Separation & Purification Reviews
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597294

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To cite this Article Rajcsanyi, P. M. and Ötvös, L.(1974) 'HIGH Speed Liquid Chromatography', Separation & Purification Reviews, 2: 1, 361 — 441

To link to this Article: DOI: 10.1080/03602547408068799

URL: http://dx.doi.org/10.1080/03602547408068799

HIGH SPEED LIQUID CHROMATOGRAPHY

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Introduction

Classical column liquid chromatography has been an effective separation method since the beginning of this century, but low column efficiencies and long separation times are characteristic of it.[1] The mobile phase flows through the column only with a linear velocity of 10^{-2}- 10^{-3} cm/sec. Regarding the

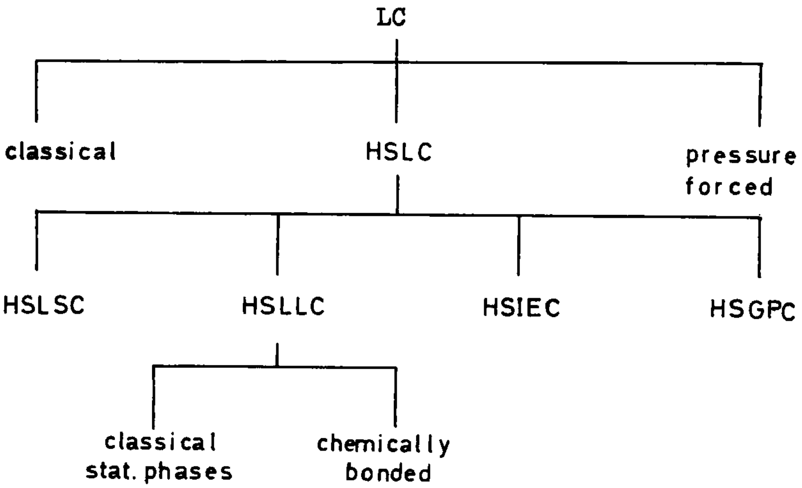

FIGURE 1
Liquid chromatographic methods

time required for chromatographic separation, gas chromatography /GC/ had undoubtedly some advantage over liquid chromatography /LC/ - at least at that stage of development. The GC method is, however, limited compared to LC because of its demand for volatile compounds. Thin layer and paper chromatography had other types of limitation. Thus, there was sufficient claim for improving separations by LC. It was clear that improving the LC technique to a point where the time and efficiency of separations by LC rival those by GC allowed widespread application of LC in the analysis of several types of compounds. Chromatographic separation of compounds with molecular weights $2x10^2$ to 10^8 was expected to be possible by modern LC, including liquid-liquid /LLC/, gel permeation /GPC/, ion exchange /IEC/, and liquid-solid /LSC/ chromatography.

In this review mainly LL partition and LS adsorption chromatography will be discussed as HSLC and the theory of GPC and IEC will not be treated here.[2,3] To reduce the analysis time and increase the separation efficiency of LC, it is necessary to consider the three main steps of development:

1. an appropriate theoretical basis
2. a satisfactory instrumentation system
3. experience in application of the system

I. Theoretical Basis for HSLC

The basis of chromatographic separation is the distribution /or partition/*of sample components between two phases which are immiscible. Thus separation depends on both the mobile or moving phase and the stationary phase. Interactions between the molecules of the two phases are negligible in GC, but play a very important role in LC. These interactions of the liquid chromatographic system determine the degree of sorption of particular substances and also the effectiveness or selectivity of the separations.

1. Thermodynamics of LLC

The sample components in a column are continuously partitioned between the mobile and stationary phases in constant ratios, named distribution or partition coefficient:[4,5]

$$K = \frac{\text{solute concentration in stat. phase}}{\text{solute concentration in mobile phase}} \qquad /1/$$

The solute distribution coefficient must be constant during the migration of the solute down the column, that is, a linear partition isotherm is preferred. /At this point several assumptions must

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* Throughout this chapter diagonal bars (/) are used instead of parentheses or brackets.

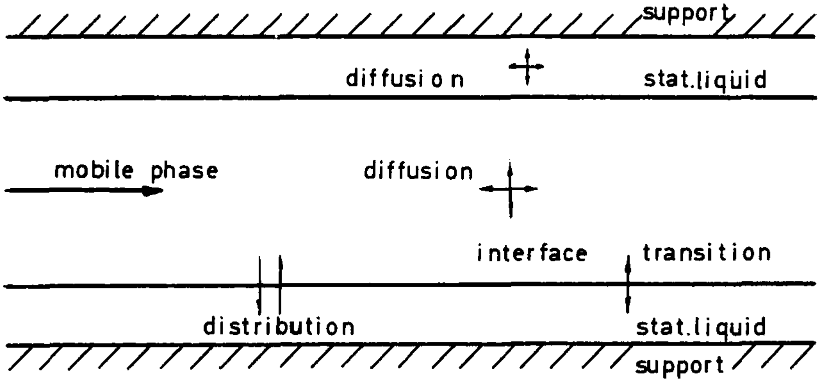

FIGURE 2
Elementary processes
in an interstitial channel of a LC column

be taken into consideration according to Locke and Martire[4].

If V_s is the volume of stationary phase in the column and V_m is the interstitial volume of the column, the retention volume of a component, for which $\underline{K}$ differs from zero, is defined as

$$V_R = V_m + KV_s \qquad /2/$$

Considering solute residence times due to solute-liquid phase interactions, the net retention volume of a component can be defined:

$$V_N = V_R - V_m = KV_s \qquad /3/$$

and from /3/ we obtain an equation for the specific retention volume

$$V_g = \frac{V_N}{w_s} = \frac{K}{\rho_s} \qquad /4/$$

where w_s is the weight of stationary phase in the column and ρ_s its density.

A new important partition parameter, the partition ratio or capacity factor, can be obtained by multiplying the partition coefficient by the phase ratio

$/\beta = V_S/V_m/$, i.e. the volume ratio of the two phases

$$k = K\beta = K\,\frac{V_s}{V_m} = \frac{V_N}{V_m} \qquad /5/$$

The partition ratio has theoretically a simple physicochemical meaning: it is the number of component molecules in the moving phase per component molecules in the fixed phase. It means practically the time an average solute molecule spends in the mobile phase relative to that spent in the stationary phase, and can be determined directly from the chromatogram.

Taking the condition of equilibrium in LLC at pressure $\underline{P}$ and temperature $\underline{T}$

$$\mu^s\,/T,P/ = \mu^m\,/T,P/ \qquad /6/$$

where μ is the solute chemical potential, $\underline{s}$ and $\underline{m}$ indicate the stationary and mobile phases, respectively, we have for the specific retention volume

$$\ln V_g = \ln \frac{\gamma^{m,\infty}\,/T,P=1/\;M_m}{\gamma^{s,\infty}\,/T,P=1/\;M_s\,\rho_m\,/T/} + \frac{\bar{P}-1}{RT}\,/v^m - v^s/$$

where $\gamma^{m,\infty}$ and $\gamma^{s,\infty}$ are the solute activity coefficients at infinite dilution, M_m and M_s are the molecular weights of the phases and $\rho_m/T/$ is the mobile phase density at temperature $\underline{T}$, $\underline{\bar{P}}$ is the mean column pressure, $\underline{v}^m$ and $\underline{v}^s$ are the solute molar volumes in the two phases.

This equation allows us to predict partition properties, partition coefficients and retention volumes a priori for systems in which the activity coefficients are known.[6-8]

In the case of two components /1 and 2/ in a solute the relative retention

$$\alpha = /\frac{\gamma^{m,\infty}}{\gamma^{s,\infty}}/_1 \; /\frac{\gamma^{s,\infty}}{\gamma^{m,\infty}}/_2 \qquad /8/$$

will depend only on the differences in the solution behavior of the components in the two phases. That is, the relative retention is really a measure of the thermodynamic differences of component distribution, or the difference in free energies of distribution for two components. In LLC one can, therefore, improve separation by changing mainly the nature of the mobile phase; not only that of the stationary phase as in GC./The improvement of separation in LLC can also be carried out by changing the temperature/ This affords a bridge between theory and practice: the use of gradient elution. All in all, from the thermodynamic basis of LLC it is clear that the resolution of two adjacent bands can be improved by changing the separation factor or relative retention /α/ and the partition ratio /k/.

In HSLC the role of the capacity factor in resolution cannot be neglected, since the low volume ratio of stationary to mobile phase involves a low k value. These low k values are indeed desired for high speed, as discussed later on, but for resolution high k values are preferable. Therefore a compromise must obviously be made, that is, there is an optimal capacity factor value - but with a single solvent k is never optimum for all components of the sample. Thermodynamically, the value of the capacity factor depends on the solvent strength, and strong solvents give smaller k values. Horvath and

Lipsky[9] noted that the peak capacity can be significantly improved by use of gradient elution.

We should, however, note that the factors -capacity ratio and relative retention- affecting separation are not independent of each other to the effect that although k was optimized for every pair of adjacent band, either a favorable change in the separation factor or a further increase in column efficiency may be necessary.

Alteration in the α value while keeping k approximately constant can be achieved by varying the solvent composition. For this purpose nowadays mainly practical approaches are at our disposal. Therefore the choice of stationary and mobile phases is one of the weakest points in the development of HSLC.[10]

2. Thermodynamics of LSC

In LSC the distribution coefficient of a component between mobile and stationary phases depends on interaction forces; mainly on dispersion forces /nonpolar/ and on hydrogen bonding /polar/ between the sample component, solvent, and adsorbent.

The selectivity of LSC is dependent on the balance established between the two types of adsorption interaction, because the distribution coefficient is determined by the sum of the polar and nonpolar effects.

In LSC there is a competition between solvent and sample molecules for the fixed "active" sites on the surface.[11,12] The adsorption of sample molecules require the desorption of solvent molecules to permit the accommodation of sample molecules on the surface. The capacity factor k is, therefore, determined by the net energy of adsorption of sample molecules, and the net energy of adsorption is given as the sum of interaction energies. But from a practical point of view, the selection of the right solvent strength is simpler in LSC than in LLC, which is a great advantage of the latter chromatographic method in HSLC.

3. Kinetic Conditions in HSLC

If N_{req} plates are necessary to obtain sufficient resolution, the time of analysis t can be expressed as

$$t = N_{req} \; / \; 1 + k \; / \; \frac{H}{\mu} \qquad /9/$$

where H is the plate height and μ is the mobile phase velocity.[13,14] The term H/μ is a fundamental measure of the speed of analysis.

Considering the pressure drop needed to obtain the required plate number, another important parameter can be defined :

$$\Psi = \frac{P_{req}}{N_{req}\,\eta} \qquad /lo/$$

where η is the viscosity of the eluent.[13,15,16]

If plots of H/μ and Ψ as a function of velocity are shown as in Figure 3., the kinetic conditions of HSLC can be well understood. The H/μ curve decreases very fast at lower velocities and flattens off at higher velocities. The Ψ-μ curve shows an inverse effect. So there is an optimum velocity range about o.8 - 5.o cm/sec in practice. Taking into consideration the general equation on band-broadening processes, with the assistance of equations /9/ and /lo/, overall conditions for high speed or high efficiency LC may be derived.[17-19]

The pressure drop ΔP is taken according to operating conditions. The maximum value of P/N is determined by the number of plates needed to achieve

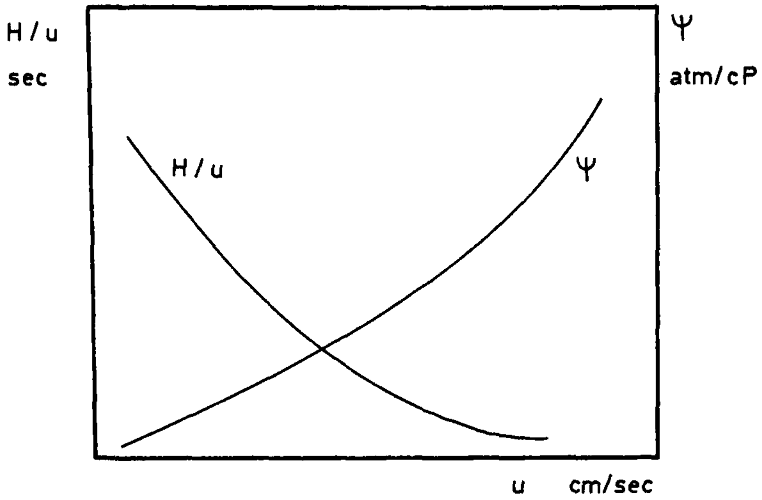

FIGURE 3
Kinetic conditions of HSLC

the required separation. A decrease of the particle diameter / $\Delta P/N$ is proportional to $1/d_p^2$ / results in a more than proportional decrease of H - but conventional packing materials have some disadvantage for use in HSLC.

The fluid velocity is practically constant along the column because of the low compressibility of the mobile phase. In HSLC, because of the coupling of the eddy diffusion and mobile phase mass transfer terms, H/μ continues to decrease with the increasing velocity. As pressure drop across the column is related to velocity through the column permeability, the more permeable the column, the shorter the analysis time. Or if analysis time t is maintained constant, a more permeable column permits the use of a longer column - but the length of the column applicable is limited by pressure effects.

Considering the relation between permeability and the particle diameter, there is an optimum value for both parameters, as permeability may be increased by increasing the particle diameter; larger particles mean, however, higher $\underline{H}$ values. This means that, in practice, use of irregular packed columns / $d_c/d_p<5$ / may increase the permeability by at least one order of magnitude, while increasing efficiency.[2o,21]

Thus the maximum resolution for a given pressure is achieved if column operating conditions /i.e. particle size, column length, mobile phase velocity/ are chosen so that the reduced plate height /H/d_p/ should be a minimum.[22]

From the discussion of the theoretical basis for HSLC it is clear that small diameter columns /1-3 mm/, solid supports of particles with diameters less than 6o µm, large pressure drop /up to 6ooo psi/, and high flow rate /ca. 1-4o ml/min/ should be used in this new method of separation. These result in high mobile phase velocity / 1-1o cm/sec /, high efficiency in column, and very rapid separation time.[23-27]

II. Instrumentation of HSLC

A block diagram of a typical high speed liquid chromatographic system is shown in Figure 4. The main parts of such a system will be discussed in the order they appear in the diagram.

1. Solvent Reservoirs

Reservoirs of stainless steel or inert polymer containing about 1.o-1.5 l liquid are optimum for analytical purposes.[28,29] For chromatographic systems used in preparative work, reservoirs of larger volume should, however, be selected.

Under particular conditions glass can also be used as reservoir material. Accessories in the reservoir are a heater, a /magnetic/ stirrer, and inlets for vacuum and nitrogen purge. Attached to the reservoir may be a cooled condenser which helps reduce losses of volatile solvents used as carrier.[30] Degassing the polar mobile phases to prevent the formation of bubbles, which can be troublesome with detectors, can be accomplished by vacuum.[31] Complete removal of dissolved oxygen may require heating and nitrogen purge.

The reservoir can also serve as a liquid pump.[32]

2. Gradient Apparatus

Gradient elution or solvent programming can be defined as changing the chemical composition of the mobile phase during an analysis.[33,34] In this way the k value of each eluted band is approximately optimum during its movement through the column, therefore gradient elution is the most useful approach in improving separation.[35] Gradients may be stepwise or continuous. Gradient elution devices can be divided into two categories:

a. mixed-stream devices
b. mixing-chamber devices

Today, most commercial manufacturers offer also gradient elution accessories - the optimum gradient for a particular separation is, however, selected mainly by trial and error.

3. Pumping Systems

The small particle sizes and small diameter columns being used in HSLC require pumps delivering mobile phase at pressures in the range of 1ooo to 6ooo psi.[36-39] Bidlingmeyer et al. used a high speed liquid chromatograph in the separation of methyl-

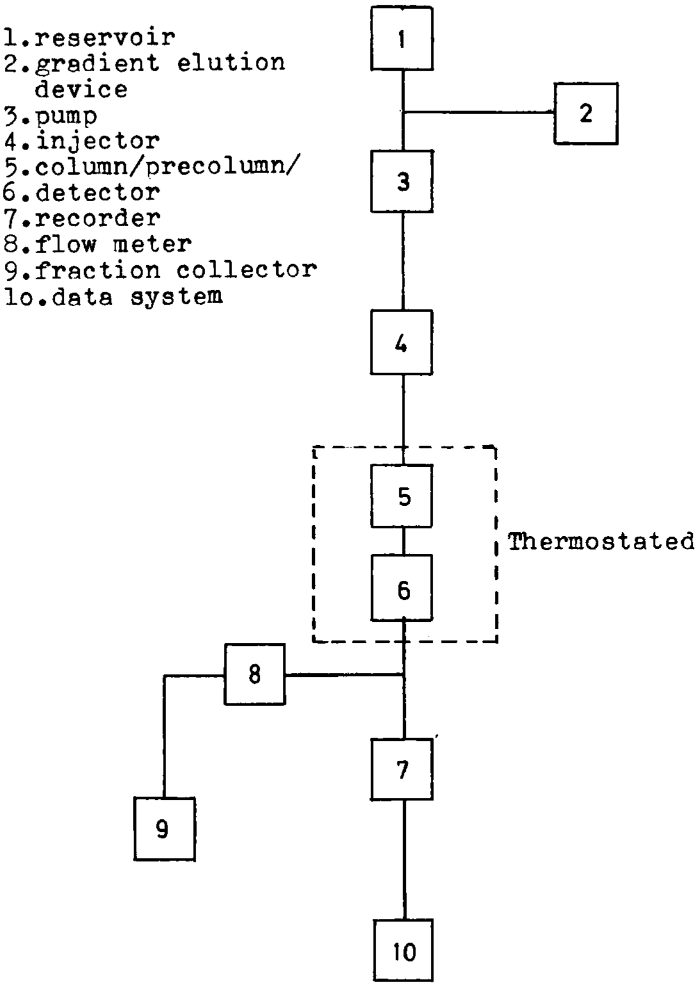

FIGURE 4

Block diagram of a liquid chromatograph

and ethylorange, which delivered eluent under pressures up to 6o,ooo psi.[4o]

The advantage of the screw-driven type mechanical pump is that it can deliver a pulse-free supply of liquid, but it has limited solvent capacity. The main drawback of the reciprocating piston and the diaphragm pumps is that these produce a pulsating flow. One of the major objections to the use of a pulsating pump for HSLC is that optimum circumstances are difficult to realize when flow is varying. A specific advantage of the reciprocating pump is that it has small internal volume and its delivery is continuous.

Pneumatic pumps are operated by gas pressure working on a container or piston that pressurizes the moving phase.[28,32,37,41,42] The advantage of these pumps are that high pressures can be easily achieved and they deliver a pulse-free supply of liquid phase. By varying the gas supply with a pressure programmer, the mobile phase flow can be easily programmed.[43-45]

Karger and Berry[46] have presented a continuous gas displacement pumping system that offers no pulsation and inexpensive construction as well as low detector noise level. This pump has been operated over 2ooo psi.

4. Sample Injector Systems

Two modes of sample injection are used:

1. with moving solvent
2. with stopped flow technique

Very high separation efficiencies can be obtained by on-column injection; injecting the sample directly onto the top of the column packing. The major advantages of this type of injection are as follows: simplicity in design, injected volume readily changed and giving only very small contribution to elution bandwidth.[38,47,48] In a swept injection port, the sample is swept into the packing by the mobile phase. The swept injection port must have a minimized dead volume to minimize brandbroadening caused by diffusion above the packing.[49] These ports accommodate large samples of 5o or 1oo µl, too.

Sample valves are often used in very HSLC above 3ooo psi or when high repeatability of sample introduction is required.[5o,51] One of the major advantages of sample valves is that they are easily adapted to automatic repetitive analysis.

Injection with stopped flow or moving stream can be carried out with either type of these systems.[52]

Injection ports are often located at the bottom of the column to decrease the time necessary to sweep all air out of a newly installed column on start-up.[28,4o]

Smuts and his coworkers[53] have designed an inlet system based on splitter principles which decreases considerably the solute band width.

A sampling system was described by Krejci et al.[54] by means of which compressed samples could be transferred into a pressure forced /up to 25 atm/ liquid chromatograph without previous sample release.

5. Column

The success of an analysis may depend on the column in great measure. The column material can be glass or stainless steel in several forms and lengths.[55,56] The influence of coiling on the efficiency of the separation has been shown to depend on column and coil radii.[57,58] The influence of coiling on column efficiency in HSLC is increased as the tube radius increases or the coil radius decreases.[59] Column lengths can vary from 15 to 3oo cm or more /longer columns generate higher back pressures/.[6o,61] The typical diameters of the analytical columns in HSLC range from 1.5 - 3.o mm. As the column internal diameter is increased, an increase in H occurs; with very small internal diameter columns, extra-column peak-broadening effects, however, become significant and it is possible that wall effects are a dominant factor.[62,63] In preparative applications, columns with larger diameter are used.[64,65] The pressure drop along the column is directly proportional to the column length and inversely to the square of the particle diameter.[24,49]

An analytical column of usual dimensions contains about 6-8 gm of a superficially porous packing material or 2-4 gm of a regular porous packing.

The influence of column packing, coiling, and length on the peak-broadening is co-determined by the surface properties, mechanical stability, and sieve fraction of the support.[66]

Effects of particle size of porous layer bead support on the performance of columns with larger diameter were discussed by Beachell and DeStefano.[67,68]

For LSC, a detailed discussion on the size and geometry of the adsorbents has been presented by Snyder.[69]

In HSGPC, semi-rigid[7o-73] or rigid gels[74,75] can be used.

5.1. Supports for HSLC

Two main categories of column packing materials in HSLC may be distinguished - porous and superficially porous /porous layer bead/ supports[76-79], although the packing materials in HSLLC, HSLSC, HSGPC[8o], and HSIEC[81] have different characteristics. Chemically bonded packing materials signify a special type of packing materials and show the main tendency of

development as discussed later on.[82-89]
Porous packing materials are either spherical or irregularly shaped, have deep pores, large surface areas /5o-4oo m^2/g / and, therefore, high sample capacities. Porous supports include silica gels, alumina, and diatomaceous earth materials.
Superficially porous packing materials in HSLLC and HSLSC consist of a solid core and a thin, porous coating, and are commercialized as Zipax, Corasil I and II, Vydac, Perisorb, Pellidon, etc.[9o-98]
Pellicular ion-exchange materials for HSIEC were developed by Horvath et al.[99] in 1967, as the first major result in the field of new supports for HSLC. A thin film of styrene-divinylbenzene resin polymerized onto a spherical glass bead was treated to make either a cation or an anion exchanger. Resins have also been coated on Zipax porous layer bead supports[35] and other supports.[1oo,1o1] Both pellicular and superficially porous resins are of 3-12 ueq/g capacity which requires sensitive detectors, but are of high efficiency. Porous ion-exchange resins previously developed mainly for amino acid analysis can also be used although high cross-linking /above 8% / is desirable.
Column packings in HSGPC differ from those of the other HSLC modes and are discussed elsewhere.[72,73,1o2,1o3]
Sizing of porous support materials by dry sieving can be achieved down to 3o-4o µm.[16] Scott has developed a continuous elutration method for preparing spherical ion-exchange beads of 5-4o µm by fractionating the material as a suspension into an upward flow of liquid.[1o4] Irregularly shaped porous silica gel supports are commercially available down

to 1o µm and can be used in HSLC.[69,1o5,1o6]
The adsorbent materials recommended for HSLSC can be coated with stationary phase and used in HSLLC. Whenever possible, the column in HSLC is dry packed; for particles below 3o µm and irregular particles below 4o µm, a slurry packing technique is recommended.[16,62,82,1o7]

5.2. Chemically Bonded and Surface-Modified Supports

The conventional packing materials to be used in HSLC have procedural limitations: the carrier must be presaturated with the stationary phases to prevent its stripping from the support[1o8]; high flow rates are to be avoided; gradient elution technique cannot be used.

To surmount these disadvantages, chemically bonded packing materials have been developed.[1o9-112]
Halász et al.[113] have developed the so-called "brushes", esterified siliceous supports which are commercialized as Durapak. The brush packings, however, tend to be thermally and hydrolitically unstable; water and alcohols limit their general usefulness.

A second type of bonded phases are prepared by polymerization after silylation of the hydroxyl groups on the silica surface as can be seen in Figure 5. This type of packing is thermally and hydrolitically stable.[82,87,88]
Locke et al.[114] have given procedures for forming silicon-carbon bond being thermally and solvolitically stable. The primarily substituted supports were also transformed into their ion-exchange forms. Reverse phase column packing materials have been

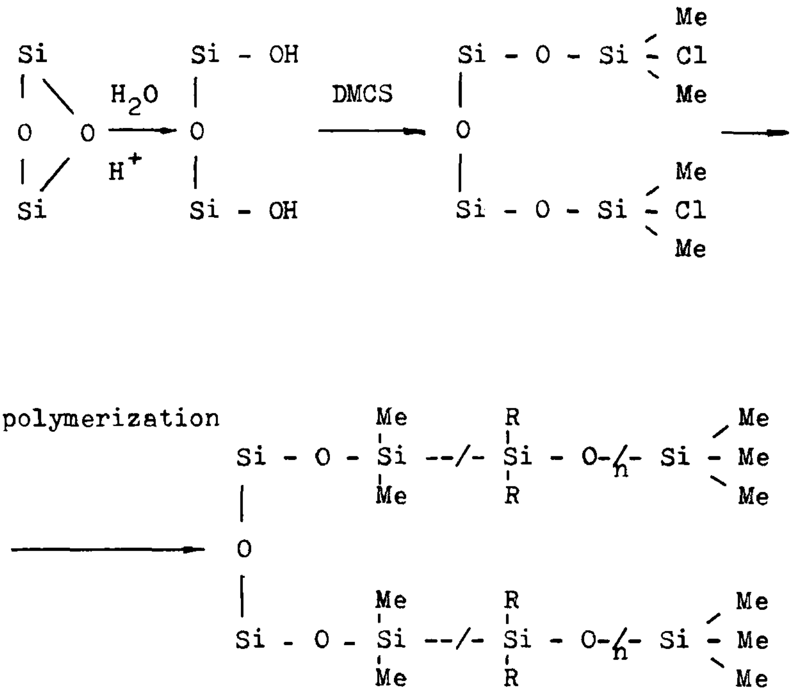

FIGURE 5
Preparation of a chemically bonded siloxane stationary phase

recently developed and commercialized as Bondapak phases.[115]

Ross and Jefferson[116] have described the use of in situ-formed open-pore polyurethane as a chromatographic support and evaluated for GC and LC separations.

Karger et al.[117] have introduced surface etched glass beads for HSLC, which are only modified and not chemically bonded packings. The results show that above a 15 ml/min flow rate significant bleeding of the stationary liquid phase occured even with careful presaturation of the mobile phase.

All in all, it is obviously clear that small-particle packings with chemically bonded phases are the next step in the development of the column packing technology.

5.3. Stationary Phase

In separations by LLC, a partition of the sample molecules occurs between the mobile and stationary phases according to the equation /1/. The choice of a stationary phase is dictated by the sample to be separated and is somewhat empirical at the present stage of development.

Normal LLC uses a polar stationary phase and a non-polar mobile phase whereas reverse phase LLC uses inverse phases. Most LLC separations capable of manipulating the character of both phases can be carried out on relatively few stationary phases. The most widely used solvent pairs are tabulated in Table 1.

The optimum level of partitioning-phase loading is largely dependent on factors as viscosity of the stationary phase, molecular weight, polarity, and peculiarities of the support.[118]

The stationary liquid phase must be uniformly coated onto the support, mainly by solvent evaporation techniques or by coating the prepacked column.[107] Suggested stationary phase loadings are tabulated in Table II.

The use of monomers or unique liquid as stationary phase is preferred to polymeric materials or liquid-mixtures, although ternary or quaternary systems mixed to form two immiscible phases were successfully used in HSLC[8]. However, as Huber[119] indicated, there are two main practical limitations: 1. the

TABLE I

Widely used solvent pairs in HSLC

Stationary phase/normal/	Mobile phase
1. β,β'-oxydipropionitrile	saturated hydrocarbons aromatic hydrocarbons di-n-butyl-ether
2. polyglycols	hexane, heptane, isooctane, benzene
3. Durapak OPN	hexane, heptane, isooctane, hexane modified with alcohols
4. Permaphase ETH	see 1.
Stationary phase/reverse/	**Mobile phase**
1. Durapak n-octane	acetonitrile
2. Vydac reverse phase	acetonitrile water-acetonitrile
3. Permaphase ODS	water-alcohols water-acetonitrile
4. Cyanoethyl silicone	water-alcohols water-acetonitrile nitromethane

more the two phases become similar, the more the stationary phase will be displaced by the moving phase, i.e. the column becomes unstable; 2. the larger the partition coefficient becomes, the less soluble the sample is in the eluent, i.e. the detection of the sample becomes more and more difficult.

5.4. Selection of the Mobile Phase

Many different organic and aqueous solvents are available as mobile phase, influencing separation.

TABLE II

Suggested stationary liquid phase loadings

Support	Maximum loading w %	Minimum loading w %
diatomaceous earth	3o	5-1o
silica gel/micro pore/	35	6
Zipax	2	o.5-1.5
Corasil I	1.5	o.5-1.o
Vydac	3	2
Pellumina	1o	1

These should be selective for components. In preparative chromatography, volatility of the mobile phase is desirable.

Resolution in a chromatographic separation, as discussed earlier, is dependent on $\underline{N}$, $\underline{\alpha}$, and $\underline{k}$. For a given stationary phase, a strong solvent will cause low $\underline{k}$ values, whereas a weak solvent will result in high $\underline{k}$ values that is, the solute prefers the stationary phase.

There are general criteria[12o] for the selection of a good mobile phase, such as: the mobile phase should

1. dissolve the sample
2. keep the column stable[121]
3. have appropriate viscosity[34,69]
4. be pure[122]
5. be compatible with the detector[123]
6. satisfy a number of special criteria /the mobile and stationary phases must be immiscible in one another; active fluorides should be avoided with glass containers; and should not contain dissolved oxygen; etc./

For HSLC, mobile phases are listed according to their solvent properties in eluotropic serie , which offers a choice in the k values. Using a solvent lower than previously applied in the eluotropic serie, the k value of an individual sample component will decrease, and inversely. Usually, there is a maximum solvent strength which can be tolerated for a given stationary phase.

In LSC, water is frequently added to both mobile phase and adsorbent to reduce the strong solute-stationary phase interactions. Snyder[124] has estimated that maximum linear capacity in LSC usually occurs for 5o-1oo % of a water monolayer, that is, o.o2-o.o4 gm of water per 1oo m^2 of adsorbent surface and for example with Porasil support this means 6-12 gm of water per 1oo gm of dry adsorbent. Addition of the right amount of water can raise adsorbent linear capacity relative to the dry adsorbent by a factor of 5-1oo.[125]

For superficially porous adsorbents, o.5-1.o % of water should be added. The water on the adsorbent must not change during separation.

HSIEC is usually carried out in aqueous solution, but sometimes organic solvents are mixed with aqueous buffers to provide unique selectivity changes and improve sample solubility.[126-128]

In HSGPC, the solute should interact with the free mobile phase, the adsorbed mobile phase, and the gel matrix to reduce resolution dispersion and skewness of elution curves.[129,13o] To improve solute solubility in an HSGPC separation, a mobile phase with lower viscosity should be chosen.

For solving the so-called general elution problem, a change in band migration rates during separation is required.[33,35] The ways whereby this can be executed are gradient elution /or solvent programing/, temperature programming, flow programming, and coupled columns.[35,131,132] Among these, only gradient elution is closely related to selection of mobile phases.

Gradient elution can provide significant improvement in resolution at all points in the chromatogram, particularly at the front-end, by adjusting the $\underline{k}$ values of individual bands so that each band is eluted from the column under near-optimum conditions. The role of gradient elution may be especially important in the analysis of unknow samples or with samples whose components show wide variation in structure. Snyder and Saunders[34] have discussed the problem of designing an optimum solvent program for a given separation and selected several requirements for this program. Scott[133] has developed theories for gradient elution using the continuous and incremental methods of solvent mixing, including a simple computer program that provides curves relating moving phase composition with time for the incremental method of mixing. Byrne et al.[134] have developed a multifunctional gradient elution device which can produce a variety of shapes and time durations without hardware changes. Using this device, liquids from two reservoirs can be mixed automatically by two electronically controlled proportioning valves.

The use of a binary mixture as mobile phase results in the concentration of the component with high polarity in the mobile phase, being at all times greater at the inlet of the column will move at a greater velocity than that at the end of the column and this will consequently cause a loss in resolution. Scott and Lawrence[135], supported by earlier experiences concerning this problem[131,132,136], discussed gradient elution under conditions of axial equilibrium, i.e. without polarity gradient, produced an apparatus for carrying out this purpose, and illustrated its applicability to the analysis of natural mixtures.

Chilcote et al.[137,138] have recently developed an automated two-chambered gradient-generating system which can provide also the automated regeneration of the chromatographic column in preparation for the next analysis.

The refractive index and micro adsorption detectors which respond to changes in solvent composition cannot usually be used with continuous gradient elution. At UV detector, since solute UV absorbance is a function of the solvation strength, calibration runs should be made for quantitative work. Using multicomponent eluents to provide the eluent strength necessary for separation, at the above mentioned detectors, spurious peaks were observed. Solms et al.[139] have set up a model to account for such behavior, used computer simulation, and verified the results of the computer study both quantitatively and qualitatively. They found that in certain cases quantitative errors could amount to 3o % of the correct result.

Relations between the Hildebrand solubility parameter and the solvent strength parameter developed for LSC should provide a better guide to choosing mobile phases and predicting solute migration also in HSLC.[14o,141]

The use of the mobile phase in HSLC involves also some practical problems, such as solvent degassing and solvent presaturation.[142]

Comparison of the gradient elution, temperature programming, and flow programming shows that gradient elution is a substantially better general technique whereas temperature programming and flow programming may prove valuable in selected situations.[35,69,132,143]

6. Detector Systems in HSLC

Reviews and evaluating studies on the detectors most often used in HSLC are nowadays frequently appearing in the literature.[144-150]

HSLC detectors may be classified as being selective or universal, destructive or nondestructive - although it is understood that unfortunately there is not a really universal and nondestructive detector on the market for the time being. Therefore, if samples range widely with respect to their properties to be measured, there is no other choice but to use several detectors at the same time.

A selective detector responds to limited classes of compounds. A universal, non-specific detector should respond to a wide range of compounds and not only to a change in composition of the eluent but to any fluctuation of the recorded bulk property of the mobile phase.

In evaluating the HSLC detectors discussed in this review, one should keep in mind that their performance depends on the relation between the measured physical quantity and the composition of the effluent as well as on the signal transfer characteristics of the whole detector system. Consequently, if a small amount of sample is measured, the output function is a signal, whose width /which is very important in comparison with the minimum detectable peak width/ depends on the sample amount, on the mixing in the sample device /on the physical distribution of the sample in the sampling device/, in the connection tube, and in the detector cell, and on the time behavior of the detector.

The primary detector parameters, such as noise drift, absolute and relative sensitivity as well as linearity, and other overall operational parameters are defined and discussed elsewhere.[151,152]

Concerning these parameters, it should be emphasized that the usefulness of a detector is strongly limited by its flow and temperature sensitivity, e.g. both micro adsorption and refractive index detectors require a high precision control of the temperature and with UV, polarographic, and micro adsorption detectors a pulse damping system must be used in order not to transmit a pulsating flow into baseline noise and drift.

If detectors are placed in series, they should be connected in the order of increasing dead volume. Using parallel detectors, sensitivity can be lost because of flow and sample splitting.

6.1. Light Absorption Detectors

These selective, nondestructive detectors are used mainly in the UV region of the spectrum, although recently there have been several attempts to extend their measuring range over the whole UV and visible spectrum.[153-157] In these detectors, the liquid stream passes through a flow cell across which light, collimated by lenses from low pressure mercury lamp is transmitted. The light is filtered or converted into specific wavelengths. High performance UV detectors are commercially available which offer detection at either 254 nm or 280 nm, and which offer simultaneous detection at both wavelengths.[158-160] Monochromators often used in place of the filter provide an infinite selection of wavelengths.

UV cells of two different types have been used in commercialized high speed liquid chromatographs. In the type of Z configuration, the mobile phase slips half of its pathway along one window of the cell, travels the distance between the two windows against the light beam, and goes out along the other window.[131] To minimize noise and baseline drift owing to the changes in mobile phase flow, an H-shape cell has also been developed.[28] As the flow inside the cell is not uniform, sometimes turbulence can be generated which tends to scatter the light in the cell, thus affecting the signal. If the absorbance of the mobile phase appears to be changing, a reference cell can be used, although a reference cell can cause more drift and noise than it corrects.

Light absorption detectors can provide absorbance output which is more convenient than transmittance output because it is directly proportional to sample concentration in accordance with Beer's Law. It is desirable for the output of the photocells to be fed to log amplifiers to make the output correctly linear with concentration. Relying on the approximate log function of the photodetector may be useful up to about 0.01 absorbance.[161] Mobile stream-monitoring by UV detectors is perhaps the simplest method, for UV absorption characteristics are usually known from previous works and because light absorbing derivatives can be formed from the compounds with no UV absorption, as in the detection of amino acids.[162]

Commercial spectrophotometers could also be easily modified for use as HSLC detectors.[44,137,163-167]
In the commercial light absorption detectors for use in HSLC, cell volumes are of 8-2o µl, path lengths are of 5-lo mm, drift stability ranges 10^{-3}-$4x10^{-4}$ AU/hr and linear dynamic range is usually of $2x10^{-4}$-1.o AU. The UV detectors with larger cell volume /up to 1.o ml/ are useful mainly for preparative work.

Thacker et al.[168] have developed a mini-size UV photometer for continuous monitoring of the effluent at 254 nm and 28o nm. The photometer operates as a double-beam instrument at both wavelengths indicated and can be used also with gradient elution.

UV detectors are frequently used for detection of nucleic acid constituents from ion exchange columns. It is advantageous to carry out these analyses by pellicular materials at temperatures of 7o-8o °C at which the exchange rate of the resin is sufficiently increased. However, high temperature and high flow rate create excessive detector noise, making the measurement of picomole amounts of these substances impossible. The stability and sensitivity of these detectors can be improved by some modification by which the mobile phase leaves the column, passes through a water-jacketed cooling coil and then enters a water cooled UV cell.[169]

Solvent systems being transparent at the analytical wavelength are preferred for use in HSLC with light absorption detectors.

6.2. Differential Refractometers

This type is a nonselective, nondestructive detector, whose response depends on the difference between the refractive indices /RI/ of the solute and mobile phase.

One of the two basic types of RI detectors, the reflection-type detector, is governed by Fresnel's Law whereas the other, the deflection-type is ruled by Snell's Law. The latter senses the deflection of the light beam, while the first measures the intensity of the reflected light.[17o-172]

Their sensitivity in milligrams of sample is roughly equal to the reciprocal of the difference in RI between solvent and sample.[147,149] The measured physical property RI is, however, affected by the temperature, consequently the sensitivity is also influenced by the stability of the eluent property considered. The refractive indices of water and organic solvents have temperature coefficients of $1o^{-3}$-$1o^{-4}$/°C. Therefore, it is of primary importance that reference cells should be used and the temperatures of the two streams should be equalized before entering the refractometer. It is also desirable to thermostat the incoming feed lines to the cell.[148]

Deininger, Walkling, and Halász have modified a commercially available RI detector by designing a heat transfer system with a minimum dead volume in the analytical stream to minimize band spreading.[173-175]

Bakken and Stenberg[176] have recently adapted and tested an interferometer as the basis of an LC detector.[176]

All commercial RI detectors /except one manufactured by the E-C Apparatus Corporation/ have cells with volumes of 3-12 µl, permit the use of solvents with RI ranges 1.31-1.6o /only the refractometer marketed by Waters Associates Inc. has a range of 1.o-1.75 RI units/ and the minimum detectable RI /equivalent to noise/ is usually 10^{-7} RI units.

Using a differential refractometer as HSLC detector, also down-scale peaks may be obtained if the mobile phase is lower in RI than the sample component. Gradient elution is very seldom used with RI detectors as it may result in a baseline drift and also cause temperature fluctuations. However, in situations where initial and end-point compositions can be chosen with similar refractive indices, separations by gradient elution can be carried out.[123,177,178] This mode of monitoring combined with gradient elution is applicable only with a reduction in sensitivity of 10 or more.

6.3. Transport Detectors

These detectors based on the ionization detectors developed for GC operate so that the volatile mobile phase should be removed before the detection process. A moving wire, belt, or chain transports a small amount of the effluent successively into an evaporator, a pyrolizing oven, and the detector. The detector may be a flame ionization /FID/, an argon ionization, or an electron capture detector, although today only the first is in use.[32,179-183] This type of detector is destructive only to a small fraction of the sample and thus permits also preparative separations.

In the detector reported by Scott and Lawrence, the sample components are burned to CO_2 and then converted to CH_4 before detection.[184-186] This modification has resulted in a more sensitive system, especially to oxygenated compounds, with predictable and linear response. The sensitivity of this type of moving wire FID has recently been enhanced approximately by a factor of 30 by spraying the

column effluent on the wire instead of coating from a block.[187] As a result of this modification the split ratio and consequently the peak areas no longer depend on the mobile phase flow rate.
Lapidus and Carmen[188] have developed and tested a FID system similar to that of Stouffer et al.[189,190] in which a buffer storage device with a 35 cm long helix made of nichrome wire moves through the flame. Noise levels were generally less than $5x10^{-13}$ amp and unaffected by flow rate changes up to 40 ml/min. The helix speed was suitable between 4.0-7.5 cm/min.
Johnson et al.[191,192] have reported a completely destructive FID using an endless belt which transports the entire column effluent into the pyrolizer to increase the sensitivity of the detector.
A disc FID reported by Dubsky[193] easily transfers the sample with a disc from the column into the combustion space of the detector system. In the detector system, a thin net-screen is placed between the electrodes of the FID and the rotating disc, functioning as a Faraday cage and stabilizer of the flow of gases and vapors. The detector signal is reported to be independent of the intensity and changes in the flow rate of the mobile phase.
Transport detectors have a detection limit of about 2-4 ug/ml.

6.4. Heat of Adsorption Detectors

When a sample component displaces solvent molecules, i.e. adsorbs on the packing material surface /or is desorbed/ causing concentration change in the composition of the effluent, the heat of adsorption is distributed between the solvent and the adsorbent and raises the temperature by an amount proportional to the change in concentration. Heat loss by the eluent from the column can distort this temperature response.[194] Also computer simulation studies suggest that asymmetric response may arise from heat loss from the detector with relatively large volume.[195]

Several approaches have been used in detecting the temperature change.[196-198] The detector system developed by Hupe and Bayer is favored and is simple, sensitive, and nonselective.[199-201] In this equipment, the detection and reference cells sensing the temperature change by thermistors are located adjacent to one another in the mobile phase stream, which significantly reduces noise and baseline drift and improves sensitivity by a factor of 10^4 over heat of adsorption detectors having been reported earlier.

These detectors have an advantage in that they are ideally suited for determining optimum separation conditions and qualitatively visualizing the elution pattern. Their disadvantage is that the detectors give for each eluted component a positive and negative going peak and the interpretation of two not completely resolved peaks is rather difficult. As this type of detector is subject to any thermal effect, e.g. thermal conductivity and heat capacity of the solvent, it should be maintained at an absolutely constant temperature and at constant flow rate.

These micro adsorption detectors can be used with gradient elution only with baseline drift and at reduced sensitivity. The detector is also sensitive to changes in mobile phase flow rate. The dominant flow sensitivity behavior can be attributed to the heating of the solvent by the upstream thermistor and reduced by decreasing the thermistor power dissipation. Munk and Raval[2o2,2o3] have discussed how this power dissipation can be eliminated. They have improved the operation of the detector either by reducing the bridge voltage or by using higher resistance thermistors or by increasing the distance between the thermistors. They have found another similar approach to achieve a lower flow sensitivity by inserting a metal heat conducting disc between the two thermistor discs. The so-called dual micro adsorption detector with two complete detectors in series has also been developed.

Smuts and his coworkers[2o4] have also modified a micro adsorption detector on an adiabatic approach by continuously adding to the detector an adsorbent slurry so that troublesome negative peaks have been eliminated. The sensitivity of the modified device has reportedly decreased and the modification has increased noise.

6.5. Fluorescence Detectors

This type of detector can measure light re-radiated by many compounds including certain metabolites, amino acids, vitamines, steroids, and many drugs excited by UV radiation.[2o5-2o7] Fluorescent derivatives of several compounds can also be made.[2o8,2o9] A reference cell should be employed to balance fluorescence radiated from the mobile phase, although it is emphasized that the solvents to be used should be transparent to both the exciting UV energy and the fluorescence wavelengths.

The fluorimeter may be very useful as a specific, nondestructive detector with sensitivity as high as $1o^{-9}$ g/ml for strongly fluorescing compounds and is also usable with gradient elution. However, fluorescence suffers from vulnerabilities such as turbidity and quenching.

Cassidy and Frei[21o] have equipped a commercial fluorimeter with a small volume flow cell. The detector exhibited a very stable baseline and had a noise level equivalent to 2 ppb. of quinine sulfate in the 7.5 µl cell. The baseline has shown no dependence on the mobile phase flow rate. An increase in the cell volume to 2o µl has not produced noticeable increase in bandwidth for a loo cm column, but the shape of the cell was found to be a significant factor with respect to efficiency. The detector had a rather slow response time as a disadvantage.

Thacker[211] has developed a miniature flow fluorimeter to detect fluorescent compounds in physiological fluids.

6.6. Polarographic Detectors

Polarography can be used as the basis of a selective and sensitive detector in HSLC, measuring continuously and recording the current between a polarizable electrode in the function of time. The solution must have electric conductivity and sometimes it is necessary to introduce a suitable electrolyte into the mobile phase before its entering the column. The sample is, therefore, dissolved generally in an aqueous solution of an indifferent electrolyte.

Koen et al.[212] have employed a micro polarographic detector with a dropping mercury electrode for the analysis of pesticides. They applied a constant voltage, separated oxygen and other electrochemically active impurities by means of a column, and carried out the measurements in a liquid flow, which resulted in a detection limit of about 2 orders of magnitude /$1o^{-7}$-$1o^{-8}$ molar/ over classical polarography.

The polarographic detector reported by Joynes and Maggs[213] consists of a carbon impregnated silicone rubber membrane used as an electrode suitable for oxidation and reduction processes for both organic and inorganic compounds.[214] Reportedly, it gave a sensitivity of about $4x10^{-1}$ Al/mol for organic and $1x10^{-2}$ Al/mol for inorganic compounds and had a detection limit of about $2x10^{-9}$ mol/l for organics. This type of detector is difficult enough to use in quantitative work, since the signal may vary with mobile phase, flow rate, voltage, and cell construction.

6.7. Radioactivity Detectors

Radioactivity measurements may be very important in the investigations of biological systems. Such detectors in HSLC are for this purpose not widely employed today, although the technique has a number of advantages such as wide linear range, adaptability to gradient elution, high sensitivity, and suitable quantitation.

In this method of detection, the effluent may pass through a flow cell or tube packed with a suitable scintillator /today mainly calcium fluoride scintillator is recommended/ so that the isotope will be in direct contact with the scintillator. The light pulses generated can be detected by a photomultiplier.

In a recent work Schram studied various types of scintillation cells and their efficiencies for ^{14}C and ^{3}H.[215] The packed cells mentioned above have efficiencies of 55% for ^{14}C and 2% for ^{3}H. The mixing system presented by Hunt[216], in which a stream of liquid scintillator miscible with the mobile phase is mixed into the effluent, has efficiencies of 7o% for ^{14}C and 3o% for ^{3}H. A similar method has been reported by McGuiness and Cullen.[217] A flow cell, filled with crystalline 2,2'-p-phenylene-bis-/5-phenyloxazole/ as scintillating material, suitable for the continuous measurement of weak beta radiation in conjunction with HSLC has been presented by Sieswerda and Polak.[218] The efficiencies for ^{14}C and ^{3}H in the case of an effective fluid volume of o.lo ml were 51% and 5%, respectively.

Heterogeneous and homogeneous scintillation countings for detecting beta radioactivity in LC effluents have been compared by Schutte.[219] With heterogeneous scintillation counting, the solution is led through a U-shaped flow cell filled with cerium-activated lithium glass beads, whereas in the homogeneous case, part of the effluent is mixed with a scintillator solution and passed through an empty helical cell. The counting efficiency is reported to be better in the homogeneous counting system. In this case the activity per peak may be as low as 2 nCi for ^{14}C and 5 nCi for ^{3}H, without splitting.

For high-energetic beta- or gamma-radiation, conventional radioactivity measurement methods can also be used in HSLC.[22o]

6.8. Other Detectors

Several other detectors have been employed in HSLC for monitoring column effluents. Their common charactarestic is: they were all used in special cases for a certain range of compounds.

Of the current monitoring techniques, the electrolytic conductivity detector is one of the simplest-to-operate and easy-to-maintain. Here the sample entrained by the mobile phase stream is indicated by a change in conductivity.[221-223] Its sensitivity depends on the difference in conductivity between solvent and sample component. Therefore, this type of detectors is useful mainly with aqueous eluent and ionic solutes /e.g. amino acid analyses/ without solvent gradient. Commercially available detectors of such type consist of a flow cell with a two- or three-electrode design, through which the total stream of effluent passes. Under appropriate conditions in temperature and flow rate, compounds as low as 10 µg are detectable and the detector response may be linear with concentration over a wide range. Pecsok and Saunders[224] have recently reported a conductivity detector. In this detector, the contribution of the strongly reduced dead volume /less than 5 µl/ to retention could be neglected. The detector response, however, was nonlinear over the entire range studied because no compensation for different cell capacitance was employed.

When the polarity of sample component and solvent are widely different, the use of dielectric constant or capacitance detector may be profitable.[225] This type of detector is nondestructive to the sample material but not recommended with gradient elution, particularly if the gradient causes a change in solvent polarity. Recently, the theory of various possibilities of employing a capacitance detector in LC has been evaluated by Haderka.[226-228]

The capacitance detector with a capacitor consisting of two brass coaxial cylindrical electrodes reported by Vespalec and Hana has been tested for several solutions.[229] The sensitivity in optimal cases is reported to be lower than 10^{-6} g/ml.

Other types of detectors proposed or applied are gas density balance[230], vapor pressure[231], polarimetry[232], and light scattering[233].

III. Applications

A variety of analytical problems have been solved by means of HSLC and recent developments in this field have given a flexible tool to investigators also for routine analyses.[234-236]

The examples given here in this section of the review are representatives of this flexibility and cover all the branches of the HSLC-separated compounds of interest for chemists and biochemists. Consistent effort has been made to present a complete display as far as possible, but some special cases, being beyond the bounds of possibility, had to be disregarded.

1. Alcohols, Aldehydes

Aliphatic alcohols are usually separated by GC. Aromatic alcohols may be, however, analyzed by HSLC. For this purpose, a Zipax porous layer bead column coated with trimethylene glycol /TMG/ was used.[237] Benzyl alcohol, its methyl- and dimethyl-substituted derivatives, 2-phenylethyl alcohol, and cinnamyl alcohol were separated, using hexane or heptane mobile phases saturated with TMG. On a β,β'-oxydipropionitrile /BOP/ column, benzyl and cinnamyl alcohols had the same retention time, i.e. were not resolved. As aliphatic alcohols are usually undetectable with the present HSLC detectors, their determination at trace level means a challenge for the investigator. Traces of low molecular weight mono and diethylene glycols as 3,5-dinitrobenzoates in polyethylene glycols were determined down to 72 ppm.[238] A porous layer bead column, heptane-ethyl acetate /3:1/ mobile phase, and UV detection have been used.

The compounds except cinnamyl alcohol can be separated on a column packed with 1.5% BOP on Vydac adsorbent.[239] The pellicular silica packing material Pellosil coated with 2% BOP has also been used successfully for separating α-methyl-benzyl alcohol, phenylethylalcohol, and benzyl alcohol.[240] The best resolution for the mixture of the five compounds mentioned above has been achieved on Permaphase ETH chemically bonded packing material.[241]

More recently, Kirkland[242] has separated aromatic alcohols by HSLSC on porous silica micro spheres /8-9 um/ and by HSLLC on this adsorbent /5-6 μm/ coated with 30% BOP.

The 2,4-dinitrophenylhydrazones of the C_1-C_7 aliphatic aldehydes were separated on a porous layer bead column, using heptane-ethyl acetate /97:3/ as mobile phase.[238]

Benzaldehyde and p-nitrobenzaldehyde were separated from aromatic amines and nitro derivatives by HSLSC using isooctane-chloroform /9:1/ as mobile phase.[239]

2. Alkaloids

As a number of alkaloids are of biological and pharmacological importance, an improved method against classical methods of analyzing these compounds is valuable.

Snyder[36] has developed a HSLSC method for rapid qualitative and quantitative analysis of hydrogenated quinoline mixtures. The separation was carried out on an alumina column, using water saturated solvent prepared by passing 35% CH_2Cl_2/pentane through a column of 30% water-silica. A CH_2Cl_2 gradient elution was employed, varying the CH_2Cl_2 concentration from 25 to 60 % by volume.

Also adsorption energies of some alkaloids were measured on acidic, neutral, and basic aluminas.[243] Mixtures of 10% diethyl ether and 10% methylene chloride with n-pentane were used for elution. The results show that the application of n-pentane-10% methylene chloride as mobile phase on acidic alumina is advantageous.

Ipecac alkaloids, Emetine and Cephaeline, were chromatographed on a cyanoethyl silicone column by using n-heptane mobile phase whereas Veratrum viride alkaloids could be separated on TMG or Carbowax 400 stationary phases.[244]

Three major purine alkaloids, caffeine, theobromine, and theophilline commonly occuring together, have been separated successfully on Corasil porous layer beads coated with 1.1% poly G-300 stationary phase, using heptane-ethanol /10:1/ as mobile phase.[245] Also on this column, strychnine and brucine could be determined.

Cation exchange chromatography has also been applied successfully for the separation of pyridine, quinoline, isoquinoline, and 8-hydroxyquinoline with 0.15 M $NaNO_3$ mobile phase.[246] Quinoline and its 8-hydroxy derivative reversed elution at a concentration of about 0.11 M $NaNO_3$.

Separation of substituted benzimidazoles on a cation exchange column has been presented by Kirkland.[55] The compounds were baseline-separated in about 15 min

Talley reported an HSIEC method for the separation of the cyano-, carboxamido-, and carboxy-substituted pyridine isomers achieving quantitative results.[247] Resolution was not sufficient, however, to permit baseline separation of all nine isomers in one chromatogram. The isomeric picolines and pyridine were also separated on a column packed with 1.0% BOP.

HSLC separations of polynuclear aza-heterocyclic compounds on a column filled with silver ion impregnated porous layer bead have been reported.[248] The compounds were separated by donor-acceptor complexing between the silver ions and heterocyclic nitrogen atoms. The elution order was influenced not only by the basicity of the compounds but steric accessibility of the nitrogen lone-pair. Basicity were the only factor, the elution order would follow the order of increasing pK_a. The results indicate that the elution order departs from this rule and does not follow the trend of increasing pK_a.

Ray and Frei[89] developed a brush-type packing material for the separation of polynuclear aza-heterocyclics. The material was prepared by bonding p-nitrophenyl isocyanate to the surface silanol groups of a porous layer bead. This brush material behaves as a good charge acceptor and this behavior for forming donor-acceptor complexes is affected by the stereochemistry of the molecules to be separated. Of these effects, donor-acceptor complex formation plays the major role in the separation process.

3. Amines and Azo Compounds

Ion exchange chromatography resins have been used for both simple and complex analyses of basic amino acids and related compounds. The procedure has been automated in amino acid analyzers for several years.

Cation exchange resin can be well employed in separation of amino acids by HSIEC.[101] The mobile phase is o.1 M ammonium acetate to which hydrochloric acid should be added until pH is 2.5. The reported results show that tyrosine and phenylalanine was not completely separated. Eighteen amino acids were, however, determined by ion exchange separation, as reported by Inglis and Nicholls.[249]

Thyroidal iodoamino acids can be separated rapidly by chromatography on columns of rigid controlled-pore glass using a mixture of ethyl acetate-methanol-2 N aqueous NH_4OH /4o:1o:4/ as eluting solvent, although the separation was classical rather than high speed chromatography.[25o]

Phenetylamines have been chromatographed on an ion exchange column, using o.2 M $NaNO_3$ /pH 3.15/ as mobile phase.[251] During the analysis, temperature was 55 °C.

A systematic survey on the elution of anilines from two brush-type /Durapak OPN and Durapak Carbowax/ and one adsorbent /Corasil II/ columns has been presented by Sleight[252] to gain an understanding of the mechanisms causing retention and the structural factors modifying it. Sleight has well utilized the earlier experiments on the separation of anilines.[63,64,84,1o7] The retention caused by any direct interaction of the substituent with stationary phase due to its electronically induced effect on amino groups was examined with some monosubstituted benzenes. The results do not show clear correlation with constants in the Hammet plot although suggest that electron donating substituents cause increased retention. Retention can be decreased by introducing ortho-substituents into anilines. N-substitution and di-N-substitution decrease retention owing to a decrease in capacity factors.

These results indicate that hydrogen bonding does not play such an important role in aniline retention as does in phenol retention.

On TMG or BOP columns, p,p'-methylene-dianiline can be purified from its impurities.[237]

Porapak Q and T as packing materials have been used for the separation of Freon, isopropanol and N,N-diethyl-m-toluamide which are the basic compounds of sprays.[54]

Substituted aniline and dianiline antioxidants have been separated on porous layer beads coated with 0.5% BOP and on a brush-type column.[93] The elution order was the same on all three columns. Selectivity for each solute was the greatest on the brush-type packing material, resolution and H values were, however, less favorable.

The separation of aniline, N-ethylaniline, and N,N-diethyl aniline can be carried out on a column packed with small diameter /10 μm/ silica gel coated with 33% BOP.[253]

HSLSC has been successfully used for the determination of nitroanilines[239] as well as xylidines.[240]

Azoaniline and substituted N,N-dimethyl- and N,N-diethyl azoanilines have been separated using 10% methylene chloride in hexane as mobile phase.[254]

A solvent program of 5-30% CH_2Cl_2 in hexane at 4%/min may improve the separation.

4. Aromatic Hydrocarbons and Substituted Polynuclear Aromatics

Although GC is very useful in the separation of hydrocarbons, some molecules have low volatility owing to their high molecular weight so that HSLC may be a valuable complementary method in this field of analysis, too.

A classwise separation of a premium gasoline into saturates, monoaromatics, and diaromatics by HSLLC was reported by Stevenson.[255] The elution times of alkylated benzene compounds vary systematically with the number of paraffinic substituents. Increasing number of exocyclic carbon atoms causes decrease in retention time along a straight line. Extrapolation of this behavior indicates that pentadecylbenzene would emerge with the saturated hydrocarbons on a Carbowax 600 column. Naphtenic substitution of the benzene ring apparently increases retention time. Separation of aromatic hydrocarbons, paraffins, and olefins as well as diolefins has been achieved by Locke[256] who used squalane as stationary phase and acetonitrile-20% water as mobile phase.
The separation of six polycyclic aromatic hydrocarbons was carried out on a 8.1% dimethylformamide-Carbowax 350 column with isooctane as the carrier phase.[257] Phenanthrene is not resolved from 7,12-dimethylbenzo/a/anthracene and benzene as the first peak may not be well detected.

Nonpolar solutes such as hydrocarbons may be separated effectively by reverse phase chromatography, i.e. using nonpolar liquid stationary phase and polar carrier liquids. The most useful mobile phases for this purpose are water-alcohol mixtures, although water-dioxane and water-butyl cellosolve may also be employed. The resolution and retention of polynuclear aromatics are ruled by the alcohol percentage. At 90% alcohol content in the mobile phase, all aromatics elute as a single peak which gives a method for the rapid estimation of total aromatics present in mixtures.
The advantage of reverse phase chromatography can

be extended to more polar solutes such as anthraquinones by increasing the concentration of water in the mobile phase.[258]

The recently developed chemically bonded reverse phase packing materials have substantially improved the efficiency and resolution for these compounds.[259,260] In addition to these compounds, chloro-, bromo-, iodobenzenes, and mixed halogen derivatives of aromatics have also been chromatographed with this system.

HSLSC is also a method considerable for the determination of polynuclear aromatic hydrocarbons. Rapid baseline separations can be achieved both with porous layer bead silica and with neutral alumina packing materials, partially deactivated with water. A change in these packing materials causes a reverse order of elution of benzanthracene and o-quaterphenyl. In the case of alumina, by changing the solvent, pure hexane for hexane-isopropyl ether /60-40%/, all components are eluted sooner and benzanthracene is eluted last, after m-quaterphenyl.

5. Compounds in Body Fluids

The normal and pathological constituents in body fluids as urine and blood are very important but are contained in complex biological mixtures.

Scott and coworkers have developed a method for separating the UV-absorbing constituents of body fluids by HSIEC.[137,261-264] They have demonstrated the separation of more than one hundred constituents from human urine. Several of these constituents have been qualitatively identified.[265]

The chromatograms obtained from such separations may be employed as fingerprints in evaluating body function. Burtis has used strongly basic anion exchange resin and linear sodium acetate gradient elution varying in concentration from o.o15 M to 6.o M at constant pH 4.4.[266,267] The results suggest that, assuming a molar absorptivity of 1o,ooo, ng quantities of some constituents can be detected. To use such fingerprint profiles for diagnostic purposes, dietary effects must be considered.[268]

The method described above can be applied for the analysis of the carbohydrates in body fluids with gradient elution of an aqueous borate buffer.[137,268] Since carbohydrates do not absorb UV light at the commonly available wavelengths, phenol/sulfuric acid color development reaction should be used.

6. Drugs and Related Compounds

In GC analyses of drugs and related compounds, preparation of chemical derivatives should be made prior to the analyses because these compounds are usually nonvolatile.

HSLC not only surpasses this problem but offers a method, ion exchange, with no analog in GC and which is ideally suited as a separation technique in pharmaceutical analysis for a large percentage of compounds.[269]

An APC tablet /aspirin, caffeine, and phenacetin/ has been examined as a test mixture for cation exchange, and optimum separation of this sample was achieved with a mobile phase of distilled water buffered to pH 6.8.[246]

Components of analgesic tablets containing other compounds in addition to the three mentioned above, can be easily separated by HSIEC, too.[27o] The mobile phase should be adjusted to a pH of 9.2 with a borate buffer and the ionic strength is adjusted by the addition of o.oo2 M $NaNO_3$. Increasing the ionic strength of the moving liquid reduces analysis time without significantly impairing the resolution of the sample components. It should be also noted that an anion exchanger elutes a typical analgesic in the following order: caffeine, phenacetin, and aspirin, whereas a cation exchanger gives an elution order of aspirin, caffeine, and phenacetin. Using an anion exchange column with 1.o M tris/hydroxymethyl/ aminomethane /pH 9.o/ as mobile phase, the elution order is caffeine, aspirin, and phenacetin. Anion exchange analysis of a single tablet of asthma and hay fever medication, containing ephedrine, theophilline, and phenobarbital can be carried out using o.ol M $NaNO_3$ as solvent.[271]

Anders and Lattore[272] have managed to apply HSIEC for the separation and determination of barbiturates, some of their metabolites, and related compounds, among others, diphenylhydantoin. Nongradient elution with KH_2PO_4 buffers has proved very suitable for separating phenobarbital, diphenylhydantoin, and their phenolic metabolites. The best conditions found for this analysis are 2o mM phophate buffer, pH 3.5, and a column temperature of 8o °C. Barbiturates and metabolites have been separated with a linear sodium chloride gradient at 8o °C.

Barbiturates can also be determined successfully by using an anion exchanger and o.ol M $NaNO_3$ mobile phase[273] or using porous layer beads and 2% methanol in heptane as mobile phase[239].

Reverse phase chromatography has proved effective in the determination of hashish, using a chemically bonded packing material and a linear gradient elution of 2% change/min. of methanol in water.[26o] However, LSD from an illicit tablet can be determined by anion exchange.[274]

A chemically bonded packing material, Permaphase ETH, has given well-separated peaks of sulfapyridine, sulfamethazone, and sulfanilamide, using n-hexane/ isopropyl alcohol /60-40%/ as mobile phase.[241] Reverse phase HSLLC has provided a highly specific and practical technique for the analysis of sulfonylurea antidiabetic agents.[275] The mobile phases were 0.01 M sodium borate containing 27.5% methanol and 0.01 M monobasic sodium citrate containing 15% methanol. The elution volumes for all the sulfonylureas investigated decreased as pH was raised, indicating that these compounds being weak acids are more soluble in solutions of higher pH.

A method for the determination of a quinazoline derivative drug, metolazone, in urine is described by Hinsvark et al.[276] A polar, brush-type packing material and chloroform modified with 5% isopropanol have been used.

Another polar, brush-type material has been employed for the analytical scale preparation of some benzodiazepines at ug level in urine.[277] The most polar carrier used in the analysis was a 70-30% mixture of hexane and isopropanol, although an optimum composition has been achieved at 10% isopropanol content.

The combination of a Zipax porous layer bead column and hexane-ethanol /75-25%/ as mobile phase has permitted low-level detection of some impurities in a 3-formylrifampin sample.[278] At the same time, it provides good measurement of the strongly polar, high-molecular weight major component.

A method for the analysis of Clopidol, an effective coccidiostat, has been developed.[279] The method involved chromatography on an alumina and on an ion exchange column in series with o.1% acetic acid gradient elution in methanol.

Also HSLSC may be very effective in the analytical chromatography of synthetic organic drugs. It can be used to determine the yield obtained under various reaction conditions and to suggest preparative schemes for purifying the products of each synthesis step. 1-/3,4-dibenzyloxyphenyl/-2-nitro-trans-prop-1-ene and 3,4-dibenzyloxybenzaldehyde were separated on a Corasil II porous layer bead column, using 1o% chloroform in isooctane as moving phase.[28o]

7. Herbicides and Insecticides

Although herbicides and insecticides may strongly vary in their chemical composition and the examples given here serve only as starting points for the selection of a suitable chromatographic system, it is obviously expected and clear that HSLC may be employed for all types of these compounds.

Substituted urea herbicides were chromatographed by HSLLC on BOP stationary phase, using several types of liquid as mobile phase.[62,99,281]

For the analysis of esters of phenoxyacetic acid-base herbicides, Permaphase ODS reverse phase packing material has been used with a 4o% water - 6o% methanol mobile phase.[282]

The free acids eluted with the solvent front under these conditions can be chromatographed by reducing the methanol content in the mobile phase and adding dilute phosphoric acid.

With a reverse phase Bondapak C_{18} column, 5o% water-5o% methanol was necessary to be used as mobile phase for the separation of fenuron, nonuron, and diuron.[282] A high-sensitivity detection of diuron /down to 5 ng/ has been achieved on a polar chemically bonded phase with hexane modified by 3% dioxane carrier liquid.[283]

Triazine herbicides are well separable on a BOP column, using a modified cyclohexane mobile phase, and ion exchange columns also proved applicable in the separation of such compounds.[284]

The separation of a mixture of thermally unstable pyrethins can be achieved rapidly and quantitatively on a silica gel adsorbent, using 20% diethylether in hexane as mobile phase.[285] Quantitative studies of pyrethins were carried out also on a reverse phase chemically bonded packing material and by HSGPC.[260]

HSLLC also proves effective in the analysis of insecticides.[237,286] Insecticide carbaryl, for example, can be separated from its primary decomposition product 1-naphtol on a BOP or TMG column, using n-heptane saturated with the stationary phases BOP and TMG, respectively.

A baseline separation of a synthetic mixture containing the active ingredient of Lannate methomyl insecticide could be obtained on a 1% BOP-porous layer bead HSLLC column with n-hexane modified by 7% chloroform.[121]

8. Metal Ions

Ion exchange chromatography is a widely accepted method in the separation of metal ions by elution with HCl or HCl-modified mobile phases.[128,287-290]

Hydrobromic acid mobile phase is also useful in certain cases.[291]

While good separations can be obtained by classical methods, the rapid, quantitative separation of substantial amounts of such elements was not presented prior to the development of HSIEC.[292]

Campbell et al. and other workers have, however, demonstrated that high-speed separation of rare earths is practical.[293-295] The technique used in the investigations consisted of loading the trivalent lanthanides and actinides in a weakly acid solution onto a cation exchanger and then eluting the metal ions with an anionic complexing agent. In the separation of elements being highly radioactive, small diameter resins should be employed to avoid radiation-induced resin damage and radiolytic gas evolution by reducing the analysis time.[296]

In addition to particle size, the type of the mobile phase can significantly influence the quality of separation. The importance of solvent gradient, particle size, and resin cross-linkage on resolution and efficiency has been examined by Sisson et al.[297]

A ternary liquid-liquid two-phase system composed of water, isooctane, and ethanol has also been used to separate metal-β-diketonates by HSLLC.[298]

Veening[299] has demonstrated the usefulness of HSLLC in separating metals as metal carbonyl complexes, using isooctane mobile phase and UV detection.[299]

9. Nucleic Acid Constituents

The constituents of nucleic acids are nowadays perhaps the most studied field of compounds in separations using HSLC. This part of the review demonstrates application of HSLC instrumentation to these investigations, but only to a limited extent in order not to overstress this field at the expense of others. It is preferred to refer to more extensive and remarkable surveys.[300-302]

All the chemical characteristics of the nucleic acid constituents suggest that HSIEC is a very functional and desirable mode of separation, al -

though the degree of ionization is subject to temperature, pH, and ionic solvent. In the case of bases and nucleosides, strongly acidic cation exchangers whereas for nucleotides, strongly basic anion exchangers are preferred. To incorporate fast mass transfer, rapid ion exchange, and rapid equilibration, resins with small diameter should be chosen.

To determine which parameter to adjust for optimum results in separations of nucleic acid constituents, one should keep in mind several useful guidelines described by Horvath[99,303], Uziel et al.[304], and Burtis[305].

10. Pesticides

Although pesticides can be analyzed by GC, these compounds frequently require preliminary time consuming clean up steps and the synthesis of derivatives for analysis. Therefore, HSLC may be a complementary method for the analysis of pesticides. The analysis of carbamates and their decomposition products has been carried out on BOP or TMG column. The TMG column has proved more selective for hydroxyl groups in the compounds. Many of the chlorinated hydrocarbon pesticides have also been chromatographed on a BOP column with heptane-chloroform or on a reverse phase column with methanol-water mobile phases.[306] Lindane, Endrin, Heptachlor, DDT, and DDD have been separated on a reverse phase chemically bonded packing material with 70% methanol-30% water mobile phase.[307] Parathion, Folpet, and Imidan have been determined on a polar chemically bonded stationary phase, using isooctane as solvent.[308] Pesticide residue analysis of Dyfonate can be carried out by HSLSC with a 2.5% chloroform in isooctane mobile phase.[309]

HSLLC has been used by Henry et al.[310] to detect and isolate impurities in the pesticide Abate. The chromatographic procedure was combined with a bioassay experiment.

11. Phenols and Related Compounds

Sleight[252] has studied not only the electronic effects of substituents on the retention of anilines but also those of phenols. The results suggest that electron-withdrawing substituents increase phenol retention and vice versa. Retention is decreased by introducing ortho-substituents into phenols, and for ortho alkyl phenols this decrease appears to be in correlation with the size of the alkyl group.

HSLSC has proved very effective in several separations of phenolic compounds. To directly extrapolate separations by TLC to HSLSC, three hindered phenolic antioxidants have been separated on a silica gel plate and on a porous silica layer bead column.[93] In both cases the mobile phase was hexane with 1% isopropanol modifier. The elution order was the same on TLC and by HSLSC.

Also on silica gel, phenol, xylenol, and p-nitrophenol have been chromatographed with dichloromethane moving liquid.[311]

The 2,6-, 2,5-, and 3,4-xylenols were separated on a column packed with Mercosorb SI 60 fine silica particles with dichloromethane solvent.[312]

Phenols, however, may be resolved also by HSLLC. Huber[105] has separated dimethyl- and trimethylphenols as well as 3-methylphenol on a porous material coated with 6% 1,2,3-tris/2-cyanoethoxy propane/, using isooctane as mobile phase.

12. Phthalates

The separation of a mixture of di-n-alkyl phthalates has been carried out on a porous layer bead column coated with o.8% BOP, using hexane as moving solvent.[95] Separation of diphenylphthalate from insecticide, EPN, has been achieved on a o.5% BOP column with hexane.[62]

Didecyl-, dibenzyl-, and decylbenzyl phthalate plasticizers, which can be determined by GC only after being saponified, have been chromatographed successfully on a porous layer bead column coated with BOP, using isooctane carrier liquid.[93]

Phthalic acid isomers make a suitable test mixture for the evaluation of a strong cation exchange column performance using water as mobile phase.[313] Near the pK of the compound, pH has a great effect on retention and resolution and this effect may cause a change in the elution order. By adjusting the pH to 2.75, the elution order is as follows: phthalic, terephthalic, and isophthalic acids. The retention times of the compounds are strongly influenced by ionic strength changes.

13. Polymer Resins

As the physical properties of polymers of relatively high-molecular weight are strongly related to their molecular weight, the separation of polymer compounds can be achieved by a technique, called steric exclusion or gel permeation chromatography, based on molecular size.[314-321] The degree of retention depends on the size of the sample component molecule relative to the size of the pores through which the selective diffusion of solute molecules proceeds. Therefore, molecules larger than the pore size may possibly be completely excluded from the pores and will elute from the column before the smaller molecules. This technique of separation is preferred at molecular weight ranges above 2000 units. From the results obtained by GPC, one can determine the polymer molecular weight distribution or an average molecular weight. Polyvinyl alcohols from various methods of production have been separated on four columns in series, each packed with gel of different pore size.[70]

The separation of surfactants by HSGPC can be carried out on gels with pores of 500 Å, using tetrahydrofuran as mobile phase.[322] The separation of crude oil before and after distillation has shown the potentiality of HSGPC as a distillation analyzer and to replace or supplement distillation and GC because the method is nondestructive and usable at ambient temperature. Aqueous steric exclusion analysis of polysodium silicate has been carried out on a porous silica column.[323]

On a column packed with 5-6 um porous silica microspheres with pores of 350 Å, polystyrene fractions with molecular weight of 2000, 51,000, and 411,000 have been separated, using tetrahydrofuran as carrier.[242] A mixture of low and high molecular weight polystyrene polymers can be separated also on glass columns.[324]

The monomers and polymers in phenol formaldehyde resins can be clearly identified by HSGPC.[325]

Ede[326] has developed a HSGPC method for determining the molecular weight distribution of Nylon 6.[326] Using this method, good agreement has been obtained by Ede between number average molecular weights determined by HSGPC and by end group determinations. The variation of distribution with polymerization time has also been studied.

Although as described and noted above, GPC is nor-

mally used for large molecules with molecular weight above 2000 units, small molecules may also be rapidly separated by this method.[322,327,328]
The HSGPC method can also be used for preparative work, sometimes with recycling.[329-331]

14. Steroids

Representative members of several classes of steroids have been separated by Siggia and Dishman using HSLC.[332] Reverse phase chromatography has been used throughout their study because the relatively labile steroids investigated are quite polar and elute slowly enough using nonpolar eluants. Therefore, the mobile phase consisted of a mixture of water-methanol or water adjusted to pH 11.5 with NaOH. The retention time of the fast eluting, more polar adrenal corticosteroids decreased with increased stationary phase loading while the less polar steroids show a minimum retention at a given loading and then increased retention at higher loadings. This behavior can be explained by the fact that as the loading is decreased the contribution of the liquid-solid adsorption is increased and when liquid-liquid partition becomes predominant, the retention times increase with loading. For the polar steroids, the adsorption process has been predominant over the loading range /18-35%/ studied by Siggia and Dishman. For the separation of nine corticosteroids, step-wise flow programming has been employed. Under the same conditions, the progestins eluted after all the corticosteroids and androgens chromatographed. Therefore, to determine these compounds, a water-methanol mixture is recommended as mobile phase. Progesterone was, however, not satisfactorily separated from 4-pregnene-20β-ol-3-one.

Huber et al.[333] carried out the quantitative analysis of trace amounts of estrogenic steroids in pregnancy urine. Two coexistent phases of the ternary system water-methanol-isooctane have been used as the stationary and mobile phases. Partition coefficients of 28 steroids in this ternary system were determined earlier.[8]

Henry et al.[334] employed normal phase, reverse phase, and ion exchange chromatography to separate 2,4-dinitrophenylhydrazine derivatives of all major types of steroids and the use of both UV and RI detectors for steroids is discussed. Conventional liquid-liquid partition chromatography has proved to be the most useful for separating mixtures of closely related steroids. Ethylene glycol, BOP as well as cyanoethylsilicone have been used as stationary phases. Progesterone represents about the least polar steroid that can be partitioned on a BOP column. For the separation of estradiol glucosiduronic acid from impurities, a strong anion exchanger has been applied with a linear gradient of $NaClO_4$ in phosphate buffer.

The separation of the compounds studied by Henry et al.[334] may be achieved also on chemically bonded phases.

HSLSC has also proved very effective in the separation of steroids, using methanol-chloroform, hexane-methanol-chloroform or isooctane-methanol-trichloromethane mobile phases.[239,312,331]

15. Vitamins

HSLC presents a well-suited method for the analysis of fat and water soluble vitamins.[325] Fat soluble vitamins are best chromatographed by the reverse phase technique whereas the ion exchange separation process can be well applied to the determination of water soluble vitamins. When a multiple vitamin sample is being separated it may be necessary to change the mobile phase by gradient elution because these vitamins vary widely in their structures and chromatographic properties. A typical gradient elution separation of six fat soluble vitamins can be carried out on a chemically bonded reverse phase packing material, using gradient from water to methanol at 5%/min. Also on this column, a tocopherol mixture may be separated using a gradient from methanol-water /80-20%/ to methanol-water /85-15%/ at 1%/min. As vitamin K is the most water soluble of the fat soluble vitamins, it is chromatographed in a solvent at 20% methanol- 80% water.

The water soluble vitamins to be chromatographed may be divided into two classes; those that separate by anion exchange and those that separate by cation exchange. The vitamins B_2, B_3, and B_6 can be determined by cation exchange at pH 4.2 in KH_2PO_4 buffer modified by sodium perchlorate. The separation of the vitamins B_1 and B_{12} requires a pH of 9.2. Ascorbic acid, folic acid, and niacin can be chromatographed on an anion exchanger with a mobile phase adjusted to pH of 7.0 with various concentrations of **ionic** modifier added. A mobile phase of 0.02 <u>M</u> sodium acetate at pH 4.6 is also useful.

HSLSC can also be employed for the analysis of vitamins. On a porous layer bead column, A, D_2, and E vitamins have been separated using chloroform as mobile phase.[336]

A mixture of vitamin A palmitate, vitamin A acetate, vitamin D_2 has been separated on a Pellumina column with 1-chlorobutane mobile phase.[337] A number of fat soluble vitamins have been successfully chromatographed on a column packed with small-particle silica adsorbent, using 1% isopropanol in hexane as mobile phase.[338] A mixture of hexane-dichloromethane-isopropanol as mobile phase has been used for the analysis of vitamin A acetate and vitamin E on silica adsorbent.[154]

List of Symbols

K	distribution/partition/ coefficient
V_R	retention volume of a component
V_m	interstitial volume of the column
V_s	the volume of stationary phase in the column
V_N	the net retention volume of a component
V_g	the specific retention volume of a component
w_s	the weight of stationary phase in the column
ρ_s	the density of stationary phase
k	partition ratio/capacity factor/
β	phase ratio
u	the solute chemical potential
T	temperature
P	pressure
γ	the solute activity coefficient
M	molecular weight
ρ_m	the density of mobile phase
v	the solute partial molar volume
	relative retention
t	the time of analysis/retention time/
N_{req}	plate number required to achieve a given separation
H	plate height
u	mobile phase velocity
ΔP_{req}	pressure drop required to N_{req}
N	plate number
ΔP	pressure drop across the column
	the viscosity of eluent

d_p particle diameter
d_c column diameter

Acknowledgment

We thank Prof. Dr. G. Schay for critical reading of the manuscript.

Trademarks

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