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POLYOL BONDED TO SILICA GEL AS STATIONARY PHASE FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A new derivatized silica gel to which a polyol is bonded is investigated as a stationary phase for high-performance liquid chromatography. Efficient separations of polar compounds (carbohydrates, alkaloids, phenols, proteins) have been realized using straight-phase or normal adsorption chromatography and also with reversed-phase solvent systems. The polarity of the new phase is similar to or higher than that of silica gel itself, but the selectivity is different. A very high percentage of modifier can be used in straight-phase chromatography on Polyol-RSiL. This stabilizes the results and increases the potential interest for straight-phase techniques.

INTRODUCTION

Several investigations of the application of hydroxy-bonded silica gel phases for high-performance liquid chromatography (HPLC) have been reported. LiChrosorb DIOL phase (Merck) has been used for protein separations[1–3] and for the analysis of carbohydrates[4]. Cyclodextrin-bonded phases have also been described[5–7]. Recently a new polyphenol phase was developed[8]. This paper presents the chromatographic properties of a new silica gel phase to which a polyol is bonded. This phase (Polyol-RSiL) was developed at this laboratory in collaboration with Alltech-RSL (Eke, Belgium).

EXPERIMENTAL

The chromatographic system consisted of a Varian 5020 LC pump, a Varian 9176 recorder and a Varian LC-50 UV detector. Carbohydrates were detected by refractive index detection (Waters Assoc. differential refractometer R-401). A 7000 p.s.i. 15-μl Valco sample loop injector was used. The columns were LiChroma tubes (15 × 0.46, 25 × 0.46 and 20 × 0.70 cm) provided with Valco fittings. Irregular-shaped-particle (5 and 10 μm) Polyol-RSiL phases were used. The total organic content of the phase as measured by thermogravimetric analysis was 15%. Solvents used were all spectroscopy grade. The columns were filled by slurry packing. The phases

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were suspended in water–methanol (1:9), and pumped into the columns with a Haskel air-driven fluid pump at a pressure of 400 bar. The same solvent was used as the pressurizing liquid. Beer proteins A were isolated from tannin–protein complexes obtained from tannin-stabilized and chillproofed beer. The sludge of precipitated tannin–protein complex is dried and decomposed with methanol. The residue is dissolvent in water and freeze-dried to give light yellow fluffy proteinic material. Beer proteins B and C were isolated from unstabilized beer by ammonium sulphate precipitation. Further separation was obtained by dialysis of the precipitate, followed by lyophilization. Sample B is obtained from the solution retained by the membrane (mol. wt. > 10000) and sample C from the solution that passed the membrane (mol. wt. < 10000).

RESULTS AND DISCUSSION

Analysis of carbohydrates

Carbohydrates are currently chromatographed on many phases. A popular system uses aminopropyl silica gel, but one disadvantage of this packing material is its short column lifetime. Retention can decrease rapidly with time. Therefore the development of new, eventually more stable stationary phases for carbohydrate analysis is important. Fig. 1a, b and c show the separation possibilities of carbohydrates on the new polyol phase.

The selectivity of the polyol phase is comparable with that of a cross-linked aminopropyl phase, *e.g.* the Carbohydrate Phase I (Alltech-RSL, Belgium). Efficiencies for fructose and sucrose are 19600 and 17600 plates per metre, respectively. The average efficiency for the carbohydrate chromatography is *ca.* 15600, which is good for the analysis of carbohydrates in general (Table I).

This phase has the additional advantage that separations between groups (mono-, di-, trisaccharides) as well as within groups are possible (Fig. 1c and b). The peak symmetries as indicated by the peak asymmetry values (*A* in Table I) are very good. Only light tailing is observed.

Fig. 2 shows the *H–u* relationship (where *u* is the linear velocity) for fructose and sucrose (conditions as in Fig. 1). The curves demonstrate a linear relation with correlation coefficients 0.96 and 0.97, respectively, down to the minimum. This means that the *B* term in the Van Deemter equation is of little or no importance for this chromatography. The slope of the line indicates an important *C* term, but still it is smaller than for many other phases used in carbohydrate analysis. A four-fold increase of the linear velocity increases the *H* value by only *ca.* 20%. A minimum in the *H–u* curve is not reached, even at linear velocities of only 0.3 mm/s. Other systems based on RSiL, *e.g.* RSiL-C_{18}-HL-D, with acetonitrile–water and pyrene as sample show an optimum linear velocity of 1.6 mm/s, which is indeed much higher. This is typical for larger sample molecules, such as the carbohydrates, and prevents large plate numbers being obtained with such systems. More efficient separation can be obtained at very low flow-rates ($u < 1$ mm/s). This is however not very realistic. The chromatograms of Fig. 1 were therefore run at usual speeds.

As indicated in the legend to Fig. 1, it is necessary to use triethylamine (TEA) in the mobile phase. if not, peaks are broadened or eluted as a doublet owing to the separation of the two anomeric forms of the sugars. This addition of TEA to the

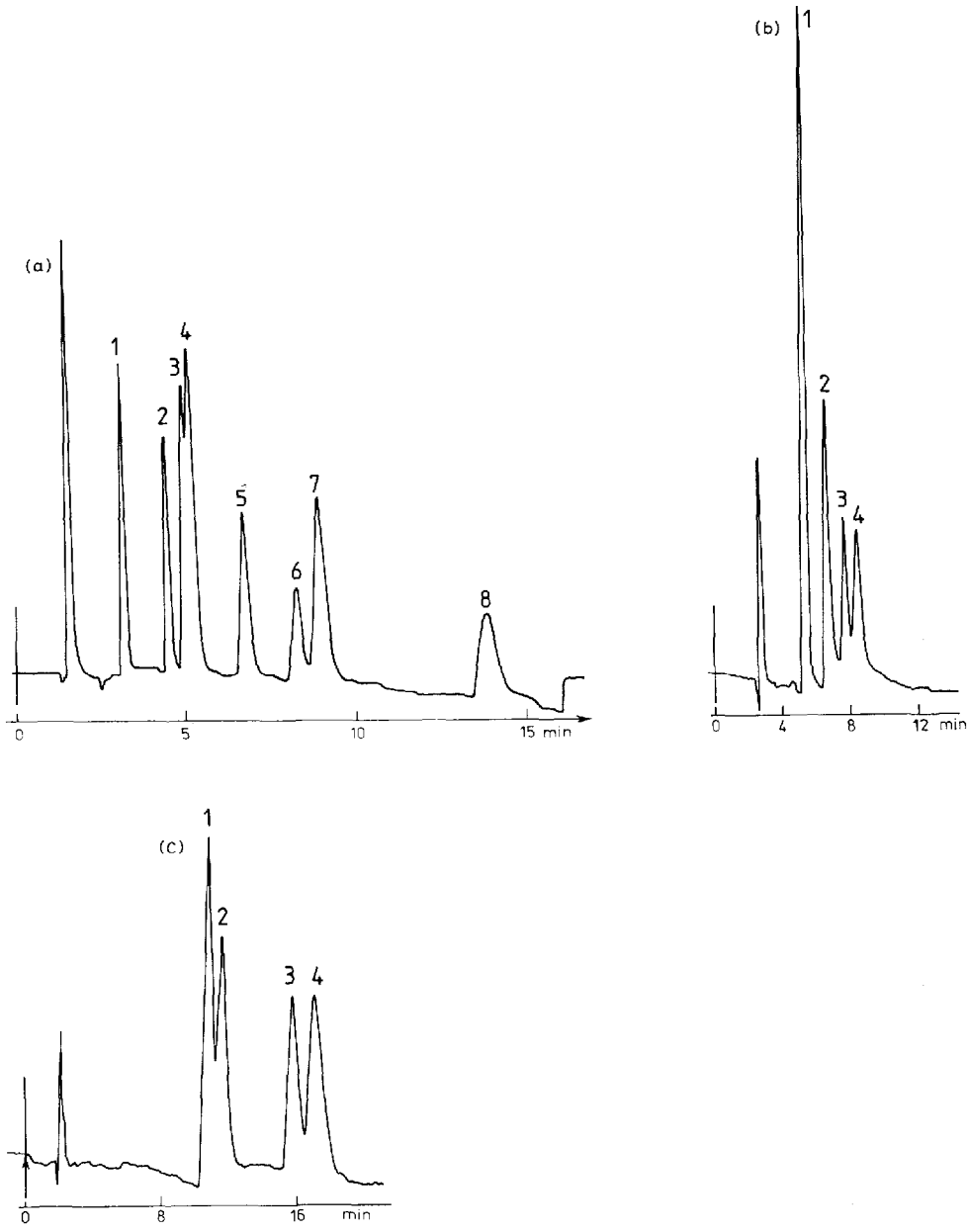

Fig. 1. (a) Separation of rhamnose (1), fructose (2), sorbitol (3), glucose (4), sucrose (5), maltose (6), lactose (7) and maltotriose (8). Column, 25 × 0.46 cm I.D. filled with 5 μm irregular Polyol-RSiL; mobile phase, acetonitrile–water (70:30) with 0.1% TEA; flow-rate; 1 ml/min; recorder speed, 0.5 cm/min; detection, refractive index; pre-saturation column, 5 × 0.46 cm I.D. filled with silica gel (Prep-RSiL, particle diameter 12–40 μm); pressure, 138 atm. (b) Separation of monosaccharides: rhamnose (1), arabinose (2), mannose (3) and galactose (4). Conditions as in (a). (c) Separation of di- and trisaccharides: maltose (1), lactose (2), maltotriose (3) and raffinose (4). Column, 5 μm Polyol-RSiL; (25 × 0.46 cm I.D.); mobile phase, acetonitrile–water (68.6:31.4) with 0.1% TEA; flow-rate 1.33 ml/min; recorder speed, 0.5 cm/min; detection, refractive index; pre-saturation column as in Fig. 1a.

TABLE I

RETENTION AND EFFICIENCY DATA FOR SUGARS ON POLYOL-RSiL

Conditions as in Fig. 1a. k' = Capacity factor; N = plate number; H = plate height; A = peak asymmetry factor.

Sugar	t_R *(mm)*	k'	N/m	H *(mm)*	A
Rhamnose	31.6	1.0	15.368	0.065	136
Fructose	44.6	1.84	19.591	0.056	125
Glucose	52.0	2.31	13.587	0.074	128
Sucrose	62.0	2.94	17.600	0.057	115
Maltose	82.8	4.27	13.142	0.076	124
Maltotriose	138.7	7.83	14.093	0.071	145

eluting solvent could rapidly lead to reduced column lifetime. To prevent this, a precolumn is placed before the injector. The stability of the combined system was tested by rinsing with 8 l of mobile phase (containing 0.1% TEA) at a flow-rate of 1.5 ml/min (corresponding to *ca.* 400 analyses). A test mixture consisting of rhamnose, sucrose and maltotriose was again injected. Unlike on aminopropyl silica gel, the compounds eluted later than they did originally (2, 15 and 25%, respectively). Use of a higher water content led to restoration of the former retention times.

Analysis of phenols, polyphenols and other polar compounds

Fig. 3 presents the chromatograms of phenols, polyphenols and some other: polar compounds. The elution sequence is the same as on other polar phases but the

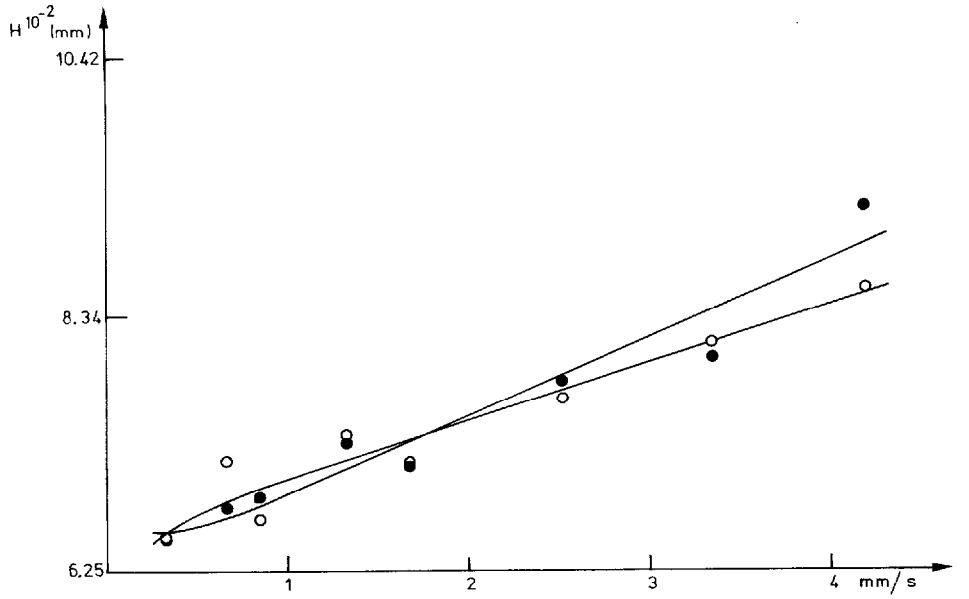

Fig. 2. *H–u* curves for fructose (○) and sucrose (●). Conditions as in Fig. 1a.

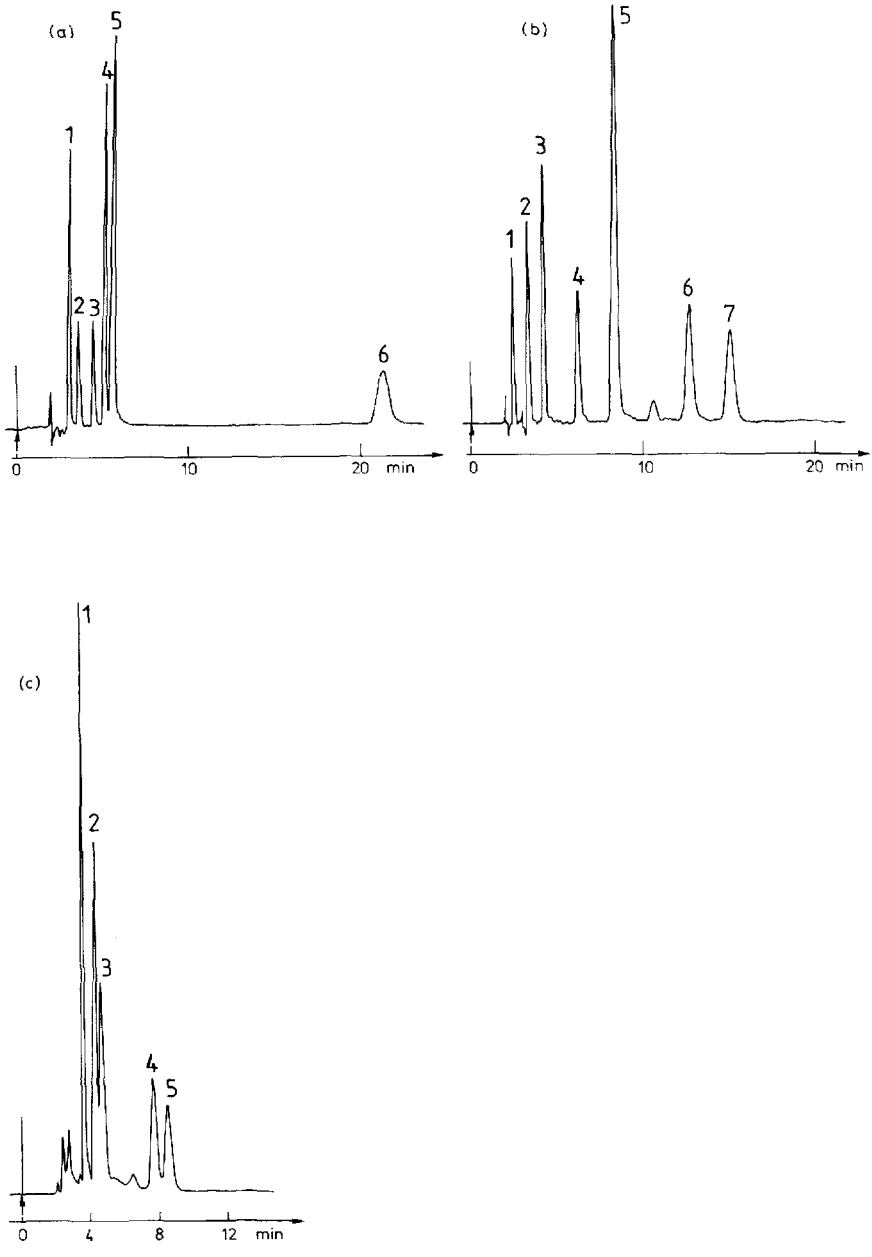

Fig. 3. (a) Analysis of chalkone (1), benzoic acid (2), aspirin (3), caffeine (4), theophyllin (5) and catechin (6). Column, 5 μm polyol-RSiL (25 × 0.46 cm I.D.); mobile phase, hexane–methanol–tetrahydrofuran (70:22.5:7.5) with 0.25% citric acid; flow-rate, 1 ml/min; recorder speed, 0.5 cm/min; detection, UV at 280 nm; pressure, 62 atm. (b) Analysis of phenolic compounds: di-*tert.*-butylphenol (1), phenol (2), catechol (3), pyrogallol (4), gallic acid (5), catechin (6) and quercitrin (7). Mobile phase, hexane–methanol–tetrahydrofuran (60:30:10) with 0.25% citric acid. Further conditions as in (a). (c) Analysis of polyphenols: kaempherol (1), quercetin (2), fisetin (3), epicatechin (4) and quercitrin (5). Mobile phase, hexane–methanol–tetrahydrofuran (50:37.5:12.5) with 0.25% citric acid. Further conditions as in (a).

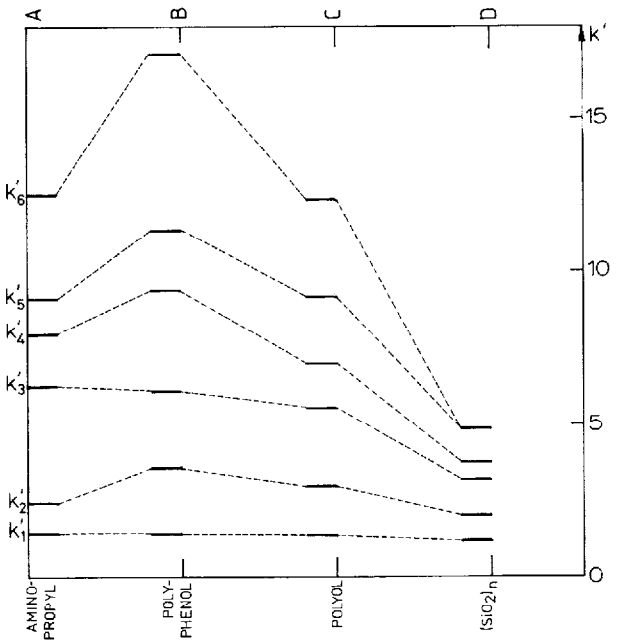

Fig. 4. Comparison of capacity factors on different straight phase silica gels: k'_1 = catechol; k'_2 = pyrogallol; k'_3 = gallic acid; k'_4 = impurity of 6; k'_5 = catechin; k'_6 = quercitrin. Columns: A = aminopropyl silica gel, 5 μm; B = polyphenol silica gel, 5 μm; C = polyol silica gel, 5 μm; D = pure silica gel, 5 μm. Dimensions, 15 × 0.46 cm I.D.; mobile phase, hexane–methanol–tetrahydrofuran (70:22.5:7.5) with 0.25% citric acid; flow-rate 1 ml/min; recorder speed, 0.5 cm/min; detection, UV at 280 nm.

selectivity is slightly changed. The polarity of the polyol phase was compared with that of silica gel, aminopropyl silica and polyphenol silica gel. Under identical conditions the magnitude of the k' values for a series of compounds was taken as a measure of polarity: the more polar the compound, the greater the retention and the higher the k' value. The results for most of the phenols of Fig. 3b are given in Fig. 4. As already reported[8] the polyphenol phase is clearly the most polar one. For these phenols, silica gel is the least polar phase. For more neutral compounds the difference between silica gel and the other phases (except the polyphenol phase) is not so great. Retention times and selectivity factors of the polyol phase are comparable with those of an aminopropyl silica gel. However, the retention times of acidic compounds, such as gallic acid, are slightly higher on the aminopropyl phase. Citric acid is used as a metal complexer and to create an acidic mobile phase that leads to more reproducible retention times.

Silica gel is not polar enough to allow large amounts of modifier (30–50% methanol) for the simple compounds used in Fig. 4. Solvent systems containing methylene chloride, benzene, ether on ethyl acetate, for example, to which a small amount (0.1–5%) of modifier is added, are more usual for silica gel. Such systems lead to unstable columns that are difficult to regenerate. This fact is the main reason for the success of reversed-phase chromatography, which is more reliable and uses easily regenerated columns. The more polar polyol and polyphenol silica gel phases allow

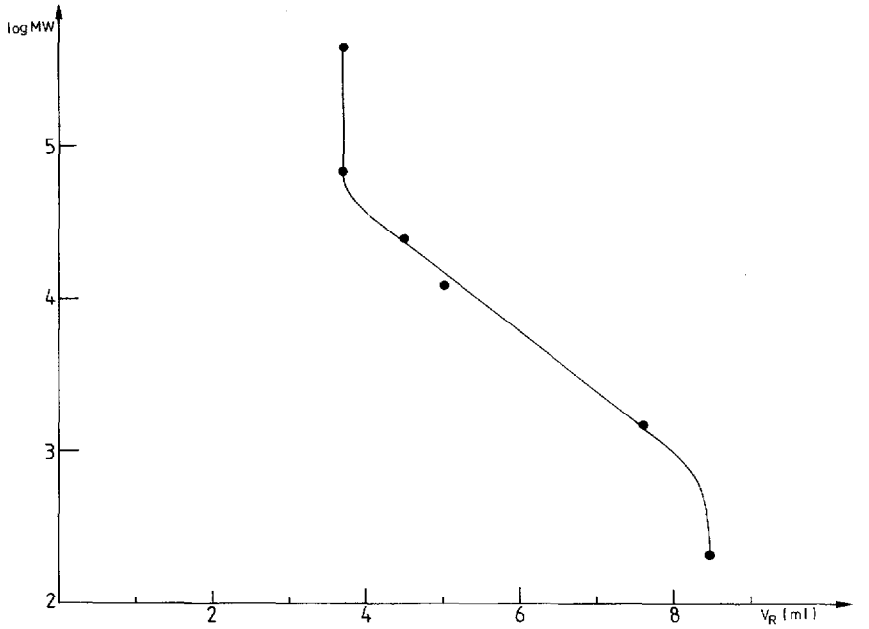

Fig. 5. Calibration curve for ferritin (mol. wt. 450000), bovine serum albumin (BSA, 67000), chymotrypsinogen A (25000), cytochrome *c* (12300), bacitracin (1500) and tryptophan (204). Conditions as in Fig. 6. MW = Molecular weight; V_R = retention volume.

high modifier content while still showing good retention power. Such systems do not have the inconvenience of earlier straight phase chromatography. Retention times are reproducible and column regeneration is fast. From 100% methanol back to 30% in hexane again leads to stable retention times after washing with only six column volumes.

Chromatography of proteins

Diol[1–3] and acetylamide phase[9,10] are two bonded phases suitable for aqueous size-exclusion chromatography (SEC) of proteins. Both are characterized by an inert hydrophilic layer at the surface. The polyol phase, which has similar properties, should also be suitable for SEC of proteins.

A mobile phase of pH 7.5 was used in order to minimize ion-exchange reactions of the silanol functions with positively charged proteins. At this pH about half of the silanol functions can be considered to be ionized. To prevent ionic bonding forces between the support and the macromolecules a high ionic strength (3 *M* sodium chloride) is needed. However, no solvent is added to the aqueous buffer.

Fig. 5 presents the SEC calibration curve for six compounds. Proteins with molecular weights higher than 67000 (BSA) elute in the same volume (3.7 ml), which is about the elution volume of the column. The curve shows clearly that size exclusion is the dominating separation mechanism. The large retention volume of tryptophan, however, is an indication that adsorption effects with the stationary phase cannot be totally suppressed. The retention mechanism must therefore be controlled by a combination of steric exclusion and an adsorption effect.

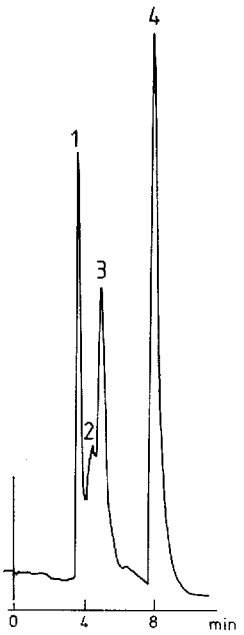

Fig. 6. Separation of BSA (mol. wt. 67000, conc. 2%), chymotrypsinogen A (25000, conc. 1%), cytochrome *c* (12300, conc. 0.2%) and tryptophan (204, conc. 0.05%) eluting in that order. Column: 10 μm Polyol-RSiL (20 × 0.7 cm I.D.); mobile phase, phosphate buffer (pH 7.5) and 3% sodium chloride; flow-rate, 1 ml/min; recorder speed, 0.5 cm/min; detection, UV at 280 nm; pre-saturation column, 5 × 0.46 cm I.D. (12–40 μm); injected volume, 15 μl; pressure, 30 atm.

Fig. 6 shows the chromatogram for a mixture of BSA, chymotrypsinogen A, cytochrome *c* and tryptophan. Fig. 7 shows the elution profiles of several beer protein fractions.

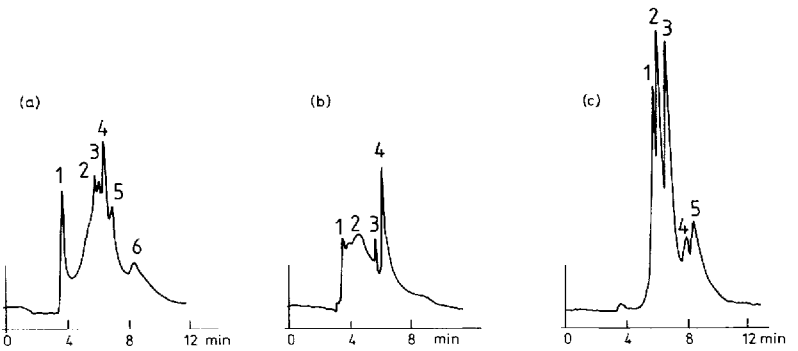

Fig. 7. (a) Chromatogram of beer proteins isolated from protein–tannin complexes: concentration, 3%; conditions as in Fig. 6. (b) Chromatogram of beer proteins, isolated from unstabilized beer: mol. wt. > 10000; concentration, 3%; conditions as in Fig. 6. (c) Chromatogram of beer proteins isolated from unstabilized beer: mol. wt. < 10000; concentration, 3%; conditions as in Fig. 6.

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