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SOLVENT-GENERATED ION-EXCHANGE SYSTEMS WITH ANIONIC SURFACTANTS FOR RAPID SEPARATIONS OF AMINO ACIDS

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SUMMARY

The retention behaviour of amino acids in phase systems consisting of a hydrophobic solid support as the stationary phase and water–organic solvent mixtures containing a small amount of an anionic detergent as the mobile phase was investigated.

Such phase systems are found to behave like conventional ion-exchange systems. The degree and order of retention of amino acids can be influenced by changing the temperature, the nature of the hydrophobic support, the pH and the nature and concentration of the anionic detergent, organic constituent and counter ion in the eluent. In many instances this solvent-generated (dynamic) ion-exchange chromatography shows a greater selectivity than conventional ion-exchange systems towards amino acids. The results obtained so far indicate that a complete separation of the 19 protein amino acids by applying solvent gradients or/and multi-column system is possible within 30 min.

INTRODUCTION

The analysis of amino acids is one of the most common applications of column liquid chromatography. Fully automated "amino acid analysers" were constructed long before modern high-performance liquid chromatographic (HPLC) instruments were developed[1,2].

Although other types of chromatography such as gas (GC)[3,4], thin-layer (TLC)[5] and paper chromatography (PC) and electrophoresis[6] have been used for the separation of amino acids, ion-exchange chromatography in columns[1,2,6–8] has been found to be superior until now.

A polystyrene–divinylbenzene matrix cation exchanger is used in nearly all systems. The compressibility of these types of soft materials is unfavourably high, however, and does not allow columns to be designed for high-speed separations. Despite this fundamental drawback of soft materials, significant improvements in the

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speed of separation have been achieved with organic matrix ion exchangers during the last few years. The speed of analysis of protein amino acids has been reduced from about 1 day[1] to about 1 h[9] as a consequence of applying modern concepts[2] and the development of suitable column packings and instrumentation. In spite of these improvements, it was considered to be useful to explore the use of solvent-generated (dynamic) ion-exchange chromatography for the separation of amino acids. In this mode of operation, pressure-stable packing materials that permit the use of very small particles and high flow velocities can be applied.

The potential of this method has been demonstrated recently[10–12]. The application of this type of chromatography to amino acids is considered in this paper.

EXPERIMENTAL

Apparatus

The apparatus used was a home-built amino acid analyzer using colorimetric detection based on the ninhydrin reaction according to Spackman *et al.*[1]. It was mostly assembled from commercially available parts.

In the separations a constant-flow pump (Varian 4100) was used as the eluent delivery system. A high-pressure sampling valve (Valco CV-6-UHP-a-C20) provided with a sample loop of 200 × 0.15 mm, resulting in an injection volume of 8.64 μl, was used. The columns were 125, 150 and 250 mm long and constructed from 3.0 mm I.D., 6.35 mm O.D. precision-bore stainless-steel 316 tubing. They were thermostated by means of a water-bath (Haake FT) or used at ambient temperature (25°).

The detector consisted of a combination of a tubular flow reactor and a UV absorbance detector, optimized for the needs of HPLC[13]. For the proportioning of the ninhydrin solution a reciprocating pump was used (Milton Roy, Type 196-39). The flow-rates of the eluent and the ninhydrin solution were 10 and 5 μl/sec, respectively. A stainless-steel 316 capillary (18 m × 0.25 mm) coiled with a diameter of 20 mm was used as the flow reactor. The reactor was thermostated at 140° in a thermostatted air-bath (part of the Perkin-Elmer 1220 liquid chromatograph) and was connected to a UV absorbance detector (Perkin-Elmer LC-55) by means of a 250 × 0.15 mm capillary cooled with tap water in order to bring the reaction mixture to about ambient temperature. A needle valve behind the spectrophotometer was used to maintain a back-pressure of about 10 bar in order to prevent boiling of the reaction mixture at temperatures above its boiling point. Using a wavelength of 570 nm, or 400 nm when proline was present, chromatograms were recorded with a linear potentiometric recorder (Goertz, Servogor RE 542). An integrator (Spectra-Physics, Autolab System I) was used to measure retention times and peak areas. An automatic flow meter as described previously[14] was used. The flow-rate of the liquid mixture was constant to within 0.2%.

Chemicals and materials

In all experiments de-ionized water and organic solvents of analytical-reagent grade were used. Ninhydrin solution was prepared according to the literature[15] and kept under nitrogen. Amino acids were chromatographically pure and obtained from Ajinomoto (Tokyo, Japan).

Hydrophobic surface packing materials RP-2, RP-8 and RP-18 (Merck,

Darmstadt, G.F.R.) with an average particle size of 5 μm were used. Methyl-bonded silicas[16], coded Si 60-Me and Si 1000-Me, were prepared from narrow-sized silica Si 60 and Si 1000 (Merck), respectively. The anionic detergents used were sodium dodecyl sulphate (SSD), sodium dodecyl sulphonate (SDS), sodium pentyl sulphonate (SPS) (Eastman-Kodak, Rochester, N.Y., U.S.A.) and dinonyl naphthalenesulphonic acid (DNNS).

Procedures

The columns were packed by a balanced slurry technique[17]. The capacity ratio, κ_i, of a component i was determined from its retention time, t_{Ri}, and the retention time, t_{R0}, of an unretarded compound. As both times are increased by the reaction time, t_r, the capacity ratio can be found from

$$\kappa_i = \frac{t_{Ri} - t_{R0}}{t_{R0} - t_r}$$

Cysteic acid was used as the unretarded compound. The reaction time was 60 sec.

THEORETICAL BACKGROUND

The exact distribution mechanism of ionizable substances in solvent-generated ion-exchange chromatography is not yet well elucidated. A great number of competing equilibria of different origin may be involved in the distribution process of ionizable substances[10,18].

At a constant detergent concentration and considering only the main equilibria involved, a simple expression describing the dependence of the overall distribution coefficient on the pH and the counter ion concentration can be derived.

(i) Dissociation and association of the amino acid $^{-}HB^{+}$ in the mobile phase:

$$H_2B^{+} \underset{K_{d1}}{\overset{+H^{+}}{\rightleftharpoons}} {}^{-}HB^{+} \underset{K_{d2}}{\overset{+H^{+}}{\rightleftharpoons}} B^{-} \qquad (1)$$

where K_{d1} = dissociation constant of H_2B^{+} and K_{d2} = dissociation constant of $^{-}HB^{+}$.

(ii) Ion-exchange of H_2B^{+} and $^{-}HB^{+}$ with the counter ion Y^{+} of the adsorbed ion pairs $\overline{RA^{-}Y^{+}}$:

$$\overline{RA^{-}Y^{+}} + H_2B^{+} \underset{K_{e1}}{\rightleftharpoons} \overline{RA^{-}H_2B^{+}} + Y^{+} \qquad (2)$$

$$\overline{RA^{-}Y^{+}} + {}^{-}HB^{+} \underset{K_{e2}}{\rightleftharpoons} \overline{RA^{-}\,{}^{+}BH^{-}} + Y^{+} \qquad (3)$$

where the bar indicates the adsorbed phase, K_{e1} = ion-exchange constant between H_2B^{+} and Y^{+} and K_{e2} = ion-exchange constant between $^{-}HB^{+}$ and Y^{+}.

It seems reasonable to assume that the physical adsorption of the charged

amino acids on a hydrophobic surface is very small compared with the ion exchange. The overall distribution coefficient, K_B, of an amino acid is then

$$K_B = \frac{\overline{[RA^-H_2B^+]} + \overline{[RA^{-+}BH^-]}}{[^-HB^+] + [B^-] + [H_2B^+]} \quad (4)$$

By substitution of the equilibria 1–3 into eqn. 4, an expression is obtained that describes the dependence of the total distribution coefficient on the pH and counter ion concentration on the one hand and the pK_{d1} and pK_{d2} values of the amino acid on the other:

$$K_B = \frac{\overline{[RA^-Y^+]}\left(K_{e1} + \frac{K_{e2}K_{d1}}{[H^+]}\right)}{[Y^-]\left(1 + \frac{K_{d1}}{[H^+]} + \frac{K_{d1}K_{d2}}{[H^+]^2}\right)} \quad (5)$$

This expression is similar to that found in ion-exchange chromatography[19]. For amino acids with more ionizable groups, more complicated expressions are obtained. In practice, however, such expressions can be simplified.

The overall distribution coefficient can also be expressed in terms of the detergent concentration in the mobile phase, as was shown by Knox and Laird[10]. The amount of detergent adsorbed on the interface, however, is determined by its adsorption isotherm. If the shape of the isotherm is not known this brings a high degree of uncertainty into the discussion. The same is valid for the effect of the organic solvent as it influences the adsorption isotherm[18].

An expression as given by eqn. 5, valid at a constant detergent concentration, should be of more practical value as it seems reasonable to assume that the amount of adsorbed detergent is not affected significantly by small changes in pH and counter ion concentration. In order to explore chromatography with anionic detergents for the separation of amino acids, the effect of the nature and concentration of the anionic detergent, the type of hydrophobic support, the type and amount of organic solvent present in the aqueous eluent, the pH, the counter ion concentration and the column temperature on the retention of amino acids were investigated.

RAPID SEPARATION OF AMINO ACIDS BY MEANS OF SOLVENT GENERATED ION-EXCHANGE CHROMATOGRAPHY

Influence of the nature and concentration of the detergent

The effect of the type and concentration of the detergent present in the mobile phase on the degree and order of retention of amino acids was investigated for a phase system consisting of C_8-bonded silica as stationary phase and a 9:1 (v/v) mixture of 0.01 *M* sodium citrate (pH 2.50) and *n*-propanol containing 1–10 g/l of an anionic detergent as the mobile phase. In order to find out whether physical adsorption of the amino acids on the interface occurs, the capacity ratios were also measured with no detergent in the eluent. The degree of retention of the amino acids was found to be insignificant (about 5%) compared with the degree of retention observed with detergent. This indicates that under the conditions chosen physical adsorption is

small, as assumed earlier. The effect of the presence of a detergent in the eluent and the influence of its nature on the degree and order of retention are shown in Fig. 1. Addition of SSD, SDS and DNNS to the eluent increases the degree of retention considerably, while addition of SPS has no significant effect. The effect of SSD, SDS and DNNS suggests that another distribution process is introduced on addition of an anionic detergent. The insignificant effects with SPS and also with 10% (v/v) *n*-propanol can be explained by the amount of detergent adsorbed decreasing with decreasing hydrophobicity of the detergent (*i.e.*, $[\overline{RA^-Y^+}]$ in eqn. 5 becomes small, and with it the value of K_B).

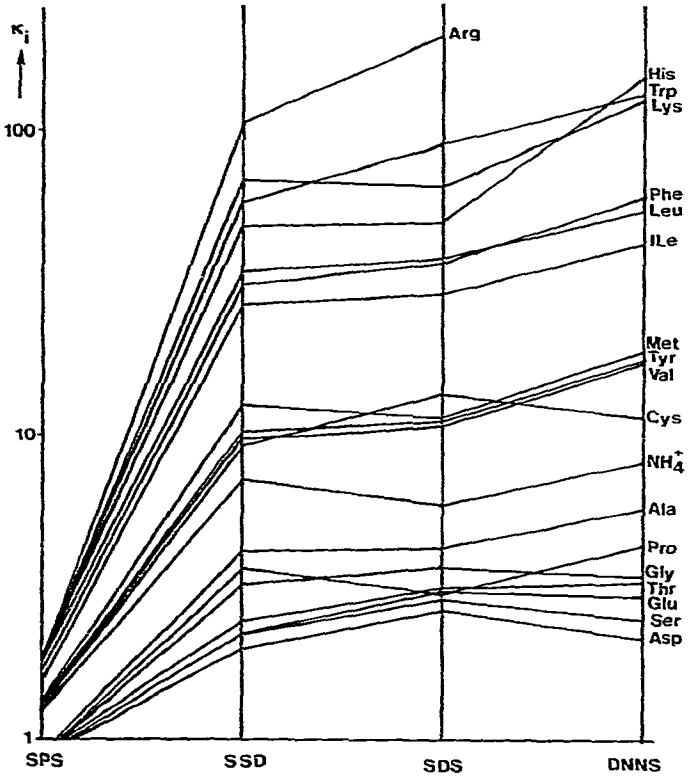

Fig. 1. Effect of the nature of the anionic detergent in the mobile phase on the capacity ratio of amino acids. Stationary phase: C_8-bonded silica (RP-8). Mobile phase: 0.01 *M* sodium citrate (pH 2.50) + *n*-propanol [9:1 (v/v)] + detergent [SPS and SSD 0.3%, SDS 0.05% and DNNS 0.01% (w/w)]. T = 25°. Sample: amino acids (Ala = alanine; Asn = asparagine; Asp = aspartic acid; Arg = arginine; Cys = cystine; CA = cysteic acid; Gln = glutamine; Glu = glutamic acid; Gly = glycine; His = histidine; Ile = isoleucine; Leu = leucine; Lys = lysine; Met = methionine; Phe = phenylalanine; Pro = proline; Ser = serine; Thr = threonine; Trp = tryptophan; Tyr = tyrosine; Val = valine).

The order of elution of the amino acids with SSD, SDS is the same as with DNNS, except for His, Trp, Lys, Phe, Cys and Pro. The degree of retention is of the same order but the selectivities differ considerably with the three types of detergent. Despite the low solubility of DNNS in the eluent (maximum *ca.* 0.01%), the capacity

ratios are drastically increased. It is known that naphthalenesulphonates are very good "ion-pairing" ions[20,21] while the bulky hydrophobic group of DNNS guarantees a strong adsorption on the hydrophobic support. These solution and adsorption properties cause a disadvantage in the use of DNNS as a large volume of eluent has to pass through the column before equilibrium is achieved. The same effect, although to a lesser extent than with DNNS, was found with SDS. With respect to its solubility in the mobile phase and the time needed to equilibrate the column, SSD is by far the best choice of the four detergents.

The order of elution of amino acids in "soap" systems differs significantly from that usually found with resin-type cation exchangers. The influence of the hydrophobic part of the amino acids on the retention, usually responsible for the retention differences of closely related amino acids on cation-exchange resins, seems to be significantly more pronounced in dynamic cation-exchange systems. This can be explained by the effect of the ion-pair formation constant, which increases significantly with increasing hydrophobicity. On resin ion exchangers, the influence of the hydrophobicity on the ion association is much smaller. The separation of pairs of amino acids such as Phe–Tyr, Leu–Ile and Gly–Ala, which are difficult to separate on resin cation exchangers, is easily achieved with detergent-based cation-exchange.

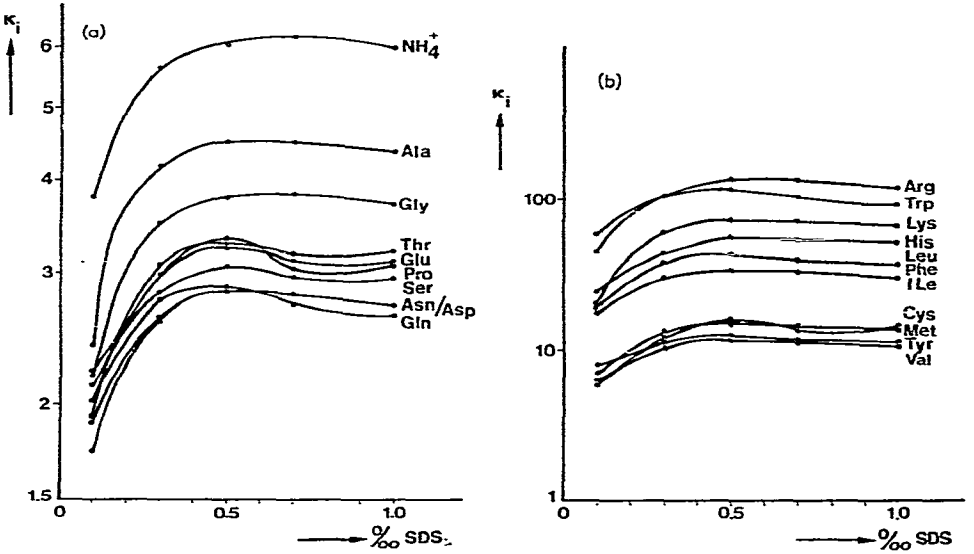

Fig. 2. Effect of the concentration of the anionic detergent in the mobile phase on the capacity ratio of amino acids. Stationary phase: C_8-bonded silica (RP-8). Mobile phase: 0.01 *M* sodium citrate (pH 2.50) + *n*-propanol [9:1 (v/v)] + SDS [0.01–0.1 % (w/w)]. $T = 25°$. Sample: amino acids (see Fig. 1).

The behaviour of ammonia, which on resin cation exchangers is one of the most retarded compounds is interesting; in dynamic cation-exchange chromatography it is eluted as a medium-retarded solute.

Figs. 2a and 2b show the effect of the SDS concentration in the mobile phase on the capacity ratio of amino acids at constant pH, Na^+ concentration and *n*-

propanol content. For most solutes the capacity ratio increases sharply on going from low to higher SDS concentrations and reaches a constant value at about 0.05% SDS. According to eqn. 5, the capacity ratio is proportional to the amount of detergent adsorbed on the interface (*i.e.*, $\kappa_i \approx [\overline{RA^-Y^+}]$). The relationship between the concentration of the detergent on the support and that in the eluent is given by its adsorption isotherm, which is usually of the Freundlich type. At a constant amount of *n*-propanol, pH and Na^+ concentration, the capacity ratio can be expected to increase with increasing detergent concentration in the eluent, leveling off to a plateau at higher concentrations (*i.e.*, the saturation point of the interface). This theoretical prediction is confirmed in practice, as is shown in Figs. 2a and 2b. The shape of the plots shown in Figs. 2a and 2b differs from those shown by Knox and Laird[10], as the counter ion concentration was not kept constant in their experiments.

As can be seen in Figs. 2a and 2b, the order of elution at low SDS concentrations differs from that at high concentrations. In particular, Pro and Cys show deviating behaviour which might be attributed to their extreme sensitivity to the *n*-propanol content and the pH of the mobile phase, respectively, which might differ very little in each experiment. The detergent concentration was found to be a powerful parameter for adjusting the degree and order of retention. A small detergent concentration, however, decreases the load capacity of the column and too small a detergent content therefore might give rise to column overloading effects and must be avoided.

Similar effects to those with SDS were obtained with SSD and, because of the advantages mentioned above, SSD is a better choice than SDS. For SSD the plateau in the capacity ratio of amino acids is reached at about 0.1%. As the resolution between Leu and Phe improves slightly at higher SSD concentration, an SSD concentration of 0.3–0.5% was chosen for further experiments.

The effects of the nature of the detergent and its concentration show that these parameters are very suitable for adjusting the degree and order of retention.

Influence of the nature and the concentration of the organic solvent

The results of the investigation of the effect of different types of organic solvents on the retention of amino acids at a constant organic solvent content, pH and SSD and Na^+ concentrations are shown in Fig. 3. The more lipophilic is the organic solvent, the less retarded are the amino acids, which would be expected as the competitive adsorption of the eluent increases (*i.e.*, $[\overline{RA^-Y^+}]$ decreases with increasing lipophilicity of the organic solvent constituent). The order of elution of the amino acids is the same, with the exception of the aromatic amino acids Trp and Tyr and Leu. The elution strength of the organic solvent constituents is in close agreement with the classification of organic solvents by the polarity index described by Snyder[22].

Fig. 3 shows that significant selectivity changes can be obtained by changing the organic solvent. In practice, the decision as to the proper choice of the organic solvent also depends on the requirements for the post-column reaction and on other considerations such as boiling point, viscosity and toxicity. In this study the ninhydrin reaction at 140° is applied and in order to avoid the formation of gas bubbles in the reactor both *n*-propanol and *tert.*-pentanol (at smaller volume percentages) would be a good compromise. In all further experiments *n*-propanol was used.

The dependence of the capacity ratio of some amino acids on the volume percentage of propanol in the eluent is shown in Fig. 4. The capacity ratio decreases

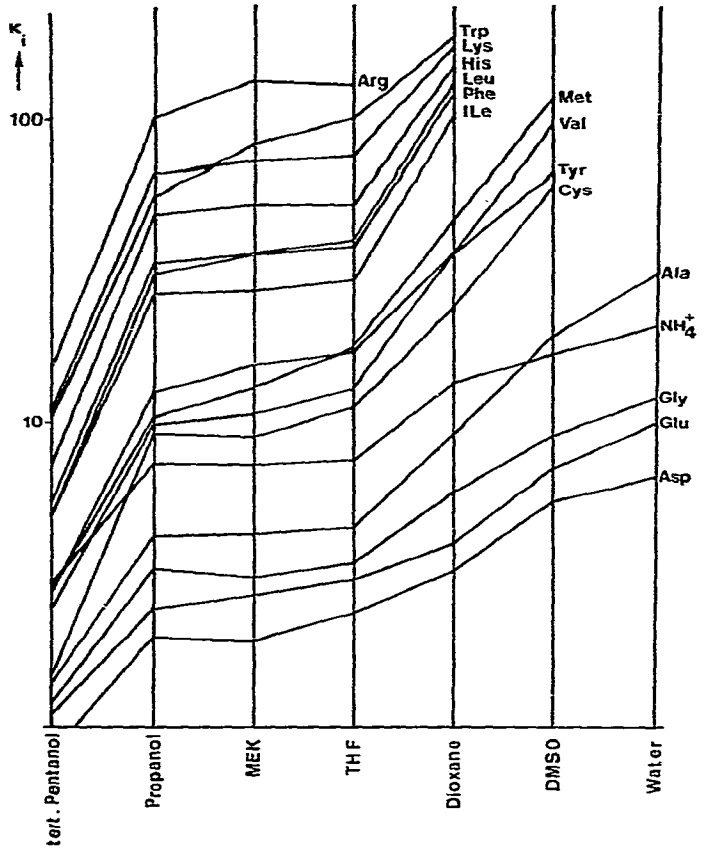

Fig. 3. Effect of the nature of the organic solvent component on the capacity ratio of amino acids. Stationary phase: C_8-bonded silica (RP-8). Mobile phase: 0.01 *M* sodium citrate (pH 2.50) + organic solvent [9:1 (v/v)] + SSD [0.3% (w/w)]. $T = 25°$; Sample: amino acids (see Fig. 1).

with increasing *n*-propanol content, which is in agreement with what is usually found in adsorption if the competitive adsorption property of a solvent component is significant[23]. In order to obtain a useful degree of retention for the less retarded amino acids, small volume percentages of *n*-propanol (below 5%) have to be applied. The strong influence of the concentration of the organic solvent on the degree and order of retention can be exploited by using gradients.

Influence of the pH of the mobile phase

The pH of the eluent has been shown to be an important parameter for adjusting retention and selectivity in ion-exchange chromatography. According to eqn. 5, the capacity ratio will decrease at high pH. At lower pH, however, the capacity ratio will also decrease because H^+ ions act as counter ions. The effect of pH on the capacity ratio for the less retarded amino acids was measured without *n*-propanol in the eluent and for the other amino acids with an *n*-propanol content of 10% (v/v). The results of these measurements are shown in Figs. 5a and 5b. With 10% of *n*-propanol the capacity ratio–pH plot shows a maximum, in agreement with what is

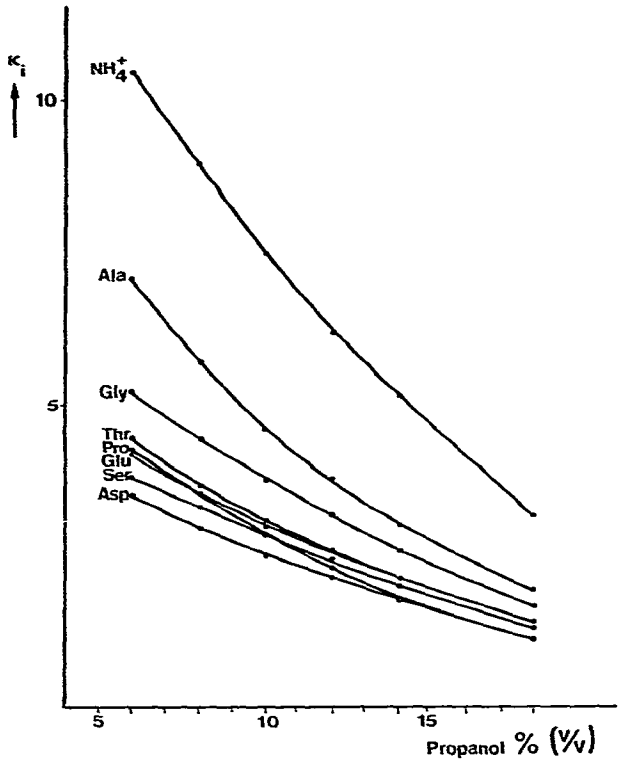

Fig. 4. Effect of the concentration of the organic solvent component on the capacity ratio of amino acids. Stationary phase: C_8-bonded silica (RP-8). Mobile phase: 0.01 *M* sodium citrate (pH 2.25) + *n*-propanol [6–18% (v/v)] + SDS [0.05% (w/w)]. $T = 25°$; Sample: amino acids (see Fig. 1).

usually found in ion exchange and as predicted by eqn. 5. The broken line in Fig. 5a indicates the fraction of amino acid present as H_2B^+ as a function of the pH. The decrease at high pH is significantly steeper than the decrease in the capacity ratio, which suggests that $^-HB^+$ is also involved in the ion-exchange process.

If no *n*-propanol is added, as for the less retarded amino acids, a maximum in the capacity ratio–pH plot is not observed. This might indicate that some physical adsorption occurs on the interface if no *n*-propanol is present. As is shown in Figs. 5a and 5b, pH is also a powerful parameter for adjusting the degree and order of retention of amino acids in dynamic cation-exchange chromatography. For example, the order of elution of the very pH-sensitive amino acid Cys can be changed considerably by small changes in the pH of the eluent. Ammonia, on the other hand, seems to be almost unaffected by the pH, as would be expected as it is fully protonated in the pH range investigated.

Influence of the concentration of the counter ion

According to eqn. 5, a linear relationship between the reciprocal of the capacity ratio and the counter ion concentration must exist if the pH, SSD and *n*-propanol concentration are kept constant. For amino acids that contain *n* basic groups, a linear relationship to the *n*th power of the counter ion concentration must be found. The

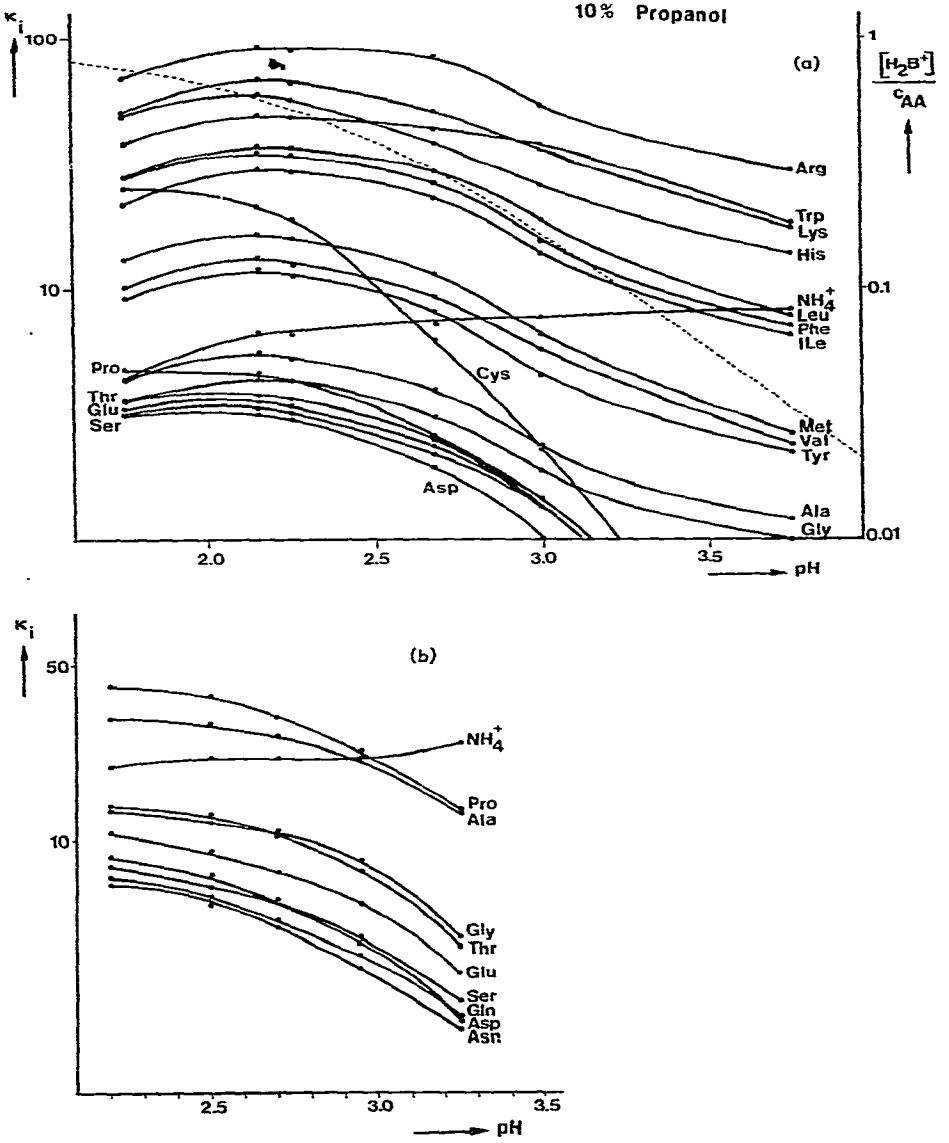

Fig. 5. Effect of the pH of the mobile phase on the capacity ratio of amino acids. Stationary phase: C_8-bonded silica (RP-8). Mobile phase: 0.01 *M* sodium citrate [pH = 1.75–3.75 (a), 2.20–3.25 (b)] + *n*-propanol [9:1 (v/v)] + SSD [0.5% (a), 0.3% (b)]. $T = 25°$. ———, Fraction $[H_2B^+]/([H_2B^+] + [^+HB^-] + [B^-])$ of the positively charged amino acid form in the solution for the neutral amino acid Trp. Samples: amino acids (see Fig. 1).

effect of the counter ion concentration was investigated with the less retarded amino acids, using Na^+ as the counter ion. The results are shown in Fig. 6, where the linear relationship suggests that the systems behave like ion-exchange systems. The value of the intercepts might indicate some residual physical adsorption. If one takes into account, however, that H^+ ions can also act as counter ions, no significant intercepts

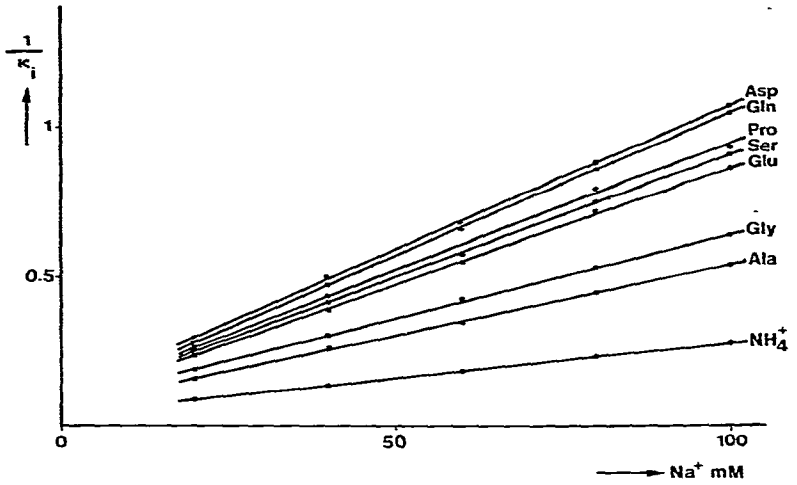

Fig. 6. Dependence of the capacity ratio of amino acids on the counter ion (Na^+) concentration in the mobile phase. Stationary phase: C_8-bonded silica (RP-8). Mobile phase: 0.01 *M* citric acid (pH = 2.25) + *n*-propanol [9:1 (v/v)] + Na_2SO_4 (0–0.08 *M*) + SSD [0,5% (w/w)]. $T = 25°$. Samples: amino acids (see Fig. 1).

are found. This result proves again that ion exchange is by far the dominant effect in "soap" chromatography if an organic solvent is present in the mobile phase. Physical adsorption, however, might contribute significantly to the distribution of non-amphoteric ionizable substances.

The counter ion concentration and possibly the nature of the counter ion which was not investigated in this work, offer further possibilities for adjusting the retentions of ionizable substances in detergent chromatography in a predictable way.

Influence of temperature

Increased column temperatures have been shown to cause favourable effects on the column efficiency and selectivity in ion-exchange[24,25] and ion-pair chromatography[12,21,26].

In order to investigate the effect of temperature in dynamic cation-exchange chromatography, the capacity ratios of amino acids were measured at different column temperatures using eluents containing 0–20% (v/v) of *n*-propanol and 0.3% (v/v) of SSD. The results of these experiments are shown in Fig. 7.

In all experiments the capacity ratio decreases with increasing temperature, while in some instances the order of elution changes considerably. The decrease in retention with increasing temperature is in agreement with the results usually found in adsorption systems. As an exception, the retention of ammonia was found to be almost unaffected by changes in column temperature, which might indicate that the effect of temperature on the capacity ratio is mainly caused by changes in the ion-exchange equilibrium rather than to a decrease in the amount of detergent adsorbed with temperature (*i.e.*, the ion-exchange capacity, $[\overline{RA^-Y^+}]$). The changes in relative retention indicate a similar effect. Therefore, the significant changes in the order of elution must be attributed to unequal shifts in the equilibrium constants. It can be

and dichlorobenzene for a given retention time was then plotted against the retention time (see Fig. 3). This plot gives the approximate fraction of the sample that is not lost in the column. As can be seen from Fig. 3, more than 50% of the sample was lost at retention times larger than 14 min. In order to correct the results for these column effects, it is essential to use an internal standard with properties similar to those of the sample.

Quantitative determination

The resolution of all isomers of the nine sugars was sufficient for a quantitative determination of the main monosaccharides in sea water, using lyxose as internal standard. For quantitative purposes it is an advantage to measure the peak heights of all anomers, as the mutarotation equilibrium in water can be shifted when changing to another solvent in the derivatization step[3].

Calibration graphs are constructed by plotting the sum of the peak heights of the anomers of each monosaccharide divided by the sum of peak heights for the anomers of lyxose against the amount of the corresponding monosaccharide, as can be seen in Fig. 4. The amount injected on the column ranged between 0.2 and 1.5 ng, and the total amount of each monosaccharide that had been derivatized ranged between 0.2 and 1.5 μg. The four graphs represent four different types of sugar, *viz.*, aldohexoses (glucose), ketohexoses (fructose), aldopentoses (arabinose) and deoxy-

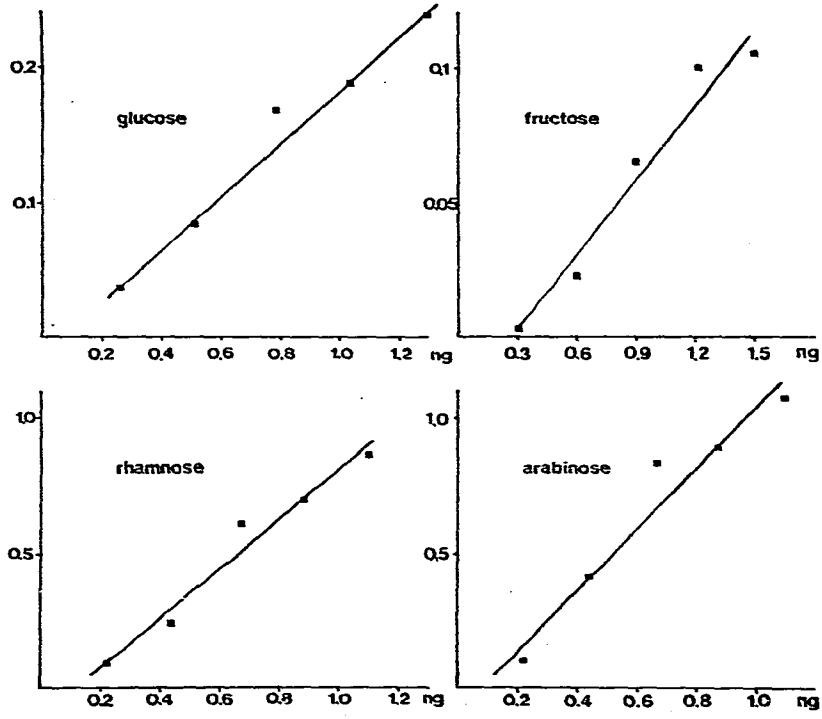

Fig. 4. GLC response plots in the picogram range for glucose, fructose, rhamnose and arabinose. The sum of the peak heights for each monosaccharide divided by the sum of the peak heights for lyxose (used as internal standard) is plotted against the amount of monosaccharide injected on the column.

Increased column temperatures have also favourable effects on the column efficiency and pressure drop.

Influence of the type of hydrophobic support

The effect of the type of hydrophobic support on the degree and order of retention was investigated with two home-prepared (Si-60-Me and Si-1000-Me) and three commercial (RP-2, RP-8, RP-18; Merck) materials. The results are shown in Fig. 8.

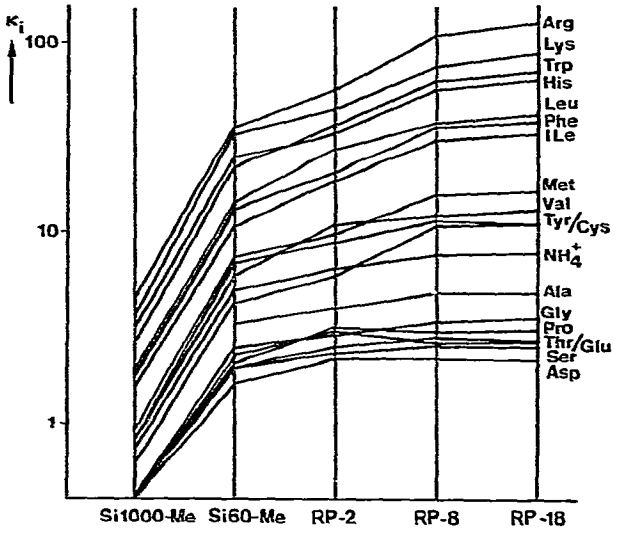

Fig. 8. Effect of the nature of the hydrophobic support used as stationary phase on the capacity ratio of amino acids. Mobile phase: 0.01 *M* sodium citrate (pH = 2.60) + *n*-propanol [9:1 (v/v)] + SSD [0.3% (w/w)]. $T = 25°$. Sample: amino acids (see Fig. 1).

The degree of retention increased in the order Si-1000-Me < Si-60-Me < RP-2 < RP-8 ≈ RP-18. This order agrees with the reported adsorption strengths of hydrophobic materials, which are caused by the difference in adsorption capacity of the alkyl layer or by the surface area[16,27,28] (Si-1000-Me = 20 m^2/g, Si-60-Me = 500 m^2/g). The difference in adsorption capacity results in a difference in the ion-exchange capacity (*i.e.*, $[\overline{RA^-Y^+}]$). Significant differences in order of elution and selectivity can be noticed between the short-chain (Si-60-Me, RP-2) and the long-chain (RP-8, RP-18) supports. The difference between RP-2 and Si-60-Me cannot be explained in terms of surface area and must be attributed to a less effective silanization of the home-prepared Si-60-Me material compared with the commercial RP-2. Proper adjustment of the degree of silanization, or the silanization of silicas with smaller surface areas, however, might be valuable in order to reduce the time needed to separate all amino acids under isocratic conditions on a multi-column system (as the distance between the amino acids is shorter).

The column efficiencies obtained with the different supports decreased in the order Si-60-Me > RP-8 > RP-2 > RP-18 > Si-1000-Me. The theoretical plate height ranged between 25 μm (Si-60-Me) and 100 μm (Si-1000-Me) at a linear fluid velocity

of 3 mm/sec. The effect of the type of support, as shown in Fig. 8, demonstrates that this parameter might be extremely important in obtaining high-speed separations of amino acids.

Separation of amino acid mixtures

The results of the systematic experiments showed that this type of chromatography might be very suitable for the separation of amino acids. Several parameters are available for adjusting the degree and order of retention.

To optimize the separation of amino acids with respect to time one is confronted, however, with problems similar to those in conventional ion-exchange chromatography. Firstly, the degree of retention on, *e.g.*, RP-8, of the acidic, neutral and basis amino acids differs considerably, which does not permit a rapid separation under isocratic conditions. Secondly, when trying to speed up the separation by applying a concentration gradient of *n*-propanol, pH, Na^+ or SSD, one has to choose the optimal shape of such a gradient. This choice is difficult as the degree of retention is effected unequally for the different amino acids.

Fig. 9 shows a first attempt to apply an *n*-propanol gradient in order to speed up the separation. The acidic and some neutral amino acids which are less retarded are eluted, starting with 2% (v/v) *n*-propanol, by a linear *n*-propanol gradient of 0.5% (v/v)/min up to 25 min. Subsequently the other neutral and the basic amino acids are separated with a linear *n*-propanol gradient of 1% (v/v) min. A complete separation of the 19 protein amino acids is achieved in about 40 min. Apart from *n*-propanol, the pH, Na^+ and SSD concentration or combinations of them can also be used as variable parameters for increasing the speed of separation by means of a gradient. It is difficult and time-consuming, however, to find the optimal gradient. Therefore, mathematical optimization procedures, such as Simplex, might be necessary to solve this problem.

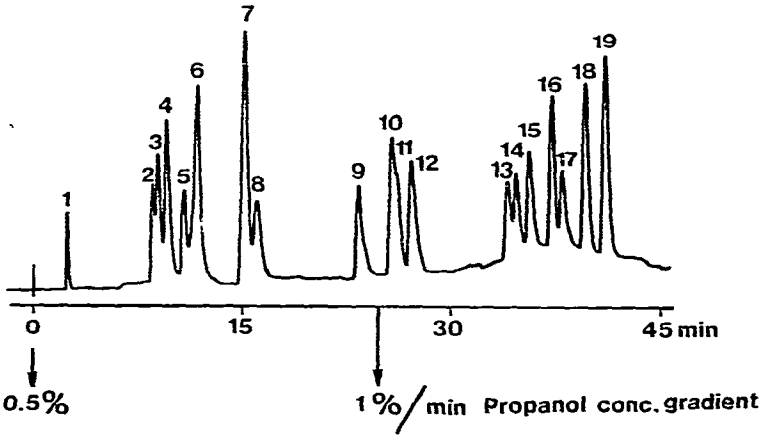

Fig. 9. Separation of a test mixture of amino acids occurring in protein hydrolysates using an *n*-propanol gradient. Column: 250 × 3 mm. Stationary phase: C_8-bonded silica (RP-8). Mobile phase: 0.017 *M* sodium citrate (pH = 2.70) + SSD [0.5% (w/w)] + *n*-propanol gradient (0–25 min, 2%/min; 25 min-end, 4%/min). $T = 25°$; $\Delta p = 250$ bar. Sample: 1 = $CySO_3H$; 2 = Asp; 3 = Ser; 4 = Glu; 5 = Thr; 6 = Gly + Pro; 7 = Ala; 8 = Cys; 9 = NH_4^+; 10 = Tyr; 11 = Val; 12 = Met; 13 = Ile; 14 = Phe; 15 = Leu; 16 = His; 17 = Lys; 18 = Trp; 19 = Arg.

As mentioned above, column supports with a smaller adsorption capacity might be useful for speeding up the separation as the distance between the groups of acidic, neutral and basic amino acids is smaller. Fig. 10 shows a rapid separation of amino acids, on the home-prepared Si-60-Me support, under isocratic conditions. Although no complete separation of the less retarded amino acids is obtained, the total separation is achieved in about 35 min.

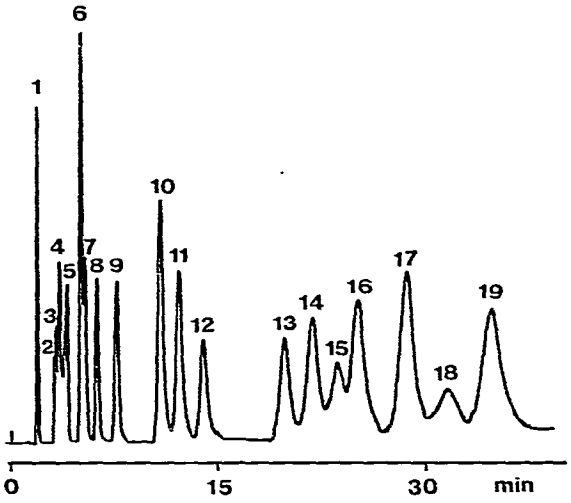

Fig. 10. Separation of a test mixture of amino acids occurring in protein hydrolysates under isocratic conditions. Column: 250 × 3 mm. Stationary phase: methyl-bonded silica (Si-60-Me). Mobile phase: 0.01 *M* sodium citrate + 0.01 *M* sodium sulphate (pH = 2.65) + *n*-propanol [49:1 (v/v)] + SSD [0.3% (w/w)]. $T = 25°$; $\Delta p = 200$ bar. Sample: 1 = $CySO_3H$; 2 = Asp; 3 = The; 4 = Ser; 5 = Glu + Gly; 6 = Ala; 7 = Pro; 8 = NH_4^+; 9 = Cys; 10 = Val; 11 = Met; 12 = Tyr; 13 = Ile; 14 = Leu; 15 = Phe; 16 = His; 17 = Lys; 18 = Trp; 19 = Arg.

Solvent-generated cation-exchange chromatography has proved to be very suitable for the separation of amino acids. The selectivity of the phase systems containing anionic detergents is, in general, higher than of conventional ion-exchange systems. One can expect to achieve a complete separation of the protein amino acids within 30 min or even less, applying multi-column operation with optimized hydrophobic supports and solvent gradients. With respect to the solvent gradient, more attention should be paid to the effect of the nature of the detergent and counter ion in order to explore these parameters.

Other phase systems for the separation of amino acids

In addition to dynamic cation-exchange chromatography, phase systems involving a resin-type cation exchanger (Aminex-A7) as well as a chemically bonded cation-exchanger (LiChrosorb KAT, Merck) were also investigated.

As the separation of the less retarded acidic and neutral amino acids has been shown to be the most difficult part of the amino acid separation, only the retention behaviour of these amino acids was investigated in more detail.

On Aminex-A7 the separation of Ser/Thr and Ala/Gly was found to be the

bottleneck for a rapid separation of the protein amino acids. These pairs of amino acids, however, can easily be resolved with the dynamic cation-exchange chromatography (see Fig. 11). On the other hand other pairs of amino acids such as Asp/Ser/Glu are more difficult to separate with the dynamic cation-exchange systems. When optimizing the pH (3.25), sodium concentration (0.2 *M*) and temperature (58°) and maximal attainable flow-rate (*i.e.* Δp = 160 bar) an isocratic separation of the ten less retained amino acids on Aminex A-7 can be achieved within 20 min. In order to elute the other more retarded amino acids a gradient has to be applied, which will take another 30 min.

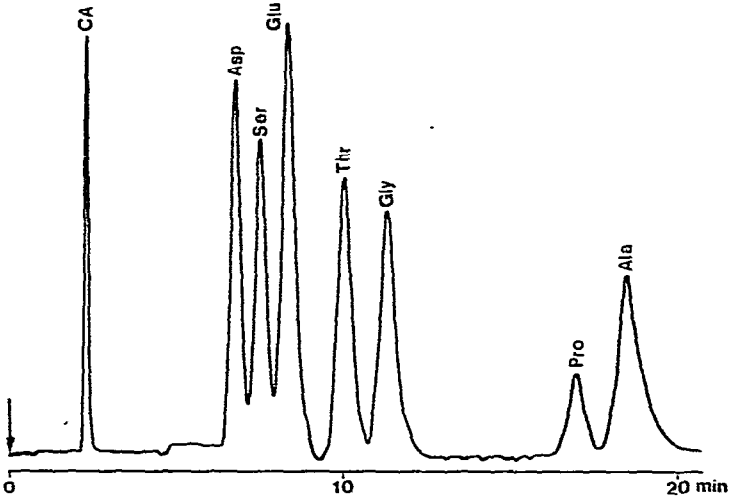

Fig. 11. Separation of the less retarded protein amino acids by dynamic cation-exchange chromatography. Column: 250 × 3 mm. Stationary phase: RP-8. Mobile phase: 0.01 *M* sodium citrate (pH 2.75) + *tert.*-pentanol [166:1 (v/v)] + SSD [0.3% (w/w)]. T = 40°; Δp = 200 bar.

On Aminex-A7, therefore, the whole amino acid analysis can be achieved within 50 min. As shown in Figs. 9 and 11 higher ultimate separation speeds can also be obtained with dynamic cation-exchange chromatography because of the higher pressure range available with this rigid material.

Chemically bonded cation exchanger (LiChrosorb KAT, Merck). This cation-exchanger consists of a silica matrix to which a benzenesulphonic acid is chemically bonded. As the ion-exchange capacity of this support is small, much lower counter ion concentrations in the mobile phase have to be used. The effects of pH and Na^+ concentration on the degree of retention of the less retarded amino acids are shown in Fig. 12. An elution behaviour as almost identical with that in detergent chromatography is found (*e.g.*, NH_4^+ and Tyr are much less retained on both the chemically bonded material and the solvent generated ion-exchange material than on the resin ion exchanger). The selectivity, however, although dependent on the pH and Na^+ concentration, is usually lower on the KAT ion exchanger than on the detergent-modified support. Nevertheless, at pH 3.10 and an Na^+ concentration of 30 m*M* a reasonably rapid separation of the less retarded amino acids can be obtained, as can be seen in Fig. 13. The resolution is disappointing, however, owing to a low column

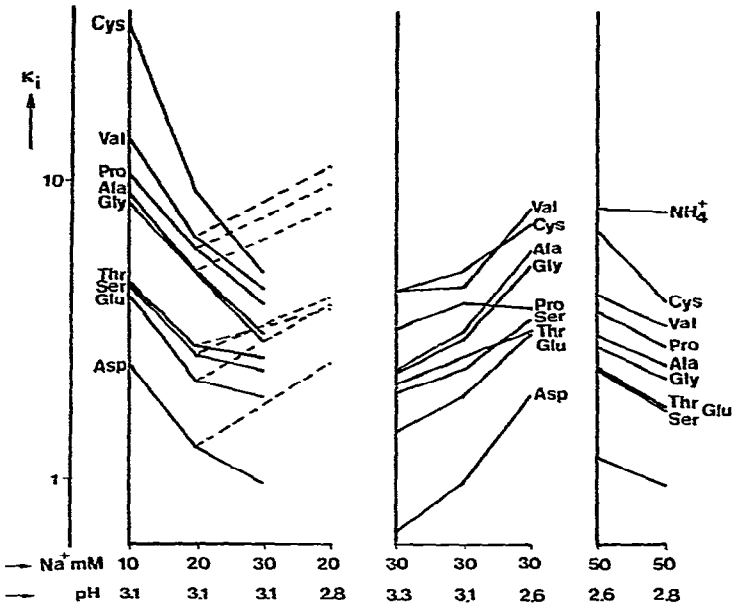

Fig. 12. Effect of Na^+ concentration and pH on the capacity ratio of amino acids on a chemically bonded silica cation exchanger (LiChrosorb KAT). $T = 55°$.

efficiency. In addition, the peaks show fronting and the pressure drop increases during the elution time. Eventually the columns become blocked as a result of stripping of the bonded phase.

To summarize, it can be concluded that the results obtained with chemically bonded silica-based cation exchangers for the rapid separation of amino acids are not promising. New types of chemically bonded materials with higher ion-exchange

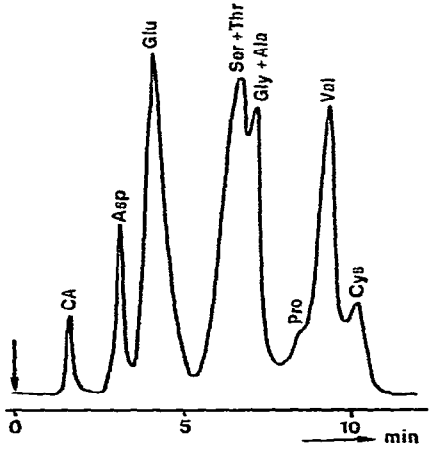

Fig. 13. Chromatogram of the less retarded protein amino acids on a chemically bonded silica cation exchanger (LiChrosorb KAT). Column: 250 × 3 mm. Mobile phase: 0.01 M sodium citrate (pH 3.10). $T = 55°$; $\Delta p = 200$ bar.

capacities and much better efficiency and stability will have to be developed. The high flow-rates attainable on these supports certainly justify such an attempt, which could yield a means of increasing further the speed of amino acid separations.

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