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CHEMICALLY BONDED STATIONARY PHASES FOR AQUEOUS HIGH-PERFORMANCE EXCLUSION CHROMATOGRAPHY

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SUMMARY

The preparation of chemically bonded stationary phases based on a silica support for aqueous exclusion chromatography is discussed. A defined amount of functional groups can be bonded on to the silica surface only if the appropriate silane is prepared prior to binding it to the surface. The properties of the prepared stationary phases, *i.e.*, wettability by water, adsorption of water-soluble polymers, etc., are described.

Stationary phases with amide bonds can be used to separate by exclusion chromatography polystyrenes and polyethylene glycols in dimethylformamide as eluent as well as polyethylene glycols and dextrans in water as eluent. The graphs of molecular weight *versus* elution volume are, with acceptable deviations, independent of the nature of the polymer.

INTRODUCTION

Aqueous exclusion chromatography is usually performed with soft gels consisting of crosslinked carbohydrates (dextrans) or of the polyacrylamide type[1]. Because of their low mechanical stability against pressure, these soft gels cannot be used in high-performance liquid chromatography (HPLC). Separations of synthetic and natural water-soluble polymers based on exclusion using inorganic stationary phases (porous glass, silica, etc.) have been described[2–4]. The disadvantage of these inorganic supports is the irreversible adsorption and/or denaturation of some of these polymers[4,5]. Modification of the silica support offers a possible means of minimizing these effects.

By binding alkyl or aryl groups on to a silica surface, stationary phases of the reversed-phase type are obtained[6–15]. These stationary phases exhibit hydrophobic or solvophobic sorption properties with organic solutes if water or aqueous mixtures are used as eluents, and the solute–stationary phase interaction is strongest in water. Therefore, the application of reversed phases as stationary phases for exclusion chromatography in aqueous eluent mixtures is limited. Through the introduction of polar functional groups into the organic moiety bonded on the surface, the hydrophobic reversed phases can be converted into hydrophilic phases. The steric exclusion chromatography of biological macromolecules on such a stationary phase with

glycerolpropylsilane as the bonded organic moiety has been described[5,16–18]. These stationary phases, however, still exhibit reversed-phase properties.

In this paper, the preparation and the properties of different hydrophilic chemically bonded phases are described. These stationary phases are optimized for: (a) maximum shielding of the unreactive surface silanol groups on the silica support; (b) minimum reversed-phase character; and (c) good chromatographic properties, *i.e.*, rapid mass transfer as achieved with monomeric coverage of the surface[19].

In considering the properties of reversed phases[9], it was shown that the shielding of the non-reactable surface silanol groups is optimal if the alkyl group is about 3–5 carbon atoms in length. The reversed-phase properties (k' for a given sample) depend on the carbon content of the stationary phase, which is a function of the length of the organic molecule bonded to the surface. In this work, only substituted propylsilanes with different functional groups were used.

The hydrophilic functional groups should be neutral and completely unreactive with different samples.

PREPARATION OF HYDROPHILIC CHEMICALLY BONDED STATIONARY PHASES

If functional groups other than alkyl or aryl groups have to be bonded on to a silica surface, the functional group has to be introduced into the organosilicon compound before binding it to the silica surface. The introduction of these groups by successive reactions with alkylsilanes already bonded to the surface is hindered for two reasons, which are discussed below.

Organic reactions with almost quantitative yields in bulk-phase chemistry give only 20–50% yields if carried out with reactants that are already surface bonded[20,21]. For example, when bonded butyl groups were sulphochlorinated, an ion-exchange sulpho group could be introduced into a maximum of one in five butyl groups[21]. The reversed-phase properties of such an ion exchanger therefore can not be neglected.

Reactions on bonded alkyl- or arylsilanes always lead to a loss in bonded material, which is measurable via organic elemental analysis. In the simple substitution reaction of bromine in a bonded alkyl bromide with hydrogen sulphite, for instance, approximately 30% of the bonded material was lost[21]. Sometimes even the total carbon content decreased, despite the introduction of further organic groups[20].

Hence a tedious and complicated method has to be adopted for synthesizing suitable organosilanes with the desired functional groups and for binding them to the surface of the silica. A possible method for such a synthesis is the preparation of an olefin in which the double bond is in the ω-position to the functional group. These olefins are subjected to reaction with silicochloroform or methyldichlorosilane in the presence of hexachloroplatinic acid or a similar catalyst[22]. This is possible, however, only with functional groups such as nitriles, which do not react with the chlorosilane bonds. By protecting the functional group in the olefin with suitable protecting groups, which are subsequently removed after binding the chlorosilane on to the surface, the scope of this type of reaction could be increased[23]. The removal of the protecting groups sometimes involves the same problems as discussed above.

Alkoxysilanes are less reactive than chlorosilanes, which is advantageous for the preparation of organosilanes with functional groups, but disadvantageous for the reaction with the surface silanol groups of silica, especially if monomeric brush-type

coverage of the surface is required. In order to bind the alkoxyorganosilanes in the same way as chloroorganosilanes on to the surface[8,9], the same precautions have to be taken; in particular, trace amounts of water have to be excluded. To improve the rate of reaction, the alcohol formed was removed by azeotropic distillation. The reaction can also be accelerated by increasing the reaction temperature and/or by the addition of a catalyst such as *p*-toluenesulphonic acid[23].

In a typical procedure, about 10 g of purified silica were suspended in 200 ml of organic solvent and *ca.* 10 ml of the alkoxysilane were added to the suspension. The mixture was heated above the boiling point of the corresponding alcohol, which was removed from the mixture by distillation through a heated reflux condenser, which was kept at a temperature above the boiling point of the alcohol and below the boiling point of the suspending solvent.

Fig. 1 demonstrates the influence of the type of suspending liquid on the rate of reaction. N-(3-Triethoxysilylpropyl)acetamide ("amide") was reacted in the described manner with silica in benzene or tetrahydrofuran (THF) at 65°. The kinetics of the reaction were measured by taking aliquots of the silica, washing and cleaning it in the described manner[8] and determining their carbon and nitrogen contents by elemental analysis. As can be seen in Fig. 1, with the less polar benzene the reaction was completed after *ca.* 12 h, and the carbon content of the chemically bonded stationary phase was over 7% (w/w). However, with the more polar THF as the suspending liquid the carbon content of the silica was only *ca.* 5% (w/w) after a reaction time of 24 h. The reaction could be accelerated further if toluene or xylene was used instead of benzene as the suspending liquid and the reaction temperature was between 100 and 120°. Under these conditions, trimethoxyoctadecylsilane reacted with silica and the reversed phase thus obtained was compared with that prepared with the appropriate chlorosilane[9]. No differences could be observed regarding the total carbon content (20.5% with toluene, 21.2% with xylene), the shape of the *h versus u* curve, if the particle diameters were identical, of course, and the retention behaviour of typical standard compounds, in polar as well as in apolar eluents[9]. When tested in

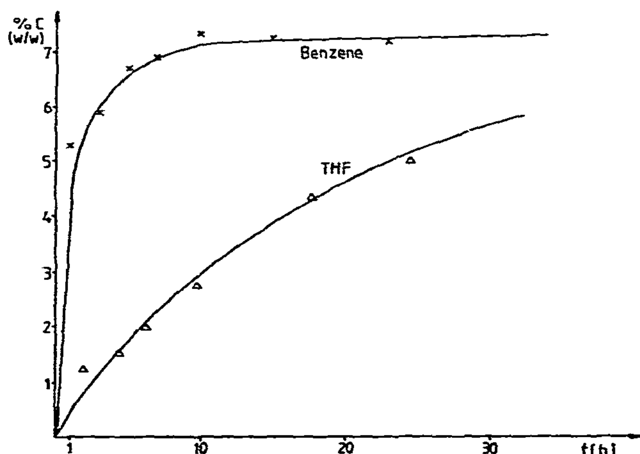

Fig. 1. Kinetics of surface bonding. Reaction of N-(3-triethoxysilylpropyl)acetamide with silica (SI-100) in benzene or tetrahydrofuran at 65°. C content determined by elemental analysis.

gas chromatography, these stationary phases showed the same characteristic properties as "brushes"[24,25].

With the method described here, chemically bonded stationary phases with different functional groups were prepared, as summarized in Table I. Silica SI-100 (Merck, Darmstadt, G.F.R.) with an average pore diameter of 100 Å, a pore volume of 1 ml/g and a specific surface area of about 350 m^2/g was used exclusively as the support. The starting silane for these stationary phases was 3-aminopropyltriethoxysilane, which was made to react with acetic anhydride to yield N-(3-triethoxysilylpropyl)acetamide ("amide"), b.p. 156°/4 mm; with trifluoroacetic anhydride the corresponding "trifluoroamide", b.p. 135°/16 mm, was obtained. The "sulphonamide" was prepared in a similar manner with methanesulphonyl chloride, and the "glycinamide" was prepared by using the cyanoester coupling method[26]. The structure of the silanes was demonstrated by spectroscopic methods before binding them to the surface.

TABLE I

CHARACTERISTICS OF CHEMICALLY BONDED STATIONARY PHASES WITH $Si-CH_2-CH_2-CH_2-X$ ON SILICA SI-100

Stationary phase (X)	*Nomenclature*	*Elemental analysis of bonded phases*		*Surface concentration (μmole/m²)*	*Average spacial requirement (Å²)*
		C (%, w/w)	*N (%, w/w)*		
$-NH_2$	"Amine"	6.28	2.25	4.97	33
$-NH-CO-CH_3$	"Amide"	8.44	1.66	4.02	41
$-NH-CO-CF_3$	"Trifluoroamide"	8.11	1.70	3.86	43
$-NH-SO_2-CH_3$	"Sulphonamide"	8.82	1.98	5.25	32
$-NH-CO-CH_2-NH-CO-CH_3$	"Glycinamide"	8.84	2.57	3.01	55
$-O-CH_2-CHOH-CH_2OH$	"Glycol"	5.48	—	2.17	76
$-(CH_2)_{14}-CH_3$	RP-C_{18}	21.03	—	2.78	60

For comparison, a glycerolpropylsilane-bonded stationary phase ("glycol") was prepared. A reversed phase (RP-C_{18}) and, of course, pure silica which was used as the support were also included in these experiments. The "amine" stationary phase itself was not studied further because it decomposes. During the use of a column packed with the free amine stationary phase, it was noticed that the column properties were changing, and the absolute and relative retentions and also the efficiency of the column changed. Elemental analysis of the unpacked column material showed a loss of more than 40% of the carbon content during the use of the column. This is not surprising, because an aqueous suspension of this packing material showed a pH value of greater than 9, which is sufficient for silica to be dissolved. If the stationary phase is neutralized with acids it is, of course, as stable as the other chemically bonded stationary phases studied in this work. The chromatographic selectivity, however, is a function of the anion used. The self-catalyzed hydrolysis of this stationary phase seems to stop when the amino groups are neutralized with the newly created acidic silanol groups. In addition, chemical reactions of the samples with the amino groups probably also cause irreversible adsorption.

EXPERIMENTAL AND RESULTS

The liquid chromatograph was constructed with an M 6000 pump and a U6K sampling device, both from Waters Assoc. (Milford, Mass., U.S.A.). A spectrophotometric detector (Spectroflow Monitor SF 770, Schoeffel Instruments, Trappenkamp, G.F.R.) and a differential refractometer (R401, Waters Assoc.) were connected in series. The fluctuation of the flow-rate of the pump was less than $\pm 0.6\%$[27]. The drilled[28] stainless-steel columns were 30 cm in length, with I.D. 4.1–4.3 mm. The volumes of the empty columns were determined volumetrically. The columns were packed by a modified slurry method[28]. As suspending liquid cyclohexanol–carbon tetrachloride (30:20, v/v) or isopropanol–methanol (35:15, v/v) was used. The flushing solvent was *n*-heptane.

Deionized water and analytical-reagent grade eluents were used. The protein samples were obtained from Serva-Feinbiochemica (Heidelberg, G.F.R.), the polyethylene glycol samples from Chemische Werke Hüls (Marl, G.F.R.) and the dextrans from Deutsche Pharmacia (Frankfurt, G.F.R.).

Characterization of the stationary phases

Wettability. A very simple test for the hydrophilic character of a chemically bonded phase is its "wettability" by water. To measure this wettability, the phase is suspended in a test-tube in water, and non-wettable phases float on the surface of the water. All stationary phases listed in Table I are wettable under these conditions, except the reversed phase (RP-C_{18}) and the "trifluoroamide". The latter is wetted if the water contains at least 10% of methanol, whereas the RP-C_{18} is wetted if the methanol concentration is greater than 60% (v/v) or the acetonitrile concentration is greater than 40% (v/v).

Exclusion chromatography with water. In exclusion chromatography, no interactions should occur between the sample and stationary phase. Only under these conditions is the elution volume of a given sample governed by its geometry and by the pore size distribution of the stationary phase. To characterize the chemically bonded stationary phases for their applicability in aqueous exclusion chromatography, the elution behaviour of water-soluble polymers was studied. Dextran standards ($\bar{M}_w$ between 10,000 and 2,000,000), Polyethyleneglycols (PEG) standards ($\bar{M}_w$ between 200 and 40,000) were used. In the dextran series glucose, saccharose and raffinose and in the PEG series di-, tri- and tetraethyleneglycol were included as low-molecular-weight samples. As protein standards ovalbumin, bovine serum albumin, myoglobin, insulin, etc., were used.

Chemically very similar polymers such as PEG and dextrans show significant differences in their elution behaviour even if the chemical structure of the bonded material is similar to that of the samples. In Fig. 2 the elution of dextrans ($\bar{M}_w$ 600, 10,000 and 500,000) and PEG 600 on a column packed with the "glycol" stationary phase with water as eluent is shown. The dextrans are eluted in front of the inert sample (D_2O) and separated according to their size. The PEG 600 standard, however, is retarded and partially resolved into its constituents of the homologous series. PEG standards with larger molecular weights are retarded much more strongly.

If a stationary phase with better hydrophilic properties is used, such as the

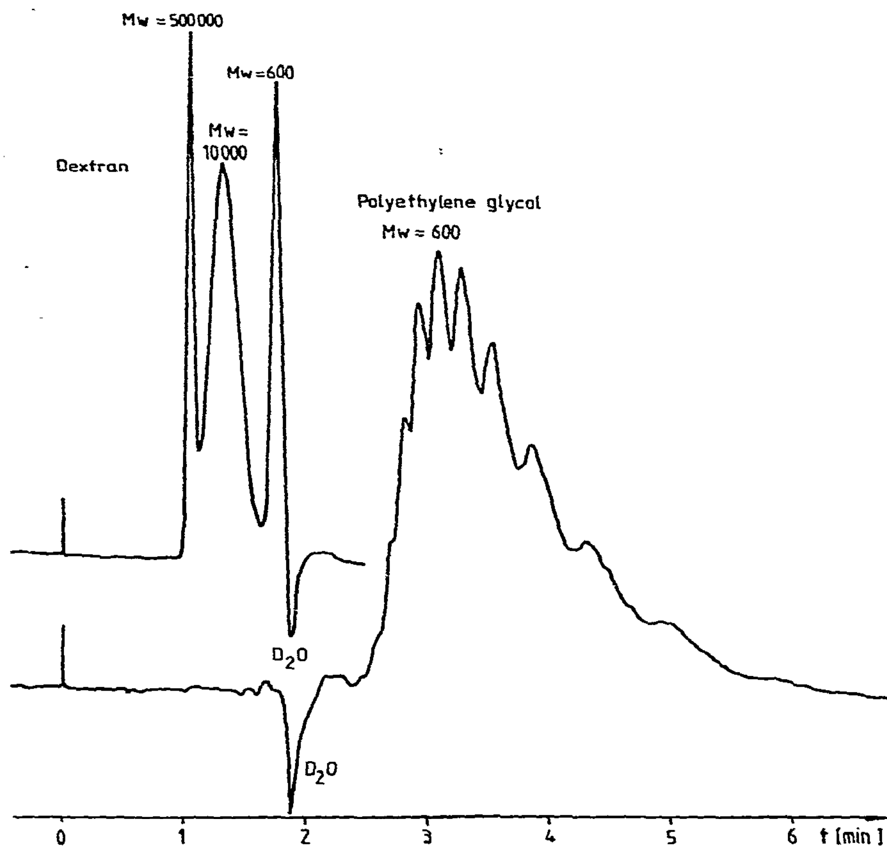

Fig. 2. Elution of dextrans and PEG 600 from "glycol" stationary phase on SI-100. Column: 30 cm × 4.3 mm I.D., drilled. Eluent: water, u = 2.6 mm/sec, F = 2.1 ml/min, Δp = 67 atm. Samples: dextrans, $\bar{M}_w$ = 500,000 and 10,000; raffinose (M = 595); PEG 600. Inert sample: D_2O.

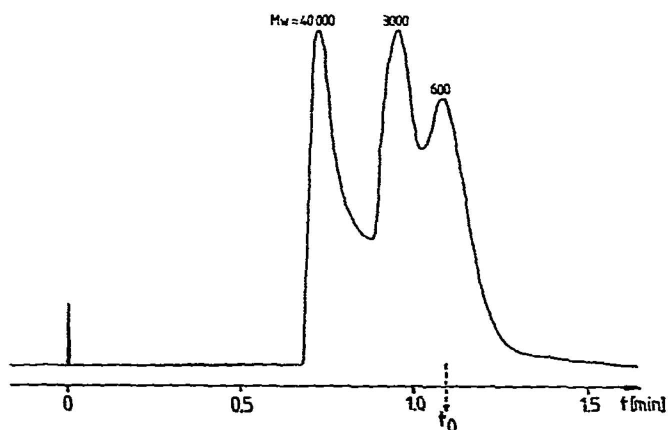

Fig. 3. Elution of PEG standards from "glycinamide" stationary phase on SI-100. Column: 30 cm × 4.3 mm I.D., drilled. Eluent: water, u = 4.6 mm/sec, F = 3.3 ml/min, Δp = 110 atm. Samples: PEG standards, $\bar{M}_w$ = 40,000, 3000, 600.

"glycinamide" phase, the PEG standards are also excluded, and eluted according to their size.

Fig. 3 shows the elution of different PEG standards on the "glycinamide" stationary phase with water as eluent. The PEG standard with $\bar{M}_w$ 600 is eluted with approximately the same elution volume as the inert D_2O.

Table II summarizes the properties of the chemically bonded stationary phases for aqueous exclusion chromatography. It is easy to find a stationary phase for the exclusion chromatography of dextrans. If the stationary phase has slightly more hydrophilic properties than a reversed phase, the dextrans are not retarded. The polyethyleneglycols are eluted in front of the inert peak (D_2O) only on two stationary phases ("glycinamide" and "amide") and their elution volume increases with decreasing molecular weight. On the other stationary phases the PEG samples are retarded and eluted after the inert peak. Their retention volume increases with increasing molecular weight as expected for hydrophobic interactions[13,14].

TABLE II

EXCLUSION CHROMATOGRAPHY OF WATER-SOLUBLE POLYMERS WITH WATER AS ELUENT

Stationary phase	*Dextrans*	*Polyethylene glycols*	*Proteins*
Silica	Excluded	Strongly retarded; PEG 200, $k' \approx 3$	—
RP-C_{18}	Retarded	Strongly retarded; PEG 200, $k' > 6$	Strongly retarded
"Trifluoroamide"	Excluded	Retarded; PEG 200; $k' \approx 0.2$	Strongly retarded
"Sulphonamide"	Excluded	Retarded; PEG 4000, $k' \approx 0.5$	Retarded
"Glycol"	Excluded	Retarded; PEG 200, $k' \approx 0.1$	Retarded and excluded
"Glycinamide"	Excluded	Excluded	Weakly retarded
"Amide"	Excluded	Excluded	Excluded

Problems in the separation of proteins

The silica support for the chemically bonded stationary phases has an average pore diameter of 100 Å, and consequently its separation capability in aqueous exclusion chromatography is limited to molecular weights between 500 and about 15,000. This range is of minor interest in protein chemistry so that for the exclusion chromatography of proteins, silica with average pore diameter of 500–1000 Å has to be used as the support.

Nevertheless, protein samples were injected into columns packed with the stationary phases described above. With water as eluent all of the protein samples were eluted in front of the inert sample (D_2O) only from the "amide" stationary phase. With the "glycol" stationary phase this was also true for some of the proteins, while others seemed to be retained[17]. With the "glycinamide" stationary phase all of the proteins were weakly retarded, *i.e.*, eluted shortly after the inert sample (D_2O). With other stationary phases, the proteins were not eluted in reasonable time when water was used as the eluent.

Calibration graphs with different eluents

The advantage of chemically bonded phases with a silica support over conventional gel-permeation material (*i.e.*, organic polymers) is that they are incompressible

up to pressures of 400 atm and above. Therefore, their pore structure is, to a first approximation, independent of the pressure. Furthermore, they do not shrink or swell as a function of the quality of the eluent.

Polystyrene standards and also PEG are not retarded on the "glycinamide" stationary phase when dimethylformamide is used as the eluent. Typical calibration graphs for exclusion chromatography (log $\bar{M}_w$ *versus* V_e) for these polymers are shown in Fig. 4. Also included are the curves for PEG and for the dextrans with water as the eluent obtained on the same column. The results demonstrate the possibility of correlating the molecular weight of some polymers with those of the easily available polystyrene standards. Of course, the error in the molecular weight determinations is large, but acceptable, as the statistical coil diameters of the different polymers depend on the type of polymer and on the quality of the eluent used. The dextran standard ($\bar{M}_w$ 10,000), which is the only one of the dextrans in the separating range of the silica support used here, is eluted at a higher elution volume than expected for its molecular weight.

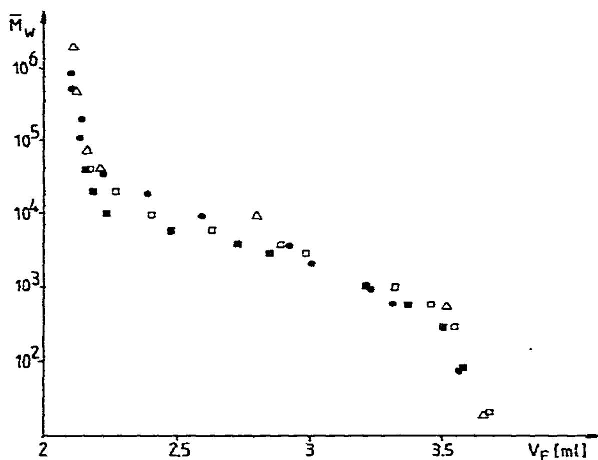

Fig. 4. Calibration graph for different eluents. Stationary phase: "Glycinamide" on SI-100. Column: 30 cm × 4.3 mm I.D., drilled. Closed symbols: eluent: dimethylformamide, u = 4.8 mm/sec, F = 3.3 ml/min, Δp = 100 atm. Samples: polystyrene standards (●). $\bar{M}_w$ = 600–867,000; PEG standards (■), $\bar{M}_w$ = 600–40,000. Open symbols: eluent: water, u = 4.5 mm/sec, F = 3.4 ml/min, Δp = 115 atm. Samples: PEG standards (□), $\bar{M}_w$ = 600–40,000; dextran (△), M_w = 10,000–500,000; and raffinose, M = 595.

Chromatographic separations

The exclusion chromatographic properties of these phases and the selective interaction of small not-excluded molecules with the chemically bonded stationary phase can be used to characterize samples containing small as well as large molecules. In Fig. 5 the elution diagramm of a cola soft drink is shown with water as eluent. The lower curve corresponds to the UV absorption at 275 nm, whereas in the upper curve the refractometer track is shown. In this curve one large peak, corresponding to the sugar of the beverage, was eluted with V_0, *i.e.* was non-retarded. In the UV trace a reasonable signal is obtained before V_0, which could be attributed to the colour added

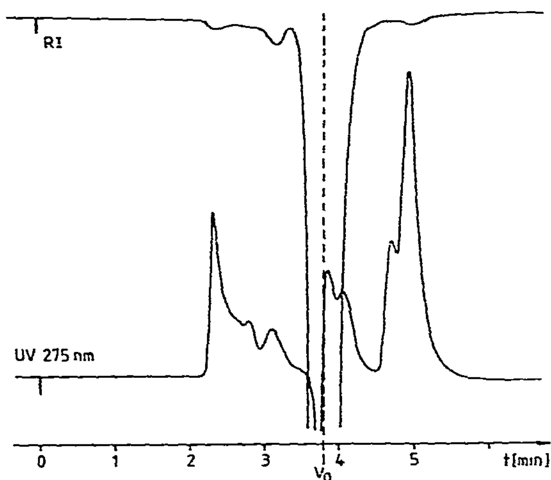

Fig. 5. Separation of a cola soft drink. Stationary phase: amide on SI-100. Column: 30 cm × 4.3 mm I.D., drilled. Eluent: water, u = 1.33 mm/sec, F = 0.89 ml/min, Δp = 36 atm. Sample: 2 μl of cola soft drink. UV detector at 275 nm, 0.1 a.u.f.s. Refractometer, × 1.

to the beverage. The finger print of this polymer content varied from brand to brand. From the peaks eluted with a larger elution volume than V_0 the last one could be identified as caffeine.

In Fig. 6 a chromatogram is shown which was obtained by applying 1 μl of filtered coffee on to the same column. In this case, with water as eluent, the amount eluted with a smaller elution volume than V_0 is even detectable with the refractometer. This part of the eluate is deeply coloured even after the dilution during the chromatographic process. The peaks eluted after V_0 could be identified as chlorogenic acid (1) and caffeine (2).

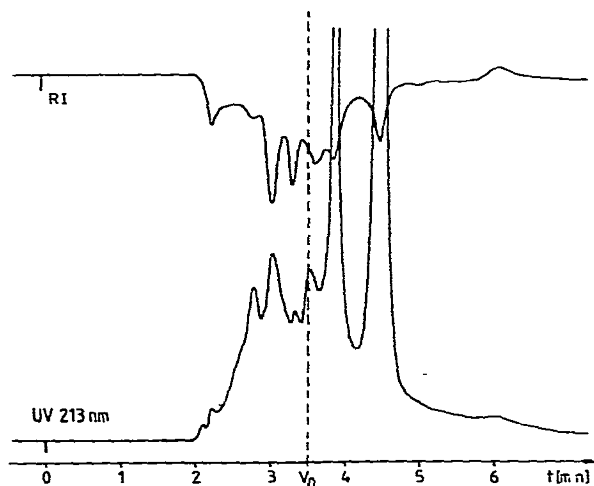

Fig. 6. Separation of filter coffee. Stationary phase: "amide" on SI-100. Column: 30 cm × 4.3 mm I.D., drilled. Eluent: water, u = 1.43 mm/sec, F = 0.93 ml/min, Δp = 35 atm. Sample: 1 μl of filter coffee. UV detector at 213 nm, 0.4 a.u.f.s. Refractometer, × 1.

Recently, the advantages of stationary phases with small particle diameter for exclusion chromatography have been emphasized[29–31]. It is very dangerous[32] to discuss plate numbers in relation with exclusion chromatography of polymolecular samples with a given finite molecular-weight distribution. Some of the problems involved here would exceed the scope of this paper and will therefore be discussed in a following paper. However, the big advantage of using stationary phases with small particle diameters in exclusion chromatography is the possible high speed of analysis.

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