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Review

Some specific problems in the practice of preparative high-performance liquid chromatography

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ABSTRACT

The practice of preparative high-performance liquid chromatography (PLC) is reviewed. Special attention is paid to problems with the use of this method in research and development which are insignificant or unfamiliar on an analytical scale. PLC column concepts, stability and related packing procedures are discussed. Guidelines to column size selection and optimum use are presented. The paramount importance of high resolution for successful PLC separation is stressed and the effect of friction heat generated by viscous flow on the column performance is described. The significance of sufficient sample solubility in the mobile phase is discussed. Possible deleterious effects of the use of strong solvents with viscosities different from that of the mobile phase are considered. The packing solubility is shown to influence product purity; various product isolation procedures are discussed and the use of solid-phase extraction is recommended.

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1. INTRODUCTION

Preparative high-performance liquid chromatography (PLC) suffers from problems that are insignificant or unfamiliar on an analytical scale and originate mainly from the different goals of analytical and preparative work. The basic difference between PLC and analytical high-performance liquid chromatography (ALC) consists in the purpose of separation. The usual ALC requirements are separation, identification and determination of components of the mixture. The purpose of PLC is the isolation of pure substances for a subsequent application that determines the amount needed and thus the scale of PLC separation. In the research and development use of PLC, tens of milligrams may be required (micropreparation) for the structure elucidation of unknown compounds by spectroscopic techniques or a few grams may be needed for subsequent synthetic work; it is required here to obtain the substance with as little overall effort as possible (including analytical work). The next PLC category, usually termed the process scale, is expected to produce hundreds of grams to hundreds (or more) of kilograms of substances for sale and the aim here is to produce the largest possible amount of product in the shortest period of time keeping the total production costs to the minimum. This mode usually requires careful optimization of economy and all stages of PLC process [1,2]; the preceding analytical work becomes only a minute part of the total costs. Research and development PLC often deals with difficult separations of unknown compounds and more than one or two products are required. Typical tasks on the process scale [3] are the isolation of one (removal of impurities) or two (often isomers) pure compounds. All the knowledge from the preceding research and development step is usually available here and, if additional parameters of solute isotherms are determined [4], considerable optimization can be achieved on the basis of recent theoretical developments in the field of mass overloading in non-linear chromatography [5,6]. Research and development PLC of an unknown compound represents a typical situation where the selection of proper conditions [7,8] is a trial-and-error procedure based on experience in the field and on the knowledge of corresponding analytical separations.

The amount of product obtainable on a column of a given size increases with increase in the allowed specific column loading, expressed per gram of packing, that gives sufficient resolution [9] to obtain the selected purity. The separation factor $\alpha = k_2'/k_1'$, where k_1' and k_2' are the capacity factors of the most closely eluted compounds ($k_2' \geqslant k_1'$), is the decisive parameter and the higher (lower) α is the higher (lower) is the possible specific loading.

The stringent requirement of high α values [2] and the necessity for the use of columns under conditions of mass overload [5,7] are unimportant in ALC. The same holds when difficulties associated with sample injection (sample solubility becomes paramount), performance and stability of large-diameter PLC columns, product isolation and its purity are considered. Some additional issues follow from differences between PLC and ALC system configurations and will be mentioned later; a pressure pulse produced by the injector six-port valve rotation, seldom noticed in ALC, is a good example. When a diaphragm pump is used in PLC [10], this pressure shock may be detrimental to the lifetime of the diaphragm. With a single-diaphragm pump, unexpected diaphragm rupture is really annoying.

This paper reviews the practice of PLC mainly from the point of view of difficult separations of unknown compounds and is focused on the topics outlined above in the elution chromatography configuration. It is hoped that this review will be useful especially to those who want to use PLC in the research and development area. Closely related to these topics are modern theories of volume and mass overload, especially of interacting bands as a function of parameters of the corresponding adsorption isotherms, reviewed in another part of this volume [82].

2. COLUMN CONCEPTS

The column is the heart of the process and consequently deserves maximum attention. The other PLC hardware will not be discussed in

detail here, but a few comments are appropriate. In research and development PLC, laboratory-made equipment is often built up from already available components with predefined parameters. Columns with diameters up to 20 mm may be run on most analytical equipment if the speed of analysis is sacrificed. Otherwise, a high-pressure, high-flow-rate pump is needed, pulse damping being less critical, and a loop injector or an auxiliary pump may be used for sample introduction. The detector must be insensitive; a less sensitive refractive index (RI) detector (in isocratic work) with a preparative cell (RI temperature dependence is not critical at low settings) and/or a UV detector with a short path length (0.5 mm) preparative cell may be used. A gradient-forming device extends the applicability with UV detection, speeds up the column reconditioning and sharpens the late-eluting peaks [11]. Nevertheless, satisfactory peak sharpening can often be achieved by a simple stepwise change of properly selected mobile phase compositions.

2.1. Dimensional extension of analytical columns

The first critical point, common to all concepts of large-diameter columns, is the inlet of mobile phase. The jet effect [12] resulting from the enormous change in linear velocity, as a result of a sudden change in inlet tubing/column diameter, increases with the square of the column diameter. In a 4.6 mm I.D. ALC column with an inlet bore of *ca.* 0.8 mm, a linear flow velocity ratio of 32 is obtained, increasing to 100 in an 8 mm I.D. column. McDonald and Bidlingmeyer [12] have shown that a parabolic profile of a sample zone is observed in this instance. A low-permeability inlet frit is sufficient as a flow/sample distributor in analytical columns but fails in large I.D. PLC columns [13] where the flow velocity ratio might be 625 and 2500 for 25 and 50 mm I.D. columns, respectively, if a 1 mm I.D. inlet capillary is used. Hence the design of proper end fittings that fulfil the requirement of a regular distribution of the sample over the entire cross-section at the column inlet and uniform collection of the sample at the column outlet becomes complex (Fig. 1). In contrast, turbulent flow in narrow tubing from the injection point to the column top is desirable [12]; the turbulent mixing cancels the parabolic velocity profile formed in laminar flow [14] and reduces extra-column band broadening. Coiling of long loops to a small diameter decreases band broadening in the laminar flow regime owing to secondary flows created by centrifugal forces [15]. If further improvement of the loop behaviour is necessary, the loop should not be filled completely [7] or should be switched off-line when one loop volume enters the column [4].

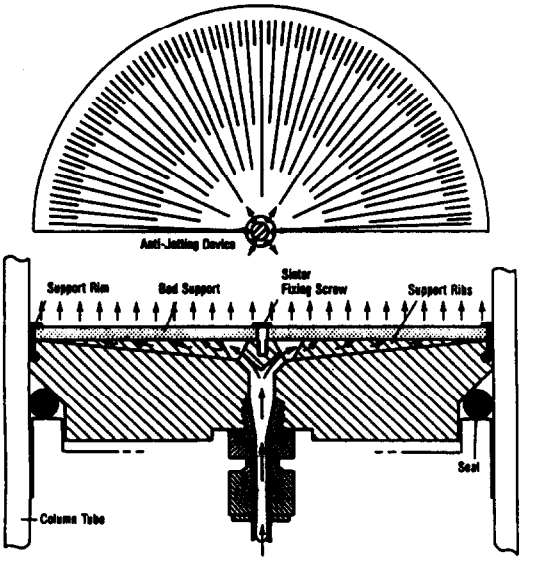

Fig. 1. Patented flow distributor with anti-jetting device (courtesy of Amicon).

The complexity of sample/mobile phase distributors is the main difference in geometrical extensions of analytical columns to larger diameters. The oldest nut-and-ferrule system of end fittings is applicable [16] up to about 22 mm I.D.; wider columns should be flanged to prevent the ferrules sliding off during the column packing. Conventional slurry packing of analytical columns is routinely done at pressures of 30–40 MPa and the use of these pressures for slurry packing of wider columns requires the use of thick-walled tubes and sturdy end fittings when the tensile strength of stainless steel and safety requirements are accounted for. The same diam-

eter limit holds for the second concept used with analytical columns, *i.e.*, a thicker column tube (as compared with the nut-and-ferrule system) with outer threads on the ends and end fittings with a corresponding inner thread. An example of a 250 × 50 mm I.D. column [13] with pressure rating 54 MPa, illustrating its complexity, is shown in Fig. 2. Although the use of high flow-rates may considerably increase the throughput [4] in PLC, the pressure drops comparable to high-pressure slurry packing pressures cannot be used during PLC experiments owing to friction heat dissipation problems [16]. Hence the selection of the slurry packing pressure is the main factor that determines the ruggedness of an extension of an ALC column concept to PLC size.

2.2. Compressed bed columns

Well known problems with the dimensional stability of packed beds in large-diameter columns have led to the widespread use of compressed systems.

A packed bed can be compressed axially and/or radially. A number of manufacturers offer various configurations; axially compressed systems differ further in the application of the force between runs only (constant or fixed compression) or continuously during separation (dynamic or continuous compression [17]). The schemes in Fig. 3 summarize different possibilities. A column in the form of a heavy-walled plastic cartridge compressed by a pressurized liquid or in a special compression holder is typical of a radial compression system (Fig. 3a). In continuous compression systems (Fig. 3b), the pressure may be applied at both column ends. Fixed compression systems differ in pressure-generation methods. An adjustable piston can be shifted down in the column by bolts moving flanges of the column and the piston part together (Fig. 3d) or by rotation of the screw-on end fitting (Fig. 3e). The tapered part of the piston in Fig. 3c extends through the whole column and can be moved down by a screw (annular expansion) exerting both axial and radial compression. The reader is referred to an excellent review of column hardware by Verzele *et al.* [16] for details.

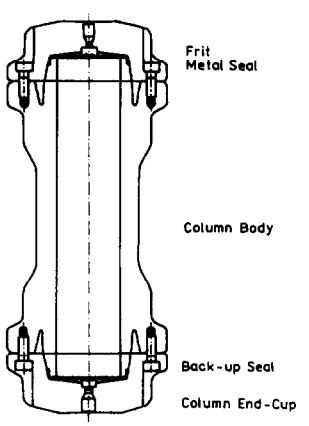

Fig. 2. Diagram of 250 × 50 mm I.D. column with pressure rating 27–55 MPa. From ref. 13.

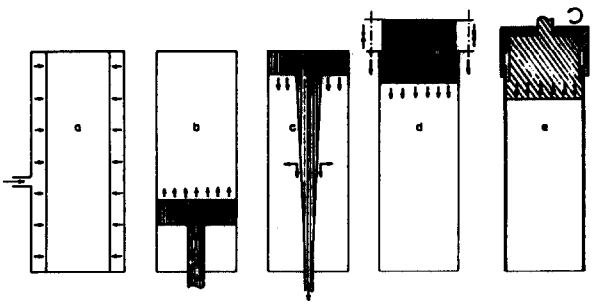

Fig. 3. Schemes of column compression techniques. (a) Radial compression, (b) dynamic axial compression, (c) annular and axial compression, (d) static compression flanged and (e) outer nut system. Compression forces indicated by arrows.

At first glance these systems are more intricate (and hence more expensive) than the fixed-bed columns described above. Nevertheless, some of them assume easy packing and unpacking by a customer, thus allowing the use of different heights of packed beds formed from different packings. One suitable apparatus of this kind substitutes a set of expensive prepacked PLC columns and the price comparison may then be entirely different. The dynamic compression apparatus of Prochrom has this versatility and seems to be the most popular at present.

2.3. Others

Owing to the limited pressure resistance of glass, its use is rare in PLC column technology.

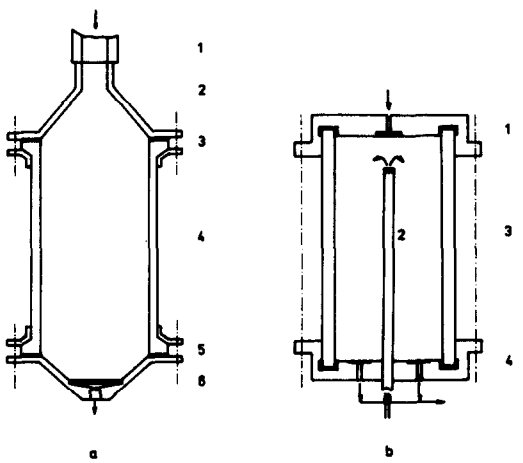

Fig. 4. (a) Glass MPLC column. 1 = Precolumn; 2 = upper stainless-steel flanged column head; 3 and 5 = seals; 4 = flanged glass column tube; 6 = bottom stainless-steel part. The conical column head with narrow precolumn is supposed to ensure sample distribution. Dotted and dashed lines indicate bolts. (b) Column with stopped-flow intra-column injection. 1 = Upper end fitting; 2 = sample injection tube; 3 = column tube; 4 = bottom end fitting. Mobile phase flow is indicated by single arrows and sample flow by double arrows. Inlet of mobile phase is closed during injection.

The scheme of a transparent glass column, used in a medium-pressure liquid chromatographic (MPLC) system, is shown in Fig. 4a. The pressure limit of this column is only 3–4 MPa but the use of 15-μm particles gives efficiencies and separations [18–20] similar to those obtained with the other techniques discussed here.

Stop-flow injection into the sorbent bed [21] was used [22] to avoid bed stability problems (Fig. 4b). The injection tube is introduced centrally through the bottom end fitting and ends at four fifths of the total column length. Owing to counter-current stop-flow injection with a closed mobile phase inlet, this system performs much better [23] than a simple point introduction of sample on to the column top.

Ion-exchange, hydrophobic interaction and affinity pH-gradient elution chromatographic separations of proteins frequently exhibit very high resolution [24,25] which allows short packed beds to be used in PLC. The unconventional radial flow column [26] looks like a usual column but its outer mobile phase introduction channel extends along its whole length. The inner wall of this channel is a porous tube. Another, but narrow, porous tube forms the outer wall of the sample/mobile phase collection channel in the column centre. Hence the effective bed height is the distance between the outer and inner porous tubes and elution takes place radially. Owing to the low pressure drop over the short column bed, this column can also be used with semi-rigid packings.

3. PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC COLUMN PACKINGS, STABILITY AND USE

The key requirement for optimum PLC column performance is homogeneity of the packing and its long-term volume stability. Darcy's law [27] conveniently describes the flow through a PLC column:

$$u = \frac{\Delta P d_p^2}{\phi \eta L} \quad (1)$$

where u is the linear velocity of the mobile phase, ΔP is the pressure drop over column length L, d_p is the particle diameter, η is the mobile phase viscosity and ϕ is a flow resistance parameter [28]. The importance of packing structure homogeneity then follows from the requirement for identical u over the entire column cross-section. Packing homogeneity is also reflected in the column plate height H through the A-term of the Van Deemter equation; different packing procedures may be compared if these terms are determined from corresponding H/u curves [29].

Having a homogeneously packed bed and well devised inlet (outlet) sample/mobile phase distributors (collectors), the column should exhibit correct PLC performance provided that no void volume is formed at the column top during the column use. Void volume formation is a crucial point because large-diameter column beds are generally unstable. Both short- and long-term instabilities are observed. The long-term instability may be explained by the slow dissolution and/or degradation of the packing, but the origin of the short-term void formation is still unclear [4]. It is believed [17,30] that the bed may contain loose, unstable regions where particle bridging forms microvoids; with small-bore

columns, the packed bed is supported by the column walls and its particles do not change the structure in the microvoids. In a large-diameter column, this wall support is lost and the bed settles. Often, this void appears at low pressures and is neither cylindrically nor horizontally oriented; cracks and gaps with loose packing structure are formed irregularly across the diameter or along the column wall. The effect of friction heat, introducing volume changes resulting from different thermal expansion of packed bed and of stainless steel mantle, was suggested as an explanation [16].

The author observed a distinct difference between 15-μm silica and C_{18}-bonded silica PLC columns packed by sedimentation in an unpublished study. The irregular void formation as above was observed only with C_{18}-bonded silica, indicating a possible effect of the bonded layer. The length of an extended C_{18} chain [31] may be estimated as 2.1 nm. Reversed-phase (RP) packing solvents are usually selected to prevent slurry aggregation, *i.e.*, thermodynamically strong; C_{18} chains should be extended and well solvated and the penetration of these surface layers is not probable. A possible surface layer structure was described [32] to form a "fur" where the surface layers of neighbouring particles might penetrate each other like a zip when thermodynamically weak solvent penetrates packed bed. Moreover, "stacked" structures [32] might be formed in a non-solvating solvent. Considering possible penetration and/or stacking (some flexibility of the C_{18} chain), an assumption of a change in effective particle diameter, *e.g.*, from 10.0042 to 10.0014 μm (to one third of the extended chain length) might be reasonable. This gives a volume change of 0.084% and for a 500-ml PLC column size the corresponding volume change could be about 0.4 ml. Any narrow gap of this size along the column wall could be very detrimental to the column performance. Yang and Gilpin [33] pointed out that bonded-chain reordering may take place but, unfortunately, no further evidence for this speculative explanation was found.

Anyhow, the selection of a proper packing procedure for large-diameter PLC columns not equipped with some void elimination facility still involves some "black magic".

3.1. Packing procedures

The current methods of packing rigid (silica-based) particles may be classified into four groups: dry packing, high-pressure slurry packing, compression slurry packing and packing by gravity sedimentation. The last method, which is less common but promising, deserves more attention.

3.1.1. Dry packing

This oldest procedure was extensively used in the past for particle diameters $d_p \geq 30$ μm; the "tap-and-fill" technique was [34,35] the most popular. It is now generally agreed [4] that dry packing does not work for particle sizes smaller than 30 μm. As an exception to this rule, a successful packing of MPLC columns with 15-μm silica with alternating vacuum and nitrogen overpressure (1 MPa) during the process has been reported [18–20].

3.1.2. Slurry packing

High-pressure slurry packing is more or less analogous to the well known [34,35] procedure used to pack ALC columns. This technique becomes increasingly complex with column diameters above 20 mm at pressures of 30–40 MPa; its use for a 250 × 50 mm I.D. PLC column has been described only once [13]. The explanation lies in the very high requirements on mechanical strength of the system and pump capacity.

Compression slurry packing uses pressures that do not exceed too much the pressure drop in PLC experiments and is applied most successfully in dynamic axial compression systems [17]. The concentrated slurry is compressed at 10 MPa until the piston stops. The column is then ready for use. It should be pointed out that a compressed bed, formed from both silica and C_{18}-bonded silica, continues to shrink during the use of the packed column, more quickly at the beginning and slowly between 3 and 100 h of use [17]. The total volume decrease was about 6% of the bed volume; this fits the experience that high-pressure slurry-packed ALC columns also soon settle if the packing pressure is low. The moving piston eliminates void formation and a

correct sample introduction is maintained. A steady and high efficiency observed [17] from the beginning of column use indicates a correct velocity profile within the column (eqn. 1) even with a less dense packing structure at the beginning of use.

3.1.3. Packing by sedimentation

The drag force applied on a particle during high-pressure slurry packing exceeds gravity by almost four orders of magnitude. This explains why slurry packing is more efficient than dry packing of small particles [4]; a higher drag force is more efficient in aggregate disruption. Hence the gravity sedimentation of small particles can be successful only if a suitable solvent prevents the formation of any aggregates. In particular, spherical particles may then form regular and dense structures. Giesche *et al.* [36] proved this to be true for 1–2-μm non-porous silica spheres. Their gravity settling in aqueous suspension of pH 10.9 at a concentration of about 1% gave the most dense structures even when compared with slurry packing at 100 MPa. The increase in particle charge at pH 10.9 increases particle repulsion, prevents irregular aggregate formation in the suspension and the resulting regular structure depends on a balance of interaction forces among individual particles in the sediment. Moreover, they observed that the same silica spheres covered with a C_{18} layer, packed into a 50 × 4.6 mm I.D. column by gravity settling from an ethanol–water suspension, gave nearly the same performance characteristics as when packed by the conventional slurry technique. A solvent preventing (supporting) aggregation is called deflocculating (flocculating) in colloid physics; Shelly and co-workers [37,38] used these concepts to modify the slurry packing of ALC microcolumns. They selected acetone as a deflocculating slurry solvent of 3-μm porous C_{18}-bonded silica spheres; methanol as a flocculating solvent was then used to consolidate the packed bed further. Acetone prevents slurry aggregation here and methanol should introduce additional attractive forces among particles in the packed bed, leading to further contraction. They also observed that the column stability is further improved if water is used as the next consolidating solvent, introducing even stronger interparticle attraction in the packed bed.

Gravity sedimentation packing of 250 (500) × 21 mm I.D. PLC columns with irregular C_{18}-bonded silica of particle size 16 μm was described recently [39]. Acetone was the optimum deflocculating slurry solvent and methanol–water (1:1) was used to consolidate the packed bed. The columns remained stable after 6300 column volumes of solvent conditioning; the appearance of leading peaks in some instances was the only drawback of unexplained origin [39]. In the author's laboratory, a very similar procedure was extensively used for packing columns of 50 mm diameter [22] with both 15-μm silica and C_{18}-bonded silica spheres. No consolidation of the packed bed was needed with plain silica if methanol was used as the slurry solvent; it is believed that interparticle forces determining the bed structure after sedimentation cannot be changed too much by the solvents used in normal phase PLC. With C_{18}-bonded silica, consolidation of the bed structure with methanol–water (15:85) was necessary after sedimentation from an acetone slurry. It is the author's opinion that sedimentation packing of PLC columns might successfully replace in particular dry-packing procedures known to exhibit low reproducibility and also in other instances represents a promising and simple alternative. The occasionally observed leading peaks [39] deserve attention; their origin might be found in the formation of an uneven velocity profile across the column diameter during deflocculating/flocculating solvent changes. A comparison of the behaviour of a packed bed formed by sedimentation in a deflocculating solvent in a dynamic compression column with that of a column conventionally packed during the first few hours of their use could be also interesting.

3.2. Column selection and use

The selection of the PLC column includes a decision concerning column size (length and/or diameter) and packing. The packing selection then determines the PLC mode. A review [30] of PLC packings may be useful in this respect; chiral stationary phases for PLC of enantiomers

were reviewed by Francotte and Junker-Buchheit [40].

There is no doubt that RP-ALC is nowadays the prevailing technique in practice; it is estimated [32] that 80–90% of ALC work is done in the RP mode. Because of the weak surface energies of RP bonded phases, analyses are rapid, column re-equilibration is quick and the technique allows the separation of a wide range of solutes differing greatly in polarity and ionization. Simple binary mixtures of water with methanol or acetonitrile, sometimes modified with small amounts of other additives, mostly suffice as mobile phases and several RP columns with different lengths of bonded RP phase cover a broad range of applications.

On the other hand, PLC requires high sample solubility in the mobile phase, which is seldom achieved in RP systems. Water removal is a second problem complicating product isolation; packing solubility and/or degradation in water-containing mobile phases also come into play. In fact, if both normal-phase and RP-ALC separations of a mixture in question already exist with similar resolution, normal-phase PLC is often to be preferred, at least for solutes of medium or low polarity [7].

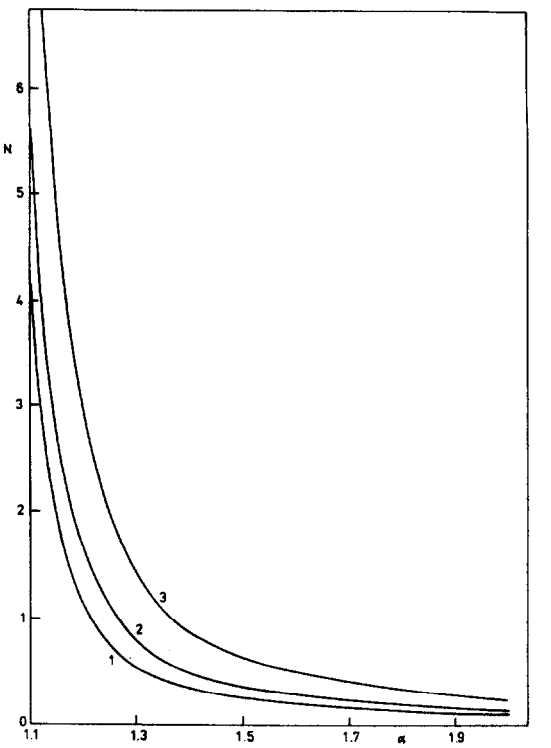

Fig. 5. Number of theoretical plates required for selected resolution as a function of α ($k_2' = 5$, $\alpha = k_2'/k_1'$). (1) $R_s = 1.25$; (2) $R_s = 1.5$; (3) $R_s = 2.0$.

3.2.1. Column size selection

An initial guess can be made on the basis of ALC separation. The analytical resolution (Gaussian peaks assumed), R_s, is [34]

$$R_s = \frac{1}{4}\left(\frac{\alpha - 1}{\alpha}\right) N^{1/2} \left(\frac{k_2'}{1 + k_2'}\right) \quad (2)$$

A value of $R_s = 1.25$ means an optimum, almost baseline ALC separation [34], but indicates a difficult preparative run; only minute overloading is possible until the resolution deteriorates too much. As pointed out by Verzele *et al.* [16], α has the greatest impact on the resolution; an increase in α of only 10% dramatically increases the resolution while a 10% increase in k_2' or N has a small influence. Fig. 5 illustrates the importance of high α in PLC and allows its values to be estimated for different R_s for fixed column and efficiency. The question arises [9,16,41] of the extent to which the theoretical plate concept can be used when a column is overloaded. Within this uncertainty, the equation [9,42]

$$1/N_{tot} = 1/N + 1/N_{th} \quad (3)$$

conveniently describes an overloaded case; here N_{tot} is measured under overload (and calculated from the second moment), N is the analytical efficiency and N_{th} is the thermodynamic contribution resulting from the non-linearity of the adsorption isotherm. Provided that $N_{tot} \ll N$, non-linearity of the isotherm determines the peak shape and N becomes unimportant. N_{th} does not depend on particle size [9] and the effect of increasing the specific load is less critical with less efficient columns but a small-particle-size column will always remain more efficient [4]. The use of efficient small-particle-size columns is a necessity in difficult separations when N and N_{th} are comparable. Even in the case of heavily

overloaded columns, smaller particles exhibiting only a marginal loss of efficiency with increasing flow-rate are more advantageous at higher flow-rates in comparison with coarser column packings [16]. Moreover, under high overload, the interaction between two overlapped bands depends on column efficiency and the self-displacement effect of interacting bands can considerably improve the performance of the more efficient column in some instances [41]. It has been shown [43] that a 20-μm column is roughly fifteen times more productive than an 80-μm column and 4.4 times more productive than a 40-μm column, all of equal length. It is now commonly accepted that a particle size of *ca.* 15 μm is the best compromise [9,16], at least when the columns are intended for a wide variety of separations, as is usually the case in research and development PLC. It is also worth noting that a wide particle size distribution of packing does not influence the column efficiency at flow-rates near to the optimum value; the only negative effect is an increased back-pressure [44].

In an easy separation ($\alpha \approx 1.5$–1.7), an allowed specific loading of 20 mg/g of packing is a realistic value, but a very difficult separation with low α might not allow any appreciable overloading if the column efficiency and dimensions are fixed. The specific loading limit could then be of the order of only 10^{-4} g/g (similar to ALC) and a factor of 200 in the specific loading ratio is obtained for these two separations. A simple rule, that PLC with $\alpha < 1.25$ should be avoided, is sometimes infringed in practical research and development PLC when only milligrams of pure substances are needed and an increase in α would require a new optimization of another phase system. An example is shown in Fig. 6; sufficiently pure isomeric products of the reaction of N,N-diglycidylaniline with N-methylaniline were separated for NMR identification. The course of the reaction was conveniently monitored by gradient RP-ALC using a 150 × 3.3 mm I.D. column ($d_p = 5$ μm, $N \approx$ 8000); an isocratic analytical separation is shown in Fig. 6a. The corresponding optimized preparative run (Fig. 6b) shows that only 80 mg ($2.7 \cdot 10^{-4}$ g/g packing) of reaction mixture as a saturated solution in the mobile phase could be

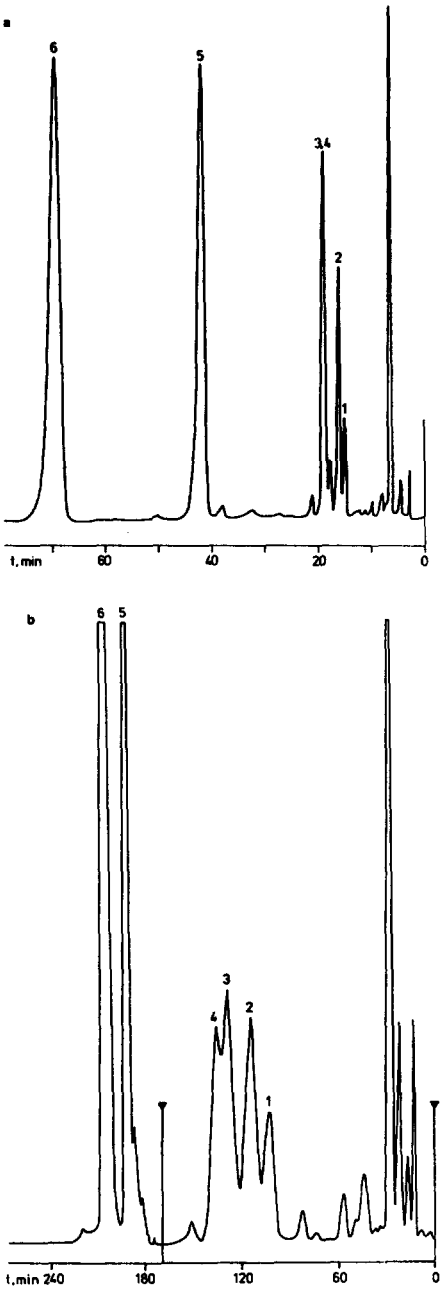

Fig. 6. (a) RP-ALC of isomeric and/or cyclization products (1–6) from N-methylaniline–N,N-diglycidylaniline reaction. Column, 150 × 3.3 mm I.D.; packing, Separon SGX C_{18}, 5 μm; flow-rate, 0.3 ml/min; mobile phase, methanol–water (7:3); sample, 2 μl of 0.4% solution in mobile phase. (b) Preparative isolation of products 1–6. Glass column [22], 250 × 43 mm I.D.; packing, Separon SGX C_{18}, 15 μm; flow-rate 23 ml/min; detection, UV at 254 nm; mobile phase, methanol–water [6:4 (0–170 min) and 8:2 (170–240 min)]; sample, 20 ml of 0.4% solution in mobile phase.

injected to achieve acceptable resolution on a 250×50 mm I.D. column ($N \approx 4000$) after proper adjustment of the mobile phase composition. On the other hand, an easy separation with an allowed specific loading of *ca.* 0.01 g/g packing could be achieved on a 250×8 mm I.D. analytical column to give the milligram amounts desired.

This example illustrates the importance of α in the column size selection; the most common situation in research and development PLC is the necessity to adapt the available column(s) size for a given task. A repetitive injection approach is then straightforward. The convenient number of repetitions depends on the reproducibility of the PLC method and on the degree of automation available; as many as 700 repetitions have been successfully used [11].

3.2.2. *Preparative high-performance liquid chromatographic runs*

Assuming a known maximum loading and a suitable flow velocity from an ALC experiment, the scaling-up to the PLC column is easy on the basis of the two columns volumes. A loading scale-up factor is

$$S = R_p^2 L_p / R_A^2 L_A \quad (4)$$

where R_P and R_A are the diameters and L_P and L_A are the lengths of the PLC and ALC (method development) columns, respectively. The flow velocity ratio scales as R_P^2/R_A^2 with friction heat evolution limitations. The use of an identical packing in the method development column is strongly recommended because different brands of the same packing may exhibit various selectivities.

Usually, the acceptable visual peak separation of the most closely separated pair in question from an ALC model development run is used to define the loading and, if partially overlapped peaks are accepted, the purity of fractions is increased by rejecting the middle fraction. A moderate increase in load may be achieved with fixed PLC column parameters if the hardware allows recycling. The loading may sometimes be considerably increased when the proper band interaction occurs [45]. This approach requires additional labour; the varying content of both components in the overlapped bands must be detected by dividing them into the set of subfractions prior to actual separation and suitable peak "heart cuts" have to be defined. It should be pointed out that the corresponding optimization on the process scale may look entirely different, depending on the task [1–3], and may lead to the use of the displacement mode as a more productive alternative [46–48], especially in biochemical separations.

Concerning the volume and concentration of sample injected, there is no doubt that concentration overload gives a much higher production than volume overload [4,41]. The meaning of this rule in practice is that smaller volumes of concentrated (saturated) solutions in the mobile phase are more advantageous. The useful condition [49,50] gives the allowed injected volume

$$V_i \leq \frac{V_0(1 + k_i')}{N^{1/2}} \quad (5)$$

(V_0 is the column void volume), which should not cause significant additional volume band broadening and V_i can be higher [41] in the case of mass overload.

The UV absorption or refractive index increment of an unknown solute may create unexpected problems. Too high UV absorption "blinds" the detector; at first glance, the wavelength shift to the adsorption curve tail could solve the problem, but this approach is rarely successful [48]. A mistaking of the product and an impurity may be a result of a low detection response of the product [7]. "Invisible" impurities almost never interfere in ALC but may be discovered in the PLC product if their presence is checked by another technique. It is useful to take the whole sample history into account before decisions concerning detection and peak selection are made and all simple (if existing) prepurification steps should be applied first.

The large volumes of mobile phase handled daily often require additional precautions concerning safety rules. Non-volatile additives to the mobile phase cause problems in product recovery; volatile buffers [51,52] should be mentioned in this respect. Solvent regeneration,

almost never done in ALC, is necessary here to keep the costs at an acceptable level. Again, a more complex picture is seen in the case of mobile phase selection in comparison with ALC.

The advantage of high loadings is the possibility of revealing trace impurities [3] not detected in routine ALC work. The ALC technique called "high–low chromatography" was suggested [53] for the determination of trace impurities by injecting the sample in two entirely different concentrations at the detector setting that corresponds to the lower concentration. This is the usual situation in a PLC run when the main components are detected off-scale. An example is shown in Fig. 7a; PLC of biphenyl (used as a differential scanning calorimetric standard), suspected of containing one minor impurity, revealed three minor impurities. A subsequent conventional ALC experiment at low sample concentration did not show any impurities; these became visible only when the sample concentration was increased twentyfold (Fig. 7b).

3.2.3. *Effect of friction heat*

All experience with large-diameter columns has demonstrated the need for a flat velocity profile over the whole cross-section of the column (plug flow) and also along the entire length of the eluent flow. Expressed on the basis of eqn. 1, a constant velocity requires constant ϕ and η. Assuming homogeneity of the packed bed, the viscosity of the mobile phase will be constant if no radial and longitudinal temperature gradients exist in the column. The viscous heat effect contradicts this requirement.

Poppe *et al.* [54] have shown that with ALC columns, a parabolic temperature profile (with maximum temperature in the column centre) varying along the column axis is formed and the maximum temperature is proportional to the square of column diameter. The column in the laminar flow regime may then act as a set of independent narrow columns, each corresponding to a certain radial position [55], and the observed peak represents a sum of elution curves from these hypothetical columns. Fortunately, some compensation appears in ALC columns due to coordinate-dependent heat transport

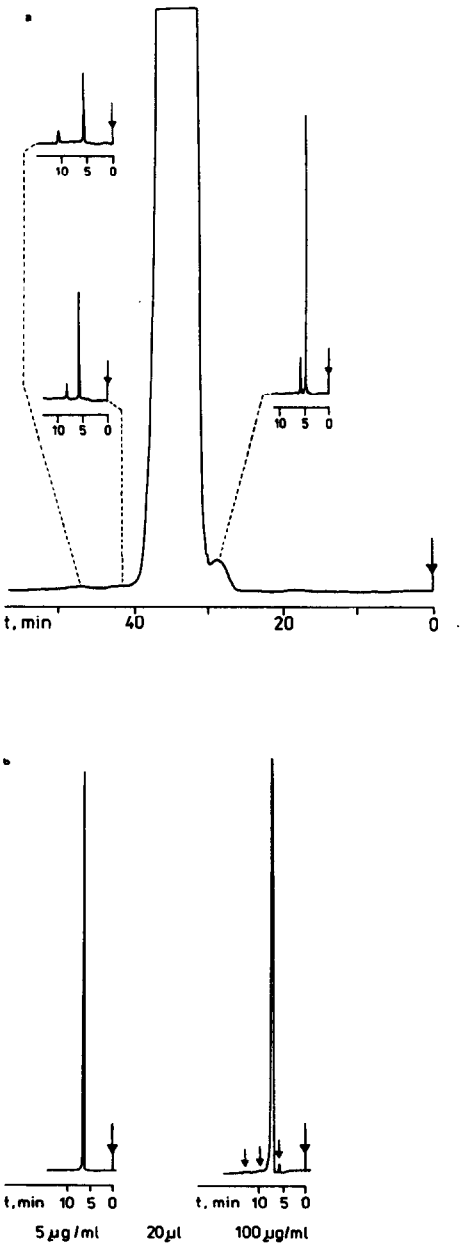

Fig. 7. Purification of biphenyl. (a) Preparative run showing minor impurities. Column and detection as in Fig. 6; flow-rate, 21.5 ml/min; mobile phase, methanol–water (5:1); sample, 100 mg in 10 ml of mobile phase. ALC confirmation of impurities: 20-µl samples of effluent as indicated (dashed lines), spiked with biphenyl; conditions identical with those in (b) except for flow-rate, 0.5 ml/min. (b) ALC of biphenyl at injection concentrations of 5 and 100 µg/ml. Column, 150 × 3.3 mm I.D.; packing Separon SGX C_{18}, 5 µm; mobile phase, methanol–water (5:1); flow-rate, 0.4 ml/min; injection, 20 µl.

through the steel column tube and end fittings [55] and to other band-broadening terms [54].

The situation is complex even with narrow columns [54,55] and, therefore, the observed peculiar behaviour of large-diameter columns is not surprising [4,16,29]. Completely different *H versus u* curves were observed [16,29] for a 44 mm I.D. column compared with columns with I.D. up to 22 mm; the increase in *H* with increase in *u* appears at much lower flow-rates than expected and goes up very steeply.

It seems that two effects may be superimposed when non-compressed beds are tested at higher flow-rates. The bed settling due to microvoids may take place progressively together with the influence of the non-uniform viscosity profile due to viscous heat evolution. The superposition of these two effects then determines the width and shape of the peak observed. Without aiming to improve the understanding of the behaviour of PLC columns at high flow-rates, the results of the separation of a large-volume model mixture at different flow-rates (giving an increased pressure drop) on a 250 × 50 mm I.D. column with injection into the sorbent bed are shown in Fig. 8a–c. It is believed that the effect of void formation is largely eliminated (if formed at the top of the column) and mainly a viscous heat influence is observed. The correct peak shapes resulted until the flow-rate was 196 ml/min at a pressure drop of 12.5 MPa. The corresponding linear velocity is 1.7 mm/s and fits well the top of the steeply increasing part of the *H versus u* curve obtained by Verzele *et al.* [16], thus indicating qualitative agreement for a column of similar size. The subsequent small-volume injection at the same flow-rate (Fig. 8d) confirms that the strange behaviour does not disappear at low sample size. When the flow was stopped for 30 min, a considerable improvement in the peak shapes was observed (Fig. 8e). When the column was left standing overnight, the initial performance (Fig. 8f) was fully restored and the behaviour in Fig. 8c could be reproduced again. It seems that some compensation of heat dissipation/flow distortion profile applies, as in the case of ALC columns, until some critical situation is reached. If this is true, such a pressure limit should be an experimental limiting factor in high-speed PLC.

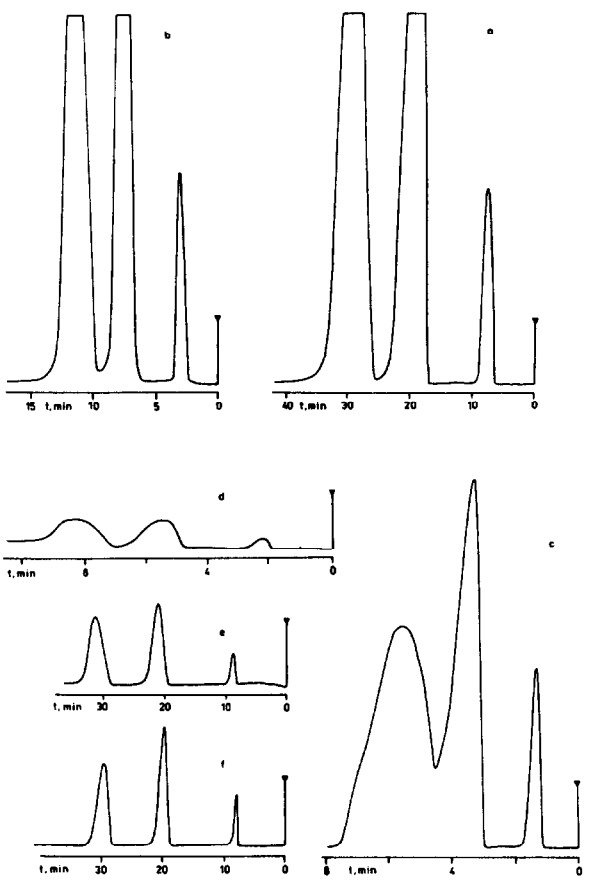

Fig. 8. Effect of friction heat on PLC column performance at different flow-rates. Flow-rates and pressure drops: (a) 37 ml/min, 3.5 MPa; (b) 90 ml/min, 8.5 MPa, 60-min recycling of mobile phase before injection; (c) 196 ml/min, 12.5 MPa, injection immediately after increase in flow-rate from 90 ml/min; (d) injection immediately after run (c) at the same flow-rate; (e) 37 ml/min, 3.5 MPa, injection after 30 min, the equipment switched off; (f) 34 ml/min, initial performance. Volume injected, (a–c) 40 ml and (d–f) 1 ml; sample, acetone (2 mg/ml), benzene (1.5 mg/ml), toluene (1.5 mg/ml); stainless-steel column [22], 250 × 50 mm I.D.; packing, Separon SGX C_{18}, 15 μm.

4. SPECIFIC PROBLEMS OF PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

It is certainly impossible to discuss the daily practice of PLC in full. A few of the issues

believed to be of more general importance will be discussed here.

4.1. Sample solubility

This is a minor problem in ALC where very sensitive detectors allow the use of very low concentrations. The opposite is true for PLC: the sample solubility determines the possible loading, the performance, the throughput of the system and the concentration of collected fractions.

The use of the mobile phase as a sample solvent is the best and simplest alternative, but unfortunately it is sometimes not feasible in practice. This is especially true if medium- or low-polarity compounds [7] are separated by RP-PLC. If possible, it is advisable to select a PLC phase system exhibiting sufficient sample solubility in the mobile phase to avoid problems. The viscosity of a concentrated sample solution in the mobile phase may differ significantly from that of the eluent. A distortion of the flow profile (eqn. 1) may be the result and severe tailing [42,56] would appear owing to the high sample viscosity; fronting could also be seen if the sample viscosity is much lower than that of the mobile phase. This effect is known from SEC [35] as "viscous fingering". As a rule of thumb, the condition that the sample solution to mobile phase viscosity ratio should be kept well below 2 is recommended to avoid these effects.

Such a high viscosity difference may seldom be achieved when low-molar-mass sample is dissolved in the mobile phase. In contrast, if the sample solvent differs from the mobile phase, it is easy to reach this difference in RP systems. For example, the ratio of methanol:water (3:1, v/v) mixture viscosity to methanol viscosity is *ca.* 2.3 and increases to 3 at the maximum of the viscosity *versus* methanol–water composition curve [57]. The same factor of 3 follows from the maximum of the viscosity plot [32] *versus* acetonitrile–water composition. It should be noted that a viscosity change due to a high solute concentration in PLC samples may occasionally decrease these ratios. SEC experiments [56] have clearly shown that a high sample viscosity (the relative viscosity of the sample against water as mobile phase was *ca.* 2.5) leads to severe tailing that increases with increasing sample volume and a low-viscosity sample injected into a viscous mobile phase exhibits severe peak fronting. A net effect of viscosity difference was observed in this study owing to the absence of solute–packing interactions in SEC.

The elution strength of the sample solvent adds in LC when the sample solvent differs from the mobile phase. The deleterious effects of a sample solvent stronger than the eluent on the performance are known from both ALC [58,59] and PLC [12,60,61]. The explanation of unusual peak shapes was found to be a natural consequence of the dynamic gradient between the composition of the sample solvent and of the mobile phase at the beginning of the column [56,62,63]. The superposition of sample solvent strength and viscosity effects is expected in PLC practice.

Therefore, anomalous peaks may be expected even when the sample solubility in the mobile phase is sufficient if the sample solvent and mobile phase differ considerably in viscosity and/or the elution power of the sample solvent substantially exceeds the mobile phase strength [23]. The viscosity difference can be lowered if a proper (non-interacting) viscosity modifier is found [56] and a too high elution power of the sample solvent should be decreased by mixing with mobile phase prior to injection [23]. When the sample solubility in the mobile phase is too low, the formation of supersaturated solutions or precipitation of the solute appears; it was shown recently [61] that solutes are spread over almost the entire column at large sample loads. Hence the sample solubility in mobile phase should always be checked prior to injection [3] and a comparison of the viscosity and elution power of the sample solvent (if different) with those of the mobile phase should not be omitted.

A procedure called "solid injection" was suggested to circumvent the low sample solubility. Dry powdered sample mixed with the column packing is introduced on to the top of a dynamic compression column [64] or the mixture is filled into a small precolumn positioned before the

main PLC column in the stream of mobile phase [65]. It may sometimes be the best of bad selections under gradient conditions [65], but it is necessary to remember that peak tailing is controlled by the rate at which the sample dissolves [66].

The addition of a sample to a solvent that has a lower elution power than the mobile phase [59] requires sufficient solubility and permits on-column concentration of the sample on the column top. The net effect of this procedure is mass overload [67]. This on-column enrichment [68] may be advantageous in the case of proteins [24] where the solubility is usually more than sufficient.

4.2. Packing solubility and degradation

Silica, mostly used in normal-phase PLC, is soluble in water [69] up to a level of *ca.* 100 μg/ml at neutral pH, but no dissolution of silica is detected in organic solvents [69,70]. Polar bonded phases based on silica behave in aqueous eluents similarly to a bare matrix [70]. A C_{18} surface-bonded layer protects silica to a great extent and decreases its solubility to the level of few μg/ml [69]. This is true in the pH range *ca.* 2–7 and the solubility in water increases strongly at pH > 9. The addition of an organic modifier decreases the solubility of silica in an alkaline aqueous eluent. It has been shown that bare silica survives [71] 100 days of regular use at pH 9.2 in methanol–ammonium acetate buffer [90:10]. On the other hand, acidic mobile phases such as 0.1% trifluoroacetic acid (pH 2) are known [72] to promote cleavage of the silane ligand from the silica surface. Hence some leakage must be expected and accepted if RP phases are used in water-containing eluents, in contrast to the normal phases in organic eluents. Moreover, oligosiloxanes, believed to be the residue from organosilane bonding procedures, were observed to leak especially from freshly prepared C_{18} packings [70,73,74].

Rigid polymeric porous packings are in general assumed to be stable between pH 2 and 12, but some monomer leakage might be observed [30]. A high price and small but noticeable swelling [75] (different in various solvents) might explain their rare use [40] in PLC.

As pointed out by Unger and Janzen [30], none of the packings in LC are totally inert. Any release of matter from the packing impairs the purity of the products and subsequent elimination of these impurities from the product may be extremely time consuming and costly.

4.3. Product isolation and purity

Many conventional sample concentration techniques, such as distillation at normal or reduced pressure, precipitation, freeze-drying, non-miscible solvent extraction and membrane filtration, may be used, depending on the product properties. Rotary evaporation and flash distillation devices are most commonly used [12] to recover pure substances and mobile phases.

The major factor in any PLC separation is sample dilution. Knox and Pyper [42] have shown that concentrations at the column outlet may range from *ca.* 0.03% to 0.3% depending on α (collection over the base peak width, $k = 4$, no band overlap) at moderate mass overloads. This imposes high requirements on the mobile phase purity. Assuming a 0.1% concentration of product in the effluent, 0.001% of non-volatile impurity in the mobile phase means only 99% purity of the product after rotary evaporation. Therefore, all newly purchased solvents should be distilled before use. Many solvents contain stabilizers [76] and, if not stabilized, some of them form peroxides. Methyl *tert.*-butyl ether is the modifier of choice in the normal phase mode [7,77] as it does not undergo peroxide formation.

Contamination with dissolved silica occurs in RP-PLC using aqueous eluents; 5 μg/ml of dissolved silica compared with 5 mg/ml of product in the eluent represents a 0.1% purity loss. This should be borne in mind when rotary evaporation is used. Moreover, rotary evaporation (to dryness) often fails with water-insoluble organics owing to their steam distillation. Extraction with a water-immiscible solvent after methanol (acetonitrile) removal usually helps.

The use of solid-phase extraction [78] for product recovery [12] seems to be underestimated. It is analogous to the widely used solid-

phase trace enrichment approach that is used to concentrate different water pollutants prior to ALC analysis [79]. The only additional step required in PLC product isolation is sufficient dilution of the effluent with a weak solvent (water in RP-PLC) to achieve a relevant increase in retention. As an example, 100 ml of toluene solution (0.17%) in methanol–water (mobile phase) (7:3) is diluted will 134 ml of water to obtain a 3:7 composition. A C_{18} packing is able to process 53 ml/g of this solution (corresponding to 23 ml of original solution), compared with 2.9 ml at the 3:7 composition, before toluene breakthrough is observed. Approximately 1.1 ml of methanol is needed to elute adsorbed toluene. Hence the volume of the original solution is reduced twentyfold and additional advantages such as dissolved silica or siloxane removal are gained free of charge. The detergent removal and desalting often needed in biopolymer separation [80] are further possible benefits. An attractive possibility seems to be the non-volatile buffer removal (if necessary for separation) using a small volume of a volatile buffer to recover the pure substance after its solid-phase adsorption.

A great potential of solid-phase extraction in PLC lies in its universality. A variety of bonded-phase silicas with entirely different functionalities exist [81] and the method is not limited to reversed phases only. Many combinations in normal-phase mode using silica or polar bonded phases are easy to envisage.

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