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HYDROPHOBIC CHROMATOGRAPHY WITH DYNAMICALLY COATED STATIONARY PHASES

II. DYNAMIC CATION-EXCHANGE SEPARATIONS OF TYROSINYL PEPTIDES

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SUMMARY

A new class of dynamically produced ion exchanger is shown to be the product of equilibration of a silica high-performance liquid chromatographic packing material and eluents composed of aqueous–organic solutions of both a non-ionic and an anionic surfactant. This new technique, conveniently called "dynamic soap chromatography", may be used to separate mixtures of peptides and non-ionic organic compounds with the same efficiency as may be obtained from the other modes of high-performance liquid chromatography now in common use.

INTRODUCTION

In the course of further[1] investigations of the effects of added surfactants on conventional high-performance liquid chromatographic (HPLC) adsorbents reported by Ghaemi and Wall[2], it was found that non-ionic surfactants such as laurylpolyoxyethyleneglycols ("Brij") and palmityl sorbitan polyoxyethylenepolyols ("Tween") interact with both acidic gel materials such as silicon(IV) and zirconium(IV) oxides and amphoteric oxide gels such as aluminium(III) oxides in aqueous methanol solution to generate a hydrophobic surface on the oxide. Surfactants such as sodium dodecyl sulphate did not react with acidic gels in aqueous methanol solution, although they did form hydrophobic surfaces on amphoteric porous alumina.

The results led to interest in whether anionic surfactants could interact with the dynamically generated hydrophobic surface formed by action of non-ionic surfactants on chromatographic silica gel in a fashion analogous to that observed with hydrocarbon chains covalently bonded to the silica matrix ("dynamic ion exchange"[3] or "soap chromatography"[4]). In the event, such interactions did take place and some separations achieved by this technique are described below.

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EXPERIMENTAL

Apparatus

The chromatographic measurements were carried out with a laboratory-made pneumatic pressure intensifier pump; columns and injectors were made in the laboratory to a design similar to that marketed by Shandon Southern Products (Runcorn, Great Britain); microsyringes and rubber septa from Scientific Glass Engineering (U.K.) (London, Great Britain); and a Model 2012 photometric detector fitted with an 8-μl flow cell (Cecil Instruments, Cambridge, Great Britain).

The columns (113 × 5 mm) were packed by the upward flow procedure of Bristow *et al.*[5], using methanol to suspend and to pack the Hypersil (Shandon Southern Products) spherical silica gel ($d_p \approx 5\ \mu m$; $S_{BET} \approx 200\ m^2\ g^{-1}$) at a pressure of *ca.* 330 bar.

Reagents and solvents

The ketone solutes were all obtained from standard commercial sources. The amino acids and peptides (all L-configuration) were purchased from Sigma London (Poole, Great Britain) with the exception of the protected tetrapeptide, N-acetyl-L-tyrosyl-L-leucyl-L-valyl-L-histidinamide, which was kindly supplied by Dr. A. P. Ryle of this University. The surfactants "Tween 40" and sodium dodecyl sulphate (SDS) were also purchased from Sigma London, but the "Tergitol 7" was supplied by Fluka (Buchs, Switzerland) —all were technical grade, and the "Tween" and "Tergitol" were stated to be mixtures rather than pure chemicals.

Methanol and acetonitrile were HPLC grade from Rathburn Chemicals (Walkerburn, Great Britain) and all water was glass-distilled. Initial acetonitrile-based eluent experiments were with the ordinary reagent grade chemical, and just as in earlier[2] work with reagent grade methanol, the unpurified acetonitrile consistently gave lower capacity ratios (k') for peptide solutes as if eluents prepared from this solvent contained about twice as much sodium ion as calculated. The mixed surfactant–buffer salt–organic modifier eluent systems were quite stable over the temperature range from 15 to 60°C, but temperatures outside this range occasionally induced cloudiness or even precipitation, which drastically affected the viscous resistance of the column system. Eluents were degassed by boiling under reflux and were subsequently filtered through filter paper before use, but this filtration did not prevent a slow accumulation of microparticulate contaminants at the top of the column, which gradually reduced column performance. This "microdirt" reduced column life to *ca.* 50 (working) hours, although two or three restorations of original efficiency were possible by careful removal of the top 2–3 mm of the silica bed and its replacement by firm tamping of a thick slurry of fresh packing in the eluent. Methanol-based eluents appeared to produce more of this column contamination than those using acetonitrile as modifier, although no *visible* contamination appeared in any of the eluent systems as long as the temperature of use and storage was above 15°C and below 60°C.

Methanol–water (40:60, v/v) was the base for all experiments with methanolic eluents. Surfactants and buffer constituents were dissolved in the aqueous portion of these mixtures before dilution with the organic modifier, and then the pH of the eluent system was adjusted by addition of small amounts of phosphoric acid (3 *M*

in water). The same procedures were also followed for the acetonitrile–water eluents, except that the modifier concentration was reduced to give a 20:80 organic solvent–water volume ratio. The eluites were dissolved in methanol–water (40:60) except for the plate height–velocity experiments, for which the peptide standards were dissolved in the acetonitrile-based eluent used for column development.

RESULTS

Variation of anionic surfactant concentration

Previous studies[1] established that the maximum retention of non-ionic solutes on silica in contact with solutions of Tween 40 in methanol–water (50:50) occurred in the concentration range from $5 \cdot 10^{-4}$ to 10^{-3} *M*, and it was easily confirmed that the same concentration range gave maximum retention in the methanol–water (40:60) eluents of these experiments. Accordingly, the concentration of SDS was varied from 10^{-3} to 10^{-2} *M* at constant ($5 \cdot 10^{-4}$ *M*) concentration of Tween 40. No retention of charged or neutral eluites was observed with the described aqueous organic solvent systems in the absence of the non-ionic detergent, either with or without SDS. Fig. 1 shows the effect of these changes on retention of three neutral eluites and a peptide which must be virtually 100% in the protonated

form at pH 2.10. Over this rather narrow concentration range k' values of the charged eluite are reduced slowly in a nearly linear relation to [SDS], whereas the ketone k' values are reduced non-linearly by a factor of *ca.* 3.5. The crossover points at $1.5 \cdot 10^{-3}$ *M* [SDS] and $6 \cdot 10^{-3}$ *M* [SDS] demonstrate analytically useful changes in *relative* retention between ionized and neutral eluites under these conditions.

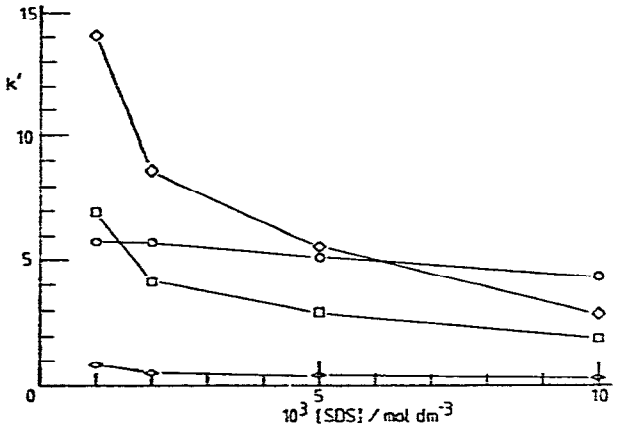

Fig. 1. Relationship of eluite retention to anionic surfactant concentration. Eluent: pH 2.0, methanol–water (40:60, v/v); [Tween 40] = 10^{-3} *M*; [SDS] as shown. Eluites: ◇ = fluorenone; □ = 2-acetylnaphthalene; ○ = GLY; ◇ = acetophenone.

Variation of pH

It is to be expected that retention of ionizable eluites (which may vary in charge with pH) will also vary with pH as the balance of solute hydrophobicity[6] and solute charge changes. In the present system the retention mechanism for the peptides is probably a mixture of hydrophobic attraction and ion exchange (of $R–NH_3^+$ for Na^+). Since ionization of the peptide C-terminal carboxyl group begins to become significant as pH rises to 3.5, k' of such solutes would be expected to decrease rapidly between pH 3 and pH 5 (ionization of RCOOH *ca.* 50% at pH 4.8). Fig. 2 shows that k' for unprotected peptides does indeed fall as predicted —simultaneous measurements of k' for neutral eluites showed virtually no sensitivity to pH, and two basic solutes, L-tyrosine methyl ester and N-acetyl-Y-V-L-H-NH_2* also demonstrated a very slight drop in retention with increased pH. Minimum values for k' of amine solutes should lie above pH 7 where silica is attacked by aqueous eluents, so this prediction was not checked.

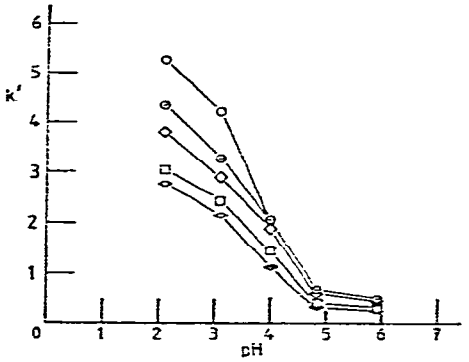

Fig. 2. Relationship of peptide retention to pH of eluent. Eluent as in Fig. 1; [Tween 40] = $5 \cdot 10^{-4}$ *M*; [SDS] = $5 \cdot 10^{-3}$ *M*; [Na^+] = $2.5 \cdot 10^{-2}$ *M*; pH as shown. Eluites: ○ = GLY; ⊙ = LY; ◇ = VYV; □ = VY; ◇ = AY.

Variation of counter-ion concentration

The sections *Variation of anionic surfactant concentration* and *Variation of pH* were reports of data from columns eluted by methanol–water solvent systems, but since the object of the present exercise was to test the versatility of a new mode of HPLC, acetonitrile–water eluents were used for the latter part of the investigation. Accordingly, in an attempt to duplicate retentive character, the acetonitrile concentration was halved from the previous methanol levels to organic–aqueous (20:80, v/v). This was the only composition change from the earlier pH 3.10 solvent ($5 \cdot 10^{-4}$ *M* Tween 40, $5 \cdot 10^{-3}$ *M* SDS, $2 \cdot 10^{-2}$ *M* Na^+) in the first experiments of the new series. Retention of neutral eluites was similar to that with 40% methanol eluents, but *peptide* retention was unexpectedly reduced by a factor of three.

Since this effect might have been caused by the relatively high solvent power

* The single letter code for amino acids used in this paper is suggested by Dayhoff[47]: A = alanine; G = glycine; H = histidine; L = leucine; M = methionine; P = proline; V = valine; Y = tyrosine.

of the acetonitrile, its concentration was progressively reduced to 15% (v/v) and 10% (v/v), but, contrary to expectations, peptides were even less retained from these more aqueous eluents. The cause of the decrease was not found until a acetonitrile–water (20:80, v/v) solution of Tergitol 7 (sodium "C_{17}" sulphate) was tested in an attempt to increase the hydrophobicity of the pairing ion/stationary phase. Previous work[1] had shown maximum retention (of neutral eluites) on alumina from methanol–water (50:50, v/v) eluents to occur in the $2–5 \cdot 10^{-3}$ *M* concentration range of Tergitol 7 (and SDS), and accordingly the acetonitrile eluents above were initially made up to be $5 \cdot 10^{-3}$ *M* in the anionic surfactant. Reduction of Tergitol concentration to 10^{-3} *M* increased retention of peptides to the same levels as previously observed for $5 \cdot 10^{-3}$ *M* SDS, methanol–water (40:60) eluents, and the reduced surfactant composition [acetonitrile–water (20:80, v/v), $5 \cdot 10^{-4}$ *M* Tween 40, 10^{-3} *M* Tergitol 7, sodium ion from NaH_2PO_4 buffer, pH 3.10] was used in subsequent experiments. Clearly, the retentive power of the dynamically generated cation exchanger must be a function of both the hydrophobicity *and* the concentration of the organic modifier, as well as added surfactant concentration.

If, as suggested above, the retention mechanism for peptides in these systems is a balance of hydrophobic attraction and ion exchange, investigation of the consequences of changes in counter-ion (Na^+) concentration on k' should shed light on details of this mechanism. If $R\overset{+}{N}H_{3(mob)}$ is the solute in the mobile phase and $Q–S\bar{O}_3Na^+_{(st)}$ is the cation exchanger of the stationary phase, we can define an ion-exchange equilibrium constant, K_{IE}, and an ion-exchange distribution constant, D_{IE}, as shown below:

$$R–\overset{+}{N}H_{3(mob)} + Q–S\bar{O}_3Na^+_{(st)} \overset{K_{IE}}{\rightleftharpoons} Q–S\bar{O}_3H_3\overset{+}{N}R_{(st)} + Na^+_{(mob)} \quad (1)$$

$$\text{so } K_{IE} = \frac{[Q–S\bar{O}_3H_3\overset{+}{N}R]_{st}\,[Na^+]_{mob}}{[R–\overset{+}{N}H_3]_{mob}\,[Q–S\bar{O}_3Na^+]_{st}} \quad (2)$$

$$\text{and } k' \propto 15 \text{ proportional to } D_{IE} = \frac{[R–\overset{+}{N}H_3]_{st}}{[R–\overset{+}{N}H_3]_{mob}} = \frac{K_{IE}[QSO_3Na^+]_{st}}{[Na^+]_{mob}} \quad (3)$$

Since the concentration of cation-exchange sites $[QSO_3^-]$ is fixed by the equilibrium between the (constant) eluent and the silica packing, k' should vary inversely as the sodium ion concentration in the eluent.

Fig. 3 illustrates that the relationship of retention to counter-ion concentration is of the form to be expected if a substantial part of the retentive mechanism is ion exchange. However, since the nearly straight lines so produced do not go through the origin, the affinity of the eluites for the "stationary phase" must be greater than can be accounted for by ion exchange alone. This additional retentive power may be due to a "salting-out" effect in which the hydrophobic solvating capacity of the eluent is reduced by increasing its ionic strength. Fig. 4 shows that such an effect is indeed present, since k' values of neutral ketonic solutes increase with increased $[Na^+]$. Data for the peptide GLY on the same plot vary in nearly mirror image fashion, again indicating the degree of control over selectivity of separation in this new mode of HPLC.

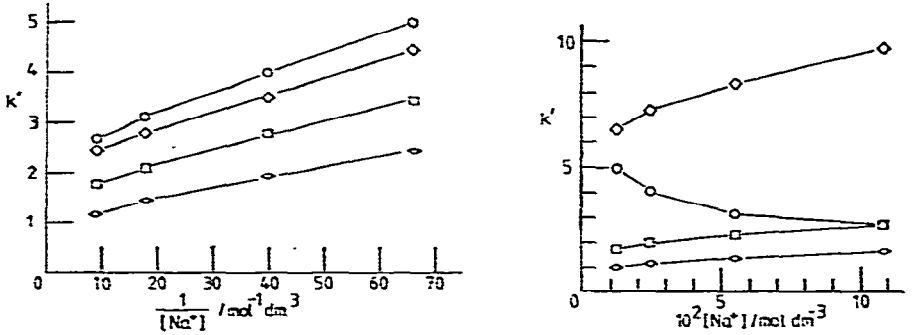

Fig. 3. Relationship of peptide retention to counter-ion (Na^+) concentration. Eluent: pH 3.10, acetonitrile–water (20:80, v/v); [Tween 40] = $5 \cdot 10^{-4}$ *M*; [Tergitol 7] = 10^{-3} *M*; NaH_2PO_4 added to shown concentration. Eluites as in Fig. 2.

Fig. 4. "Salting-out" effects on non-polar eluites. Eluents as in Fig. 3. Eluites: ◇ = 2-acetylnaphthalene; ○ = GLY; □ = 1,2,3,4-tetrahydronaphthalen-1-one; ◇ = acetophenone.

Separation efficiency

No description of a chromatographic procedure is complete without an indication of the efficiency of the system; accordingly, the acetonitrile–water (20:80) eluent system (pH 3.10, $5 \cdot 10^{-4}$ *M* Tween 40, 10^{-3} *M* Tergitol 7, 10^{-2} *M* NaH_2PO_4) was used to separate samples of a mixture of three peptides (AY, $k' = 2.4$; VY, $k' = 3.4$; VYV, $k' = 4.5$) at flow-rates from 0.2 to 1.7 $cm^3\ min^{-1}$. At the lowest flow-rate both AY and VYV were transported as zones whose width was equivalent to *ca.* 9000 plates. The column efficiency for the third peptide VY was slightly poorer at 7500 plates, but this eluite was present at twice the concentration of the other two. Similar efficiencies were recorded for neutral eluites such as the acetylnaphthalenes ($k' = 6.00, 6.5$) at a higher flow-rate, 0.5 $cm^3\ min^{-1}$. These best efficiencies represent plate heights equal to 2.5 particle diameters, and are fully comparable with good quality results from conventional alkyl-bonded phase ion-pair chromatography.

Fig. 5 is a plot of the plate height, *H*, of the peptide VYV *versus* the linear velocity, *u*, of an unretained solute in the column. Values of diffusion constants of the eluites VYV and 1-acetylnaphthalene were estimated by the Wilke–Chang[5]

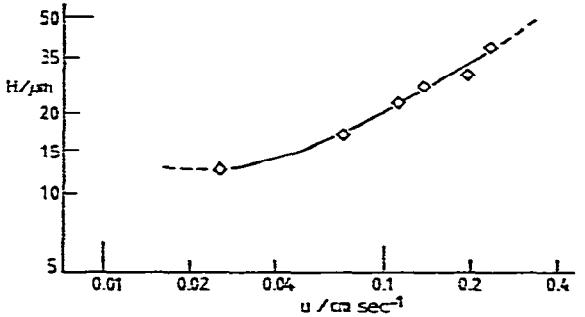

Fig. 5. Relationship of theoretical plate height, *H*, to linear velocity, *u*, of an unretained solute for peptide VYV. Eluent: pH 3.10; acetonitrile–water (20:80, v/v); [Tween 40] = $5 \cdot 10^{-4}$ *M*; [Tergitol 7] = 10^{-3} *M*; [NaH_2PO_4] = $1.2 \cdot 10^{-2}$ *M*.

approximation; this calculation suggests that the rate of diffusion in the mobile phase of acetylnaphthalene should be approximately twice that for the peptide, and accordingly it is to be expected that H(VYV) at 0.2 $cm^3\ min^{-1}$ should approximate H(acetylnaph.) at 0.5 $cm^3\ min^{-1}$. The H *versus* u data for the peptide do not extend to low enough flow-rates to establish the position of the expected minimum in H, but they do follow the course that would be expected for a reasonably well-packed column of these dimensions[9].

DISCUSSION

Nature of the dynamically coated surface

In the first report[2] of this work the authors suggested that the "stationary phase" produced on interaction of a cationic surfactant with acidic silica and zirconia gel column packing materials was a close analogue of the covalently bonded alkyl-modified silicas of commercial use. The supposition was that the surface presented to a solute molecule in the mobile phase was covered with a "brush" of hydrocarbon chains attached to the silica by electrostatic attraction of $R\text{–}N^+(CH_3)_3$ for $\equiv SiO^-$. This simplistic explanation was reinforced by the lack of apparent reaction of the same acidic silica and zirconia with anionic surfactants, compounds which did react with alumina, an oxide known to possess active sites of basic function.

However, discussions with colleagues and publication of a review article by Rupprecht[10] clarified the picture of the structure of the surface layers "seen" by eluite molecules. The initial surface monolayer (an incomplete layer[2]) is formed rapidly by interaction of polar groups of cationic and non-ionic surfactants with gel hydroxyls and then a partial bilayer is formed more slowly by hydrophobic interaction of the long alkyl chains of the bound surfactant and those of the surfactants dissolved in the eluent. The final equilibrium surface would then be better described as resembling a synthetic ion-exchange resin: *i.e.*, brushes bearing $-N^+(CH_3)_3$ or $-OCH_2CH_2OH$ groups on the ends exposed to the mobile phase. Synthetic ion-exchange resins are well known to retain non-polar (and polar) solutes according to the solute hydrophobicity: indeed, conventional amino acid and sugar analyser separations proceed very largely on that basis.

Such a surface would explain these present results, since the primary layer (which is relatively difficult to remove by washing) would be formed by interaction of silanols with some of the three alcohol termini per Tween molecule and the secondary layer would be formed by interaction of the anionic surfactant alkyl chain with the exposed alkyl chain of the Tween as in Fig. 6. Reduction in retention of neutral solutes with increasing surfactant concentration would be expected, since strongly polar sulphate groups would increasingly dominate the stationary phase surface, decreasing the attraction to hydrophobic solutes, as shown in Fig. 1.

Applications

Conventional alkyl-bonded phase dynamic ion-exchange chromatography is probably the most powerful technique now available for separation of mixtures of ionizable and neutral compounds, since both relative and absolute retention may be controlled by variation of organic modifier concentration, ionic surfactant concentration, counter-ion concentration, hydrogen ion concentration and temperature.

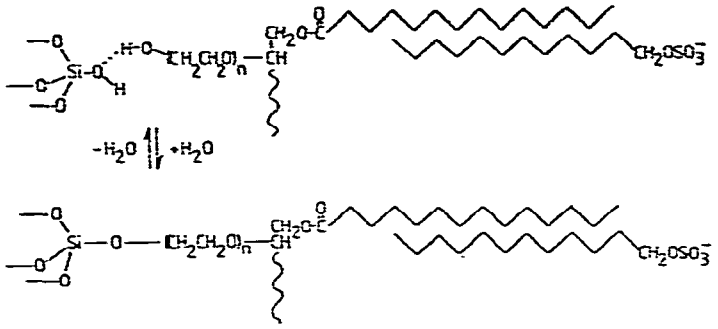

Fig. 6. Possible chemical structure of dynamically generated ion exchanger, showing interaction of silica surface, Tween 40, and SDS.

The technique presented here adds to these an element of control of stationary phase hydrophobicity by choice of surfactant to the above list, so it was of interest to see how well a range of compounds of multiple polar functionality could be separated by the new method, since such compounds have been used[11] to demonstrate lack of homogeneous surfaces on conventional alkyl-bonded packings.

A range of simple tyrosinyl peptides was chosen as test substances both because of personal interest[12] and of a recent increase in publications[13–19] on separations of peptides by HPLC. Figs. 7 and 8 show separations of some of these di- and tripeptides at the same pH: note that selectivity is strongly dependent on the organic modifier. Fig. 7 shows that LY can be easily separated from GLY with a methanol-based eluent, but hardly at all with an acetonitrile eluent system: precisely opposite

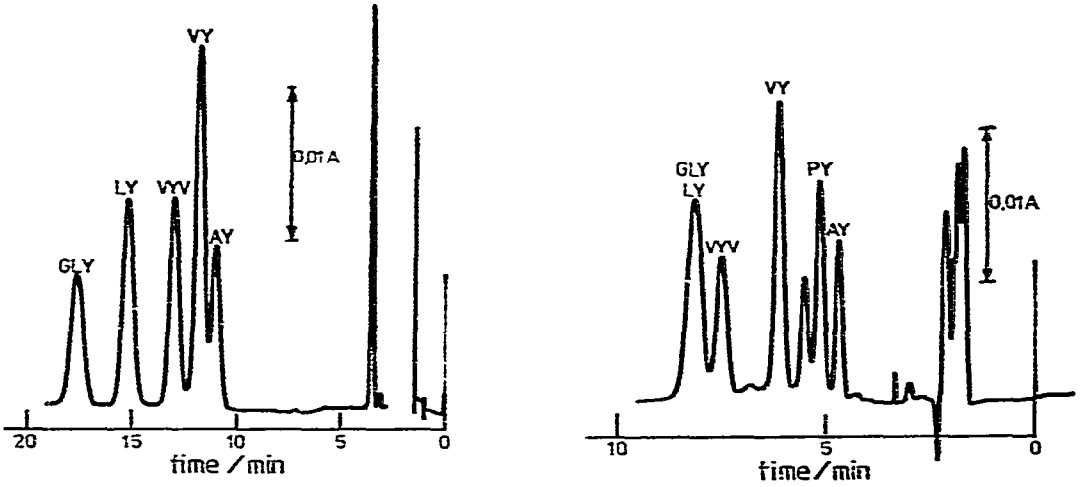

Fig. 7. Separation of tyrosinyl peptides. Eluent: pH 3.08, methanol–water (40:60, v/v); [Tween 40] = $5 \cdot 10^{-4}$ *M*; [SDS] = $5 \cdot 10^{-3}$ *M*; no added buffer salts. Flow-rate 0.6 cm^3 min^{-1}. Detection at 275 nm.

Fig. 8. Separation of tyrosinyl peptides. Eluent: pH 3.10, acetonitrile–water (20:80, v/v); [Tween 40] = $5 \cdot 10^{-4}$ *M*; [Tergitol 7] = 10^{-3} *M*; [NaH_2PO_4] = $5.5 \cdot 10^{-2}$ *M*. Flow-rate 0.8 cm^3 min^{-1}. Detection at 272 nm.

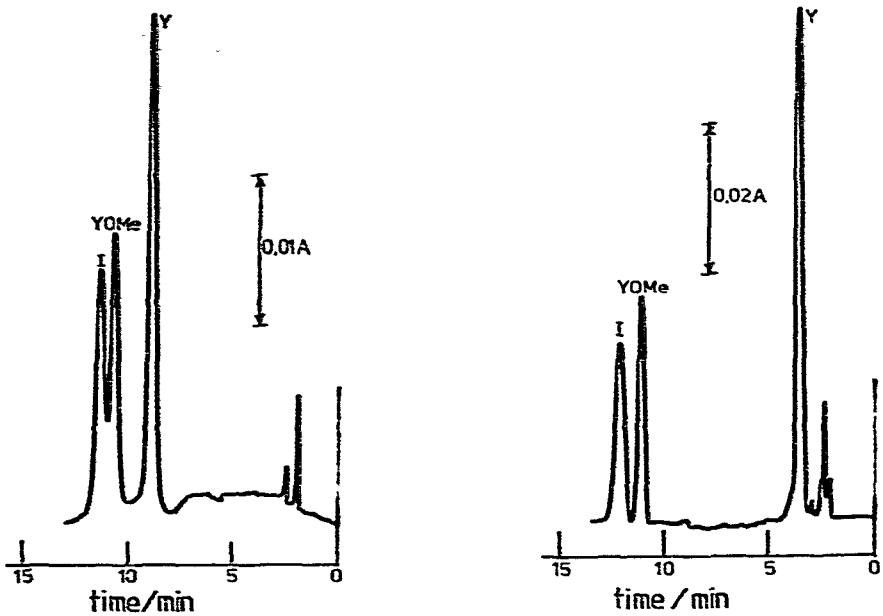

Fig. 9. Separation of tyrosine and derivatives. Eluent: pH 2.10, composition as in Fig. 2. Eluites: YOMe = L-tyrosine methyl ester; I = N-acetyl-Y-V-L-H-amide. Flow-rate 0.7 cm^3 min^{-1}. Detection at 223 nm.

Fig. 10. Separation of tyrosine and derivatives. Conditions as in Fig. 9, except eluent pH 4.00 and flow-rate 0.6 cm^3 min^{-1}.

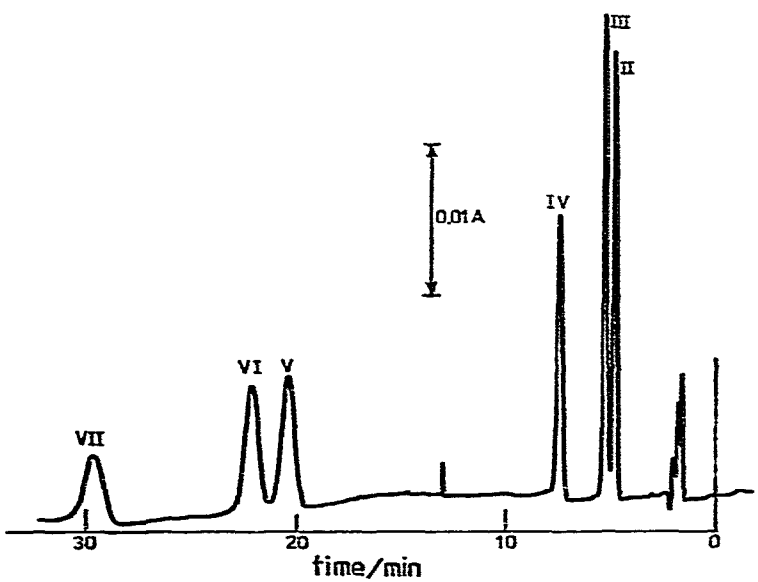

Fig. 11. Separation of aromatic ketones. Conditions as in Fig. 8. Eluites: II = 4-methoxyacetophenone; III = acetophenone; IV = 1,2,3,4-tetrahydronaphthalen-1-one; V = 1-acetylnaphthalene; VI = 2-acetylnaphthalene; VII = benzophenone.

effects may be noted with AY/VY pair. All separations of the four dipeptides AY, PY, VY and LY gave the elution order shown, different from that observed by Hearn *et al.*[18] on a C_{18} alkyl-bonded column. Both solvent systems are sufficiently transparent at 210 nm to allow detection of peptides by carbonyl absorption bands when made up from HPLC grade UV-transparent methanol or acetonitrile.

Figs. 9 and 10 are records of separations of mixtures of the parent amino acid, tyrosine, Y, its methyl ester, YOMe and the protected tetrapeptide N-acetyl-YVLH-NH_2 at pH 2.10, where ionization of the amino acid carboxyl group is nearly completely suppressed and at pH 4.00 where the Y is a zwitterion.

Fig. 11 is a simple demonstration that peak shape and column efficiencies in separations of neutral compounds are comparable to those achieved by conventional alkyl-bonded phase HPLC. The solutes are retained over the k' range from 1.2 to 11.5.

Fig. 12 demonstrates that this dynamically coated stationary phase system has the same sort of selectivity towards dipeptide diastereomers as the C_8 alkyl-bonded phase used by Pietrzyk[16]. Discrimination between the D,L- and L,L-forms of leucyl-tyrosine is slightly less ($k'_{L,L}/k'_{D,L} = 1.36$) by the new technique than by classical hydrophobic chromatography on a C_8 phase ($k'_{D,L}/k'_{L,L} = 1.51$), and, surprisingly, the order of elution is reversed in the separation presented here, but this new mode of separating such mixtures will clearly be of use in monitoring the stereochemical purity of synthetic peptides both during and after their synthesis.

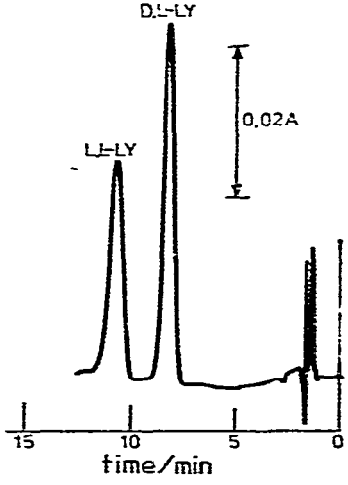

Fig. 12. Separation of diastereomeric dipeptides LY. Conditions as in Fig. 8, except flow-rate 0.9 $cm^3\ min^{-1}$ and detection at 223 nm.

CONCLUSIONS

As suggested in the first paper[2] derived from this current study, cationic and non-ionic surfactants do apparently react with acidic sites on the surfaces of acidic HPLC oxide gel packing materials to give hydrophobic stationary phases whose properties are a function of surfactant concentration in the mobile phase. The retentive characteristics of such dynamically coated stationary phases may be further modified

by incorporation of anionic surfactants in the eluent; such modification converts the uncharged hydrophobic surface derived from a non-ionic surfactant into a cation exchanger, probably by means of a secondary layer hydrophobically bonded to the non-ionic surfactant/oxide primary layer. This dynamically generated cation exchanger is shown to be able to separate mixtures of neutral and multi-polar functional organic compounds with the same efficiencies as conventional alkyl-bonded silica packing/surfactant systems.

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