JOURNAL OF LIQUID CHROMATOGRAPHY

March 1983

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Manuscript Preparation and Submission. See the last page of this issue.

Subscription Information. *Journal of Liquid Chromatography* is published in fourteen numbers and two supplements in January, February, March (3 numbers), April, May, June (2 numbers), July, August, September (2 numbers), October, November, and December by Marcel Dekker, Inc., 270 Madison Avenue, New York, New York 10016. The subscription rate for Volume 6 (1983), containing fourteen numbers and two supplements, is $298.00 per volume (prepaid). The special discounted rate for individual professionals and students is $149.00* per volume. To secure this special rate, your order must be prepaid by personal check or may be charged to MasterCard or VISA. Add $36.80 for surface postage outside the United States. For airmail to Europe, add $72.32; to Asia, add $91.52.

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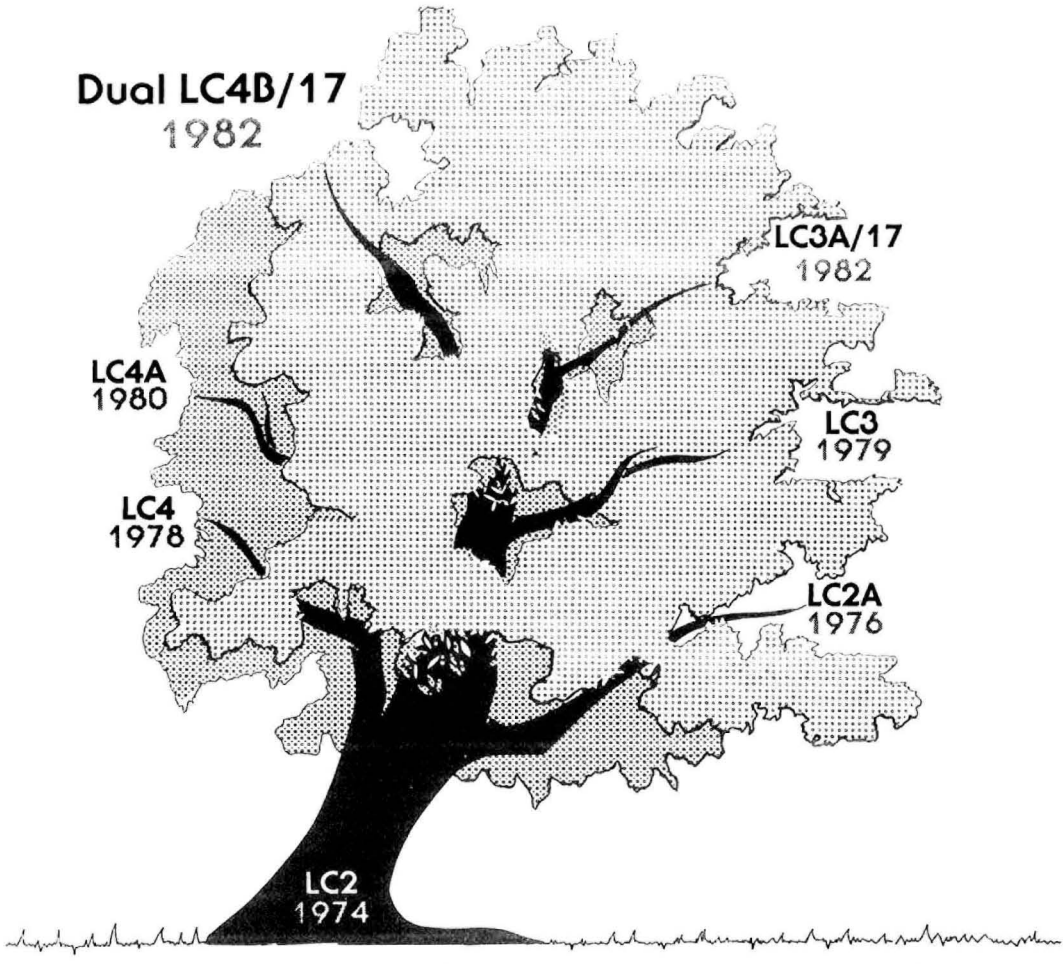

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JOURNAL OF LIQUID CHROMATOGRAPHY, 6(S-1), 1-36 (1983)

PROSPECTS FOR CARBON AS PACKING MATERIAL IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Early work on use of carbons for liquid chromatography shows that special carbons must be developed if carbon is to be useful in HPLC. The processes used to make carbons, the properties of such carbons and the requirements for HPLC carbons are discussed. The preparation of HPLC carbons is reviewed in detail and the results which have been obtained are assessed in terms of their kinetic and thermodynamic performance. Applications are reviewed.

It is concluded that none of the existing HPLC carbons combine all the desired features of particle rigidity, adequate surface area and uniformity of surface chemistry but that there is no reason to doubt that such a material can eventually be prepared. The kinetic performance of existing materials is probably adequate but peaks are often wide due to inadequate control of the surface chemistry. Carbons from different sources exhibit similar but not identical selectivity. Broadly

 0148-3919/83/06S1-0001$3.50/0

carbons exhibit different selectivity from reversed phase silica gels. In particular they show greater discrimination of isomers and members of homologous series but less discrimination of functional groups. Carbons show much stronger retention with any eluent than do reversed phase silica gels but different eluents (other than water) show rather a small range of eluotropic strength.

1. INTRODUCTION

Active carbon adsorbents have for many years played an important role in industrial purification and refinement processes (1). Carbon was therefore an obvious choice for the early fundamental studies in column liquid chromatography by Tiselius and co-workers (2,3). The carbons used at that time and indeed until quite recently, were made from industrial products and were not specifically designed for chromatography. Amongst the substances separated on carbon using polar eluents were oligosaccharides and their methylated derivatives (4-7), amino acids (8-10), fatty acids and fatty alcohols (11) and even 5'-nucleotides obtained from the enzymatic digestion of RNA and DNA respectively (12). Analysis of retention patterns revealed that aromatic derivatives were more strongly adsorbed than aliphatic derivatives: this was attributed to the graphite-like surface of active carbons. In order to prevent irreversible adsorption of the solutes, carbons were often pre-treated with modifiers, e.g. stearic acid, n-octadecane, n-octadecylamine etc. (10, 13). With stearic acid as deactivating reagent, for example, aromatic amines could be eluted from carbon columns with high recoveries (10). Detailed studies were carried out on the effect of molecular size and eluent composition on the adsorption of methyl derivatives of pyridine (14,15). During this period the main theoretical interest in liquid chromatography centred on the treatment of adsorption phenomena and the evaluation of separation techniques: the status of carbon in this context was reviewed by Snyder in 1968 (16).

During the years following the early chromatographic experiments on carbon, manufacturing processes for carbons were extended and improved; carbon blacks in various grades and with defined properties were produced in large quantities for the rubber industry; the high-temperature treatment of carbon materials, including graphitization, was thoroughly examined. Concurrently gas chromatography was developed as a potential separation technique (17, 18). In gas-solid chromatography active carbon was employed as adsorbent for hydrocarbon separations (19) and graphitized carbon blacks were chosen as model adsorbents for examining gas-solid interactions (20,21), due to their non-polar and homogeneous surfaces. Graphitized carbon blacks were also employed as supports in gas-liquid chromatography (22,23) and as stationary phases in capillary columns, (24,25). Research between 1960 and 1970 in gas chromatography resulted in excellent separations of volatile hydrocarbons and provided a much better understanding of the phenomena occurring at the gas-solid interface.

For completeness' sake it may also be noted that a few experiments have been performed on active carbon as a support in thin-layer chromatography (26,27). The black colour of the plates, however, made a simple visualization of the spots difficult, although the drawback could be avoided by using radio-labelled solutes or by subsequent elution from the carbon into a silica layer.

Early in the 1970's a new era in chromatography opened with the development of high performance column liquid chromatography for which microparticulate silicas and their surface modified derivatives rapidly became the preferred column packings. However, in the search for new supports showing different selectivities and greater resistance to hostile eluents, especially alkalis, attention has once again turned to carbon. In contrast to 30 years ago, highly developed technologies now exist for producing various kinds of carbon: active carbons,

pyrolytic carbons, glassy carbons, carbon blacks and graphitized carbons. Between them these materials possess a wide range of surface and pore structures. Nevertheless, such carbons still, in the main, have been developed for purposes other than chromatography, e.g. as adsorbents in water treatment, as coatings, as fibres, as fillers etc. It is not therefore surprising that the development of carbon packings for HPLC has had to be carried out independently. The present review describes the work of the last decade and concludes with a critical discussion of what has been achieved.

2. PREPARATION OF CARBON PACKINGS

2.1. Desirable Characteristics for a Column Packing Material

To be applicable as a packing in HPLC a particulate carbon should possess the following characteristics: (i) sufficient hardness to withstand high pressures: (ii) a well-defined, reproducible and stable surface which shows no change during chromatographic work or storage: (iii) a specific surface area in the range of 50 to 500m^2/g to give adequate retention of solutes and to maintain a reasonable linear sample capacity: (iv) a mean pore size not less than 10nm and an absence of micropores in order to ensure rapid mass transfer of solutes into and out of the particles: (v) ease of preparation by a simply controlled, low-cost process.

The extent to which these often conflicting characteristics can be achieved will be dependent upon the type of starting material, upon the procedure chosen for preparation of the product and upon the conditions under which it is used. Evidently numerous routes can be taken in the synthesis of tailor-made porous carbon products and much work is still required to provide carbons with optimum properties for HPLC.

2.2. Processes involved in Carbon Formation

In principle four consecutive stages may be distinguished in the formation of carbons from naturally occurring or synthetic precursors: homogenization, carbonization, volatilization of inorganic impurities, and graphitization.

The term "homogenization" covers all operations which lead to an improved ordering of the structure of any solid or liquid carbonaceous starting material. It usually consists of a thermal treatment of the starting material at 700 to 1000 K in an inert atmosphere. It is well known that the degree of structural order of the carbon precursor essentially determines the extent to which the penultimate material is converted into a graphitic or an amorphous carbon, the two limiting cases.

Carbonization covers a number of processes including coking, charring and reaction with oxidising gases such as oxygen, carbon dioxide and water vapour. It is carried out between 1000 and 1500 K (28). Carbonization increases the percentage carbon content and introduces pores. The products so formed are termed active carbons and possess a high adsorptive capacity. Carbonization also covers processes whereby a gaseous hydrocarbon is pyrolysed between 1300 and 2000 K to yield dense non-porous layers of pyrolytic carbon (29).

Active carbons may still contain inorganic impurities such as sulphur and silica depending upon their origin. These can be removed by volatilization at 1500 to 2000 K. This process leaves a large number of defect sites in the structure and causes a disordering of the mutual arrangement of layers. Microscopic holes may even be formed within the particles.

Graphitization covers the subsequent heat-treatment in an inert atmosphere at 2000 K to 3200 K. Such heat treatment brings about densification with concurrent removal of structural defects, and forms a three dimensionally ordered graphitic

structure (30). The degree of graphitization of any carbon brought about by high temperature treatment depends strongly on its initial source and structure. Thus treatment of some active carbons at temperatures as low as 1500 K can greatly reduce or even completely eliminate the porosity of the material, whereas some glassy carbons may not convert to graphite even on heating to 3200 K.

2.3. Consideration Relevant to the Synthesis of Carbon Packings for HPLC

Particle Size and Shape

With a granular starting material of sufficient hardness, angular microparticles can readily be obtained by milling and size-grading into narrow cuts (31). Alternatively soft carbons such as carbon black agglomerates can be reinforced by depositing pyrolytic carbon as binder (32,33). Spherically shaped carbon particles in the 5-200μm size range can be obtained by thermal treatment of polymer beads (34-36). With all pre-sized materials note must be taken of the extent to which consecutive treatments may change the shape, size and size distribution of the original particles.

An essential requirement of any final material for HPLC is that it must possess sufficient hardness to withstand high pressures and high eluent flow rates during column packing and operation. The hardness of carbon is directly related to its molecular and pore structures: thus layer-type materials such as graphite are soft whereas amorphous glassy carbons possess high mechanical strength due to their microcrystalline mosaic structure. Between these two limiting cases a large number of structural intermediates exists. One of them is the so-called turbostratic structure proposed by Biscoe and Warren (37). The layers in such carbon materials are 'arranged roughly parallel and equidistant but are not otherwise mutually oriented'. It may be concluded that because of their low

mechanical strength highly ordered graphite-like carbons are less likely to be suited as packings in HPLC than microcrystalline or amorphous carbons.

Particle porosity also has an important effect on mechanical strength. In general particles are too fragile for HPLC when the particle porosity exceeds about 70% (a typical silica gel, for example, has a particle porosity of about 55%). A complicated situation arises with carbons of composite structure, e.g. reinforced carbon blacks. These have an inhomogeneous molecular structure as well as being porous. No predictions can be made for such materials: they must be tested under experimental conditions.

Surface composition

Apart from its primarily aromatic character the carbon surface carries a variety of functional groups. The majority are acidic groups such as carbonyl, carboxyl and phenolic hydroxyl but some basic groups (38,39) may also be present. These functional groups are located at defect sites which occur randomly at the edges of crystallites. Thus the less crystalline the product the higher will be the concentration of polar surface functional groups, although even crystalline graphite rapidly chemisorbs oxygen when exposed to the atmosphere. The polar surface functional groups can be eliminated by annealing at about 750 K (40) but some will immediately reform on exposure to air. A more effective means of removal is hydrogenation at 1300 K. Alternatively, more specific surface reactions can be carried out to mask the polar functional groups, as is done in surface modification of silica (41). Graphitization followed by chemical reduction of the surface will apparently offer the best chances of success in preparing a good adsorbent surface.

However, given the considerations of the last two sections, it becomes apparent that there are serious difficulties in

making robust carbons with stable and well defined surfaces for, while graphitization may improve surface properties, it will reduce the particle strength. A compromise may have to be accepted, or alternatively a composite structure may be required.

Origin of Porosity, Pore Size and Specific Surface Area.

The origin and characterization of porosity in carbons including graphite has been the subject of several detailed studies (42-45). Evans and Marsh (42) claim that the "shape, size and distribution of porosity are intimately associated with the (molecular) structure of carbons and graphites". Isotropic and non-graphitizable carbons are microporous, whereas anisotropic graphitizable carbons generally exhibit little microporosity. Pore size and shape can be observed by optical microscopy, scanning electron microscopy and phase-contrast high resolution electron microscopy. Characteristic parameters describing the size and volume of micropores of below 3nm diameter are derived from sorption measurements (44). Apart from open and accessible pores carbon may also contain closed pores which are indirectly evidenced by comparing the apparent density measured by helium with that of pure graphite.

Each population of pores contributes to the total specific surface area. Micropores can potentially contribute surface areas exceeding $1000m^2/g$. So-called supermicropores will provide even higher areas although by this stage the concept of surface area loses its physical meaning. In general active carbons have high surface areas whereas graphitized materials have low surface areas. Carbons having high surface areas due to micropores are not suitable for use in column liquid chromatography on account of slow mass transfer, high activity in adsorption, heterogeneity of surface activity and too high retention. Undoubtedly one of the major problems in the manufacture of porous carbons suitable for HPLC arises from the extreme difficulty of eliminating micropores.

The above discussion indicates that it will be very difficult to obtain an ideal material having the correct combinations of particle rigidity, adequate surface area, uniformity of pore structure and uniformity of surface composition. Inevitable compromises will have to be accepted. The best solution may rest with a composite material having a hard microcrystalline framework with 10nm pores, the internal surface of which is coated with a thin dense layer of graphite.

2.4. Preparation Procedures

The most extensive work on the preparation and characterisation of carbons for HPLC has been that of Guiochon and co-workers (32,33) who have devoted much effort to the modification of carbon black agglomerates. These agglomerates are made up from independent spherical particles of submicron size: they possess a specific surface area of between 10 and 200m^2/g (46) and significant porosity. Owing to their origin, carbon blacks consist of small crystallites of graphitic structure, sometimes containing intrusions of amorphous carbon (47), and they possess a variety of functional groups chemically bound to the surface which give rise to their oxygen and hydrogen content. Heat treatment at about 3000 K in an inert atmosphere removes these surface groups. It also leads to an intense growth of crystals, and improves their mutual arrangement and orientation. The density of particles of graphitized thermal carbon blacks (GTCB) approaches that of graphite and the surface becomes non polar and homogeneous (47). However, the aggregates of these polyhedric microparticles are mechanically very fragile. Reinforcement of aggregated GTCB particles can be performed by depositing a thin film of pyrolytic carbon through pyrolysis of benzene at 1200 K (32,33,48). The hardness and specific surface area of pyrocarbon-modified carbon black (PMCB) are closely related to the amount of pyrocarbon deposited (32,33). While coating is traditionally performed in fluidized beds (49), Colin et al (32,33) used a quartz tube containing a crucible filled with carbon powder.

Inert gas containing a low partial pressure of benzene was passed while the tube was heated in a furnace at about 1200 K. The properties of pyrolytic carbon, namely its anisotropy, apparent density, crystallite size and microstructure, are strongly influenced by the temperature, the gas composition, the contact time and the bed surface area (49), but in general it possesses a turbostratic structure. For low surface area carbon blacks, e.g. Sterling FTFF, the amount of pyrocarbon deposited was proportional to the contact time and the concentration of benzene circulated through the furnace. The load could be increased to 50% w/w or more, but high loading resulted in a sharp drop from the initial surface area and in a heterogeneous surface (49). The optimum deposit was about 15-20% (w/w) which still provided sufficient mechanical strength. Surface purification was achieved by hydrogen treatment at 1300 K or by thermal treatment at 3000 - 3300 K. Surprisingly such treatments gave only slight improvement in chromatographic properties, such as peak profile and loadability (32,33,50). In producing materials for HPLC, problems arose in sizing batches of 8-10μm of carbon blacks, in coating these agglomerates and in sizing the resulting powder. Consequently, most studies were performed on particles with dp > 15μm. Later Colin and Guiochon (51,52) adopted the benzene pyrolysis procedure to deposit pyrocarbon on the surface of silica as developed by Bebris et al (53, 54). Thermally pre-treated silica gels were employed and again the optimum carbon deposit for avoiding particle agglomeration and low chromatographic performance was about 15% (w/w). Unfortunately the coating of the silica surface was not continuous and free of defect sites, and the retention of solutes was not the same as with pure carbon.

The pyrolysis of organic compounds on porous silica was further examined by Leboda (55-58), using aliphatic and aromatic alcohols and dichloromethane. Two-stage pyrolysis was sometimes used. The carbon-silica adsorbents were characterized by

sorption-measurements, infrared spectroscopy and gas chromatography. However, no separation by liquid-solid chromatography was carried out.

A novel carbon material has recently been developed by Czechoslovakian researchers (59,60). It is unusual in being prepared not by high-temperature carbonization but by room temperature reduction of polytetrafluoroethylene (PTFE). Non-porous angular microparticles of PTFE were first degreased and dried under vacuum at 423 K. The resulting crumbs were then treated with lithium-amalgam at 293 K, forming lithium fluoride and a carbon residue. The lithium fluoride was extracted and the product, called JADO-carbon, dried under vacuum at 423 to 623 K. Samples were prepared partially reduced (59), completely reduced (60), loaded with lithium fluoride, and with LiF fully extracted. Elemental analysis (by weight) of the dry product gave C 86%; H 1%; oxygen (residue) 13% (59). The apparent (helium) density was 2.17g/cm^3, the specific surface area according to the BET method employing argon S_{BET} was 2500m^2/g! Uptake of benzene at 298 K amounted to 1.25ml/g, indicating a high particle porosity of $\varepsilon = 0.73$. Although the molecular structure of JADO-carbon is amorphous, the micro-structure comprises fibres joining nodules. The nodules are 1.1 $\pm$ 0.35nm in diameter and the distance between them is 1.9 $\pm$ 0.3nm. The resulting cavities in the carbon skeleton are 2.3 $\pm$ 0.3nm in diameter. The final structure is determined by the primary structure of PTFE.

Attempts were subsequently made to decrease the specific surface area and oxygen content by various treatments (61): (i) after leaching the lithium fluoride the microparticles were dried under vacuum at 573 K and annealed at 1223 K under helium giving: $S_{BET} = 2000m^2/g$; (ii) the LiF loaded carbon was heated at 1223 K under helium. After cooling the material was immersed in a 0.1% w/w solution of aqueous detergent. Excess of solution was removed, the sample washed, dried and heated at 973 K in

hydrogen giving $S_{BET} = 1300 m^2/g$: $\varepsilon = 0.76$; oxygen 0.5%; (iii) the LiF loaded carbon was heated at 1223 K in helium. After washing out the LiF residue and drying under vacuum at 573 K the product was annealed at 2673 K under argon giving: $S_{BET} = 20 m^2/g$; $\varepsilon \simeq 0$; oxygen 0.

By a procedure analogous to that of Plzak et al (59), Zwier and Burke (62) prepared a high-strength carbon packing by reduction of the fluorocarbon Kel-F 300 LD with lithium amalgam. As the surface of this KFD carbon exhibited polar behaviour due to the presence of hydroxyl, carbonyl and carboxyl groups the material was treated (i) with trimethylchlorosilane to mask the hydroxyls and (ii) with thionylchloride followed by reacting the chlorinated carbon with the Grignard reagent C_8H_{17} MgBr, giving an octyl bonded carbon. The effect of silane and Grignard modification was monitored by means of ESCA measurements. The surface modified KFD carbon showed behaviour intermediate between that of the unmodified KFD carbon and typical silica based reverse phase materials.

Recognising the disadvantages of PMCB, Ciccioli et al (63) examined a commercial graphitized carbon black, Carbopack B marketed by Supelco and made from saran active carbon (64). The material was first ground to a particle size of around 20μm and the fraction of dp > 20μm used as packing. Although the material was extremely fragile and required very careful handling its LC properties were encouraging, and confirmed that the graphitic surface is likely to be the optimum for HPLC applications if it can be provided on a more robust structure.

Unger et al (31,65) employed cokes and active carbons as precursors. The starting materials had adequate hardness for HPLC and could be milled and fractioned to give narrow cuts of the desired particle size. Batches were then treated to remove the non-carbonaceous constituents:

(i) by leaching of inorganic impurities with HCl/HF mixtures and subsequent annealing at 2073 to 3073 K,

(ii) by annealing at 2073 to 2873 K without extraction,

(iii) by annealing according to (ii) followed by treatment with iron salts, partial degassing at 1273 K, removal of added salts and final reduction by hydrogen at 673 K.

Procedures (i) and (ii) resulted in products having only meso- and macropores but with very low surface area. Procedure (iii) gave S_{BET} values in the range of 50 to 200m^2/g, but the products still contained a proportion of micropores.

Another form of carbon has recently been described by Knox and Gilbert (66). The material is a porous glassy carbon (PGC) and is made by pyrolysing a phenol formaldehyde mixture within the pores of a template material (66) such as silica gel. The composite polymer/silica gel is gradually heated from 400 K to 1300 K to carbonize the polymer. The silica template is then dissolved out by alkali or by hydrofluoric acid and the resulting porous carbon heated to a temperature in excess of 2500 K. The area of the porous carbon when initially prepared at 1300°C is 50 to 500m^2/g depending upon the surface area and pore dimensions of the silica gel initially used. After heating to > 2500 K the area is reduced to between a half and a quarter of the original value. Carbon produced in this way from a typical silica gel has a rather high porosity of around 80% and is structurally weak. However, by using a more porous gel, materials capable of withstanding high pressures can be produced. The chromatographic characteristics of the PGC's so far produced are somewhat similar to those of other high temperature carbons, especially the pyrolytic carbons of Guiochon (32,33,50). It is also applicable in gas chromatography where its chromatographic properties are similar to those of Carbopack B except that the material itself is very much more robust.

3. CHROMATOGRAPHIC STUDIES ON CARBON PACKINGS

3.1. Kinetic Performance

The term "kinetic performance" implies a combination of speed of elution, plate efficiency, and economy of pressure drop. It is best measured by the separation impedance E (67) defined by equation (1):

$$E \equiv h^2\phi' = \frac{N}{t_m} \times \frac{N}{\Delta p} \times \frac{1}{\eta} \qquad (1)$$

where h = reduced plate height, ϕ' = flow resistance factor, N = number of theoretical plates to which column is equivalent, t_m = elution time of unretained fully permeating solute, Δp = pressure and η = eluent viscosity. The flow resistance factor ϕ' is dimensionless and given by equation (2)

$$\phi' = \Delta p \, d_p^2/u\eta L \qquad (2)$$

where d_p = mean particle diameter, u = linear velocity of eluent and L = column length. Where the mean particle size is well established, ϕ' is found to be in the range 500-1000. For well behaved liquid chromatography the reduced plate height h is found to obey the approximate equation

$$h = B/\nu + A\nu^{1/3} + C\nu \qquad (3)$$

The best columns show $B \simeq 2$, $A \simeq 1$, $C \simeq 0.1$ and have minimum h of about 2-3 at ν in the range 2-5 (67,68).

The performance of any material packed into a column is summarized by quoting its ϕ'-value and either showing the (h,ν) plot, giving values of A, B and C, or giving optimum values of h.

Implicit in the term "well behaved liquid chromatography", however, is the assumption that peak dispersion arises entirely from the kinetic effects covered in equation (3). For the carbon adsorbents so far tested this is rarely the case. Peaks are often asymmetric as a consequence of non-linear isotherms for the adsorption of solutes from eluent onto the surface. While it may be useful to treat the data according to equation (3) it is not necessarily legitimate to interpret A, B and C in terms of the kinetic processes with which they are usually associated. The goodness-of-packing of a column largely determines the parameter A. Microparticulate carbons have usually been packed by the well-known slurry technique (31,32,67, 69) using suspension media adapted to the wetting characteristics of carbon. Mixtures of dibromoethane and acetonitrile (32), and of dioxane and paraffin oil (31) have been used. Recently PGC's have been successfully packed using a 1% aqueous solution of the nonionic detergent Tween 20 as suspension medium (70). Ciccioli et al (63), however, dry packed their columns using Carbopack B.

Colin et al (33) determined column efficienices of PMCB using particles of 15μm and above. Optimum performance was achieved with a 20% loading of pyrocarbon. Optimum h-values of 3-7 were found for solutes with k'-values up to 1.1. However, for k'-values above 2 column efficiency generally dropped rapidly, probably due to surface inhomogeneity and overloading (52). Graphitization of PMCB affected a very small improvement in lowering A, B and C for an unretained solute (50) but significant improvement for retained solutes (32). Other carbons (31,61) showed similar effects. One of the best chromatograms published using PMCB is shown in Figure 1. Figure 2 shows a separation on carbon produced by purification and high temperature treatment of active carbon (31).

By contrast to the hard carbons, Carbopack B studied by Ciccioli et al (62) with particle size of 20μm gave $h = 4$ at $k' = 7$ and excellent peak symmetry as shown by Figure 3.

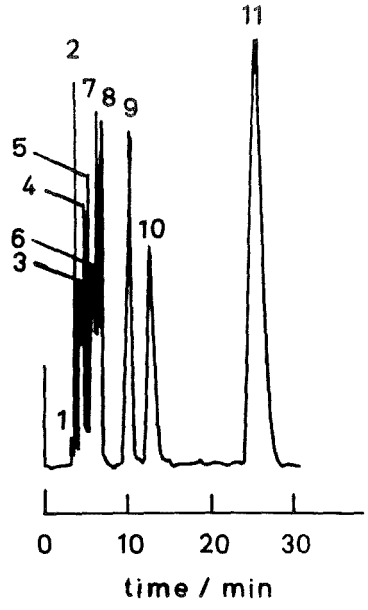

FIGURE 1. Separation of methyl benzenes. Column 550mm. Packings: 25-31μm, Black Pearls L + 44% pyrocarbon, graphitized, ~ $40m^2/g$. Eluent: acetonitrile 0.5ml/min. Detector: UV. h for last peak = 15. Solutes: 1 = unretained; 2 = benzene; 3 = 1,3 dimethylbenzene (DMB); 4 = 1,2 DMB; 5 = 1,3,5 trimethylbenzene (TMB); 6 = impurity; 7 = 1,2,4 TMB; 8 = 1,2,3 TMB. 9 = 1,2,4,5 tetramethyl benzene (TeMB); 10 = 1,2,3,4 TeMB; 11 = pentamethylbenzene. (from ref. 33).

PGC falls between the PMCB and Carbopack and gives reasonably symmetrical peaks at k'-values up to 15, as shown in Figure 3, but h appears to be large at about 25 with the 5μm particles used.

The permeability of carbon columns is much as expected with $300 < \phi' < 1000$ provided that well sized fractions free of fines are used and that the material is sufficiently robust to resist compression.

In some studies (31, 71) column loadability was studied using Snyder's parameter $\theta_{0.1}$ (16), defined as the mass of solute injected per gram of packing material which produces a 10%

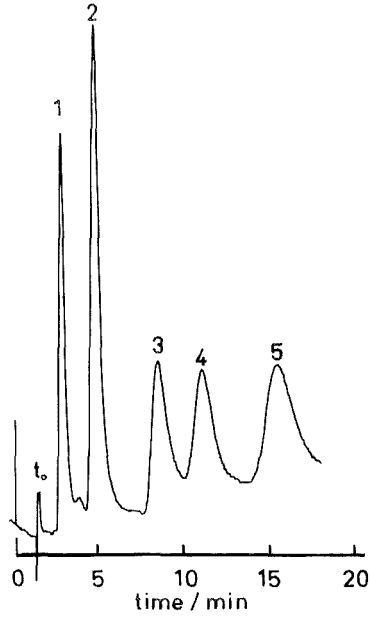

FIGURE 2. Separation of benzene carboxylic acids. Column: 100 x 10mm. Packings: 9µm active carbon calcined 2073 K,~ $5m^2/g$. Eluent: methanol/water (30/70 v/v), pH = 6.0 containing 5mM tetrabutylammonium,2.7ml/min. Detector: UV. h for last peak = 45. Solutes: 1 = pentacarboxybenzene; 2 = 1,4 dicarboxybenzene-3 = 1 methyl 3 carboxy benzene; 4 = 1,3,5 tricarboxybenzene, 5 = 1,2 dihydroxy, 3 carboxybenzene (ref. 71).

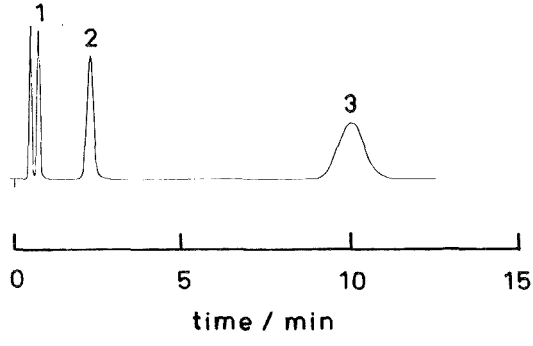

FIGURE 3. Separation of analgesics. Column: 250 x 2mm. Packing: 25-33µm Carbopack B,~ $80m^2g$. Eluent: Methanol, 1ml/min. Detector: UV. h for last peak = 10. Solutes: 1 = phenylacetamide; 2 = phenacetin; 3 = caffeine (from ref. 63).

decrease in k'. For silicas and reversed-phase silicas $\theta_{0.1}$ normally lies between 100 and 1000μg/g. An alternative measure $\theta'_{0.5}$ is the mass of solute per gram of packing which produces a 50% reduction in the plate efficiency of the column. Both $\theta_{0.1}$ and $\theta'_{0.5}$ depend upon the type of adsorbent, the surface heterogeneity, the nature of the solute and of its interaction with the adsorbent surface, and the nature of the eluent.

Colin et al (71) reported $\theta'_{0.5}$ values of 10-60μg for PMCB with acetonitrile as solvent. Unger et al (31) obtained values of around 10μg/g on carbons of very low surface areas ($\sim 5 m^2/g$). Higher values more in line with those for silica gels would be anticipated from carbons of higher specific surface areas. They also showed that $\theta_{0.1}$ and $\theta'_{0.5}$ values were different for different esters using methanol as eluent.

It may be concluded that carbons can be produced to give good chromatographic efficiencies which in some cases are comparable to those obtained with silica gels. However, the efficiency in general is significantly less as is the column loading. These two features are closely associated; both probably arise from the as-yet unsatisfactory surface chemistry of the present carbons. However, there is every reason to suppose that high efficiency carbons can be produced which will eventually compete on equal terms with existing silica gel based materials.

3.2. Retention and Selectivity

Solute retention on carbon arises from adsorption of solutes onto the carbon surface. Solvent molecules adsorbed at the surface are thought of as being displaced by solute molecules during elution, the balance between solvent and solute adsorption providing the observed retention. According to Snyder (16) the column capacity ratio for an adsorbent can be expressed by equation (4):

$$\log_{10}k' = \log_{10}\frac{V_s}{V_m} + \beta(S^o - A_s\varepsilon^o) \qquad (4)$$

where V_s = volume of a monolayer of eluent covering the active surface, V_m = volume of eluent, β = surface activity factor, S^o = dimensionless free energy of adsorption of solute ($\Delta G/2.303RT$) from a standard eluent, A_s = molecular cross sectional area of that part of the solute in contact with the surface measured in units of $8.5\mathring{A}^2$, ε^o = dimensionless free energy of adsorption of a quantity of eluent sufficient to cover $8.5\mathring{A}^2$. Both S^o and ε^o are measured relative to the value for a standard eluent since absolute values are not directly measurable. In the case of silica gel, pentane is taken as the standard having $\varepsilon^o = 0$, giving ε^o values in the range 0 to 1. The parameters V_s and β are affected by the water coverage of the surface. For carbons, methanol has been taken as the standard eluent with $\varepsilon_o = 0$ and in the light of the very limited data available on surface activity β may be taken as unity. With these conventions ε^o-values for carbon are positive for solvents such as dichloromethane, alkanes, benzene, which are stronger than methanol, and are negative for eluents containing water. The extension of the series towards water and neat aqueous buffers is, however, limited by the fact some carbons are not wetted by water.

Eluotropic series have been reported on charcoal (16), PMCB (33) and carbons made from thermally treated cokes and active carbons (31) and are listed in Table 1. It is noted that there are significant differences in the sequence which can probably be attributed to the different surface characteristics of the materials and the different test solutes used.

Values for ε^o have been calculated by Colin et al (71,72) and a selection of these values are shown in Figure 5. The range of eluotropic strength is substantially less on carbon than on silica gel especially if aqueous mixtures are discounted. For methanol/water mixtures, $\log_{10}k'$ increases

TABLE 1

Eluotropic Series for Carbons
(in order of increasing solvent strength)

Charcoal (16)	PMCB (for alkyl benzenes) (33)	Thermally treated coke and active carbon (for naphthalene) (31)
water		
methanol	methanol	methanol
ethanol	acetonitrile	hexane
acetone	ethylacetate	tetrachloromethane
propanol	hexane	acetonitrile
diethylether		dimethylsulphoxide
butanol	dichloromethane	tetrahydrofuran
ethylacetate	butylchloride	trichloromethane
hexane	tetrahydrofuran	dichloromethane
	heptane	
	octane	
	nonane	
benzene	benzene	benzene

linearly with water content as in reversed-phase chromatography to a maximum water content of about 50% corresponding to the composition at which carbon ceases to be wetted by this mixture (31,71). As found in classical adsorption chromatography on carbons, modifiers such as aromatic hydrocarbons, bases and acids are often necessary to improve peakshape. With PGC for example (see Figure 4) the addition of terphenyl (70) was necessary

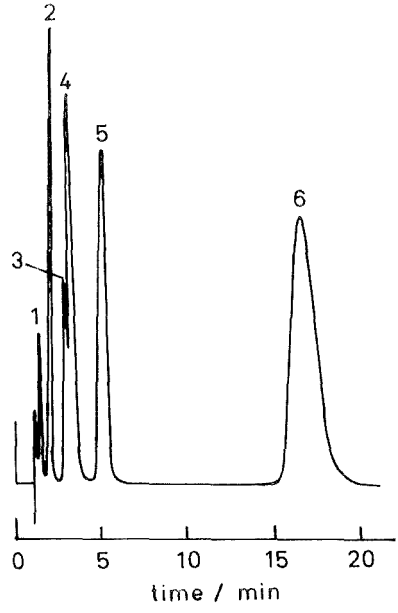

FIGURE 4. Separation of methylbenzenes. Column: 100 x 2mm. Packing: 5µm porous glassy carbon PGC26 heated to 2600 K. 380m^2/g. Eluent: methanol. Detector: UV. h for last peak = 25. Solutes: 1 = benzene; 2 = toluene + 1,4 DMB; 3 = 1,3 DMB; 4 = 1,2 DMB; 5 = 1,2,4 TMB; 6 = 1,2,4,5 TeMB. (ref. 70).

to give symmetrical elution peaks for the more retained alkyl benzenes and polynuclear aromatics while the addition of acetic acid was necessary for successful elution of aromatic acids. Similar behaviour was observed with thermally treated active carbons (73). It seems likely that the modifier occupies specific active sites on the surface of the carbons, such sites being responsible for surface heterogeneity and the extreme curvature of the adsorption isotherm at low surface coverages. The situation is familiar to those involved in adsorption chromatography with silica gel (16). Intelligent control of eluent composition is evidently necessary with currently available carbons if good peak shape is to be obtained.

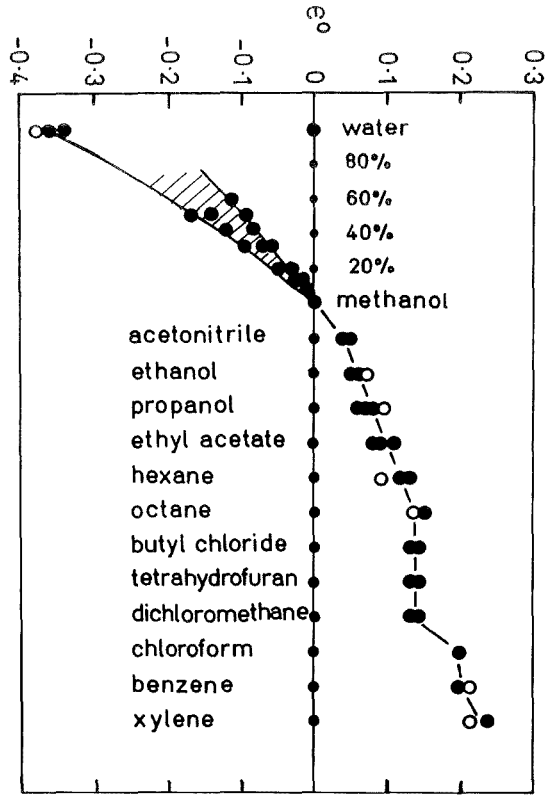

FIGURE 5. Mean eluotropic strength of organic eluents on PMCB using alkyl benzenes, methyl benzenes, methyl phenols and polynuclear aromatics: ● determined by chromatography, O calculated from interfacial surface tension data. (Data selected from Table 3 of ref. 72).

Carbons offer a special advantage over silica-based materials in that they can be used over a much wider range of pH. JADO carbon was used with neat buffers of pH from 1 to 8 (61,74) while Unger et al (31) separated alkaloids using methanol/water buffers with pH from 6-12. An example is shown in Figure 6. As with bonded silicas, separations based on ion-pair formation are also possible as seen from Figure 2.

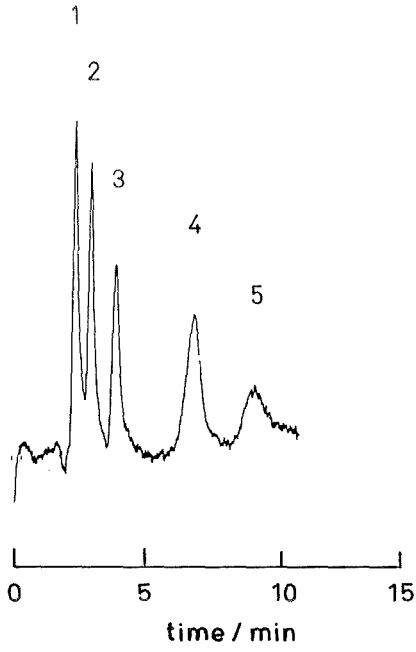

FIGURE 6. Separation of alkaloids. Column 100 X 10mm. Packing: 9µm active carbon calcined at 2073 K, ~ $5m^2/g$. Eluent: methanol/water (80/20 v/v), pH = 11.2, 1.5ml/min. Detector: UV. h for last peak = 20. Solutes: 1 = ephedrine; 2 = codeine + anaestesin; 3 = theobromine; 4 = caffeine; 5 = strychnine. (from ref. 31).

A widely used method of quantitating the hydrophobic balance between a stationary and mobile phase is to plot $\log_{10}k'$ for members of a homologous series against the number of carbon atoms, n, in the molecule or alkyl chain. A linear relationship is often found. Such measurements in reversed-phase systems give a scale of eluotropic strength for the CH_2 group, as can be seen by differencing equation (4) for two successive homologues.

$$\alpha' \equiv \log_{10}(k'_{n+1}/k'_n) = (S^{o}_{CH_2} - A_{CH_2}\varepsilon^{o}) \qquad (5)$$

where $S^{o}_{CH_2}$ is the dimensionless free energy of adsorption of a CH_2 group from the standard eluent, and A_{CH_2} is the area occupied by a CH_2 group on the surface. Several authors (32,33,61) have shown linear plots of $\log_{10}k'_n$ against n, from which the values of α' may be calculated. These values are collected in Table 2. They are substantially greater than those obtained in reversed phase silica packings with the same eluent composition (31, 65). For example, Colin et al quote $\alpha' = 0.15$ for alkyl benzenes eluted by methanol from Partisil ODS-2 whereas the values for carbon are about 0.25.

The α'-values for different carbons are self consistent and clearly show the increasing eluotropic strength (decreasing α' value) from methanol and acetonitrile to pentane and heptane. Typical α'-values for the quasi-homologous series of methyl substituted benzenes are 0.4 to 0.45 (33, 70).

Increase in the number of conjugated rings in the solute molecule leads to a marked increase in retention as seen from the data of Table 3. It is noted that in spite of a very wide range of surface area within the carbons studied the elution order is always the same. Values of k' relative to fluorene are generally comparable and when plotted against the number of carbon atoms in the solute give a fair correlation as shown in Figure 7. The gradient gives an α'-value of 0.32 for addition of each carbon atom . This falls closer to the value for addition of CH_2 groups (0.25) than to the value for addition of CH_3 groups (0.4 to 0.45) and indicates that contrary to general belief graphitized carbons do not show any special selectivity towards conjugated rings.

The behaviour of carbon is distinctly different from that of reversed phase silicas (76,77) in regard to retention of aromatic compounds carrying polar functional groups. As shown by Figure 8, polar derivatives of benzene, especially those having large dipoles,are much more strongly retained by carbons

TABLE 2

Values of $\alpha' = \log_{10}(k'_{n+1}/k'_n)$ for Homologous Series eluted from Carbons

Form of Carbon	Homologous Series	Eluent	range of n (1)	α'	Ref.
PMCB 15% Pyrocarbon on Sterling Carbon Black ~ $6m^2/g$	alkyl benzenes	acetonitrile/water 46:54 v/v	9 - 15	0.26	32
	n alkanols	acetonitrile	5 - 11	0.25	32
PMCB 55% Pyrocarbon on Black Pearls ~$40m^2/g$	alkyl benzenes bromo-n-alkanes chloro-n-alkanes n-alkanols	acetonitrile " " "	7 - 15 3 - 9 4 - 7 5 - 15	0.24 0.24 0.24 0.24	33 " " "
Carbon II from PTFE (1300 m^2/g)	n-alkanes n-alkanes	diethyl ether pentane methanol	12 - 14 8 - 16 8 - 16	0.13 0.14 0.32	61
Carbon I from PTFE (2000 m^2/g)	n-chloroalkanes n-alkenes n-alkanes	heptane " "	7 - 16 10 - 18 8 - 19	0.11 0.11 0.12	61
Carbon III from PTFE (20 m^2/g)	n-alkanols	methanol acetonitrile dichloromethane diethyl ether pentane heptane		0.32 0.24 0.20 0.13 0.14 0.11	61
Porous glassy carbon 16 380 m^2/g	alkyl benzenes	methanol	4 - 9	0.23	70

Notes: (1). n is total number of carbon atoms in molecule.

TABLE 3

Absolute values of k' and values relative to fluorene (bracketted) for elution of Polynuclear Aromatic Hydrocarbons from Carbons

Form of Carbon	Eluent	k' or k'/k'$_{fluorene}$ (bracketted)												Ref.
		benzene		naph-thalene		acenaph-thene		fluorene		phenanth-rene		anthra-cene		
PMCB + 15% pyrocarbon on sterling CB. ~6m^2/g	acetonitrile			0.4	(0.08)			5.0	(1.0)					32
PMCB + 55% pyrocarbon on Black Pearls ~40m^2/g	benzene			0.06	(0.18)	0.25	(0.8)	0.33	(1.0)	0.8	(2.4)	1.0	(3.0)	33
Purified coke heated 3073 K 3 hrs ~5m^2/g	methanol			0.07	(0.21)			0.35	(1.0)	0.52	(1.5)			31
Porous Glassy Carbon 7 350m^2/g	methanol	1.3	(0.02)	4.9	(0.08)	24	(0.4)	61	(1.0)					70
	acetonitrile	0.7	(0.04)	1.5	(0.09)	6.3	(0.4)	17	(1.0)					
	ethyl acetate			0.6		2.1								
	hexane			0.6	(0.12)	1.7	(0.3)	5.5	(1.0)					
	dichloromethane			0.6	(0.25)	1.0	(0.4)	2.4	(1.0)	8.5	(3.5)	16	(6.7)	
Porous Glassy Carbon 26 380m^2/g	dichloromethane	0.04	(0.003)	1.7	(0.13)	4.7	(0.4)	13	(1.0)					70
PGC26 + adsorbed terphenyl	dichloromethane	0.04	(0.005)	1.0	(0.13)	3.2	(0.4)	8	(1.0)					70
	methanol	0.2		16										
Number of Carbon Atoms in Solute		6		10		12		13		14		14		

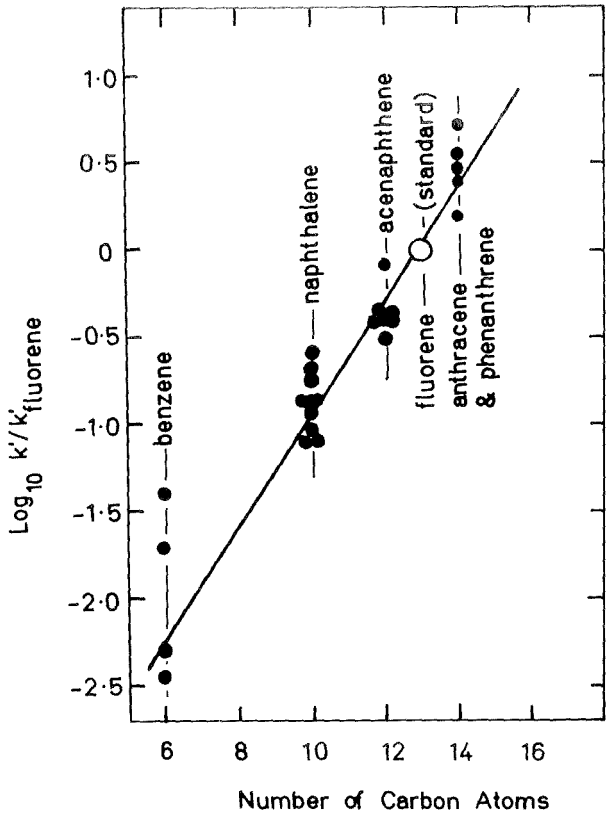

FIGURE 7. Dependence of $\log_{10} k'/k'_{fluorene}$ upon carbon number, n, for elution of polynuclear aromatic hydrocarbons from carbons. Data from Table 3.

than benzene itself whereas the opposite is true with reversed-phase bonded silica gels. In general, however, the effect of added groups whether polar or not gives approximately the same increase in retention when considered on the basis of the number of heavy atoms (C,N,O) in the group, provided that the stereochemistry of the adsorbate does prevent adsorption of all the groups. This again emphasises the importance of dispersive rather than polar interactions at the carbon surface.

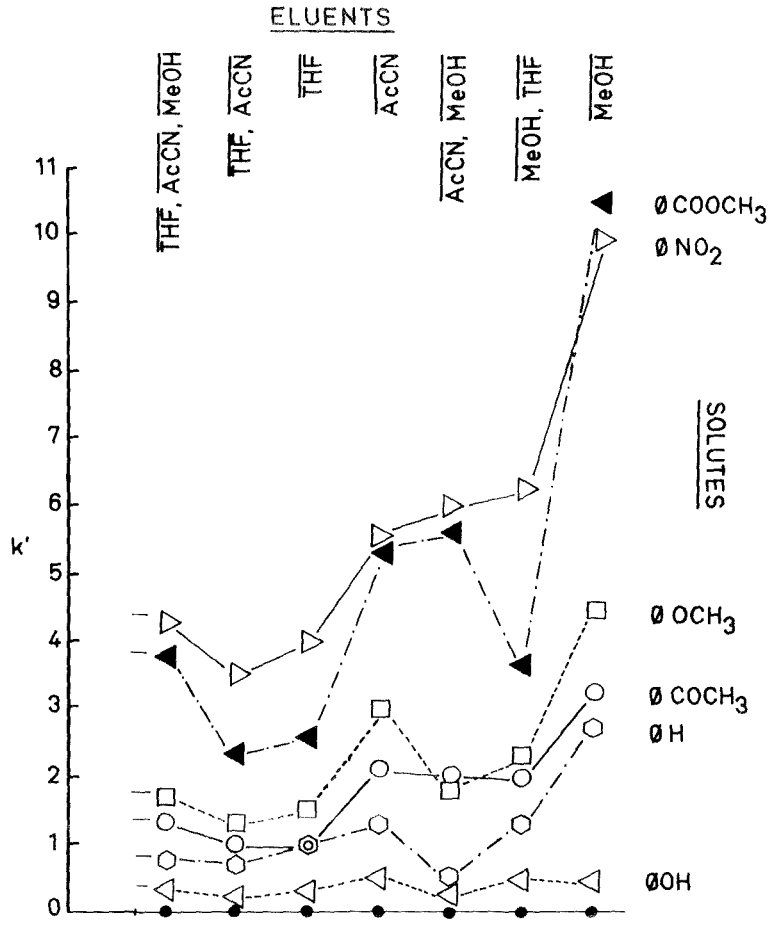

FIGURE 8. Solvent selectivity for different eluents on carbon. Column and packing: as for Figures 2 and 6. Eluents: MeOH = methanol/water (70/30 v/v): THF = tetrahydrofuran/water (40/60 v/v); AcCN = acetonitrile/water (50/50 v/v). Eluents marked THF, MeOH or THF, AcCN, MeOH etc. are made by mixing equal volumes of the individual eluents. Solutes: monofunctional derivatives of benzenes as shown (ref. 73).

The discrimating behaviour of carbons towards solutes of different polarity is relatively poor. On the other hand discrimination on the basis of molecular size appears to be higher than that of reversed phase silicas. Carbon also offers superior selectivity with respect to positional isomers such as cis-trans, aromatics containing hetero atoms etc. Figure 4 illustrates this selectivity in the separation of xylenes using PGC. While the differences in eluotropic strength of the common eluents on carbon seems relatively small (Figure 5), the rather special selectivity of carbons requires detailed examination and may well show strong dependence on eluent composition. This area requires much more study. The results of Colin et al (79) indicate that such work is likely to be rewarding.

In summary, carbon packings offer selectivity characteristics which appear to be significantly different from those of alkyl-bonded silicas. In particular they show greater selectivity for members of homologous series and isomeric compounds and lower selectivity in respect of functional groups of similar molecular weight.

4. APPLICATION OF CARBON PACKINGS

Although carbons for HPLC are not yet available as commercial products, a great diversity of solute types have been separated on carbons. A unique study was carried out by Telepchak (78) who employed a column packed with 10μm diamond dust by resolve aromatic hydrocarbons. Bebris et al (53) used methanol/water mixtures and pyrocarbons modified silica gel (PMS) to separate monoalkyl benzenes, polymethyl benzenes, alkylnaphthalenes, methyl phenols and benzoic acid esters.

The most comprehensive studies have been carried out by Guiochon and co-workers on PMCB and PMS. Aromatic hydrocarbons and methyl benzenes were well separated using acetonitrile as eluent, naphthalenes required a stronger solvent, dichloromethane,

and diphenols required ethylacetate. Other compounds subsequently separated were polychlorinated biphenyls (PCB's), steroids, sulphur and nitrogen-containing compounds (71, 79). The selectivity of PMCB towards isomers was examined with mixtures of undecen-1-ol isomers, isomeric aromatics, and isomeric compounds used in the synthesis of liquid crystals (52). Methanol/water and acetonitrile/water were used to separate adamantanes (80).

Hanai and Walton (81) applied PMCB's and PMS's to the separation of PCB's and other pesticides using methanol/water as eluent and also suggested their use for trace enrichment and preconcentration of pesticides in environmental samples. This suggestion was exploited by Frei et al (82) who compared the effectiveness of ODS-silica and carbons of different origins in the enrichment of samples containing traces of chlorinated phenols.

The Czeckoslovakian group (61) found JADO carbon suitable for separation of amino acids in neat aqueous buffers. They carried out detailed studies on the effect of the pH and ionic strength of the eluent to predict optimum separation conditions (74). Ciccioli et al (62) obtained excellent separations of alkyl substituted benzenes and naphthalene derivatives, triazine isomers, phthalate esters, analgesics and amino acids on Carbopack B.

Unger et al (31) demonstrated the utility of carbon in highly alkaline solution by resolving alkaloids using a methanol/water buffer at pH 11-12. They also discriminated various carboxylic acids using reversed-phase ion-pair chromatography (75).

Carbon packings have thus been used for a wide range of chemically different solutes ranging from non-polar to highly polar and ionic. It appears to be possible to elute compounds

containing virtually all common functional groups if the eluent is appropriately chosen. However, a serious limitation of most of the carbons is that their peak capacity is limited by growing asymmetry as k' increases. However, certain carbons do show promise which suggests that this problem can be overcome.

5. CONCLUSIONS

Carbons have been produced having some but not all of the following desirable features:

(a) adequate hardness
(b) appropriate particle size
(c) good surface characteristics

The hard carbons are superior in regard to (a) and (b) and less good in regard to (c) whereas Carbopack B, a soft carbon, is superior in regard to (c) but unsatisfactory in regard to (a). There is no reason to believe that a carbon cannot be developed which possesses all features to a sufficient degree to be useful in routine HPLC.

Carbon offers a unique selectivity which is different from that of reversed phase alkyl-bonded silica gels. Although it has been applied to a fair range of compounds its application to biochemistry has hardly begun, and an enormous field remains to be explored.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 6(S-1), 37-59 (1983)

POST-COLUMN REACTION DETECTION SYSTEMS IN HPLC

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ABSTRACT

Designs of post-column reaction detection systems for HPLC are discussed with respect to their effect on sensitivity and resolution on the basis of systems theory. The most promising post-column reactor types are the packed-bed reactor and the electronically desegmented liquid-segmented reactor. No experimental data on the latter are available, however.

The choice of post-column reaction detector system is greatly influenced by the reaction type. An overview of these reaction types with suggested systems are given.

For two examples, corticorsteroids and catecholamines in plasma, a comparison is made of the different detection possibilities.

INTRODUCTION

The use of on-line post-column reactions for specific and sensitive detection after separation is increasing to develop HPLC into an even more powerful analytical tool. Derivatisation should be considered if just separation and detection of native compounds will not suffice due to low signal to noise ratio or interfering peaks. Pre-column derivatisation requires a neat quantitative reaction giving stable and single well-defined reaction products

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unlike post-column derivatisation where not even a knowledge of the nature of the products is essential [1,2] as long as the reaction is reproducible and there is a difference in the detected property of the reagent and product(s) (unless an extraction is incorporated). Reactions can be performed off-line (not keeping pace with the HPLC separation) or on-line, and batch-wise or continuous. Pre-column derivatisation is usually performed batch-wise, because there is only one sample per chromatogram, and off-line since off-line reaction is subject to less time and solvent constraints. For post-column derivatisation only a fraction size of one tenth of the peak volume or less causes minimal information loss[3]. so, most often continuous reaction, which is conveniently done in an on-line mode and is easily automated for routine analyses, is chosen. The choice for off-line batch-wise pre-column or automated on-line continuous post-column derivatisation will be determined by the specifics of each case.

REACTOR DESIGN

The latest HPLC column technology imposes demands on the design of post-column reactors (PCR's) with respect to dispersion characteristics.

The influence of the PCR for a homogeneous reaction and a concentration sensitive detector on sensitivity for a component in the sample is given by:

$$\frac{I_{pm}}{I_s} = \frac{.4\ V_i\ E_M\ t_{RC}\ F_C}{AL(1 + k'(\sigma t_{icd} + \sigma t_{PCR})F_{PCR}} = \frac{.4\ V_i\ E_M}{(\sigma t_{icd} + \sigma t_{PCR})\ F_{PCR}} \quad (1)$$

in which: I_{pm} = signal height at peakmaximum in total system
I_s = signal hight for component in sample
V_i = injection volume
E_M = enhancement of signal due to derivatisation
t_{Rc} = retention time on the column
F_c = flow rate through the column

A = cross-sectional area of the column occupied by mobile phase

L = length of the column

k' = capacity factor of the component in the system

$\sigma_{t_{icd}}$ =dispersion in the chromatographic system without PCR

$\sigma_{t_{PCR}}$ =dispersion in PCR

F_{PCR}= flowrate of eluent plus reagents through PCR (excluding segmentation fluid)

The resolution in the complete system for two adjacent peaks, R_a, decreases relative to the resolution in the chromatograph without PCR, R_c:

$$R_a = \frac{R_c}{\sqrt{1 + \frac{\sigma^2_{t_{PCR}}}{\sigma^2_{t_{icd}}}}} \qquad (2)$$

From equation (1) and (2) it can be deduced that the sensitivity and (even more) the resolution will be impaired if the despersion in the PCR approaches the dispersion in the rest of the system- which for a well designed HPLC system equals the peak dispersion in the column (see below).

According to systems theory the variance of the dispersion in the total system is the sum of the variances of the independent dispersions in the parts. The parts that can cause dispersion are identified in fig 1.

The dispersion due to injection, σ_{t_i}, is for a good injector less than the duration of the injection. For injection times longer than a second the dispersion can usually be reduced by on-column concentration so that $\sigma_t \leq 1s$.

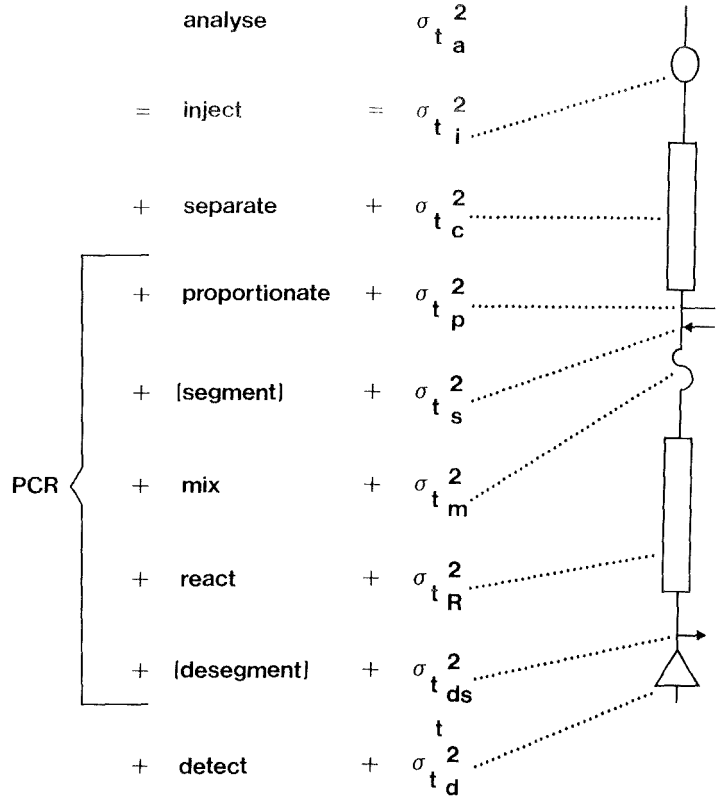


FIGURE 1

The variance of the peakdispersion in the system is the sum of the variances due to the consecutive functional units.

The dispersion in a modern UV or fluorimetric detector is less than 100 ms, but in (diffusion limited) electrochemical detection generally larger than 1s.

For 3000 theoretical plates and a retention time of 3-10 min. the column peakbroadening is σ_{t_c} = 3.3-11 s, thus:

$$\sigma_{t_{icd}}^2 = \sigma_{t_i}^2 + \sigma_{t_c}^2 + \sigma_{t_d}^2 = \sigma_{t_c}^2 \qquad (3)$$

In most PCR's reagent is added and mixed with column effluent. Proportioning can be done via a tee and should not contribute to overall dispersion. Improper proportioning, however, is a source of noise, drift or variable response. A tee with 30^{o} angle is capable of acting as a mixer [4]; a rotating flow mixing chamber [5] and a stirred mixing chamber [6] were documented to give better mixing. If their volume is reduced to less than a few microliter these mixers should show acceptable dispersion (a conical Kobayashi-type mixer is presently used in a commerc. PCR).

The most favorable mixing and dispersion are obtained in packed-bed (pb) mixers [7,8]. The minimum length for a pb mixer, L_m, is given by [8]:

$$L_m = 10\ d_t^2/d_p$$

in which: d_t = inside diameter of the mixer
d_p = particle diameter

e.g. a 10x3 mm tube packed with 100 µm glass beads will give adequate mixing and a (longitudinal) peakdispersion of less than .1 s (at .1 ml/min flowrate).

Segmentation may be introduced at the reagent tee and hence will not contribute to dispersion.

So, the total peakdispersion in the PCR simplifies to:

$$\sigma^2_{PCR} = \sigma^2_{t_R} + \sigma^2_{t_{ds}} \qquad (4)$$

There are three types of PCR design: the capillary (cap), the pb and the segmented flow (sf) reactor (see fig. 2). Dispersion in the reactor proper for cap reactors can be described by [8]:

$$\sigma^2_{t_R} = .04/D_m \left(\frac{\eta . F_{PCR}}{\Delta P} \right)^{1/3} t_R^{4/3} \quad \text{if } \varkappa > 1 \qquad (5A)$$

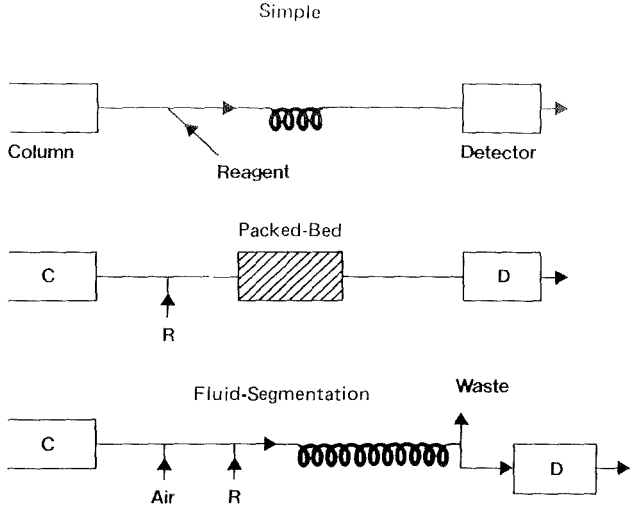


FIGURE 2

Post-column reaction detector designs.
a capillary PCR
b packed-bed PCR
c segmented flow PCR

$$\sigma^2_{t_R} = .004/D_m \left(\frac{\eta \cdot F_{PCR}}{\Delta P}\right)^{1/3} t_R^{4/3} \quad \text{if } \kappa < .1 \quad (5B)$$

$$\sigma^2_{t_R} = .23 \left(\frac{\eta^{13} \cdot F_{PCR}^{2} d_c^{6}}{D_m^{12} \Delta P \rho}\right)^{1/18} t_R^{25/18} \quad \text{if } .1 \leq \kappa \leq 1 \quad (5C)$$

in which [9]:

$$\kappa = 4.8 \left(\frac{d_t\, d_c\, \eta\, Dm}{\rho^3 F_{PCR}^2}\right)^{1/3} \quad (5D)$$

η = dynamic viscosity of the reaction solvent
D_m = diffusion coefficient of the analyte
Δp = pressure drop over the reactor

t_R = reaction time
d_c = diameter of the coil of reaction capillary
ρ = density of the reaction solvent

For pb reactors of non-porous packing material the dispersion is [8]:

$$\sigma^2_{tR} = 40\,\lambda\,\frac{\eta^{1/2}}{\Delta P^{1/2}}\,t_R^{3/2} \qquad (6)$$

if A_r 100 d_p^2 and d_p D_m/u.

in which: λ = dispersion factor; λ=3 for a well packed reactor
A_r = cross-sectional area of the reactor
u = linear velocity in the reactor

A semi empirical equation was derived for sf reactors in which a gas is used for segmentation [10]:

$$\sigma^2_{t_R} = \left(\frac{538\,d_t^{2/3}\,.\,\varkappa\,.\,\eta^{5/3}}{D_m} + 1/n\right)\left(\frac{2.35\,\varkappa\,t_R}{d_t^{4/3}}\right) \qquad (7A)$$

in which: $$\varkappa = \frac{(F_{PCR} + .92\,d_t^3\,n)^{5/3}\,\eta^{2/3}}{\gamma^{2/3}\,F_{PCR}}$$

γ = surface tension
n = segmentation frequency
D'_m = mass transfer coefficient=$4\times10^{-4}\,\eta^{-1.67}D_m$

Unfortunately a like equation has not been derived yet for solvent segmented reactors.

Using equations 5, 6 and 7 a good idea of the relative performance of the repective types of reactors is obtained. Examples are given in fig 3, showing the dispersion as a function of reaction time at several flowrates and a wide range of other experimental conditions.

The peakdispersion due to desegmentation (debubbling or phase separation) in sf reactors should be taken into account

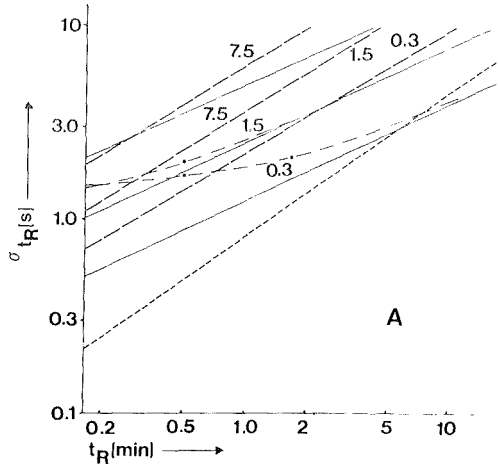


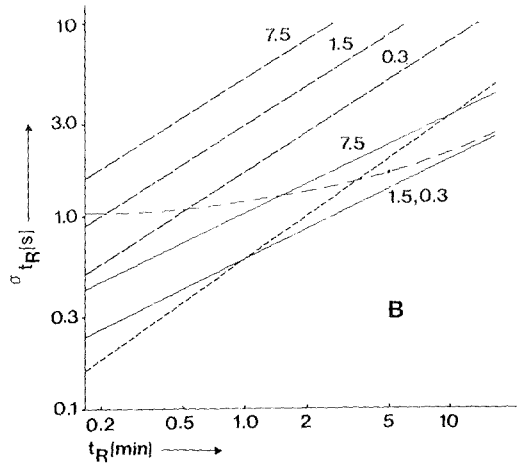
FIGURE 3

The peakdispersion in packed-bed(- - - -),capillary(—— ——), and segmented-flow(—•—) reactors.
Experimental conditions:
$\Delta p \leqslant 100$ bar; $d_t \geqslant 0.01$ cm(cap,pb)or $0.05 \leqslant d_t \leqslant 0.4$(sf); $d_c/d_t \geqslant 5$; $d_p \geqslant 5\mu m$; $K_o = 2.10^{-3}$; $\varepsilon_p = 0.4$; $\lambda = 3$; $n \leqslant 5$.

	A	B	C
D_m (x10^5 cm/s)	0.3	0.3	3
η (x100 g/cm.s)	1.5	0.4	0.4
ρ (g/cm^3)	0.8	1.4	0.8

The numbers next to the curves gave the respective PCR flowrates (in ml/min).

when comparing PCR's (see eq. 4). Recently it was found that peakdispersion in solvent segmented reactos may be caused mainly by (physical) desegmentation [11]. Over a limited range of flowrates: $\sigma_{V,ds} = 12 \pm 1$ µl for a miniaturized phase separator. This was confirmed for gas desegmentation [12].



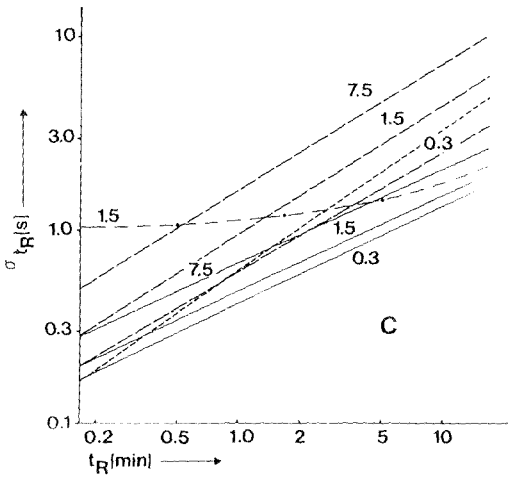


FIGURE 3B&C

An alternative to physical desegmentation is modification of the detector in order to measure only the segments of interest [13,14]. Segment volumes of ca 1 µl seem within the capabilities of present equipment.

COMPARISON

Pb PCR's give less dispersion than cap or sf PCR's at high flowrates and viscosities of the reaction solvent (c.f. fig 3). The only pb PCR's demonstrated [8,13], however, were not suitable for aggressive solvents (e.g. extreme pH).

Cap reactors are commercially available and simpler than pb reactors. They should only be used when dispersion is not an issue (i.e. for short reaction times).

Sf reactors show better dispersion performance than cap PCR's except for very short reaction times ($<$30 s) when physical de-segmentation is applied (broken lines in figs 3).

At reaction times larger than 120 s and low viscosity sf reactors should be preferred even over pb reactors for their commercial availability and versatility.

A way of increasing reaction time without appreciable peak dispersion is storage of the reaction mixture in a holding reactor [16] (see fig 4). A part of the chromatogram can be stored in each

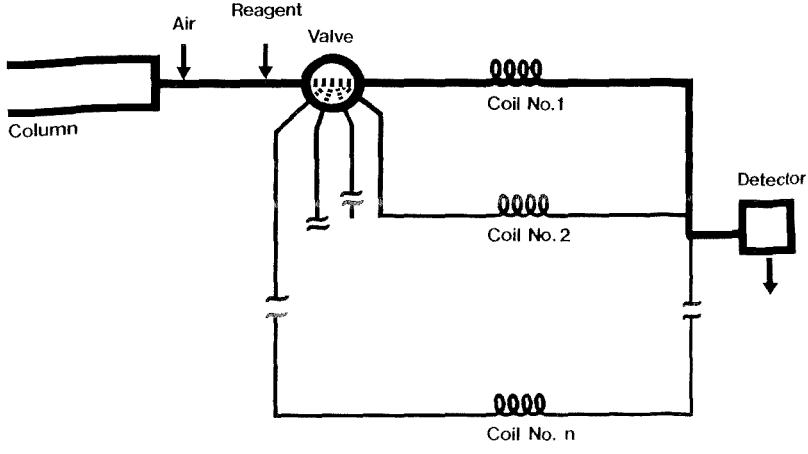


FIGURE 4

PCR for extended time and/or multiple reactions.

reactor via a switching valve, increasing the reaction time. This method proposed for sf but should also work for pb reactors[17].

Flow-splitting to obtain a low flowrate and increased reaction time without changing the volume of the reactor chould be used too, but with a loss in sensitivity proportional to the split ratio.

The versatility of sf and pb reactors can be seen clearly in fig 4: except for extended reaction times this PCR can also be used to apply different reactions with multiple reactiontimes to consecutive parts of the chromatogram without sacrificing resolution.

REACTION TYPES

Specific reactions for post-column derivatisation have been extensively reviewed [18, 19] and will not be summarized here. General PCR considerations with respect to reaction conditions are:

1. A high reagent concentration is needed to keep the dilution factor and the reaction time as small as possible.
2. Since the reaction rate increases usually 2-3 fold per 10°C, the highest reactiontemperature that the solvents permit will be used unless the analytes or products are unstable or side reactions are limiting.
3. The reaction solvent should have a high boiling point, low viscosity and should not slow the reaction or quench the signal of the product. Impurities in the solvent are known to determine the detection limit in fluormetric detection.

Most applied derivatisations are homogeneous (pseudo) first order reactions. They can be performed to create e.g. a chromophore [20], fluorophore[21], electrochemically active compound [22], luminescence or precipitation.

Post-column redox reactions based on an indicator technic e.g. carboxylic acids with nitrophenol or phenols and carbohydrates with Ce, will likely lose importance to electro-chemical detection. In the case of thioridazines the analyte products are

specifically detected and not the reagent products, giving additional selectivity not matched by electrochemical detection[23].

Efficient chemiluminescence can be obtained in several ways (see figure 5):

1. When a reaction can be made to produce a luminol oxidant like H_2O_2.
2. When a fluorophore can be produced that reacts in high yield with peroxyoxalate.
3. When a metal ion is generated that catalizes the reaction of luminol with H_2O_2 (e.g. detection limit CoII: 10 pg [24]).

A post-column precipitation reaction with nephelometric detection has been used with triglycerides by changing the polarity of the mobile phase by addition of ammonium sulfate [25]. The specificity, sensitivity and linearity of this method is likely much better when a real reaction with formation of an insoluble product is performed [25].

Some reactions do not even need addition of reagents. Energy in the form of heat or photons or the permanent presence of a

a oxidant + luminol $\xrightarrow{M^{n+}}$ luminescence

b H_2O_2 + TCPO $\Longrightarrow$ peroxyoxalate

peroxyoxalate + fuorophore $\Longrightarrow$ luminescence

oxidant = H_2O_2, OCl^-, I_2, MnO_4^-
luminol = 5-amino-2,3dihydrophtalazine-1,4dione
TCPO = bis-trichlorophenyloxalate
fluorophore: e.g. perylene

FIGURE 5

Chemiluminescence reactions

catalyst in the reactor may be sufficient. Solid phase reactors, in which the reagent is immobilized in a reactor have a definite drawback: depending on the analyte to immobilized reagent ratio the reagent is depleted with time and the reactor will have to be reloaded.

Photochemical reaction was applied for increase as well as extinction of signal in a UV detector [26], fluorimeter[26,27,28] and conductivity detector [29] with differential detection to enhance selectivity and sensitivity. Although the application of quartz or teflon pb reactors for photochemical reaction may be feasible [28], it has not yet been demonstrated. A class of catalytic PCR's of growing importance is enzymatic reactors. While their selectivity makes them attractive, temperature, pH and inhibitor/activitor sensitivity are not favorable. Most enzyme catalysed reactions have a temperature and pH otimum resp. slightly above and at the in vivo temperature and pH. Organic solvents like those used in HPLC tend to decrease the enzymatic activity. The pH optimum neccessitates buffering and limits sensitivity and linearity for pH-indicator aided detection of oxidoreductases [30].

Often enzymatic reactions obey Michaelis-Menton kinetics. The rate of product formation is given by:

$$\frac{d[P]}{dt} = \frac{k[E_0][S]}{K_m + [S]} \qquad (8)$$

in which: k = specific activity
$[P]$ = product concentration
$[E_0]$ = enzyme concentration
$[S]$ = substrate concentration
K_m = Michaelis constant

Two types of enzymatic reactors exist for:

Enzyme Analysis

Essential is that the PCR does not adsorb the analytes. For enzyme detection the substrate (reagent) concentration will

be as high as possible: $[S] \gg K_m$, so

$$t_R = [P]/\ k.\ [E_0] \qquad (9)$$

According the eq. 9 the detection limit is proportional to the reaction time. For example, LDH isoenzymes ($k > 100\ s^{-1}$) can be determined with NAD as substrate and UV detection with a detection limit of less than 1 nM per second of reaction time. Another application is the measurement of insecticide residues by their cholinesterase inhibiting effect [31].

Substrate Analysis

The price of enzymes and simplicity of design make it desirable to immobilize enzymes in the reactor. The stability of these reactors is good ("lifetime" larger than fifty days) but the bound enzymes have usually much less activity and a larger K_m than the free enzyme. When $K_m \gg [S]$ then from eq 8:

$$t_R = \frac{K_m}{k[E_0]} \ln \frac{[S] - [P]}{[S]}$$

For short reaction times a high enzyme concentration is required, therefore, pb reactors are preferred over cap reactors by virtue of their larger surface area. For example, bile acids have been detected by 3-hydroxysteroiddehydrogenase with $t_R < 6$ s and a detection limit of 10 ng [32] (see fig 6).

The sf reactor is the only PCR that employes multi (mobile) phases. This has been successfully utilized in the extraction of ion-pair complexes [33] and for separation of reagents and products [34]. The phase ratio and composition of the phases have to be carefully optimized. The distribution of solvents of intermediate polarity (e.g. the organic modifier in reverse phase separations) over the phases is often problematic. The combination of neat aqueous mobile phases and polar bonded stationary phases in HPLC may offer a solution [35].

An important aspect of the multi phase reactor is that reactions can be performed in liquid bubbles interspaced by an

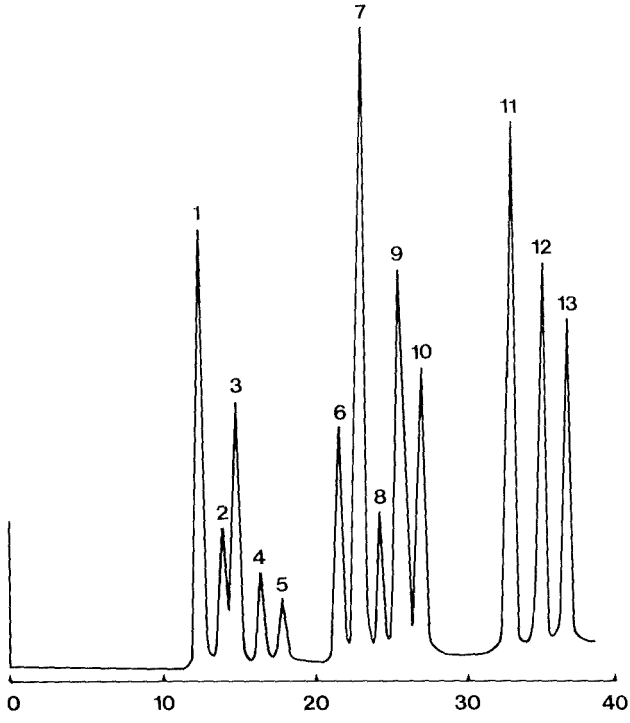


FIGURE 6

Analysis of bile acid standards.
Identity of the peaks:1 ursodeoxyC;2 C;3 glycoursodeoxyC; 4 glycoC;5 tauroC;6 chenodeoxyC; 7 deoxyC;8 glycochenodeoxyC;9 glycodeoxyC, taurochenodeoxyC;10 taurodeoxyC;11 lithoC;12 glycolithoC;13 taurolithoC. C=cholic acid. (From ref. 32, reproduced with permission of The Chemical Society of Japan.)

immiscible solvent-that may contain reagent or not-wetting the tube wall. In this case adsorption of the sample to the tube material is prevented and dispersion should be minimized.

The multiphase reactor opens possibilities for coupling of HPLC with mass spectrometry via ion-pair extraction [36], with

infra-red detection, flame ionisation detection or with HPLC in another mode.

TWO EXAMPLES

Corticosteroids

Here the analyte (e.g. cortisol) is a complex molecule with many fuctional groups and the analyst is faced with a choice (see fig 7). The Δ4-3 keto chromophore has ε_M=16000 at 240 nm[37]. The Zimmermann (Z) reaction attacks the 3- and 20-keto group[38] but also many other ketones. For the analysis of complex mixtures these detection methods may not be specific enough-UV detection at 240 nm is in ca 20% of the samples unfit for determining cortisol in urine [39].

Reaction with isonicotinoylhydrazine (INH) on the Δ4-3 keto group [40] or the multi-stage Porter-Silber (PS) reaction with phenylhydrazine on the 17-hydroxy (= keto) group[41] are more specific. The fastest and most sensitive reaction for corticosteriods is the Blue Tetrazolium (BT) reaction on the α-ketol chain [42]. Reducing sugars also react with BT, but do not interfere with corticosteroids when HPLC is applied. The INH and PS reaction do not allow water in the reactionsolvent, with the BT reaction less than 20% water is optimum. As for bile acids these endogenous corticosteroids could be enzymatically

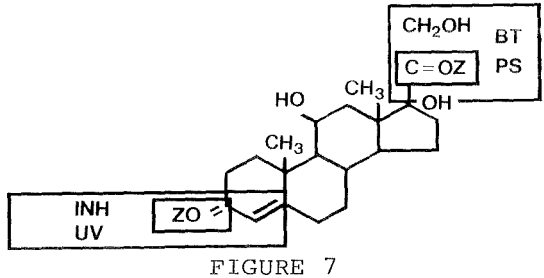


FIGURE 7

Chemical structure and reactive sites of cortisol.

detected. The convenience, sensitivity and selectivity of the BT reaction apparently has prompted no attempts to do so.

Reaction conditions for corticosteroids are summarized in Table I.

Catecholamines

Catecholamines are even more extensively studied and offer therefore greater choice in detection methods (see table II).

TABLE I

DETECTION OF CORTICOSTEROIDS

Method	λ (nm)	t_R(s)	T(^{0}C)	det.lim.	Specific	Ref.
UV	240	0	25	5 ng	-	37
Z	520	10	50		-	38
INH	370/450	55	25	7 ng	+	40
PS	420	960	60	20 ng	+	41
BT	525	20	55	1 ng	+	42
Enzy.			40		+	

TABLE II

DETECTION LIMITS FOR CATECHOLAMINES (pg)

Method	NE	E	DA	t_R	ref.
UV	10^3	10^3	10^3	0	43
Fluor.	300	300	300	0	43
Electrochem.	25	25	25	0	43
OPA	75	-	130	9	43
Borate	250	250	-	90	44
THI	1	1	800	230	43
Fl-lumin.	10	-	10	10	5
PABA	-	-	20	?	45

While cortisol normal values in plasma are 0.3-3 ng/ml, the range for epinephrine (E) is 10-80 pg/ml, norepinephrine (NE) is 100-600 pg/ml and dopamine (DA) is 10-150 pg/ml[46]. For accurate determination of normal plasma levels of catecholamines UV detection, fluorimetry and a borate complexation technic are not sensitive enough. O-phtalaldehyde (OPA) and fluorescamine (Fl) do also react with all other primary amines in the plasma but not with the secondary amine E. Even if this is overcome by a preceding demethylation of E,OPA sensitivity will be marginal and Fl fluorophores will have to be enhanced by chemiluminescence [5]. The Trihydroxyindole (THI) method[43], is sensitive for NE and E, selective and fast (t_R < 4 min). It is a multistage reaction involving oxidation to adrenochrome and alkaline rearrangement to fluorescent trihydroxyindoles. Usually ascorbic acid is added which prevents oxidation of the fluorophore. The detection limit for DA in plasma is not low enough but NE and E can be determined with relatively little sample preparation (see fig. 8). A complimentary specific and sensitive method for DA in plasma was reported[45]. Electrochemical detection is well suited for detection of the higher concentrations of catecholamines in urine after multicolumn separation [47].

PRESENT AND FUTURE

At commonly used HPLC flowrates commerical systems of the cap PCR type having a volume of 0.3-2 ml will cause an additional peakbroadening of 0.5-4 s, which is for most analyses acceptable. For longer reaction times or extractions sf has to be used. Peak dispersion in these PCR's was shown to be 1-10 s [11,34].

Problems arise at the application of PCR's for high speed separations[48,47] since peakwidths leaving the HPLC column are of the order of 0.5-2 s. From figs 3 it is clear that for high speed HPLC only sf reactors with electronic debubbling or pb reactors and very rapid reaction should be used. At the

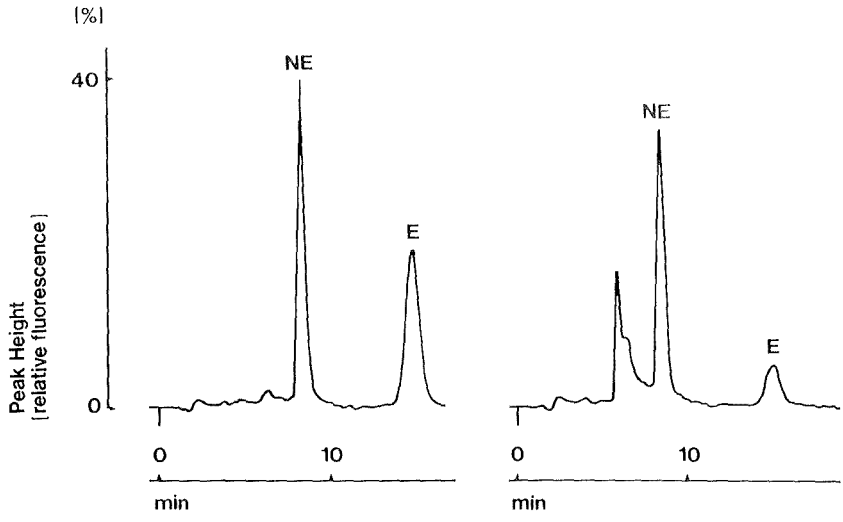


FIGURE 8

Catecholamine analysis in human plasma.
Chromatogram of standards (200 pg each of norepinephrine and epinephrine) (left) and of 1 ml of human plasma (right). (From ref. 50, reproduced with permission of The American Association for Clinical Chemistry.)

flowrates optimal for microbore and capillary HPLC columns no data are available and extrapolation seems futile. It is therefore, imperative that efficient pb reactors are developed that do not dissolve or corrode at extreme pH (e.g. made of titanium).

The type of catalytic PCR in which an analyte (or analyte product) is acting as a catalyst holds the promise that with increasing possibilities of extending the reaction time in PCR's the detection limits of the analytes will be proportionally decreased.

The application of new derivatives, different immobilized enzymes and electrochemical detection is expected to increase dramatically in the near future.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 6(S-1), 61-75 (1983)

A SIMPLIFIED SOLUTION OF TUNG'S INSTRUMENTAL SPREADING EQUATION IN SIZE-EXCLUSION CHROMATOGRAPHY

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ABSTRACT

A method of instrumental spreading correction in size-exclusion chromatography is described, which is simple, precise, and easy to calculate with a simple desk-top calculator. A Gaussian-type instrumental spreading function with variable or fixed Tung's constants is assumed. No assumption of any functions for uncorrected (observed) and corrected chromatograms for polymers is made in advance, instead, these chromatograms are assumed to be the assembly of several Gaussian distributions. After an uncorrected chromatogram being devided into several Gaussian distributions correction of instrumental spreading is made on each Gaussian function and then the corrected Gaussian distributions are assembled into the corrected chromatogram. Examples for correction are demonstrated. Even in high performance SEC, this correction is still needed.

INTRODUCTION

Size-exclusion chromatography (SEC, GPC) is one of the best tools for the determination of molecular weight averages of

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polymers. Calculation of molecular weight averages and molecular weight distributions from SEC chromatograms, however, is not straightforward as far as instrumental spreading should be taken into account. The phenomena of the instrumental spreading in SEC have been described adequetly by Tung's integral equation [1]

$$F(v) = \int_{-\infty}^{\infty} W(x)\ G(v - x)\ dx \qquad (1)$$

where F(v) represents the observed chromatogram, W(x) the true chromatogram (the corrected chromatogram), G(v - x) the instrumental spreading function which has often been approximated by a Gaussian distribution, and v and x the retention volume.

A number of procedures to solve this equation have been described in the literature: the approximation of the equation by a set of linear algebraic equations [1-4]; minimization by numerical methods [5-8]; the Fourier analysis method [9,10]; the polynomial method [10]; the iteration method by matrices [11]; and the use of partial differential equations [12].

The correction of instrumental spreading becomes significant when the efficiency of the column system is lower and molecular weight distributions of polymer samples are narrower. In order to solve the equation (1), the use of a computer is the first requisite. Danielewicz et al. [13] have tested the published methods of data correction, compared these methods with respect to correction efficiency, to the sensitivity to experimental errors, to the computer time requirements, and showed they have both merits and demerits. The remarkable progress of SEC enables this technique to spread over even small laboratories where limits fast computer with large storage space. The method described here is relatively simple and easy to calculate, if needed, even with a simple desk calculator, and there is no restriction on a Gaussian spreading function with variable or fixed resolution factor (Tung's constant). In high performance SEC (HP SEC), Tung's constant is large compared with that in conventional SEC (e.g., about 50 fold)

and instrumental spreading correction is considered not to be necessary. However, even in HP SEC, this correction is still needed. This problemis also discussed here.

THEORETICAL

When probability variables, x and y, are independent and the probability densities f(x), g(y) are Gaussian expressed as

$$f(x) = \frac{1}{\sqrt{2\pi}\,\sigma_1} \exp\left[-\frac{x^2}{2\,\sigma_1^2}\right] \tag{2}$$

$$g(y) = \frac{1}{\sqrt{2\pi}\,\sigma} \exp\left[-\frac{y^2}{2\,\sigma^2}\right] \tag{3}$$

the probability density of a variable z (= x + y) is expressed as

$$h(z) = \int f(x)\, g(z - x)\, dx$$

$$= \frac{1}{\sqrt{2\pi}\,s} \exp\left[-\frac{z^2}{2\,s^2}\right] \tag{4}$$

where $s^2 = \sigma_1^2 + \sigma^2$, $-\infty < x,y,z < +\infty$

Similarly, when the distribution function of a polymer that would be obtained if instrumental spreading effects are absent is a Gaussian as

$$P(x) = \frac{1}{\sqrt{2\pi}\,\sigma_1} \exp\left[-\frac{x^2}{2\,\sigma_1^2}\right] \tag{5}$$

and when the instrumental spreading function is also expressed as

$$G(y) = \frac{1}{\sqrt{2\pi}\,\sigma} \exp\left[-\frac{y^2}{2\,\sigma^2}\right] \tag{6}$$

it can readily be shown that the observed chromatogram is also Gaussian as

$$R(v) = \int P(x)\ G(v - x)\ dx$$

$$= \frac{1}{\sqrt{2\pi}\ s_1} \exp\ [-\frac{v^2}{2\ s_1^2}] \qquad (7)$$

where

$$s_1^2 = \sigma_1^2 + \sigma^2$$

Here x, y, v are the retention volumes, σ_1 , σ , s are the standard deviations of Gaussian functions P(x), G(y), and R(v).

The exact SEC chromatogram of a polymer, F(v), is not always Gaussian in form, however, it might be able to assume that it is expressed as the sum of several Gaussian functions

$$F(v) = \Sigma\ R_i(v - \mu_i) \qquad (8)$$

The function R_i is expressed as

$$R_i(v - \mu_i) = \int P_i(x - \mu_i)\ G(v - x)\ dx$$

$$= \frac{a_i}{\sqrt{2\pi}\ s_i} \exp\ [-\frac{(v - \mu_i)^2}{2\ s_i^2}] \qquad (9)$$

and

$$s_i^2 = \sigma_i^2 + \sigma^2 \qquad (10)$$

where μ_i is the peak retention volume of the i th distribution, σ_i the standard deviation of the function $P_i(x - \mu_i)$, a_i the area of the function $R_i(v - \mu_i)$ between the trace and the base-line. When the function R_i is Gaussian, the function P_i is also Gaussian as expressed in equations (5) - (7).

Assuming instrumental spreading to be Gaussian, next relation is obtained from equations (1), (7) - (9)

$$F(v) = \Sigma \int P_i(x - \mu_i)\ G(v - x)\ dx$$

$$= \int (\ \Sigma P_i(x - \mu_i))\ G(v - x)\ dx$$

$$= \int W(x)\ G(v-x)\ dx \qquad (11)$$

and

$$W(x) = \sum P_i(x - \mu_i)$$

$$= \sum \left(\frac{a_i}{\sqrt{2\pi}\ \sigma_i} \exp \left[- \frac{(x - \mu_i)^2}{2\ \sigma_i^2} \right] \right) \qquad (12)$$

The results explain that the true chromatogram is also expressed as the sum of several Gaussian functions.

CALCULATION

The algorithm of this method is very simple. First, the peak retention volume μ_1 of the experimental chromatogram F(v) and the peak height y_1 (= $a_1 / \sqrt{2\pi}\ s_1$) at μ_1 are estimated. Knowing the retention volume v_1 at the height equivalent to 0.607 y_1 of the chromatogram F(v), $s_1 = | v_1 - \mu_1 |$ and a_1 are calculated and $R_1(v - \mu_1)$ (eq. (9) for i = 1) is obtained. The difference between F(v) and $R_1(v - \mu_1)$ is then calculated

$$\Delta F_1(v) = F(v) - R_1(v - \mu_1) \qquad (13)$$

Repeat the above for $\Delta F_1(v)$

$$\Delta F_2(v) = \Delta F_1(v) - R_2(v - \mu_2) \qquad (14)$$

For the i th operation we have

$$\Delta F_i(v) = \Delta F_{i-1}(v) - R_i(v - \mu_i) \qquad (15)$$

Now, sum up equation (15) from i = 1 to n and we obtain the equation (8).

The values σ_i in equation (10) from i = 1 to n are calculated by knowing the standard deviation σ of the instrumental spreading function. Introducing these values into equation (12) gives the corrected chromatogram.

RESULTS AND DISCUSSCION

It might be wise to discuss the influence of instrumental spreading effects on the calculated values of the molecular weight averages and the molecular weight distributions prior to the evaluation of the proposed correction method. An SEC system with two columns (8 mm i.d. x 50 cm long x 2) [14] was considered for the computer simulation. For simplicity, two artificial chromatograms which are Gaussian distributions were used (W(x) = P(x)), in both examples with known molecular weight averages. One chromatogram (A) (= $P_1(x)$) has the narrow molecular weight distribution ($\bar{M}_w/\bar{M}_n$ = 1.05) with σ_1 = 0.45 (ml) in equation (5) and the other (B) the broader molecular weight distribution ($\bar{M}_w/\bar{M}_n$ = 1.93) with σ_1 = 1.70 (ml).

$$\text{(A)} \qquad P_1(x) = \frac{1}{\sqrt{2\pi}\ 0.45} \exp\left[-\frac{(x-28)^2}{2(0.45)^2}\right] \qquad (16)$$

$$\text{(B)} \qquad P_1(x) = \frac{1}{\sqrt{2\pi}\ 1.70} \exp\left[-\frac{(x-28)^2}{2(1.70)^2}\right] \qquad (17)$$

Five standard deviations (σ) in equation (6) were taken into consideration: 0.37, 0.45, 0.61, 1.0, 1.5. These values were calculated by assuming that the value σ of a polymer (MW = 160,000) is 1.77 times that of benzene [15]. The number of theoretical plates of the systems having these standard deviations corresponds to 36000, 24000, 11200, 4200, 1850, respectively, by benzene injection.

The artificial observed chromatograms R(v) (eq. (7)) having different standard deviations (σ) for instrumental spreading are shown in Figure 1. The significant influence of instrumental spreading on the differential and integral molecular weight distributions is observed, especially in case of samples having narrow molecular weight distributions (Figure 1(A)).

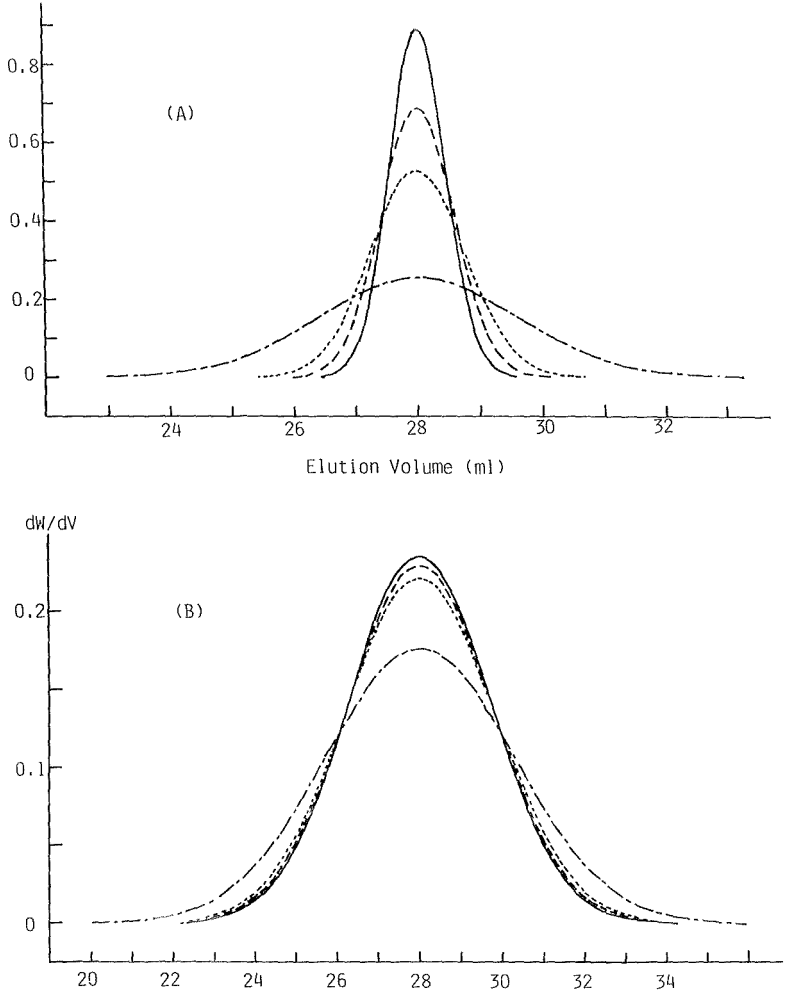

FIGURE 1. Effect of instrumental spreading for polymers of narrow (A) and broad (B) molecular weight distributions. (A) σ_1 (in eq. (5)) = 0.45 . (B) σ_1 = 1.70. (———) P(x); (— — —) σ = 0.37; (- - - - - -) σ = 0.61; (—·—·—) σ = 1.50.

In Table 1, molecular weight averages of fictitious polymer samples and those calculated from artificial observed chromatograms in different instrumental spreading parameters (σ) are shown in conjunction with standard deviations of P(x), G(v-x), and R(v) and the number of theoretical plates. The values of $\bar{M}_w$ for R(v) increase and those of $\bar{M}_n$ decrease with increasing the values of σ. When the SEC system has 24000 plates, the calculated $\bar{M}_w$ increased 3.0% for P(x) of $\sigma_1 = 0.45$ and 2.0% for P(x) of $\sigma_1 = 1.70$ compared to the original fictitious value. Correction of instrumental spreading will be required except the case of $\sigma = 0.37$ (N=36000).

A fictitious two-peak distribution which is a superimposed chromatogram of two Gaussian distributions was first used to test the our correction method.

$$W(x) = \frac{5}{\sqrt{2\pi}\ 1.70} \exp\left[-\frac{(x-28)^2}{2(1.70)^2}\right] + \frac{1}{\sqrt{2\pi}\ 0.45} \exp\left[-\frac{(x-32)^2}{2(0.45)^2}\right] \quad \text{----} \quad (18)$$

Note that in the correction method normalization of chromatogram is not required. Figure 2 shows W(x) (eq. (18)) and F(v) (eq. (19)) and corrected W(x) (eq. (12)) for the case where $\sigma = 1.0$ in equation (6). The uncorrected chromatogram F(v) is obtained by substituting equation (18) and equation (6) into equation (11)

$$F(v) = \frac{5}{\sqrt{2\pi}\ 1.972} \exp\left[-\frac{(x-28)^2}{2(1.972)^2}\right] + \frac{1}{\sqrt{2\pi}\ 1.097} \exp\left[-\frac{(x-32)^2}{2(1.097)^2}\right] \quad \text{----} \quad (19)$$

A good agreement with the original W(x) (eq.(18)) was obtained. The corrected function of W(x) is

$$W(x) = \frac{4.997}{\sqrt{2\pi}\ 1.697} \exp\left[-\frac{(x-28)^2}{2(1.697)^2}\right] + \frac{1.007}{\sqrt{2\pi}\ 0.465} \exp\left[-\frac{(x-32)^2}{2(0.465)^2}\right] \quad \text{----} \quad (20)$$

TABLE 1
The Influence of Instrumental Spreading
on the Molecular Weight Averages

Artificial narrow molecular weight distribution (A)

P(x)		G(v - x)				
σ_1 = 0.45		σ = 0.37	0.45	0.61	1.00	1.50
		N = 36000	24000	11200	4200	1850
		R(v)				
		s_1 = 0.583	0.636	0.758	1.097	1.566
$\bar{M}_w \times 10^{-4}$	9.81	9.99	10.10	10.28	11.03	11.92
$\bar{M}_n \times 10^{-4}$	9.32	9.18	9.10	8.93	8.29	7.14
$\bar{M}_w/\bar{M}_n$	1.052	1.088	1.11	1.15	1.33	1.67
*	(%)	1.8	3.0	4.8	12.4	21.5

Artificial broad molecular weight distribution (B)

P(x)		G(v - x)				
σ_1 = 1.70		σ = 0.37	0.45	0.61	1.00	1.50
		N = 36000	24000	11200	4200	1850
		R(v)				
		s_1 = 1.74	1.76	1.81	1.97	2.27
$\bar{M}_w \times 10^{-5}$	1.307	1.324	1.333	1.356	1.448	1.687
$\bar{M}_n \times 10^{-5}$	0.677	0.666	0.661	0.648	0.600	0.507
$\bar{M}_w/\bar{M}_n$	1.93	1.99	2.02	2.09	2.41	3.33
*	(%)	1.3	2.0	3.8	10.8	29.0

* [$\bar{M}_w$ of R(v) - $\bar{M}_w$ of P(x)]/$\bar{M}_w$ of P(x) x 100

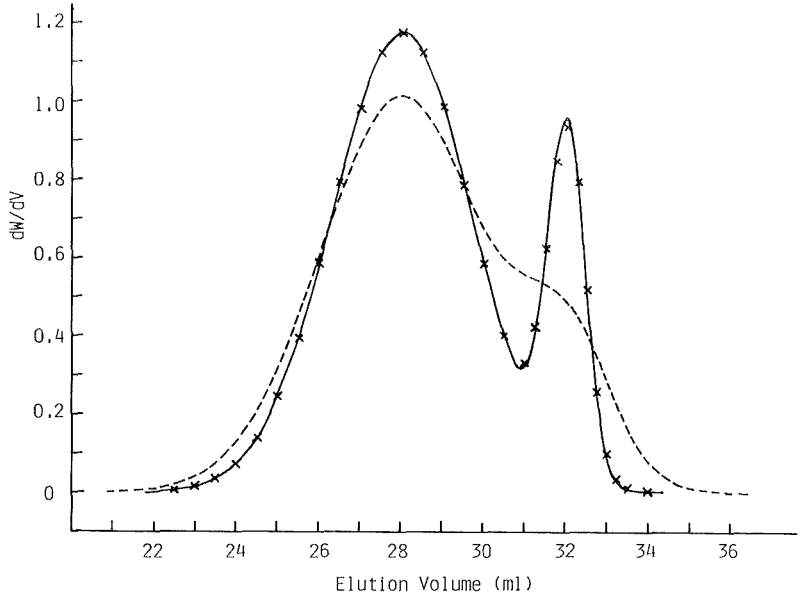

FIGURE 2. Evaluation of the instrumental spreading correction to a fictitious chromatogram having bimodal distribution. The instrumental spreading parameter σ = 1.0. (———) W(x) (eq. (18)); (— — —) F(v) (eq. (19)); (x x x) corrected values for W(x).

A fictitious superimposed chromatogram (eq. (21)) of three equivalent Gaussian distributions (eq. (16)) was then tested for the case where σ is 0.45

$$W(x) = \frac{1}{\sqrt{2\pi}\ 0.45}\left\{\exp\left[-\frac{(x-25)^2}{2(0.45)^2}\right] + \exp\left[-\frac{(x-26.5)^2}{2(0.45)^2}\right] + \exp\left[-\frac{(x-28)^2}{2(0.45)^2}\right]\right\} \quad \text{----} \quad (21)$$

Calculation was started from the midpeak, because it was the highest of the three. Good resolution into original three peaks was obtained, but the height of the corrected peak was higher and the area smaller than other two peaks. Results are shown in Figure 3. A fictitious chromatogram for the case where σ was 1.0

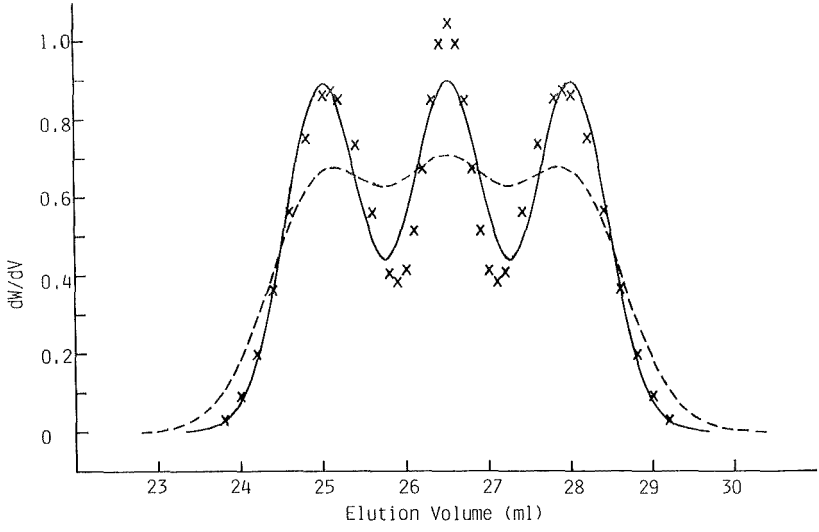

FIGURE 3. Evaluation of the instrumental spreading correction to a fictitious chromatogram having tri-modal distribution. The instrumental spreading parameter $\sigma = 0.45$. Notation as in FIGURE 2.

was not a tri-modal distribution, but one broad peak and the correction failed to show any trace of a second peak.

Figure 4 shows the examples for a combination of next six Gaussian distributions

$$W(x) = \frac{10}{\sqrt{2\pi}\,1.70}\exp\left[-\frac{(x-28)^2}{2(1.70)^2}\right] + \frac{1}{\sqrt{2\pi}\,0.45}\left\{0.1\exp\left[-\frac{(x-23.5)^2}{2(0.45)^2}\right]\right.$$
$$+ 0.5\exp\left[-\frac{(x-25)^2}{2(0.45)^2}\right] + 0.5\exp\left[-\frac{(x-30)^2}{2(0.45)^2}\right]$$
$$\left. + 0.3\exp\left[-\frac{(x-31)^2}{2(0.45)^2}\right] + 0.1\exp\left[-\frac{(x-32)^2}{2(0.45)^2}\right]\right\}$$

---- (22)

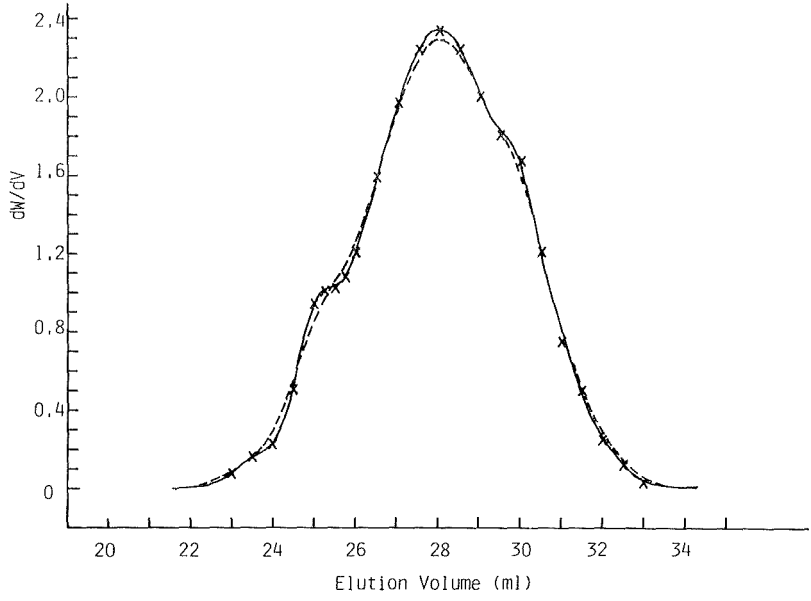

FIGURE 4. Evaluation of the instrumental spreading correction to a fictitious chromatogram of a combination of six Gaussian distributions. The instrumental spreading parameter σ = 0.37. Notation as in FIGURE 2.

Correction was applied to F(v) for the case where σ = 0.37. The corrected chromatogram fitted precisely the original function W(x) (eq. (22)). A slight oscillation of the computed function was observed for the case where σ = 1.0.

The proposed correction procedure was applied to a real chromatogram obtained with a standard polystyrene NBS 706 from the HP SEC system [14]. The instrumental spreading parameter was assumed as σ = 0.45. Molecular weight averages calculated from the experimentally obtained chromatogram were $\bar{M}_w = 2.62 \times 10^5$, $\bar{M}_n = 1.29 \times 10^5$, and $\bar{M}_w/\bar{M}_n = 2.03$ and those obtained from the corrected chromatogram were $\bar{M}_w = 2.59 \times 10^5$, $\bar{M}_n = 1.35 \times 10^5$, and $\bar{M}_w/\bar{M}_n = 1.92$, respectively. Figure 5 shows the observed and corrected chromatograms of NBS 706 polystyrene. Instrumental

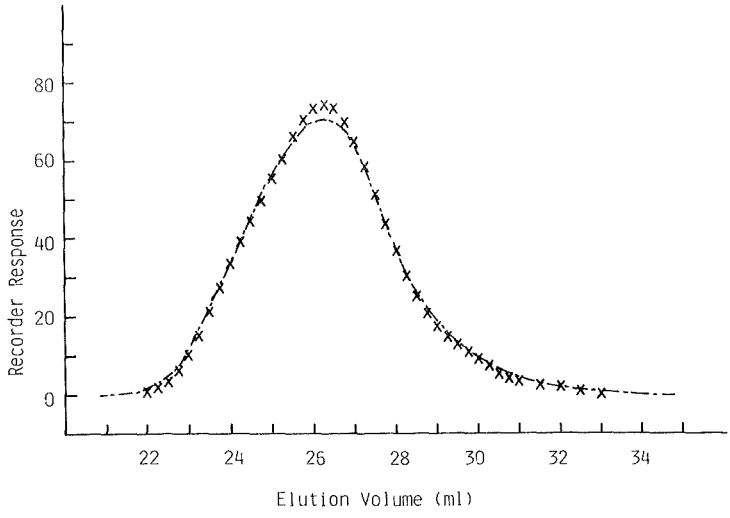

Figure 5. Observed and corrected chromatograms of NBS 706 polystyrene. (—·—·—) observed; (x x x x) corrected.

spreading correction makes a relatively small contribution to molecular weight averages when the SEC system has large efficiency in N, but still large influence on differential and integral molecular weight distributions.

In conclusion, the proposed method to correct instrumental spreading, which assumes that several Gaussian distributions are assembled into uncorrected and corrected chromatograms for polymers, is simple and precise and can be carried out with a simple desk-top calculator. Though this method has some limitations of instrumental spreading parameter σ , but it is still worth appling.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 6(S-1), 77-104 (1983)

RECENT ADVANCES IN HPLC OPTICAL DETECTION

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ABSTRACT

HPLC optical detector advances are reviewed in the field of absorbance, fluorescence, phosphorescence, and elemental emission detection. The development of UV-doped silicon photodiodes having excellent response throughout the UV, miniature gas discharge lamps (Hg, Zn, Cd) with high output UV lines, and high throughput UV interference filters allow optical systems with shot noise levels below 10^{-5} au. However, the fundamental noise limitations in such systems are now thermal changes in the optoelectronic components and flow cells. Future absorbance detectors may require thermostatting to achieve the minimal noise and drift performance inherent in the optical design. The emerging development of UV-doped photodiode arrays at reasonable cost offers multichannel absorbance detection and on-the-fly spectral information. Data system design for processing the multichannel data is critical in this field. On-column absorbance detection using packed microbore fused silica columns has been demonstrated, allowing a flow cell volume of <10 nanoliters, compatible with micro HPLC. In a similar development, on column fluorescence detection using an open tubular glass capillary column has been demonstrated in zone electrophoresis. A significant amount of research in fluorescence detection using laser sources is now underway. Sensitivity advantages of approximately 10-fold have been demonstrated due to high laser output and another 10-fold improvement for detecting long radiative lifetime molecules due to pulsed laser/temporal discrimination. Dual photon excited fluorescence, offering unique selectivities, has been demonstrated. This technique requires the high output power of the laser source. Room temperature phosphorescence HPLC detection has been demonstrated, utilizing triplet→triplet energy transfer from a donor solute molecule to a mobile phase acceptor additive (e.g., biacetyl). This energy transfer technique offers the expansion of the emission technique to phosphorescent molecules at nanogram sensitivities. Finally, the development of micro HPLC columns operating at 1-10 μℓ/min offers potential compatibility with element specific GC detectors such as the flame photometric detector (S, P specific).

0148-3919/83/06S1-0077$3.50/0

I. Introduction

Through a historical quirk, the development of HPLC occurred subsequent to that of GC. Much of the early development of HPLC was thus done by researchers trained in GC. This led to an expectation on the part of these scientists that HPLC detection should provide the sensitivity, selectivity and qualitative information inherent in gas phase detectors such as the structure specific mass spectrometer and the element-specific flame photometric detector.

Researchers in the field of coupling HPLC to the latter detectors have been frustrated by the problems of introducing 1 mℓ/min flow rates of aqueous or aqueous-organic solvents into gas phase detectors. A ray of hope for this field has recently appeared with the development of micro HPLC column technology, providing a reduction in operating flow rates to 1-25 μℓ/min.[1,2]

While academic research has focussed appropriately on the higher risk problem of interfacing gas phase detectors to HPLC, industrial research has focussed on the lower risk task of adapting classical liquid phase optical detection techniques to the specific needs of HPLC. Research has centered on UV-VIS absorbance and emission (fluorescence) detection.

As optical designers began to understand the peculiar needs of HPLC such as microvolume flow cells, low flow sensitivity and ultra-low detector noise, dramatic performance improvements were achieved. In the decade between 1970 and 1980, the noise level of UV absorbance detectors was reduced from $\sim 5 \times 10^{-4}$ au to $\sim 5 \times 10^{-5}$ au and typical flow sensitivity was reduced from 2×10^{-3} au per mℓ/min organic solvent to 2×10^{-4} au per mℓ/min. Concomitant with these improvements were a reduction in cell volume from 10 μℓ to 0.5-5 μℓ and a reduction in detector time constant from 1 second to 25-50 milliseconds, allowing optimum performance with "microbore" and "fast" HPLC.

HPLC detectors have also benefitted significantly from advances in peripheral optoelectronic technologies in meeting these performance improvements. Examples are the development and use of small, stable, high intensity discrete line gas discharge UV sources, UV-enhanced silicon photodiodes and UV-

enhanced photodiode arrays in UV absorbance detectors and the use of the laser as an excitation source in fluorescence detection. New experimental techniques in optical detection based on the laser as a source, such as photoacoustic spectroscopy and two-photon fluorescence are now under active study.

Finally, the difficulties encountered by researchers in interfacing mass spectrometers and element-specific gas phase detectors to HPLC have provided a driving force to the development of narrow bore HPLC columns operating at lower flow rates and reducing the degree of the liquid/gas interface problem. 1 mm stainless steel columns ("microbore") run at 25-50 μℓ/min and 0.3 mm fused silica columns ("narrow bore") run at 2-5 μℓ/min have thus spurred a new wave of HPLC detector research.[3-5]

Advances in current HPLC optical detection and the development of new optical detection techniques are discussed from the viewpoint of technological advances supporting these developments in the body of this report.

II. Impact of New Technology on HPLC Optical Detectors

A. Light Source

(1) Optical Absorbance Detection

Significant advances in HPLC detector source technology have occurred in both discrete line gas discharge and deuterium continuum sources. The first generation of optical absorbance detectors were fixed wavelength UV detectors which utilized a rather large, low pressure mercury arc lamp to provide 254 nm detection. The use of interference filters to isolate weaker mercury lines above 254 nm (e.g., 365 nm) provided an enhancement to detector selectivity albeit with poor sensitivity. Miniaturization of the mercury lamp soon followed, allowing improved optical design with microvolume flow cells. Finally, the need for lower wavelength UV lines to expand detector universality was met by the development of miniaturized zinc (214 nm) and cadmium (229 nm) gas discharge lamps. The miniature Hg, Zn, and Cd lamps are now widely used in fixed wavelength HPLC detectors. The 214 nm zinc line, in particular, provides

a near-universal wavelength for which relatively strong absorption is observed for ~90% of all organic molecules.

After the introduction of the fixed wavelength 254 nm Hg lamp detectors, a need for a selectable or variable wavelength detector was perceived by the chromatographer. Early versions of this detector were simply standard spectrophotometers, jury-rigged with small volume (10-15 $\mu\ell$) flow cells. The classical UV source, a deuterium lamp, was used in these detectors. However, HPLC needs for higher lamp stability and improved output in the low UV (sub 220 nm) region soon resulted in manufacturers' improvements in the deuterium source. Lamp stability has greatly improved in recent years through better understanding of optimum cathode temperature for operation and control of lamp filament and anode supply voltages.[6] Low UV output has been improved by reduction in absorbance by the lamp envelope.

Current deuterium lamps allow detector operation at low noise levels in both the UV and visible regions of the spectrum. Figure 1 shows the radiant intensity spectral distribution of commercial fused silica envelope deuterium lamps. Output is ~10x lower in the visible (350-700 nm) than the peak of the UV (~220 nm). If the detector noise were shot limited this would yield ~$\sqrt{10}$ or 3.1x greater noise in the visible than UV. However, most absorbance detectors

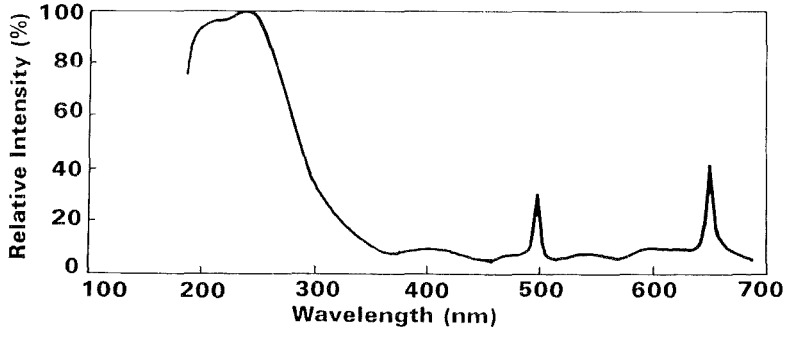

Figure 1. Radiant intensity spectral distribution of deuterium lamp (reprinted with permission from reference 6).

are not shot noise limited (see later section on thermal noise), and since the quantum efficiency of silicon photodiodes increases with wavelength, visible noise is generally within a factor of two of that achieved in the UV.

(2) Emission Detection - The laser as a source

Laser-Induced Fluorescence with Temporal Resolution

The development of the laser offers several features to the HPLC detector designer. Its high intensity allows improved fluorescence sensitivity for the case of shot noise limited detection,[7] and also has allowed the development of two-photon fluorescence excitation, a new optical detection technique. In addition to its high intensity, the pulsed laser allows the chromatographer to utilize temporal resolution in discriminating fluorescence of the analyte versus that of interferences (solvent, flow cell walls, sample matrix) having significantly different emission lifetimes.[9] It is often stated that the coherent output beam of the laser can be focussed down to a diffraction - limited spot, and that the laser therefore offers enhanced compatibility with narrow, microvolume flow cells. It should be noted that standard sources can also be focussed down to a diffraction - limited spot, albeit with more sophisticated focussing optics.

Work to date on laser-induced fluorescence has demonstrated approximately 10-fold sensitivity improvement versus standard deuterium, tungsten or xenon lamp-based fluorometers.[7] This has been disappointing in that the laser output is ~10^4x larger than that available from the typical fluorometer source (watts vs. milliwatts).[8] The use of temporal resolution with pulsed lasers has recently been shown to offer up to 100-fold improvement in detection sensitivity for compounds with long fluorescence lifetimes[9] (see data in Table I). For example, Richardson has demonstrated 0.5 ppt detection of the long-lived fluorescence of pyrene (690 msec lifetime). For the typical 100 $\mu\ell$ peak observed in HPLC, this represents detection of 50 femtograms of pyrene. Temporal resolution also provides dramatic selectivity against co-eluting interferences, as seen in Figure 2.

TABLE 1
DETECTION LIMITS OF LASER INDUCED MOLECULAR FLUORESCENCE*

COMPOUND	τ_F^0	LIMF	CONVENTIONAL
NAPHTHALENE	—	1 PPT	—
ANTHRACENE	4 nsec	4	30
FLUORANTHENE	45	1	100
PYRENE	690	0.5	100
TRYPTOPHAN	—	50 (270 exc)	25 (220 exc)
ARGININE/FLURAM	—	10	25
RIBOFLAVIN	—	0.5	5
SOLVENT IMPURITIES	≤ 2	—	—

LIMF data from J. Richardson et al, NBS PUB. 519, 1979.
Conventional data from Varian, Fluorichrom Filter Fluorometer.
Solvent impurity τ_F from Matthews and Lytle, Anal. Chem., *51*,583 (1979).

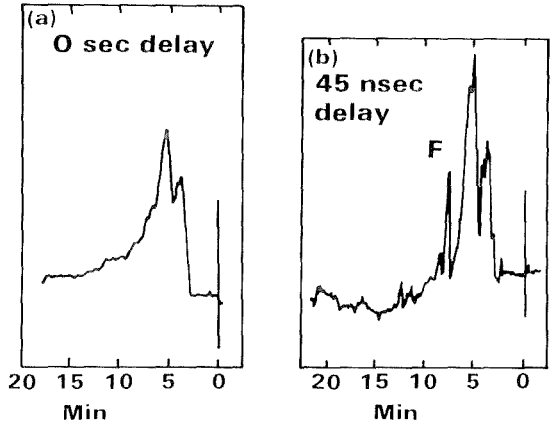

Figure 2. HPLC of coal gasification burn distillate using temporal resolution LIMB. (a) 0 sec delay (b) 45 nsec delay between excitation pulse and emission detection. F=fluoranthene (reprinted with permission from reference 9).

Finally, temporal resolution of fluorescence based on pulsed laser sources offers a potential sensitivity enhancement in post-column reaction detection for HPLC. In this case, one would choose a fluorescence label with a long fluorescence lifetime and excite its fluorescence with an appropriately pulsed laser.

The future utility of laser sources in HPLC fluorescence detection will depend on improvements in laser cost (currently >15K for a tunable dye laser) and reliability. Improved communication between chromatographers and spectroscopists searching for areas of laser applications in analytical chemistry is also required. Laser spectroscopists must stop claiming that the ppt sensitivity achieved with experimental laser based HPLC detectors is 10^3 better than that obtained with conventional HPLC fluorescence detectors. This implies ppb sensitivity of the conventional detectors. Commercially available, conventional HPLC fluorescence detectors provide 10 ppt sensitivity to highly fluorescent molecules.

Two-Photon Excited Fluorescence

The high power of the laser allows the use of two-photon excited fluorescence for HPLC detection. Quantum mechanical selection rules for two-photon excitation differ from those governing single photon excitation, thus expanding the potential selectivity of fluorescence detection. Since one can excite with visible photons yet detect fluorescence in the UV, background due to stray incident light, Rayleigh scatter and Raman scatter is greatly reduced. Matrix interferences are also relatively weak due to the restrictive selection rules of the two-photon effect.

The extinction coefficient for two-photon absorption is relatively small and is proportional to the square of the incident source power at the excitation wavelength. Thus, the technique requires both the high source power of the laser and high source stability. Yueng has demonstrated 100 parts per trillion sensitivity in the HPLC two-photon fluorescence technique using a relatively simple argon ion (Ar^+) laser source.[8] Use of a mode-locked, pulsed laser to

enhance incident power and stability is expected to bring sensitivity to the 1 ppt level. Figure 3 compares single and two-photon fluorescence chromatograms of a coal extract sample and demonstrates the different selectivities of both fluorescence techniques. It should be noted that one can simultaneously monitor single and two-photon excited fluorescence by collecting emitted light on different sides of the flow cell, with appropriate filters.

HPLC-Photoacoustic Detection

Absorption of a photon of light by a molecule produces a high energy electronic "excited" state. The molecule rapidly dissipates this energy, reverting to a lower energy "ground" state by several processes: (1) re-emission of a photon as in fluorescence or phosphorescence; (2) breaking of

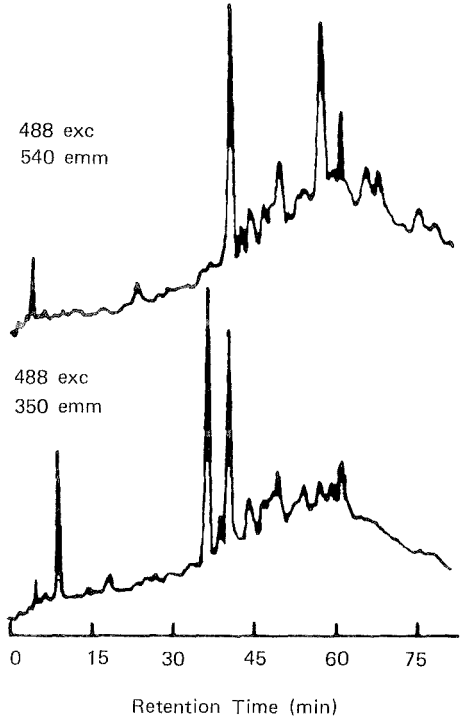

Figure 3. HPLC of PAH in coal liquids. One and two photon fluorescence detection (reprinted with permission from reference 8).

covalent bonds as in photolysis; (3) conversion into thermal energy (heat). The last non-radiative process causes localized heating which results in a pressure fluctuation in the sample, providing the basis for "photoacoustic" detection.

In the photoacoustic detector, the photon source is modulated and the resultant periodic temperature rise in the flow cell due to absorption/heat conversion produces a periodic pressure variation which is detected by a sensitive microphone (typically a ceramic piezoelectric transducer). To obtain optimum signal to noise ratio in photoacoustic detection, one requires a high source power (to increase signal) and vibration-free modulation (to reduce noise). A pulsed laser has thus been utilized as the source in experimental photoacoustic HPLC detectors studied to date.

An HPLC-photoacoustic system[10] based on a pulsed nitrogen laser with output at 337 nm yielded sensitivity for PAH molecules of approximately 100 ppb. Since these molecules are relatively strong fluorescers, one would expect at least a 5-fold sensitivity improvement for highly absorbing, non-fluorescent molecules.

An HPLC-photoacoustic system based on a pulsed argon ion laser[11] with output at 488 nm yielded detectivity down to ~10 ppb for azobenzene dyes. The 10 ppb dye concentration corresponded to an absorbance of 8×10^{-6} au/cm at 488 nm. A comparable state of the art absorbance detector detects down to $\sim 2 \times 10^{-5}$ au/cm.

In evaluating the potential of photoacoustic detection, one should note that most organic molecules studied in HPLC do not absorb above 300 nm. Thus, the argon ion and nitrogen lasers are not adequate sources for a commercial HPLC detector. State of the art lasers with output in the 200-280 nm region are very expensive and complex.

Finally, photoacoustic response of a sample molecule is solvent-dependent and the detector background signal is highly solvent dependent being a function of the solvent's absorbance and acoustical properties. Thus, gradient compatibility is a potential problem.

Advantages of photoacoustic detection are:

(1) choice of a high modulation frequency eliminates noise due to reciprocating pump pulsations, which have a much lower frequency.

(2) noise limit currently appears to be electronics (phase detection system) limited and might be improved ~10-fold with future development.

(3) signal is not dependent on optical pathlength as in absorbance. As in fluorescence, it is dependent on number of molecules excited and thus is more compatible with microvolume flow cell usage.

Future Absorbance Detector Thermal Stability Requirements

In Table 2, calculations of the photon flux that is optically collectable from miniature, intense gas discharge fixed wavelength UV lamps is compared to that of a deuterium lamp which provided a shot limited noise of 5×10^{-6} au (1 sec τ) on an experimental absorbance detector. These calculations indicate that the shot noise of properly designed fixed wavelength detectors can approach 5×10^{-7} au. However, to achieve the low noise inherent to the use of these intense sources, one must reduce non-shot generated noise components to the 5×10^{-7} au level.

The challenge involved in this task is best grasped by considering thermal noise sources. The temperature coefficients of UV-doped silicon photodiodes are in the range of 10^{-4} to 10^{-3} au per ^{o}C. Diode pairs (for reference and

TABLE 2

COLLECTABLE PHOTON FLUX OF FIXED WAVELENGTH LAMP VS. DEUTERIUM CONTINUUM LAMP

LAMP	λ	PHOTON FLUX RELATIVE TO DEUTERIUM LAMP	ESTIMATED SHOT NOISE*
Hg	254nm	~ 200	3.5×10^{-7} au
Zn	214nm	~ 50	7.1×10^{-7} au
Cd	229	~150	4.1×10^{-7} au

*SHOT NOISE WITH D LAMP IS 5×10^{-6} au

sample paths) can be obtained commercially which are matched in temperature coefficient to within $5x10^{-5}$ au per ^{o}C. Thus, to insure that thermal noise due to a differential temperature change between reference and sample diodes be of the order of $5x10^{-7}$ au will require a differential thermal stability of 10 millidegrees.

The temperature coefficient of mobile phase absorbance increases significantly as one approaches its "end absorption." The end absorbance of acetonitrile is shown in Figure 4. Acetonitrile absorbance temperature coefficient data obtained experimentally on an optical detector for which the incident light beam traversed the flow cell without striking cell walls (minimizing RI generated thermal effects) are presented in Table 3. The data suggest that reduction of solvent absorbance generated thermal noise to ~$5x10^{-7}$ au will require control of the flow cell solvent temperature to 400, 8 and 4 millidegrees at 254, 210 and 195 nm respectively (Table 4).

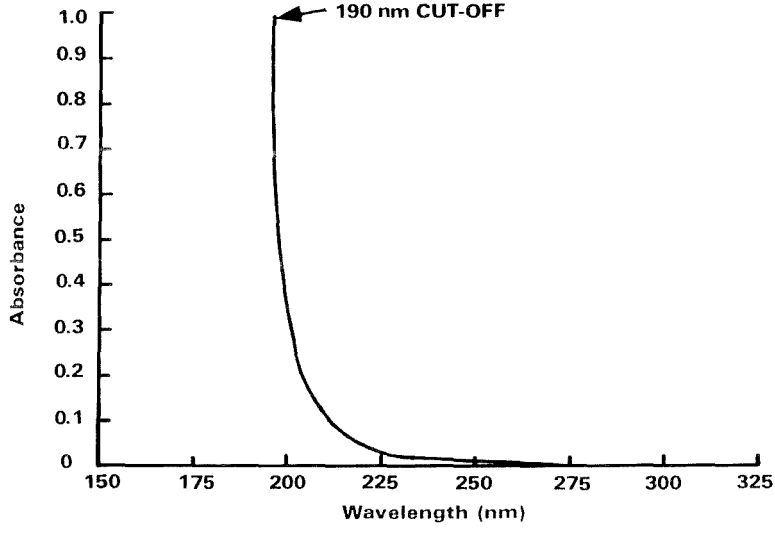

Figure 4. Absorption spectrum of acetonitrile (1 cm optical path).

TABLE 3
MOBILE PHASE TEMPERATURE COEFFICIENTS

— ACETONITRILE, 1 ml/min
— LIGHT BEAM TRAVERSES FLOW CELL WITHOUT STRIKING CELL WALLS

λ	TEMPCO
195 nm	$\leq 1.3 \times 10^{-4}$ au / °C
210 nm	$\leq 6 \times 10^{-5}$ au / °C
254 nm	$\leq 3 \times 10^{-6}$ au / °C

TABLE 4
THERMAL STABILITY REQUIREMENTS OF MOBILE PHASE TO REDUCE THERMAL NOISE BELOW SHOT NOISE

SHOT NOISE	195 nm	210 nm	254 nm
1×10^{-4} au	0.8°C	1.6°C	34°C
1×10^{-5} au	0.08	0.16	3.4
5×10^{-6} au	0.04	0.08	1.7
1×10^{-6} au	0.008	0.016	0.8
5×10^{-7} au	0.004	0.008	0.4

Clearly, in order to take advantage of current and future lamp intensity advances, HPLC detector thermal stability will require significant improvement. The placement of the detector itself in a well-controlled oven may become necessary.

B. Photodetectors

Photodetectors used in early fixed wavelength detectors utilized photoconductive films (e.g., CdS) and were characterized by response times varying from approximately 0.5 sec to several seconds, dependent on the amount of light incident on the film. Early variable wavelength detectors utilized fast response photomultiplier tubes optimized for low UV response. Whereas silicon photodiodes (fast response) initially suffered from poor quantum efficiency below 350 nm, advances in UV-doping semiconductor technology soon

increased photodiode Q.E. to ~2x that of the photomultiplier tube throughout the UV region. The dramatic improvements in silicon photodiode UV response from first through third (current) generations are shown in Figure 5. The improved UV response, lower thermal sensitivity, and very small size of the silicon photodiode have led to its incorporation as a photodetector in current fixed and variable wavelength absorbance detectors.

A silicon photodiode is simply a photosensitive p-n junction. It can thus be manufactured with a very small and precise active area (typically a few mm^2). Of further importance, linear arrays of such photodiodes can be produced on a single chip. The ability of semi-conductor manufacturers to produce linear photodiode arrays compatible in size and active area spacing with both the flat field focal plane of holographic concave gratings and the spectral bandwidth (2-5 nm) requirements of HPLC has led to the development of the diode array absorbance detector, providing multichannel absorbance information and on-the-fly spectra of eluting peaks.

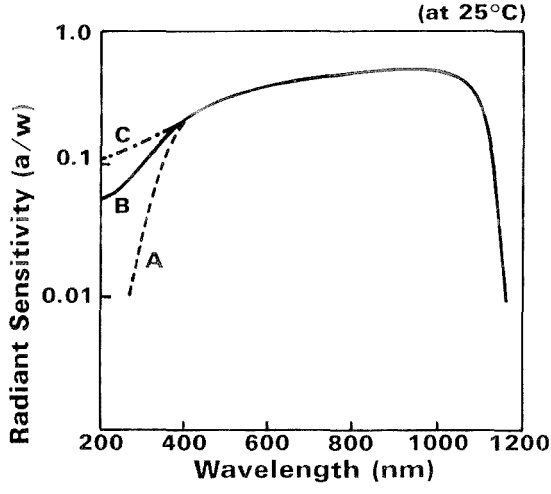

Figure 5. Radiant sensitivity as f(λ) of 3 successive generations of silicon photodiodes. Generation C is state of art 1982.

Time Programmable Diode Array and Absorbance Detectors

The period in which the development of variable wavelength detection in HPLC occurred has coincided fortuitously with developments in optoelectronic technology which have greatly expanded the utility of this detection technique. As discussed above, the development of the silicon photodiode has provided an inexpensive, compact, high performance photodetector. Advances in holographic etch manufacture of diffraction gratings produced inexpensive, low stray light and high efficiency plano and concave gratings. In addition, the development of the microprocessor allowed inexpensive control and hence time programmability of the wavelength drive of the monochromator of the variable wavelength detector.

The time programmable variable wavelength detector has allowed the chromatographer to set each section of the chromatogram to the detection wavelength that is optimum (in terms of sensitivity and/or selectivity) for the peaks eluting in that section. This feature is demonstrated in Figure 6. Time programming also has facilitated the stop-flow scanning technique in which the HPLC pump is stopped during the elution of a peak of interest, trapping the given peak segment in the flow cell, and the monochromator drive is then scanned, yielding a spectrum of that peak section resident in the flow cell.[13]

Diode Array Detection

The absorbance spectra of peak segments passing through a flow cell provide several types of qualitative information:

(1) absorbance ratios - the ratio of absorbance at any two wavelengths is determined by the spectrum of a molecule and is not concentration dependent. Thus for a pure peak, a given absorbance ratio value will be invariant at all points along the peak. Variation in this value indicates the co-elution of another compound which absorbs at least one of the wavelengths chosen in the ratio.

(2) peak confirmation (calculated) - the absolute value of several absorbance ratios can be used as a molecular "fingerprint" to confirm the suspected (based on retention time) identity of a peak.[15]

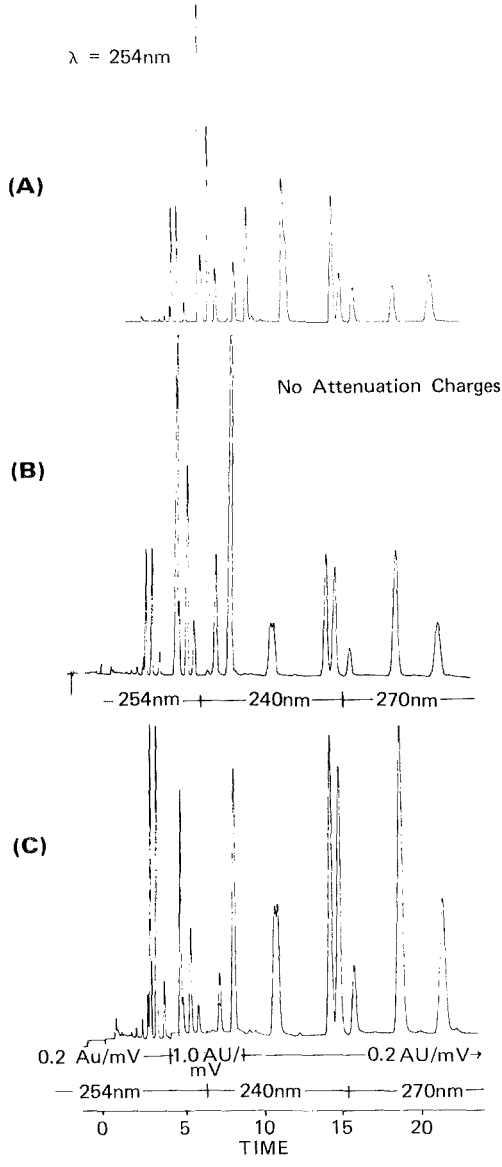

Figure 6. Chromatograms of mixture of PAH's. (a) 254 nm absorbance detection. (b) absorbance wavelength time programmed between 254, 240, 270 nm to optimize detectivity. (c) absorbance wavelength and absorbance range time-programmed (reprinted with permission from reference 12).

(3) peak confirmation (visual) - overlay of the spectrum with that of a known standard (after spectral normalization to a given wavelength) allows a visual confirmation of suspected peak identity.

Spectral information can be obtained with the stop-flow scanning technique available with most current microprocessor-controlled variable wavelength detectors. However, the need to stop the mobile phase flow becomes inconvenient as the number of spectra required per chromatogram increases. In addition, as peak volumes decrease with advances in column technology (e.g., fast LC, microbore LC), the ability to "freeze" a desired peak section in the flow cell becomes critical. Stopping the pump can result in decompression of the pump hydraulic volume and the column internal volume with resultant expansion of the compressible mobile phase through the flow cell, displacing the desired peak segment. This effect can be minimized by the addition of hydraulic elements in the system to seal the expansion volume from the flow cell path, increasing system cost/complexity.

Ideally, one would like to obtain spectral information "on-the-fly" during a chromatogram. This requirement can be met by the use of a linear photodiode array as the photodetector element in the relatively flat field focal plane produced by a concave grating.[15,16] Instead of mechanically rotating a grating so as to serially move successive wavelengths across a fixed slit/single detector element as in scanning a monochromator, one fixes the grating and locates the array so as to intercept the whole spectral region of interest (e.g., the UV). This optical system is called a "polychromator." The polychromator-array system also differs from a monochromator-single photodetector system in that the output of the source (e.g., deuterium lamp) is focussed through the flow cell prior to entering the polychromator and being dispersed by the grating. This configuration is called "reverse optics".

Linear UV-sensitive photodiode arrays are now commercially available with from 2 to 4096 elements. 1024, 512 and 211 element arrays are currently used in commercial HPLC diode array detectors.[17,18] The deluge of multichannel data

produced by the arrays is processed by a microcomputer to provide the chromatographer with wavelength chromatograms, ratio chromatograms and "spectro-chromatograms."[18]

The value of array detectors will grow as these information-rich detectors are applied to HPLC, and user feedback to the detector manufacturers yields improvements in the algorithms by which the spectral data is condensed, interpreted and presented. A certain amount of confusion already exists as to whether the information resident in a solution UV spectrum is useful for peak identification (determine identity of an unknown) or peak confirmation (confirm suspected identity of a peak at a known retention time). This author believes that the array detector will be valuable in providing impurity information based on ratios and peak confirmation based either on comparison of a pattern of several ratios with that of a standard or on the overlay of a peak spectrum and a standard spectrum. Ratio and spectro-chromatograms currently produced by array detection are shown in Figure 7 and 8.

C. Column Technology - HPLC

On-Column Detection Technique

Advances in HPLC column technology have resulted in significant reductions in peak volumes. In "fast HPLC," highly efficient 3 micron packings allow the use of short (4-7.5 cm) columns of standard diameter (4.6 mmID). Typical peak volumes on these columns are 20-100 $\mu\ell$. In microbore HPLC, column diameter is reduced to 1 mm and typical column lengths are 25-50 cm. To date, 5 μ and 10 μ packing technology has been developed for 1 mm columns, yielding typical peak volumes of 10-100 $\mu\ell$. Thus, fast and microbore HPLC techniques have required a reduction in detector flow cell values from 10-15 $\mu\ell$ to 0.5-5 $\mu\ell$, which has been achieved in current absorbance detectors.

In packed capillary HPLC, 1.0-4.6 mmID stainless steel columns are replaced by 0.2-0.3 mmID fused silica columns. These columns are now in the experimental stage of development.[2] Typical peak volumes obtained on 30-100 cm long packed

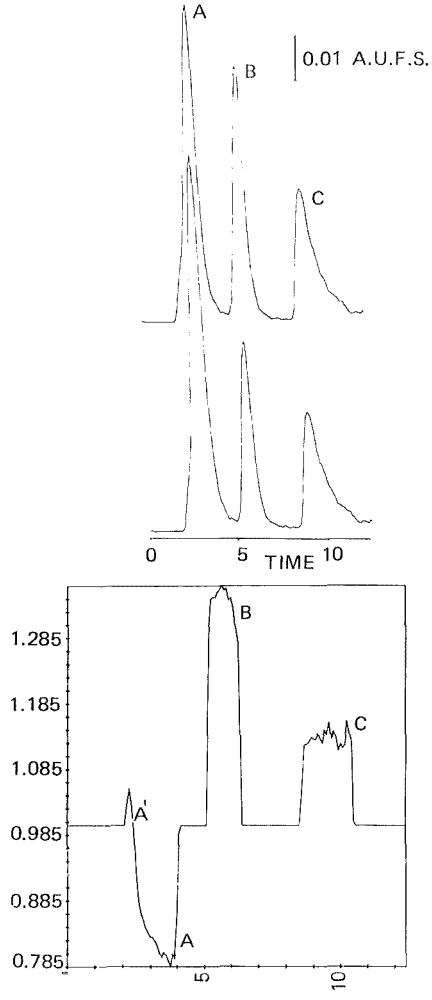

Figure 7. 230/240 nm ratio chromatogram and standard 230, 240 nm chromatograms of N-hydroxythienopyridine sample (reprinted with permission from reference 14).

capillary columns have been of the order of 2-10 $\mu\ell$. Thus, detector flow cell volumes below 200 nℓ are required for packed capillary HPLC. An ingenious solution to this problem has been the use of "on-column detection."[19]

On-column detection has been implemented by inserting the bottom of the optically transparent fused silica capillary column into the light path between

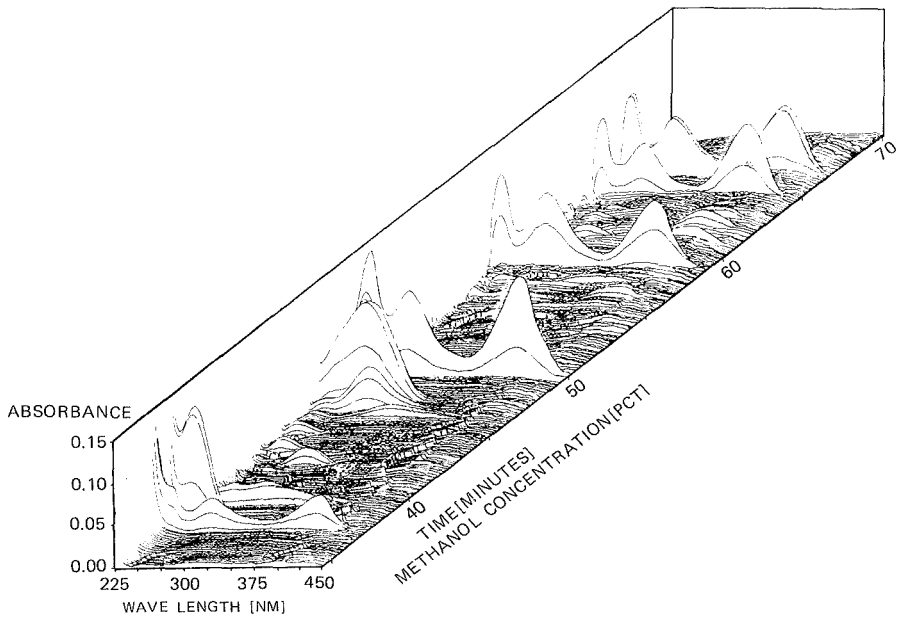

Figure 8. 225-450 nm spectrochromatograms of drug and metabolites in dog bile. (reprinted with permission from reference 14).

the monochromator and photodetector of a standard optical detector (absorbance or fluorescence) as shown in Figure 9. The column itself thus becomes the flow cell, eliminating the need for coupling tubing and providing an extremely small illuminated detection volume. For example, Yang removed 0.2 mm in length of the polyimide cladding at the bottom of a 0.3 mmID fused silica capillary column to form a 0.3x0.2 mm flow cell of 14 nanoliter volume. This produced insignificant band broadening with column generated peak volumes of 2 $\mu\ell$ (see Figure 10).

The disadvantages of the on-column absorbance technique are two-fold. The reduction in the optical path from a standard flow cell length of 5-10 mm to one of 0.3 mm, reduces signal by x15-30. This washes out most of the signal improvement inherent to capillary HPLC's lower dilution of an injected analyte. (100-500 $\mu\ell$ peak values of standard HPLC reduced to 2-10 $\mu\ell$ peak values.) In

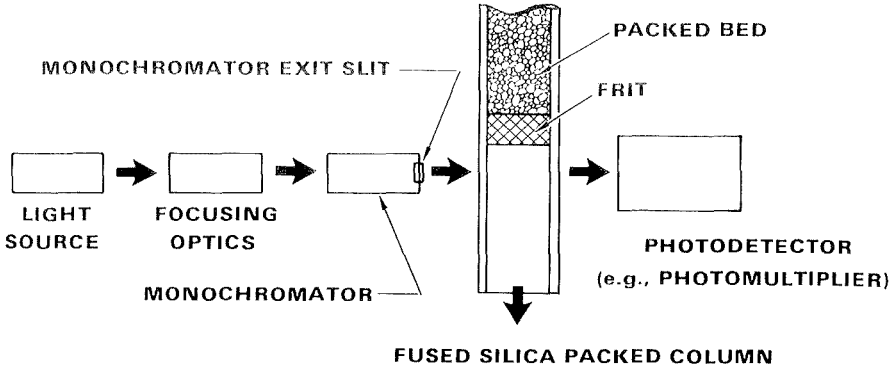

Figure 9. Schematic diagram for on-column absorbance detection in packed capillary micro HPLC.

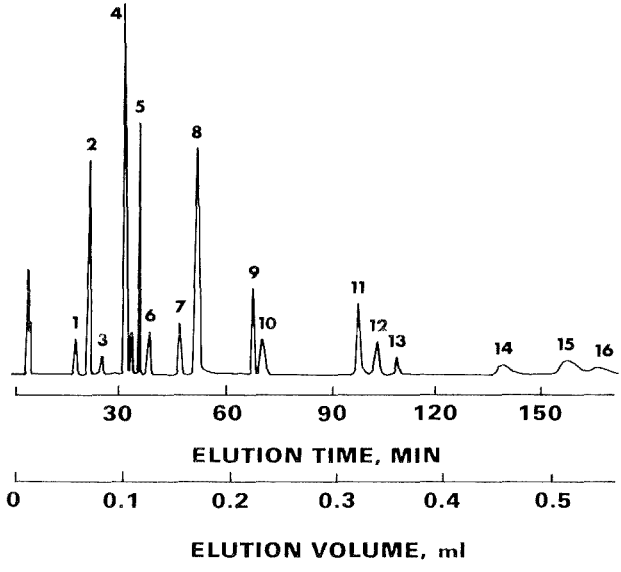

Figure 10. Chromatogram of 16 component PAH mixture separated on 50,000 plate, 45 cm x 330 μID 3 μC18 packed column, used "on-column" absorbance detection. Peak identifications are: 1, naphthalene; 2, acenaphthalene; 3, acenaphthene; 4, fluorene; 5, phenanthrene; 6, anthracene; 7, fluoranthene; 8, pyrene; 9, benzo[a]anthracene; 10, chrysene; 11, benzo[b]fluoranthene; 12, benzo[k]fluoranthene; 13, benzo[a]pyrene; 14, dibenzo[a,h]anthracene; 15, benzo[ghi]perylene, 16, indeno[1,2,3-cd]pyrene.

addition, the focussing optics of the detector into which the "on-column" flow cell was inserted was designed for imaging into larger cells. Thus the on-column flow cell is "over-filled" with incident light. This results in non-optimum light throughput and more importantly in high refractive index and thermal sensitivity of the detection system.* It is now well known in HPLC absorbance detector design,[20] that the incident light beam must traverse the flow cell without striking the cell walls in order to avoid high refractive index and thermal sensitivity.

In Figure 11, we see capillary chromatograms obtained using on-column absorbance and fluorescence detection.[21] Note the long term "wavy" drift of the absorbance detector baseline. This is due to slow variations in ambient temperature and the high thermal sensitivity of the optically overfilled on-column flow cell. The fluorescence on-column technique shows a flat baseline despite the fact that the cell was optically overfilled. The thermal sensitivity of the fluorescence technique is far less than that of absorbance. A further example of on-column fluorescence is shown in Figure 12. In this case, the on-column technique is utilized with a .075 mmID open tubular capillary operated in an electrophoretic mode,[22] yielding sub-$\mu\ell$ peak volumes.

Interfacing Micro HPLC to Optical Gas Phase Detectors

The low flow rates of packed capillary micro HPLC has allowed direct interfacing to the element-specific flame photometric detector[5] (see Figure 13). Initial experimental results demonstrated 100 pg/sec detectivity for phosphorous. For a 10 second micro HPLC peak and a typical phosphorous content in a molecule of ~10% by weight, this translates to detection of 10 ng of a P-

*Although flow sensitivity is also increasd by light striking the cell walls, the absorbance detector's response to a flow fluctuation is directly proportional to the absolute value of that fluctuation. Since the flow rates of capillary HPLC are extremely low (<10 $\mu\ell$/min), the baseline response to a micro-flow fluctuation is too low to be observed.

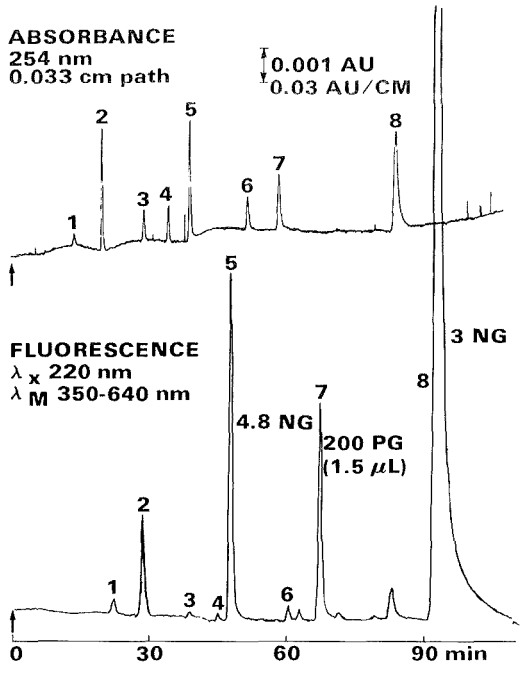

Figure 11. On column absorbance and fluorescence detection of PAH's in micro HPLC. 3 μ C18 reverse phase column, 0.33 mmID x 50 cm. 1.65 μℓ/min 80/20 acetonitrile/water. 250 atm. Absorbance data on Jasco Unidec-III spectrometer. Fluorescence data on Varian Fluorichrom filter fluorometer.

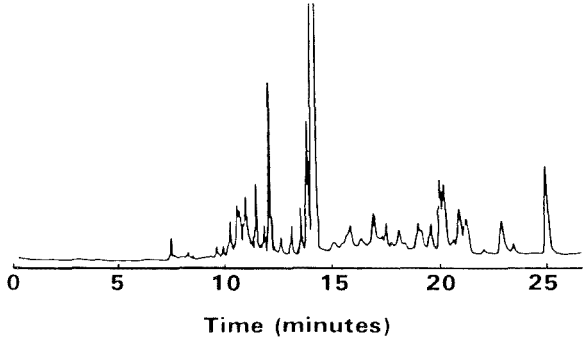

Figure 12. On-column fluorescence detection in glass capillary electrophoresis. .075 mmID x 100 cm column, 30 kilovolts applied voltage. Sample is human urine labelled with fluram tag (reprinted with permission from reference 22).

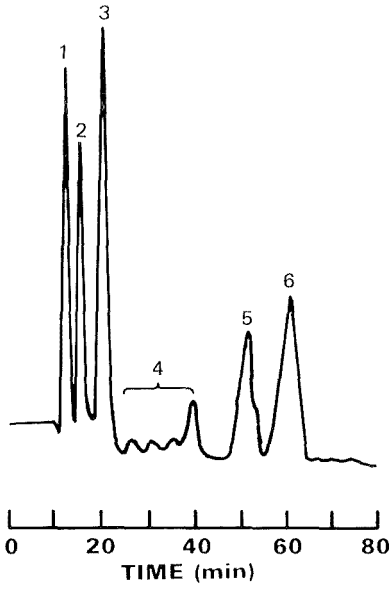

Figure 13. Micro-HPLC-FPD of organophosphorus pesticides. Column-0.07 mmID x 10 meters C18. 1 μℓ/min 42/58 methanol water. Peak identities. 1,4=impurity. 2=80 ng cygon. 3=280 ng DDVP. 5=230 ng malathion. 6=200 ng guthion (reprinted with permission from reference 5).

containing compound. P-specific detection is of great interest to phospholipid research. Although initial work is encouraging, one should note that micro HPLC-FPD success using aqueous buffer mobile phases has not yet been demonstrated. Potential problems in this case would involve precipitation of salts and non-volatile solutes in the detector system. Other potential problems could be flow sensitivity (since FPD is a mass-sensitive detector) necessitating a non-reciprocating or syringe pump to deliver pulseless flow, and gradient elution incompatibility due to dependence of sample response on mobile phase composition.

III. New Optical Detection Techniques in HPLC

Sensitized Room Temperature Phosphorescence

Only 10-20% of organic molecules fluoresce strongly enough ($\epsilon > 10^3$, $\phi_F > 0.10$) to dictate use of fluorescence detection in HPLC. The low quantum yields of the

other absorbing organic molecules are typically due to intersystem crossing from the first excited electronic singlet state (the state capable of fluorescence) to an excited triplet state. Whereas singlet state lifetimes are of the order of nanoseconds, triplet state radiative lifetimes are millisecond or longer. Thus, in solution, collisions with solvent and solvent impurities (e.g., O_2) will deactivate the triplet state before it can emit a photon. Thus, with few exceptions, triplet state emission (phosphorescence) of organic molecules is not observed in room temperature solution.

An exception to the above rule is the molecule biacetyl. Strong biacetyl phosphorescence (~10% quantum yield) is observed at room temperature in solution if the solution has been thoroughly deoxygenated ($<10^{-8}$M). This observation has been utilized for the detection of molecules such as polychlorobiphenyls, that are characterized by high intersystem crossing rates. The technique, sensitized room temperature phosphorescence (SRTP), is based on energy transfer from the analyte triplet state to the triplet state of biacetyl which has been added as a dopant to the HPLC mobile phase. The diffusion-controlled energy transfer requires that the analyte donor triplet be $\gtrsim$5 kcal/mole higher in energy than that of the biacetyl acceptor. A standard fluorescence detector is set up to excite the analyte donor at a wavelength at which biacetyl acceptor absorption is negligible (e.g., 300 nm) and to collect biacetyl phosphorescence (520 nm). Biacetyl phosphorescence can thus be used as a direct measure of analyte concentration. Detection of both halonaphthalenes and polychlorinated biphenyls at ~10 ppb levels has been achieved. A typical HPLC-SRTP chromatogram is shown in Figure 14.

Inductively Coupled Plasma (ICP) Detection in HPLC

In an ICP source, an electrodeless argon plasma is formed at atmospheric pressure and is sustained by inductive coupling to a high frequency magnetic field. A liquid stream fed into the plasma by a nebulizer is rapidly vaporized and the vapor is dissociated into free atoms which are excited and ionized by

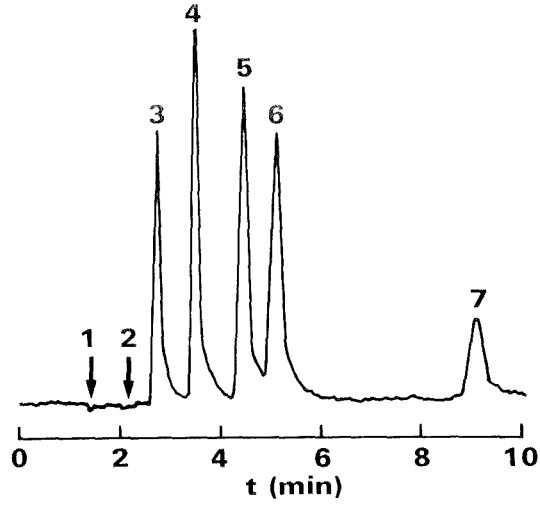

Figure 14. HPLC-SRTP of PCB's by reverse phase. 1 mℓ/min 83.7/16.3 acetonitrile/water. Biacetyl added at 10^{-4}M. λ×260 nm. λ_M 552 nm. Peak identities: 1. solvent peak. 2. oxygen. 3. 15 ng biphenyl(B). 4. 23 ng 3-Cℓ-B. 5. 25 ng 3,3'-$Cℓ_2$B. 6. 22 ng 3,5-$Cℓ_2$ B. 7.39 ng 3,5,3',5'-$Cℓ_4$ B.

the 6000-10,000°K plasma temperature. Elemental emission lines emanating from the plasma are collected by a polychromator and detected at suitable wavelengths by photomultiplier tubes appropriately located in the focal plane of the polychromator grating.

ICP, developed as a technique for simultaneous multi-element detection, is widely used for analysis of metals in complex solid and liquid sample matrices. ICP sensitivity for metals is typically ~ppb, and sensitivity to non-metals (e.g., C,P,N) is ~100 ppb. Compatibility with liquid sampling led to interfacing of ICP spectrometers to HPLC in several research laboratories.[24,25]

Results to date demonstrate the advantages of simultaneous multi-element detection:[25] the compatibility of standard HPLC flow rates with pneumatic nebulization into the ICP plasma; plasma stability in aqueous mobile phases; and an element-specific response independent of molecular form and matrix effects.

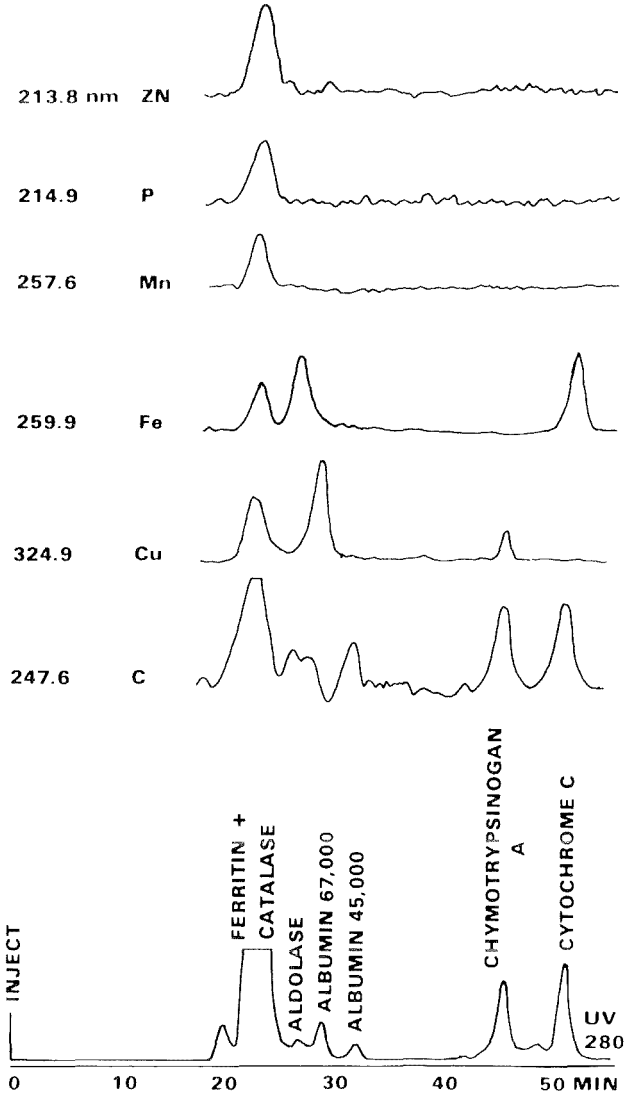

Figure 15. HPLC-ICP of proteins. 100 μg each protein. Steric exclusion separation on two TSK 3000 SW columns, 7.5 mm x 60 cm. 1 mℓ/min 0.9% NaCℓin water (reprinted with permission from reference 25).

TABLE 5
ICP ELEMENTAL LINES FOR HPLC*

NON-METALS		METALS	
C	193.1, *247.9* nm	Fe	*259.9*, 240.5
P	178.3, *214.9*	Zn	*213.9*, 202.6
N	174.3, 411.0	Cu	*324.8*, 224.7
S	180.7, 182.4	Pb	220.4, 217.0
Br	163.4, 157.7	Mn	*257.6*, 294.9
Cl	741.4, 452.6		

Wavelengths in italics are lines used in work to date.

**First wavelength corresponds to strongest line.*

However, work to date also indicates several disadvantages of ICP as an HPLC detector: it is quite expensive (~$100K); observed sensitivity to non-metals of ~ppm[26] is marginal for HPLC significant elements such as phosphorous; and plasma stability problems have been observed with hydrocarbon type organic solvents used in straight phase chromatography.

An HPLC-ICP chromatogram demonstrating the qualitative information inherent to multi-element specific detection is shown in the separation of metal-containing proteins of Figure 15. Note that ICP sensitivity to metals is 100x better than that observed for non-metals. Since non-metals are of greatest significance in HPLC, research aimed at enhanced non-metal ICP detection is required. The use of vacuum UV detection is now under study since the optimum emission lines of most non-metals lie in the 160-195 nm region (see Table 5).

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JOURNAL OF LIQUID CHROMATOGRAPHY, 6(S-1), 105-118 (1983)

ANALYSIS OF FLAVONOIDS BY HPLC

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INTRODUCTION

During the past ten years there has been a dramatic increase of interest in the field of flavonoid chemistry. This was mainly due to the application of HPLC to the study of flavonoids. These compounds, natural plant constituents with a structure based on the aromatic heterocycle, 3-phenylbenzopyrone, are ideally suited for analysis by HPLC. The basic complex structure can be varied by the number and position of hydroxyl substituents and other derivatives (sugars, methyl). These variations cause changes in the way the compounds react to solvents and columns commonly used in HPLC allowing separation and identification.

Since 1979 some pertinent and excellent reviews have been reported: Kingston [88], the application of HPLC to secondary metabolites; Adams and Nakanishi [89], selected examples of HPLC separation of natural products; Van Sumere et al [90], use of HPLC in the separation of plant phenolics; Roston and Kissinger [91], HPLC determination of phenolic acids of vegetable origin;

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Schwartz and von Elbe [92], HPLC of plant pigments; Rouseff and Ting [93], analysis of polymethoxyelated flavones in citrus; Hrazdina [94], analyses of anthocyanins in fruits and beverages; and K. and M. Hostettmann [95], the application of HPLC techniques to flavonoids analysis.

SILICA GEL COLUMNS

Various types and brands of columns have been used for flavonoid analysis using isocratic or gradient solvent systems. Early work was done on silica gel columns with and without derivatization prior to analysis. Although a major advantage of HPLC is the lack of derivatization prior to analysis, Hermann and co-workers cited several advantages to HPLC analysis of acetylated flavonoids. Among these were: durability of the column, definite identification and quantitation, and isocratic separation of the flavonoids. The isocratic run does not require solvent re-equilibration for each sample and this increases the rate of analysis.

Although silica gel columns have not been used often, they are well suited for the separation of non-polar or weakly polar flavonoid aglycones such as: polymethoxylated flavones [86,87], isoflavones [38,48,49,52], and biflavonoids [38]. The normal phase liquid chromatography on Lichrosorb Si 60 of the acetates of numerous flavonoids permitted the application of flavonoid analysis to celery [77-79], orange juice [81,82], tomatoes [79], plums [80], and cherries [83].

Reverse Phase Versus Normal Phase. By using the above discussed packing materials, (columns) the stationary phase is less polar than the mobile phase and the procedure is called reverse phase chromatography (RPLC). Thus highly polar solutes possess shorter retention times than less polar solutes. The retention time of the same polymethoxylated flavones shows this difference between RPLC [84,85] and normal phase HPLC (NPLC) [86]. However, with both types of columns, excellent separation was obtained in less than 30 minutes.

The use of RPLC [46] for the resolution of isoflavones was criticized [48] for the observed band spreading which would make the separation of multicomponent mixtures virtually impossible. A number of recent reports [53-56], however, have established a RPLC procedure for the separation and quantitation of the naturally occurring soybean isoflavone glycosides and aglycones.

REVERSE PHASE COLUMNS

In reverse phase columns, the stationary phases are prepared by bonding various organosilane molecules to the hydroxylic groups of a silica type surface. The most common of the organosilanes are octadecyltrichlorosilane, octyltrichlorosilane, and phenyltrichlorosilane. The simple procedures for preparing octadecylsilyl bonded stationary phases have been described [96,97] and may be employed, for example, for the preparation of preparative scale columns. Currently there are a number of commercially available columns possessing a high degree of reproducibility as reflected in the table.

Although the C_{18} or more specifically the μBondapak C_{18} column has been the dominant choice for RPLC of flavonoids, another type of column may be better dependent upon the class of flavonoid. In a comparison of Lichrosorb RP-18 and RP-8, Strack and Krause [41] obtained better resolution of glycosylflavone aglycones and glycosides on the RP-8 with a gradient methanol : acetic acid : water solvent system. Becker et al [39] in the only reported use of a Lichrosorb NH_2 column, achieved an excellent separation of isomeric O-glycosides of C-glycosylflavones. This type of column had been used for the separation of monosaccarides and for this reason chosen as the ideal phase for separation of C-glycosylflavones. There seemed to be no apparent differences in efficiency of separation of the flavonoids of Silybum marianum between the Lichrosorb RP-18 [33] and RP-8 [34,35] columns. However, two different solvent systems were used and an objective comparison cannot be made.

A μBondapak C_{18} with a acetonitrile : water solvent system failed to adequately separate methoxylated flavones but a Zorbax C_8 column with a tetrahydrofuran : water system did [84].

The difference in columns with the same generic name and a lack of consistent specifications make the choice of reverse phase columns difficult. The initial choice of a column for a particular reverse phase separation would involve matching the type of substance (class of flavonoid) to be separated to the column capable of providing good retentivity and selectivity characteristics toward the particular sample. Because few papers contain negative information on columns and this field is relatively new, the researchers should be aware of any available commercial literature [98].

Aromatic Acids and Flavonoid Separation. Extraction of phenolics from plant tissue usually means that both single ring phenolics and the flavonoids (C_6-C_3-C_6 carbon skeleton) are isolated in the same mixture. Two reports [72,74] advised carrying out prior separation of these groups for HPLC. Where a μBondapak alkylphenyl column was used, some of the aromatic acids and phenols interfered with separation of the flavonoids [72]. However, RPLC on a μBondapak C_{18} column proved to be effective in the separation of both the plant phenols and flavonols of tobacco [65]. A gradient elution system was found to be most effective and necessary for the separation of the aromatic acids, phenols, and flavonoids in the same mixtures [63]. There are also two other communications that have dealt with the separation of aromatic acids, phenols, and a few flavonoid compounds [66,67].

Solvent Systems. Methanol : water containing small amounts of acetic acid is one of the more commonly used solvent systems for RPLC of flavonoids. The addition of acetic or any acid improves the separation, but the amount of acid or pH of the eluting system is dependent upon the column's stability as a Chrompack Nucleosiel C_{18} column ruptured probably due to a low pH [31]. In lieu of acetic acid other compounds which have been used are: phosphoric acid

[34,35,50,64,71], perchloric acid [16,21,24], potassium dihydrogen phosphate [5,22,61,65], ammonium dihydrogen phosphate [36], and formic acid [4,7]. The absence of acid in the methanol : water system is rare [42] but such a solvent system appears to be very successful in the separation of isoflavones [51,53-56].

The acetonitrite : water system was successful in the quantitative determination of naringin and hesperidin in citrus juice [58-60,62] and in the separation of both isomeric C-glycosylflavones [39] and isomeric isoflavones [46]. The acetonitrite : acetic acid : water solvent system was most popular for the separation of flavonoids of flowers [44,70].

Other solvent systems use in RPLC are: tetrahydrofuran (THF) : acetonitrile : water [86]; THF : water [45]; acetone : acetic acid : water [8,9,11]; methanol : dimethylformamide : acetic acid : water [29]; and ethanol : acetic acid : water [72].

Effect of Structure on Retention. The effect of structure of flavonoids on their elution behaviour was first disclosed by Wulf and Nagel [57]. These data were confirmed and extended by Daigle and Conkerton [37]. In both cases a μBondapak C_{18} and a methanol: acetic acid : water solvent system was used. The influence of isomerization and the glycosylation pattern of some C-glycosylflavones on retention time was studied [40] and some of these findings were confirmed in a later report [43]. The chromatographic behavior of proanthocyanidins was investigated using several packing materials (columns) [21,24]. Lea concluded that RPLC offered the greatest potential for the separation of proanthocyanidins.

GENERAL

Detection. The high sensitivity of ultraviolet-visible detectors make analysis of sub-microgram samples possible. The wavelength must be compatible with the solvent system and, of course, suitable for the compound to be

Flavonoid Analysis By HPLC

Flavonoid Type	Column	Mobile Phase	Commodity Studied	Reference
ANTHOCYANIDINS	µBondapak C_{18}	methanol:acetic acid:water (20:5:75)	Std.[1]	1
	µBondapak C_{18}	methanol:acetic acid:water (19:10:71)	Muscadine grape skins (Vitis rotundifolia)	2
			Roselle (Hebiscus sabdariffa)	
ANTHOCYANINS				
3-G[2]/(AC)[3]	Pellidon	chloroform:methanol (87:13)	Grapes	3
3-G,E[4]	Lichrosorb ODS	10% formic acid in water:methanol (GR[5])	Grapes (Cabernet Sauvignon and Pinot noir)	4
3-G	Lichrosorb RP-18	0.1 M KH_2PO_4 in water:methanol (GR)	Wine	5
3-G; 3,5-Di G[6],E	µBondapak C_{18}	acetic acid:water (15:85) 0.1% H_3PO_4 in acetic acid:water (10:90) methanol:acetic acid:water (20:15:65)	Grapes (concord, Ives, DeChaunac)	6
3-G, E	Lichrosorb ODS	10% formic acid in water:methanol (GR)	Wine (Merlot and Cabernet Sauvignon grapes)	7
3-G	µBondapak C_{18}	acetone:acetic acid:water (7:10:83)	Poinsettia (Amy, V-10)	8
		" " " " (")	Poinsettia (28 cultivars)	9
3-G	µBondapak C_{18}	methanol:acetic acid:water (37:10:53)	Cranberry	10
3-G, 3,5-Di G	µBondapak C_{18}	acetone:acetic acid:water (7:10:83)	Rhododendron Simsii Planch (Azalea Indica L.), 7 cultivars	11
A	Lichrosorb RP-8	acetonitrile:acetic acid:H_3PO_4:water (GR)	Gladiolus	12
3-G; 3,5-Di G; E	Lichrosorb RP-18	acetonitrile:acetic acid:H_3PO_4:water (GR)	Std.	13
3-G, 3,5-Di G; E	µBondapak C_{18}	10% formic acid in water:methanol (GR)	Perilla	14
3-G; 3,5-Di G; E	Whatman ODS	methanol:formic acid 5 or 10%:water (GR)	Wine (Zinfandel and Cabernet Sauvignon)	15
3-G, Chalcones	Spherisorb Hexyl	0.6% $HClO_4$ in water:methanol (GR)	Std.	16
3-G, 3,5-Di G	Lichrosorb RP-18	acetonitrile:acetic acid:H_3PO_4:water	Gladiolus	17
Proanthacyanidins	VYDAC	hexane:methanol:chloroform:acetic acid (GR)	Beer	18,19
	SiC_{18} (RSL-Belgium)	acetic acid:water (GR)	Beer	20
	SAS Hypersheres	methanol:water:$HClO_4$ (20:80:0.1)	Cider	21
	Spherisorb S5-ODS	methanol:0.075 M KH_2PO_4:water (GR)	Beer	22
	Zorbax ODS	1% acetic acid in water:acetonitrile (GR)	Wine (Merlot and Cabernet Sauvignon)	23
	Spherisorb Hexyl	.1% $HClO_4$ in water:methanol (GR)	Cider	24
	SiC_{18} (RSL-Belgium)	acetic acid:water (GR)	Barley	25

	μBondapak C_{18}	methanol:water (GR)	Barley (Hordeum vulgare, 10 cultivars) Hops (Humulus Lupulus, 8 cultivars)	26
	Lichrosorb RP-8 Lichrosorb Si 60	methanol:water (GR) tetrahydorfuran:methanol:acetic acid:hexane (1:3:0.4:4)	Beer Sorghum	27 28
theaflavins	μBondapak C_{18}	methanol:dimethylformanide:acetic acid:water (2:1:40:157)	Tea	29
	Partilsil 5 C_{22}	methanol:acetone:water (GR)	Tea	30
Dihydroflavonols	μBondapak C_{18}	methanol:0.1% acetic acid:water (GR)	Larix (7 species)	31
	μBondapak C_{18}	methanol:acetic acid:water (40:5:60)	Silybum marianium Gaertn.	32,33
	Lichrosorb RP-18	methanol:acetic acid:water (40:5:60)	Silybum marianum Gaertn.	33
	Lichrosorb RP-8	0.02 M H_3PO_4 in water:methanol (GR)	Silybum marianum	34,35
	Partisil-10 ODS	2% $NH_4H_2PO_4$ in water:methanol (1:1)	Virginia pine (Pinus virginiana Mill.)	36
	μBondapak C_{18}	methanol:acetic acid:water (30:1:69)	Std.	37
Biflavonoids	Pellosil-HC	isopropylether containing 8% methanol	Std.	38
C-Glycosylflavones				
G	Lichrosorb NH_2	acetonitrile:water (GR)	Std.	37
A, G	Zorbax ODS	methanol:0.1% acetic acid:water (GR)	Larix (seven species)	31
	Zorbax ODS	0.1M H_3PO_4 in water:ethanol (GR)	Std.	40
A,G	Lichrosorb RP-8	methanol:acetic acid:water (GR)	Duckweed (Spirodela), Buchwheat (Fagopyrum)	41
A,G	Lichrosorb RP-18			
A	Zorbax ODS	methanol:water (GR)	Passiflora incarnata	42
A,G	Lichrosorb RP-8	methanol:acetic acid:water (GR)	Oats (Avena sativa)	43
A	μBondapak C_{18}	acetonitrile:acetic acid:water (GR)	Passion Flower; Hawthorn	44
A,E	Copell ODS	tetrahydrofuran:water (4:53)	Zizyphus vulgaria var. spinosus	45
Isoflavones				
A	Merkosorb Si 60	hexane:tetrahydrofuran (2:1) or (9:1)	Std.	38
A	Portisil-10 ODS	acetonitrile:water (1:4)	Soybeans	46
GAc	μBondapak C_{18}	methanol:water (GR)	Soybeans	47
A	μPorasil	dichloromethane:ethanol:acetic acid (97:3:2)	Soybeans :hexanes (8:2)	48,49

(continued)

Flavonoid Analysis By HPLC

Flavonoid Type	Column	Mobile Phase	Commodity Studied	Reference
A	Lichrosorb RP-8	water (pH = 2.8, H_3PO_4):acetonitrile (GR)	Std.	50
G	µBondapak C_{18}	methanol:water (GR)	Soybeans	51
A	µPorasil	dichloromethane:ethanol:acetic acid (97:3:2):hexanes (8:2)	Garden peas (Pisum sativum)	52
A,G	Zorbax ODS	methanol:water (GR)	Soybeans	53,54,55
A	Sperisorb-5 ODS	methanol:water (GR)	Bengalgram	56
Flavanones				
A,G	µBondapak C_{18}	methanol:acetic acid:water (30:5:65)	Std.	57
G	µBondapak C_{18}	acetonitrile:water (20:80)	Grapefruit	58,59,60
A,G	Zorbax ODS	methanol : 0.1%:acetic acid:water (GR)	Larix (7 species)	31
A,G	µBondapak C_{18}	0.03M KH_2PO_4 in water:methanol (GR)	Orange	61
G	Lichrosorb RP-18	acetonitrile:water (20:80)	Orange, Grapefruit	62
A	µBondapak C_{18}	butanol:methanol:acetic acid:water (5:25:2:6)	Soybeans (Glycine max L., Forrest)	63
A,G	µBondapak C_{18}	methanol:acetic acid:water (30:1:69)	Std.	37
Flavonols				
A,G	µBondapak C_{18}	methanol:acetic acid:water (30:5:65)	Std.	57
G,E	Zorbax ODS	ethanol:0.1% H_3PO_4:water (GR)	Cedrus atlantica c.v. Glauca	64
A,G	µBondapak C_{18}	0.1N KH_2PO_4 in water:methanol (GR)	Tobacco (Nicotiana tabacum L.)	65
G	Zorbax ODS	methanol:0.1% acetic acid:water (GR)	Larix (7 species)	31
G	µBondapak C_{18}	2% acetic acid in water:tetrahydrofuran (GR)	Poinsettia (2 cultivars)	66
G	µBondapak C_{18}	methanol:water:acetic acid:tetrabutylammonium-phosphate (GR)	Std.	67
G	Zipax HCP	0.5M KH_2PO_4:ethanol:ethyl acetate (GR)	Geranium Thunbergii Sieb. et Zucc.	68
G	Zorbax ODS	methanol:water (GR)	Tetrapanax papyriferum	69
G	µBondapak C_{18}	2% acetic acid in water:acetonitrile (GR)	Poinsettia (38 cultivars)	70
G,E	Zorbax ODS	2% acetic acid in water:tetrahydrofuran (GR) methanol:0.1% H_3PO_4:water (GR)	Larix (L. gmelinii)	71
A	µBondapak alkylphenyl	ethanol:acetic acid:water (47.5:5.0:47.5)	Std.	72

A	Partisil-10 ODS	methanol:NH_4 H_2PO_4:water (49:1:49)	Virginia pine (Pinus virginiana mill.)	36
A	μBondapak C_{18}	methanol:acetic acid:water (GR)	Opuntia (fulgida, spinosior, and acarthocarpa	73
A,G	Lichrosorb RP-8	water (pH = 2.8, H_3PO_4):acetonitrile (GR)	Std.	50
G	Zorbax ODS	1% acetic acid in water:acetonitrile (GR)	Wine (Merlot and Cabernet Sauvignon)	23
A	μBondapak C_{18}	acetonitrile:acetic acid:water (30:2:68)	Pine (Pinus elliotti)	74
A,G	μBondapak C_{18}	2.5% acetic acid in water:tetrahydrofuran (GR)	Hops (10 cultivars) and Barley (8 cultivars)	26
A,G,E	HC-ODS-Sil-X	2% acetic acid in water:acetonitrile (GR)	Matricaria chamomilla L.	75
A,G	μBondapak C_{18}	methanol:acetic acid:water (30:1:69)	Std.	37
Flavones				
A	μBondapak C_{18}	methanol:acetic acid:water (30:5:65)	Std.	57
A,G	Zorbax ODS	methanol:0.1% acetic acid:water (GR)	Larix (7 species)	31
A,G	μBondapak C_{18}	methanol:acetic acid:water (30:1:69)	Std.	37
G	μBondapak phenyl	methanol:acetic acid:water (GR)	Sugar cane	76
Flavonoid Acetates flavonols, flavonones, and their G	Lichrosorb Si 60	benzene:acetonitrile (85:22) benzene:acetone (90:15) benzene:ethanol (80:0.7)	Celery, Orange, and Tomatoe	77,78,79
flavonol G	Lichrosorb Si 60	benzene:acetonitile (80:20)	Plum	80
flavanone G	Lichrosorb Si 60	benzene:acetonitrile (85:20)	Orange	81,82
flavonols and G	Lichrosorb Si 60	isooctane:ethanol:acetonitrile (70:16:5.5) benzene:acetonitrile (80:20)	Cherries (Prunus avium L. and Prunus cerasus L., 7 cultivars)	83
Methoxylated Flavones				
A	Micropak C_{18}	acetonitrile:water (40:60)	Orange (Valencia), Tangerine (Dancy)	84
A	Zorbax C_8	tetrahydrofuran:water (25:75)		
A	Zorbax C_8	tetrahydrofuran:acetonitrile:water (22:6:72)	Orange	85
A	Lichrosorb Si 60	heptane:isopropanol (60:40)	Orange, Tangerine	86
A	Lichrosorb Si 60	heptane:ethanol (90:10) and (75:25) heptane:isopropanol (70:30) and (60:40)	Orange, Tangerine	87

1/ Std. = Standards 2/ G = glycosides 3/ Ac = acetates 4/ E = esters 5/ GR = gradient 6/ Di G = diglycosides 7/ Aglycones

detected at maximum sensitivity. For the flavonols, flavanones, flavones, and isoflavones and their respective glycosides, the range of wavelength between 254 nm and 280 nm was the most popular. A few researchers have also chosen the 340 nm to 360 nm range [8,26]. The anthocyanins and proanthycyanidins were detected either in the range between 520 nm and 546 nm or at 280 nm. The acetate derivatives of anthocyanidins were detected at 254 nm [3] and their chalcones at 340 nm [16]. The acetates of flavonols, and flavones and flavanones, however, were detected at 300 nm (77-83). Rouseff and Ting [10,93] employed a dual UV-fluorescence detector to determine the presence of interfering substances. The wavelength, 313 nm, was for all five polymethoxylated flavones since the impurity absorbed weakly at this wavelength. The C-glycosylflonones have been detected at a variety of wavelengths: 254 nm [39,40]; 270 [42]; 312 [41]; 330 [45]; 335 [22]; and 365 [43].

Qualitative Analysis of Flavonoids. In the HPLC analyses of complex mixtures researchers have used more than retention time data to identify, positively or negatively, the compound of interest. However, off line techniques are relatively time consuming. Recently on line techniques such as HPLC-UV/Vis and HPLC/MS spectroscopy have been found, in some cases, to be sufficient to obtain postive identification of compounds with an overall reduction in analyses time [50].

The identification of the chalcones of malvidin 3-glucoside and malvidin 3,5-diglucoside was carried out by using detectors monitoring simultaneously first at 280 nm and 525 nm, then 280 nm and 340 nm. As a result, these chalcones could be collected in sufficient amounts to measure their UV-visible absorption spectra directly and to follow their conversion to their corresponding flavylium cations [16].

SUMMARY

In the flavonoid field, HPLC has been used for the quantitative determination of plant constituents, purity verification of isolated compounds, and

chemotaxonomical comparisons. The practical applications in the beer, wine, and citrus industries also attest to its potential as an analytical tool.

Acknowledgment

The authors acknowledge the excellent assistance of Mrs. D. B. Skau, Librarian.

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LC CALENDAR

1983

MARCH 7-12: Pittsburgh Conference on Anal. Chem. & Applied Spectroscopy, Convention Hall, Atlantic City, NJ, USA. Contact: Mrs. Linda Briggs, Program Secretary, 437 Donald Rd., Pittsburgh, PA, 15235, USA.

MARCH 20-25: National Amer. Chem. Soc. Meeting, Seattle, WA, USA. Contact: A. T. Winstead, Amer. Chem. Soc., 1155 Sixteenth St., NW, Washington, DC, 20036, USA.

APRIL 18-21: Fundamentals of Chromatographic Analysis, Kent State University, Kent, Ohio. Contact: University Conference Bureau, 211-A Kent Student Center, Kent State University, Kent, OH, 44242, USA.

APRIL 25-27: Fifth International Symposium on Coal Slurry Combustion and Technology, Hyatt-Regency Hotel, Tampa, Florida. Contact: N. Maceil, JWK Int'l Corp., 275 Curry Hollow Rd., Pittsburgh, PA, 15236.

APRIL 26-28: Capillary Chromatography: 5th Int'l Symposium, Riva del Garda, Italy. Contact: Dr. P. Sandra, Laboratory of Organic Chem., University of Ghent, Krijgslaan 281(S4), B-9000 Ghent, Belgium.

MAY 2-6: VIIth International Symposium On Column Liquid Chromatography, Baden-Baden, West Germany. Contact: K. Begitt, Ges. Deutscher Chemiker, Postfach 90 04 40, Varrentrappstrasse 40-42, D-6000 Frankfurt (Main), West Germany.

MAY 15-17: International Symposium on LCEC and Voltammetry, Hyatt - Regency Hotel, Indianapolis, Indiana, USA. Contact: 1983 LCEC Symposium, P. O. Box 2206, West Lafayette, IN, 47906, USA.

MAY 30 - JUNE 3: International Conference on Chromatographic Detectors, Melbourne University. Contact: The Secretary, .np International Conference on Chromatographic Detectors, University of Melbourne, Parkville, Victoria, Australia 3052.

JUNE 1-3: The Budapest Chromatography Conference, Budapest, Hungary. Contact: Dr. T. Devenyi, Institute of Enzymology, Hungarian Academy of Sciences, Budapest, Hungary or Dr. H. Issaq, Frederick Cancer Research Facility, P.O.Box B, Frederick, MD, 21701, USA.

JUNE 22: ASTM Symposium: Computers in Chemical Analysis, Kansas City, MO, Contact: K. Greene, ASTM Publications Div., 1916 Race Street, Philadelphia, PA, 19103, USA.

JULY: 3rd Int'l. Flavor Conf., Amer. Chem. Soc., The Corfu Hilton, Corfu, Greece. Contact: Dr. S. S. Kazeniac, Campbell Inst. for Food Research, Campbell Place, Camden, NJ, 08101, USA.

JULY 17-23: SAC 1983 International Conference and Exhibition on Analytical Chemistry, The University of Edinburgh, United Kingdom. Contact: The Secretary, Analytical Division, Royal Society of Chemistry, Burlington House, London W1V OBV, United Kingdom.

AUGUST 14-19: 25th Rocky Mountain Cinference, Denver Convention Complex, Denver, Colorado. Contact: E. A. Brovsky, Rockwell International, P. O. Box 464, Golden, CO, 80401, USA.

AUGUST 15-19: Coal Science: 1983 Int'l Conference, Pittsburgh, PA. Contact: N. Maceil, JWK Int'l Corp., 275 Curry Hollow Road, Pittsburgh, PA, 15236, USA.

AUGUST 22-26: 7th Australian Symposium on Analytical Chemistry, Adelaide, Australia. Contact: D. Patterson, AMDEL, P.O.Box 114, Eastwood S.A. 5063, Australia.

AUGUST 29 - SEPTEMBER 2: 4th Danube Symposium on Chromatography & 7th I'nt'l. Sympos. on Advances & Applications of Chromatography in Indudtry, Bratislava, Czech. Contact: Dr. J. Remen, Anal. Sect., Czech. Scientific & Techn. Soc., Slovnaft, 823 00 Bratislava, Czechoslovakia.

OCTOBER 12-14: Analyticon'83 - Conference for Analytical Science, sponsored by the Royal Society of Chemistry and the Scientific Instrument Manufacturers' Ass'n of Great Britain, Barbican Centre, London. Contact: G. C. Young, SIMA, Leicester House, 8 Leicester Street, London WC2H 7BN, England.

1984

OCTOBER 1-5: 15th Int'l Symposium on Chromatography, Nurenberg, West Germany. Contact: K. Begitt, Ges. Deutscher Chemiker, Postfach 90 04 40, Varrentrappstrasse 40-42, D-6000 Frankfurt (Main), West Germany.

The Journal of Liquid Chromatography will publish announcements of LC meetings and symposia in each issue of The Journal. To be listed in the LC Calendar, we will need to know: Name of meeting or symposium, sponsoring organization, when and where it will be held, and whom to contact for additional details. You are invited to send announcements for inclusion in the LC Calendar to Dr. Jack Cazes, Editor, Journal of Liquid Chromatography, P. O. Box 1440-SMS, Fairfield, CT, 06430, USA.

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