I.D.; eluent, methanol-water 5 mM/sodium 1-pentanesulfonate) has been optimized for trimethyl-, triethyl-, tripropyl-, tributyl-, and triphenyltin separation and determination by ICP-MS equipped with an ultrasonic nebulizer [80]. Detection limits ranged between 2.8 and 16 pg Sn for various tin species, and the entire procedure required less than 6 min. An HPLC method (column, Kromasil-100, 5-|im, C18, 150 X 21 mm I.D.; eluent, 0.05% triethylamine in acetonitrile-acetic acid-water, 65:10:25) has been developed to achieve both
molecular information and good sensitivity and selectivity for organo-tin species [236]. The optimized procedure enabled detection with both API and ICP mass spectrometry of dibutyl-, tributyl-, diphenyl-, and triphenyltin in sediments. Rivas et al. investigated the effect of different spray chambers in HPLC-ICP-MS [237] on the detection limits for organotin compounds. The instrumental interface, i.e., nebulizer and spray chambers, appeared to be the critical point. HPLC has been deeply discussed for the speciation of organotin compounds and the performance of the method of detection by Harrington et al. [238].

F. Chromium

Chromium is a ubiquitous element, not only for its occurrence in nature but also for the numerous anthropogenic influences resulting from its widespread industrial applications. In environmental studies, its analytical determination is connected with the differences in biological and toxicological behavior of its two main oxidation states, Cr(III) and Cr(VI). Cr(III) is essential for the maintenance of the glucose tolerance factor in the human body. Cr(VI), due to its high
oxidation potential and to its relatively small size, which enables its penetration through cell membranes, is toxic and carcinogenic. On the other hand, the occurrence of Cr(III) in the biotic environment as the aquo-hydroxocomplexes \([\text{Cr(H}_2\text{O)}_n\text{OH}_n\text{F}^{-n}\text{H}^+\text{]}\), due to its size, makes it almost entirely excluded from penetrating cell membranes. Discussions and reference lists for chromatographic techniques with off-line separation and preconcentration, or on-line methods for chromium, can be found [239-241]. The possibility of using a complexing agent in the mobile phase for the determination of chromium speciation has been shown [242-245], including reversed-phase chromatography after the formation of neutral chelates [246,247], ion chromatography [248], and ion-pair chromatography [249-254]. Studies on chromium speciation with ion chromatographic separation of its EDTA complexes involves, in addition to common parameters, a detailed evaluation of the temperature effect. This is due to the slow formation rate of the complex between Cr(III) and EDTA. An anion chromatographic separation (polymer-based anion exchanger; eluent, EDTA-oxalic acid) has been optimized by working
at 40°C [255]. Detection was performed by direct introduction of eluate into an ICP-MS system. This method enabled 80-88 ng/L detection limits for Cr(III) and Cr(VI), respectively, within a linear range from 0.5 to 5000 \( \mu \text{g} \) Cr per liter and simultaneous determination of chromium speciation and Mn, Fe, Ni, Cu, Mg, and Ca in water samples. Arar et al. [256] developed a method using a reversed-phase guard column NG1 coupled with an IonPac AS7 anion-exchange column. The eluent used was 250 mM ammonium sulfate and 100 mM ammonium hydroxide, as developed by Dionex. In these conditions, good sensitivity has been obtained by spectrophotometric detection \([0.3 \mu\text{g/L for Cr(VI) after postcolumn reaction with diphenylcarbazide}]\). The method has proven suitable to be applied in wastewater analysis. Postcolumn catalytic oxidation of luminol allowed the chemiluminescence detection of Cr(III) and Cr(VI) at detection limits of 0.1 and 0.3 \( \mu\text{g/L}, \) respectively [257], after separation by anion exchange on an IonPac AS4A column and an acidic eluent of 0.28 M KC1. The technique developed was not applied to a real environment.
tal matrix, but to a Certified Reference Material, IAEA/W4
Simulated Fresh Water. More recently, Derbyshire et al. [258] obtained
excellent resolution of the two chromium species using a single
mixed-bed ion-exchange column (IonPac CS5) with continuous elution. Detection limits of 0.002 ng/mL for both Cr(III) and Cr(VI) were obtained after the optimization of postcolumn reactions [reduction of Cr(III) and oxidation of luminol]. The method gave results in very good agreement with certified values for water reference materials. Examples of different approaches for chromium speciation with anion exchange are two ion chromatographic procedures developed by Pobozy et al. [259]. In the first method (column, anion exchanger; eluent, potassium hydrogenphthalate), Cr(VI) was retained and Cr(III) was eluted in the void peak and postcolumn oxidized to Cr(VI). In this manner both Cr(III) and Cr(VI) were spectrophotometrically detected after postcolumn reaction with diphenylcarbazide (DPC). The second procedure was based on Cr(VI)-Cr(III) anion species separation after Cr(III) precomplexation with 1,2-diamineclohexane-N,N,N',N'-tetraacetic acid (DCTA). Detection
limits eval

uated for the first and second methods were 2.5 and 4.5 ng/mL for Cr(III) and 1.8 and 1.5 ng/L for Cr(VI), respectively. Among ion-pair applications, Posta et al. [252] optimized the Cr(VI)-Cr(III) separa
tion on a RP C18 column (length, 5 cm) by using a TBA-acetate, ammonium acetate, phosphoric acid, and methanol-based eluent at 2.5 mL/min flow rate. The eluent composition enabled the chromium species separation, enhancing the sensitivity of detection obtained by coupling HPLC to a flame AAS by a high-pressure capillary with a hydraulic high-pressure nebulization. Detection limits of 0.03 and 0.02 mg/L for Cr(III) and Cr(VI), respectively, were reduced to 0.5 (Lt/L for Cr(VI) after a preconcentration step. Detection limits of 0.3-0.5 [ig/L can be achieved using HPLC in combination with ICP-MS [260]. An anion-exchange column (Waters IC-Pak A, 50 X 4.6 mm, 10 |um) containing trimethylammonium functionalized groups on polymethacrylate and a cation-exchange column (Waters Guard-Pak, 5 X 3.9 mm, 5 |Lim) containing sulfonic groups on polybutadiene maleic anhydride silica were coupled for the simultaneous chromatographic separation of chromium
species

[260]. A step gradient with increasing nitric acid concentration and decreasing pH was used as the eluent. The analytical method has been optimized through the use of different Cr isotopes for data acquisition, comparing the interference of some species (e.g., chloride, chlorate, perchlorate, sulfate, sulfite, sulfide, tiosulfate, carbonate, cyanide, and organic species) at different ml/z values. An ml/z of 52 was chosen as the ideal isotope for Cr speciation in wastewaters. Since in all cases the main interest is in lowering detection limits and reducing analysis time, for chromium speciation great attention has been paid to interfacing ICP-MS detectors with HPLC separation modes. Despite the good detection limits, ICP-MS is prone to some interference when real samples have to be analyzed, the most serious problem being the formation of polyatomic ions, especially below atomic mass number 80. For example, with organic liquids, owing to the presence of carbon, the abundant molecule 40 Ar 12 C + ob scures 52 Cr + , the main isotope of chromium. Ion-pair chromatography with tetrabutylammonium acetate and 25% methanol on a Eurospher 100 C i8 5-jim column was applied
for the separation of Cr(III) and Cr(VI) [253]. Speciation analysis was studied using hydraulic high-pressure nebulization in combination with ICP-MS. Addition of oxygen to the aerosol gas and effective desolvation were necessary prerequisites in order to apply ICP-MS as a selective and sensitive detection technique, thus reducing polyatomic interference from carbon, due to the presence of methanol. Exploiting the residual cationic-exchange capacity of a conventional anion-exchange column (IonPac AS5, 50 X 4 mm, 15 μm, Cr(VI) anions and Cr(III) cations were retained and eluted with discontinuous elution in two steps by 0.3 M nitric acid for Cr(VI) and 1.0 M nitric acid for Cr(III) [261]. Detection was achieved by coupling ICP-MS with a sample introduction technique using hydraulic high pressure nebulization, and the relative detection limits were 0.1 fg/L for (Mill) and 0.2 μg/L for Cr(VI). Total chromium, Cr(III) and Cr(VI), speciation was achieved by Powell et al. [262] by coupling HPLC anion chromatography (eluent, nitric acid) with direct-injection nebulization and ICP-MS. The detection limits obtained were 60 and 180 ng/L for Cr(III) and Cr(VI), respectively. A procedure developed for Cr(VI)
determination [263] has been modified by Caruso et al. [264]. They used mixed-mode columns, namely, IonPac AS7, for Cr(III) and Cr(VI) separation with (NH₄)₂SO₄ eluent (pH 9.2). The Cr(III) species was stabilized with EDTA before sample analysis and detection was performed both by ICP-AES and ICP-MS equipped with a high-performance interface and a concentric nebulizer. To avoid polyatomic interference at m/z 52 from 36 S 16 O + from the eluent, an m/z 53 value was chosen for detection. Relative detection limits were 0.40 ppb for Cr(III) and 1.0 ppb for Cr(VI) within a 4% RSD and a linear dynamic range from 3 to 600 ppb and from 5 to 1000 ppb for Cr(III) and Cr(VI), respectively, in aqueous media. An automated, on-line, two-column ion-exchange system has been proposed more recently (265). Cation and anion species of chromium are sequentially retained by eluting samples through a chelating column (Chelex 100) and an anion-exchange (AG MP-1) column. Recoveries were obtained by eluting Cr(III) with 2.0 M nitric acid and Cr(VI) with a NH₄ OH/NH₄NO₃ mixture. Synthetic samples (75 350 ng/mL Cr) gave recoveries of 91% and 100% for Cr(III).
and

Cr(VI), respectively, at a flame AAS detector, but anomalous results were obtained analyzing real samples, e.g., tap water. It must be pointed out that this and similar methods, more than chromatographic procedures, could be defined as flow-injection analysis (FIA).

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