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Analytical- and preparative-scale chromatographic separation of phenylalanine from aspartame using a new polymeric sorbent

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ABSTRACT

A new, large-pore, cross-linked, polymethacrylate stationary phase separates Phe from Aspartame in 10% aqueous ethanol by reversed-phase chromatography. Batch equilibrium data at 30, 50 and 70°C, obtained with 165-μm particle size material showed linear sorption at loadings of up to 140 mg/g stationary phase, and corresponded to a mobile phase adsorbate concentration approaching the solubility limits. Column runs with 40-, 60-, 117-, and 165-μm particle size materials at 30–70°C showed retention behavior that was predictable from batch equilibrium data, and was independent of particle size at sample volumes as high as 80% of the column void volume and at outlet concentrations of 5–10 mg/ml. The relatively large pores (250 Å) of the stationary phase allowed free access of small molecules, with the methacrylate structure promoting strong sorption of aromatic amino acids. These characteristics permitted the ready calculation of column retention times, and facilitated extrapolation of analytical-scale results obtained with small particle size material to preparative-scale separations carried out under volume overload conditions with a larger particle size stationary phase.

INTRODUCTION

The chromatography of free amino acids is of both analytical and commercial importance. The traditional method for analytical-scale separation of amino acids is based on fractionation of free amino acids using cation-exchange chromatography followed by post-column derivatization with ninhydrin for detection purposes [1]. An alternate approach is precolumn derivatization using reagents such as dabsyl or dansyl chlorides, *o*-phthalaldehyde, or 9-fluorophenylmethylchloroformate [2–6].

Chromatography of non-derivatized amino acids on a preparative scale is of particular interest in the biotechnology area. The objective is often the recovery of a free amino acid from other components present from the fermentation, or the

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biochemical or chemical production step [7,8], rather than resolution of all the components in a complex mixture. In this context derivatization is undesirable at preparative scale, since it would be necessary to regenerate the free amino acid from its derivatized form, as well as to separate the derivatization reagents from the amino acid. Commercially important amino acids include lysine, monosodium glutamate, methionine, glycine, phenylalanine and tryptophan [9,10]; as well as Aspartame, the phenyl methyl ester of aspartic acid and phenylalanine. Aspartame sales were estimated to be in excess of US$ 500 million per year [11]. The overall production of amino acids and selected dipeptides has an estimated market value of over US$ $2 \cdot 10^9$ worldwide [12]. Improvements in the separation and scale-up of these small molecules continue to be of obvious importance.

Non-derivatized amino acids are readily separated by ion-exchange chromatography in acetate. Experimental methods for the separation of acidic and aromatic amino acids by anion-exchange resin [13,14], and neutral and basic amino acids by thin-layer chromatography on cellulose [15], are well known. Fully automated, high-speed ion-exchange chromatography of amino acids over cation-exchange resin was reported in 1969 [16].

We report separation characteristics of a new polymethacrylate ester, macroreticular stationary phase. This stationary phase has an average pore size of 250 Å and has no charged groups. Retention behavior for amino acids, alcohols, ethers and sugars in water and 10% aqueous ethanol show that solutes with an ether bond or with an aromatic character strongly adsorb, while molecules with aliphatic character elute at close to the void volume. This property is of practical importance in separation of aromatic amino acids from aliphatic precursors, coproducts, salts and other constituents present from the chemical or biological synthesis of the amino acids. Analytical-scale chromatography showed baseline separations for several test mixtures and led to a case study for preparative-scale separation of Aspartame from phenylalanine where the effects of particle size, eluent velocity, temperature and column dimensions on separation were observed for sample volumes of up to 80% of the column void volume. We confirmed experimentally that a properly conceived and synthesized stationary phase should be easy to scale up, and that macroporous polymeric sorbents have a special niche in this context.

EXPERIMENTAL

Apparatus

The liquid chromatography (LC) system used in this study consisted of a Milton Roy pump (LDC, Riviera Beach, FL, U.S.A.), with flow ranges of 16–160 ml/h, a Rheodyne (Cotati, CA, U.S.A.) injector Model 7125 and a jacketed column which was controlled to the desired temperature setting $\pm$ 0.1°C by circulating water through the jacket using a Haake Model FE water bath. Eluting peaks were detected by UV absorbance at 240 nm using a Varian (Palo Alto, CA, U.S.A.) VUV-10 detector, or by a differential refractometer (Waters type R401, Milford, MA, U.S.A.).

Stationary phase

The stationary phase was AmberChrom CG-71 and is a new methacrylic, macroporous, polymeric sorbent. The sorbent is stable from pH 1 to 14, and is

steam-sterilizable. Other properties are summarized in Table I. Part of this work addressed particle size effects, and for this purpose, wet particle sizes of 38.7 ± 6.9, 60.3 ± 4.9, 117 ± 15, 165 ± 15 and 164 ± 24 μm were used. The non-standard sized fractions were specially prepared for the present work by the Rohm and Haas Company. All particle sizes are for stationary phase equilibrated in the mobile phase and were determined using a Zeiss inverted stage microscope coupled to a Zeiss IBAS 2000 image analysis system (Thornwood, NY, U.S.A.) through a Dage-MTI series 68 video camera (Dage, Michigan, IN, U.S.A.). Some samples were also analyzed using a Leitz analyzer. The materials to be measured were equilibrated in water or 10% ethanol for several hours, placed on a slide as an aqueous slurry, and then analyzed while wet for average particle size, particle size range and shape.

Stationary phase preparation

Sorbents were prepared for packing in 50–100 ml quantities by (1) hydrating with deionized (DI) water for 2 h or more, (2) washing with 1 l 5% NaCl, DI water, methanol, DI water and 10% ethanol, respectively, over a filter funnel and (3) air-drying for 1–2 days, and then storing until use. Prior to batch equilibrium measurements or packing of the chromatography columns, the sorbent was hydrated in aqueous ethanol which had the same composition as the mobile phase. In some cases the sorbent was used as supplied, with no washing, and was directly slurried in the aqueous alcohol used for packing. The chromatographic properties of the washed sorbent were the same as the material which was not washed.

Columns and packing procedures

All columns used in this work were made out of 316 stainless steel. Column end fittings were of 316 stainless steel fitted with 5-μm sintered frits (Indianapolis Valve and Fitting, Indianapolis, IN, U.S.A.). Column dimensions ranged from 49.8 cm × 0.71–0.78 cm I.D. to 70 × 1.09 cm. Volume and concentration overload runs were carried out in the 70 × 1.09 cm column.

The packing procedure for the 0.71, 0.78 and 1.09 cm I.D. columns is based on a standard method [17] and was carried out in 10% ethanol for at least 4 h at room temperature and 50 p.s.i.g. For the smaller particle size sorbent, the pump was set at a flow-rate of 2–3 ml/min. For the largest particle size materials, flow-rates of up to 7 ml/min were needed to obtain a 50 p.s.i.g. pressure drop in the 0.71-, 0.78- and 1.09-cm columns. After packing, extraparticle (ε_b), and overall (α) void fractions were determined from retention times of blue dextran and salt. For this stationary phase, values were 0.34–0.42 for ε_b and 0.65–0.80 for α. The packing density (0.19–0.22 g dry

TABLE I

SUMMARY OF STATIONARY PHASE PROPERTIES

Surface area (dry by BET) (m^2/g)	450
Pore size (Å)	
Range	20–600
Average	200–300
Porosity (%, v/v)	58–63

stationary phase per ml of column volume), ρ_{pack}, was determined by packing a number of columns with CG-71, then unloading the column, drying the stationary phase and weighing the amount which was packed.

Choice of mobile phase

Water, 50% aqueous methanol, and 10, 25 and 50% (v/v) aqueous ethanol were considered as eluents. These were tested using analytical-scale columns by injecting 100 μl of a mixture of L-aspartic acid (*ca.* 1 g/l), L-phenylalanine (6 g/l) and Aspartame (4 g/l), all dissolved in the eluent, into a 49.8 × 0.71 cm I.D. column packed with 38.7 ± 6.9 μm AmberChrom CG-71. When water was used as eluent, Aspartame did not elute even after the column had been flushed with 4.5 void volumes of water. Eluents of 50% methanol and 10% ethanol gave the chromatograms shown in Fig. 1a and b. Subsequent runs showed that the peak between L-Phe and L-Asp was from an unidentified component in the L-Phe. An increase in ethanol concentration caused a decrease in capacity factors (Fig. 2), and therefore all three components, and particularly Aspartame, eluted more quickly, as would be expected in reversed-phase chromatography. While both 10 and 25% ethanol gave excellent resolution between the three components, 10% ethanol was chosen since it gave higher amino acid solubility than 25% ethanol.

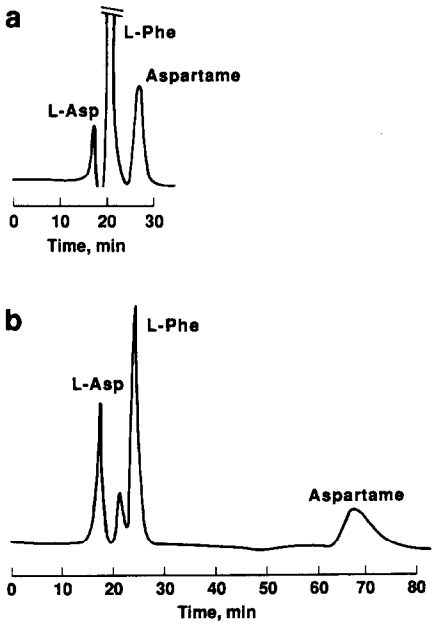

Fig. 1. Chromatograms of a mixture of L-Asp (*ca.* 1 g/l), L-Phe (6 g/l) and Aspartame (4 g/l) dissolved in mobile phase for (a) 50% methanol and (b) 10% ethanol as eluent. Injection volume of 100 μl. Column (49.8 × 0.71 cm) packed with 38.7 ± 6.9 μm material. Eluent of 50% (v/v) methanol or 10% ethanol, respectively, in DI water. Column at ambient temperature. Flow-rate at (a) 0.97 ml/min and (b) 0.95 ml/min. Detection by differential refractometer (RI) at 16× attenuation.

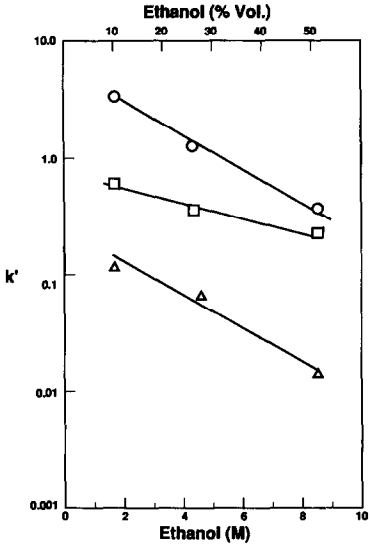

Fig. 2. Plot of capacity factors for Aspartame, L-Phe and L-Asp determined at room temperature with respect to eluents of increasing ethanol concentration. Calculations based on overall mobile phase volume. ○ = Aspartame; □ = L-Phe; △ = L-Asp. Particle size = 38.7 ± 6.9 μm.

Batch equilibrium studies

Batch equilibrium studies were carried out at 30, 50 and 70°C for phenylalanine and Aspartame with sorbent in the 165 ± 15 μm particle size range. Air-dried sorbent (4 g) was added to 20 ml of 10% ethanol in a 125-ml, capped polypropylene bottle. The bottle was placed into an incubator/shaker (New Brunswick Model G24, New Brunswick, NJ, U.S.A.), where it was heated to the required temperature (*i.e.*, 30, 50 or 70°C) while shaking for at least 30 min. A separate 40-ml volume of 10% ethanol containing a known amount of amino acid was preheated to the same temperature. The run was initiated by adding the preheated amino acid solution to the preheated sorbent slurry. The mixture was then kept at temperature and swirled by the action of the shaker for 0.5–24 h. Upon completion of a run, the shaking was stopped and the sorbent allowed to settle at the temperature of the equilibrium study. A sample of the supernatant was then taken, also at the same temperature, for subsequent analysis by a spectrophotometric assay (at 257 nm) in the case of phenylalanine and a liquid chromatography assay for Aspartame. The solubility of amino acids increases with increasing temperature (Table II) [18,19]. Since analyses of the supernatant were done at room temperature, the samples taken at 50 and 70°C needed to be appropriately diluted immediately upon sampling in order to avoid formation of a precipitate or gel upon cooling from 70°C to room temperature. An initial set of runs was carried out where the bottles were sampled at 30–60 min intervals for up to 24 h. The results showed equilibrium is attained in less than 30 min. It should be noted that batch equilibrium measurements were necessitated by the difficulty in handling small volumes of samples having concentrations above 7 g/l in an analytical chromatograph. At the higher concentrations, the amino acids tend to precipitate or form a gel in the syringe, the syringe needle, injector port and other parts of the LC system, if the

TABLE II

SOLUBILITIES OF SELECTED COMPONENTS IN WATER AND 10% ETHANOL

Component	Solubility (g/l)		
	In water (20–30°C)	In 10% ethanol	
		30°C	70°C
Aspartame	10.0[g]	7.0[a]	67.5[d]
L-Phenylalanine	30.0[h]	8.0[b]	35.0[e]
L-Aspartic acid	6.7[i]	1.0[c]	12.5[f]

[a] Solution pH 4.7.
[b] pH 4.6.
[c] pH 3.2.
[d] pH 3.9.
[e] pH 4.7.
[f] pH 2.8.
[g] From ref. 20 (at 20°C).
[h] From ref. 18 (at 25°C).
[i] From ref. 19 (at 30°C).

temperature dropped even slightly below the column temperature for runs carried out at 50 and 70°C.

According to Homler [20], at some conditions Aspartame degrades to aspartyl-phenylalanine, diketopiperazine, aspartic acid and phenylalanine. Degradation is accelerated at pH values above and below the 4–5 range, and at temperatures above ambient. At 80°C, detectable changes occur within several hours [20]. Since the equilibrium loadings were determined by measuring the difference between initial and equilibrium Aspartame concentrations, it was necessary to correct for a small Aspartame loss due to degradation for the 70°C data.

Reagents and chemicals

Research-grade L-aspartic acid, L-phenylalanine and Aspartame were obtained from Serva (Heidelberg, Germany). The L-amino acids were obtained from Sigma Chemical (St. Louis, MO, U.S.A.) in kit No. LAA-21 (Lot No. 78F-9009-8) and were listed as 99 + % pure by TLC assay. Blue dextran was from Pharmacia (Piscataway, NJ, U.S.A.). Other reagents were from Mallinckrodt (carboxylic acids) and Aldrich (methyl-*tert.*-butyl ether, MTBE; and ethyl-*tert.*-butyl ether, ETBE).

Volume- and concentration-overload runs

A column of 70 × 1.09 cm, packed with 60.3-μm stationary phase with 10% ethanol as eluent, was used in these runs. Volume-overload chromatograms were obtained at 20°C and a flow-rate of 2 ml/min by collecting fractions, diluting the fractions, measuring the absorbance at 240 nm and comparing against the appropriate standard curves. The concentration-overload run required the injection sample, column and detector cell to be heated by a circulating water bath to maintain an 82°C temperature, thus preventing the Aspartame and phenylalanine (each at 50 mg/ml) from precipitating out of solution. The sample which was extremely viscous was

loaded at 1.07 ml/min. Elution was at 2 ml/min. Fractions of the effluent were again collected, appropriately diluted and analyzed. Unlike the volume-overload run, the peaks in the concentration-overload case overlapped and all samples were thus analyzed by LC.

RESULTS AND DISCUSSION

A correlation for distribution of a dissolved solid (*i.e.*, the amino acid) between liquid and solid (stationary) phases was used to fit the batch equilibrium data which was unexpectedly linear at loadings of up to 140 mg/g stationary phase (Fig. 3). The correlation is based on the work of Davies and Rideal [21] as presented by Kipling [22], for cases where adsorption is independent of the fraction of surface covered. The solute, n, taken up by the stationary phase from a mobile phase with a solute concentration of C, is:

$$n = [n_0 K]C = \left[n_0 \kappa \exp\left(\frac{\Delta W}{kT}\right)\right] C \tag{1}$$

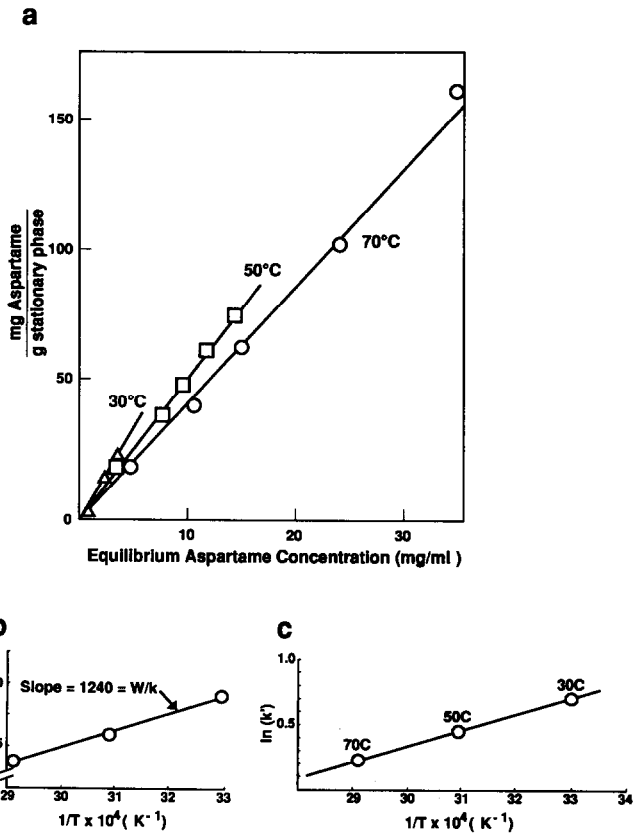

Fig. 3. (a) Equilibrium data for Aspartame in 10% ethanol with respect to CG-71 at 30, 50 and 70°C. Data fitted to eqn. 1 to obtain K as a function of temperature. Replots based on (b) eqn. 2 and (c) eqn. 3.

where n_0 represents maximum surface coverage of the solute on the stationary phase, K represents an equilibrium constant, κ a ratio of solute adsorption and desorption rates, ΔW the difference between energies of adsorption and desorption, k a constant, and T the absolute temperature.

The adsorption equilibria for Aspartame, determined by batch measurements in capped polypropylene bottles, is linear at all the temperatures used in this study and at concentrations in excess of 30 mg/ml at 70°C. The data fit eqn. 1, as is shown by Fig. 3a. The plot of the natural log of the slopes ($= n_0K$) of the lines in Fig. 3a *vs.* inverse absolute temperature (K) gives the value of $\Delta W/k$ ($= 1240$ K) from the slope in Fig. 3b, as well as the value of $n_0\kappa$ ($= 0.112$ ml/g). The temperature-dependent equation for the weight-based Aspartame distribution coefficient, K_{Aspt}, for CG-71 in 10% ethanol is then

$$K_{Aspt} = n_0K = 0.112\exp[1240/(273+T)] \quad (2)$$

where T is in °C, and the subscript Aspt denotes Aspartame. The capacity factor is

$$k' = \varphi\, K_{Aspt} = \varphi\, \{0.112\exp[1240/(273+T)]\} \quad (3)$$

where φ is the phase ratio ($= \rho_{pack}/\alpha$) and where α denotes the total void fraction and ρ_{pack} the packing density (kg sorbent/l column volume). The logarithmic form of eqn. 3 gives the temperature dependence of the equivalent capacity factors for a liquid chromatography column. This is shown in Fig. 3c using an average value of φ ($=0.31$ g/ml) for this stationary phase.

The equilibrium data for L-Phe gives a temperature-independent, weight-based distribution coefficient of K_{Phe} ($=n_0K$) of 0.931 ml/g (Fig. 4).

Retention of other amino acids

The retention behavior of a series of amino acids, carboxylic acids, alcohols, ethers and dipeptides at 30°C using either water or 10% ethanol as eluent at a flow-rate

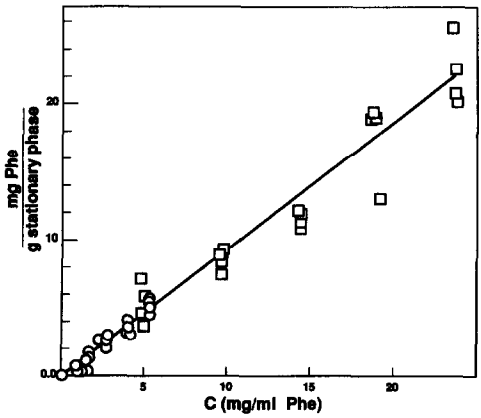

Fig. 4. Equilibrium data for L-Phe in 10% ethanol with respect to CG-71 at 30 and 50°C. Data fitted to eqn. 1. ○ = 30°C; □ = 50°C.

of 0.95–1 ml/min (Table III) shows that most amino acids elute quickly (*i.e.*, $k' < 0.1$) in water except for those with aromatic character (L-phenylalanine, L-tryptophan, and Aspartame). When 10% ethanol is the eluent instead of water, the more weakly retained species (*i.e.*, glycine, cellobiose, L-asparagine, L-serine, L-hydroxyproline, glucose, fructose and citric acid) increase slightly in retention time while the more strongly held species (*i.e.*, phenylalanine, succinic acid, butanol, L-tryptophan, MTBE, ETBE, Aspartame and benzoic acid) decrease significantly. Some components, which

TABLE III

CAPACITY FACTORS FOR SELECTED SOLUTES WITH RESPECT TO CG-71 AT 30°C

Capacity factor: $k' = (t_r - t_m)/t_m$ where t_m = retention time of NaCl; $\alpha = 0.77$. Column dimensions, 49.8 × 0.71 cm. CG-71 particle size, 60.3 ± 4.9 μm.

Compound	Capacity factor (measured)	
	In water	In 10% ethanol
NaCl	0	0
Fumarate, disodium	0	–
L-Aspartic acid	**0**	**0.13**
Glutathione	0	0.108
L-Lysine	0.018	–
L-Glutamic acid	0.021	1.000
L-Cystine	0.023	–
Diglycine HCl	0.026	–
L-Arginine	0.028	–
L-Alanine	0.031	–
Glycine	0.038	0.037
Cellobiose	0.041	0.066
L-Asparaginine	0.041	0.161
L-Serine	0.044	0.056
L-Hydroxy proline	0.046	0.111
L-Proline	0.048	0.000
Glucose	0.051	0.069
Fructose	0.067	0.140
Citric acid	0.100	0.244
L-Valine	0.107	–
L-Isoleucine	0.197	–
L-Methionine	0.225	0.323
L-Leucine	0.230	0.220
Methanol	0.238	0.196
Glycine anhydride	0.263	0.257
Ethanol	0.494	0.302
L-Tyrosine	0.618	–
L-Phenylalanine	**0.829**	**0.614**
Succinic acid	1.40	0.896
1-Butanol	1.89	0.331
L-Tryptophan	4.40	1.770
MTBE	Did not elute[a]	0.323
ETBE	Did not elute[a]	0.365
Aspartame	**Did not elute[a]**	**3.600**
Benzoic acid	Did not elute[a]	6.930

[a] As measured after more than 4.5 void volumes of eluent.

TABLE IV

COMPARISON OF RETENTION TIMES CALCULATED FROM BATCH EQUILIBRIA DATA TO THOSE OBSERVED FROM COLUMN RUNS

Temperature of column jacket (°C)	Particle diameter $d_p \pm 1\sigma$ (μm)	Sample volume (ml)	Flow-rate (ml/min)	Void fractions		ρ_{pack}	Aspartame			Phenylalanine		
				ε_b	α	$1-\alpha$ (g/ml)	K_D	Retention time (min)		K_D	Retention time (min)	
								Calculated	Observed		Calculated	Observed
30	117 ± 15.0[a]	0.2	1.32	0.43	0.65	0.626	4.19	104	130	0.58	42	55
30	60 ± 4.9[b]	1.0	0.19	0.42	0.80	1.09	7.29	234	279	1.0	104	119
30	60 ± 4.9[b]	1.0	0.58	0.42	0.80	1.09	7.29	77	92	1.0	34	39
30	60 ± 4.9[b]	1.0	4.50	0.42	0.80	1.09	7.29	10	12	1.0	4	5
50	60 ± 4.9[b]	0.1	1.30	0.39	0.76	0.917	4.76	29	29	0.85	15	16
70	117 ± 15.0[a]	0.2	1.32	0.38	0.71	0.755	3.13	80	85	0.70	45	49
70	60 ± 4.9[b]	0.1	1.30	0.38	0.74	0.846	3.51	25	29	0.79	14	15

[a] 70 × 1.09 cm column with measured empty volume of 64.9 ml.
[b] 50 × 0.71 cm column with measured empty volume of 19.7 ml.

gave sharp peaks in water, tended to tail in 10% ethanol. An extreme example is glutamic acid, which eluted as a sharp peak close to the void volume when water was the eluent, yet came out as a tailing peak in 10% ethanol. The data in Table III suggest that a shallow gradient of water to aqueous ethanol will achieve clean separation of many non-aromatic from aromatic species.

Retention behavior

The retention time, t_r, is given by the well-known equation:

$$t_r = \frac{V_r}{q} = \frac{V_0 + K_D V_s}{q} \tag{4}$$

where V_r is the retention volume, q the volumetric flow-rate, V_0 the overall void volume, V_s the volume displaced by the solid content of the stationary phase, and K_D the distribution coefficient, given by k' (V_0/V_s), or $[\rho_{pack}/(1-\alpha)]K_{Aspt}$ or $[\rho_{pack}/(1-\alpha)]K_{Phe}$.

A small sample volume, injected into an analytical column, encounters a significant degree of dilution. Consequently, linear equilibria, associated with low mobile phase concentrations of the solute, would be expected [23] and measured and calculated retention volumes are similar (Table IV). A chromatography run carried out at volume overload with a sample volume of 40 ml (equivalent to 80% of total column void volume), gave well-separated, symmetrical peaks (Fig. 5) and is consistent with theory [23]. The elution times of the leading edges at half the maximal concentration were 38 and 86 min for Phe and Aspartame, respectively. This is similar to the values of 32 min calculated from eqn. 4 with $k' = (0.931)\varphi$ for Phe; and 78 min from eqns. 2, 3 and 4 for Aspartame at 20°C.

Equilibrium constants determined for a 165-μm particle size apply to 60- and 117-μm particles since the surface area for adsorption is primarily internal to the stationary phase, and the phase ratio is proportional to the packing density for different particle sizes in this range. Retention time is also independent of particle size

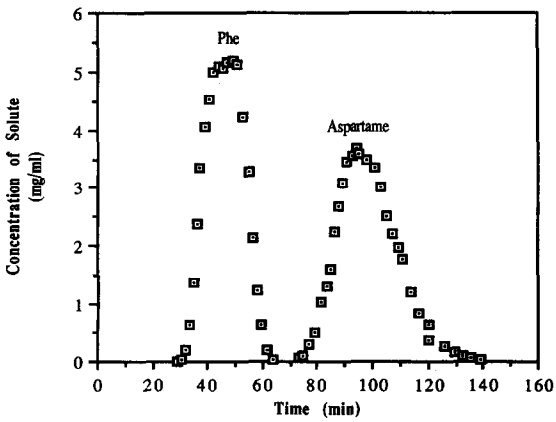

Fig. 5. Chromatogram for volume-overload run carried out at room temperature with a sample volume of 40 ml injected onto a 70 × 1.09 cm jacketed column. Column temperature at 20°C. Sample concentration of 5 mg/ml of each component in 10% ethanol. Flow-rate, 2 ml/min. Elution with 10% ethanol.

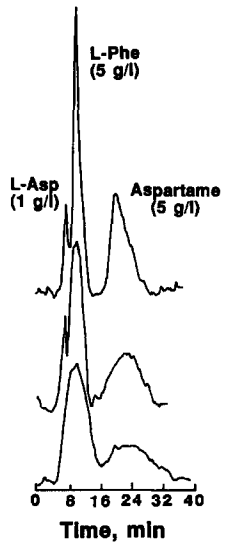

Fig. 6. Retention of L-Asp (1 g/l), L-Phe (5 g/l) and Aspartame (5 g/l) at different particle sizes. Column temperature, 50°C. Sample size, 100 μl. Flow-rate, 2.4 ml/min. Eluent, 10% ethanol. UV detection at 240 nm. $d_p \pm \sigma$ (wet): top curve, 60.3 $\pm$ 4.9 μm; middle curve, 117 $\pm$ 15 μm; bottom curve, 164 $\pm$ 24 μm.

(Fig. 6) since the packing density, and therefore the total surface area per unit column volume, changes little with respect to particle size. In comparison, peak broadening increases with increasing particle size, thus causing loss of resolution particularly for the L-Asp and L-Phe peaks. These data, taken together, are consistent with the previously developed theoretical framework of Knox and Pyper for maximizing throughput in preparative LC [23].

Peak overlap and skew is significant when volume and concentration overload

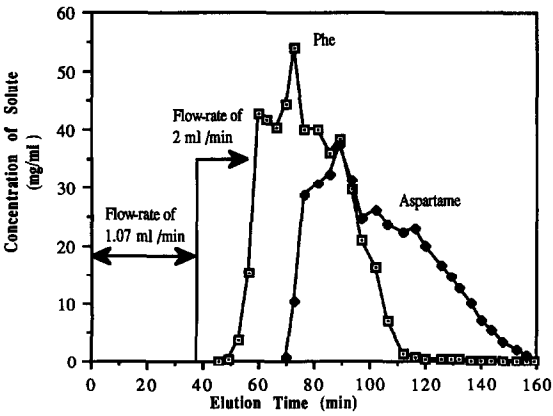

Fig. 7. Chromatogram for concentration and volume overload at 82°C. Sample concentration of 50 mg/ml of each component. Sample loaded at a flow-rate of 1.07 ml/min. Pumping of 10% ethanol initiated at 38 min at a flow-rate of 2 ml/min. Other conditions as in Fig. 5, except Aspartame degradation likely (20).

occur simultaneously as shown in Fig. 7. When 80% of the column is filled with 40 ml of sample containing 50 mg/ml of each component, a separation is still obtained. Peak skew is again consistent with theory [23].

Plate heights at moderate loading

Following the discussions of Grushka *et al.* [24], Pieri *et al.* [25] suggested that definitions for reduced velocity, $v\,(= ud_p/D_m)$, and reduced plate height, $h\,(= H/d_p)$, be combined with the reduced form of the Snyder equation

$$h = m\,v^n \tag{5}$$

to obtain the expression

$$N = \frac{D_m^n}{m}\,\frac{L}{u^n d_p^{1+n}} \tag{6}$$

where H is the plate height, d_p the particle diameter, L the column length, N the plate count, m a scaling constant, u the interstitial (linear) velocity (cm/min) and D_m the diffusion constant (cm^2/min). The value of the column characteristic constant, n may vary from 0.2 to 1.0, with pellicular packings having lower values of n and porous packings having values of n of 0.6 [24] to 1.0 [23]. Eqn. 5 is a special case of the Knox equation

$$h = \frac{2\gamma}{v} + m\,v^n \tag{7}$$

where $v > 30$ and the second term dominates. The parameter γ represents a structural constant. The first term in eqn. 7 gives the contribution to bandspreading from axial dispersion. The second term is empirical, and gives the combined contributions of dispersion arising from the disequilibrium induced by flow of eluent over the packing, and the complex flow which occurs in the column [24]. Grushka *et al.* [24], noted that m and n cannot be assumed constant over a wide range of v, so that eqn. 7 may not be reliable when d_p is varied. The framework of Knox and Pyper recommends $n = 1$, and $m \cong 0.1$ at large values of v, and these values of n and m are later shown to be applicable to the data presented here.

The temperature dependence of v is due to the temperature dependence of the diffusion coefficient, D_{Am}, as well as that of the viscosity, η_m. The diffusion constant, D_{Am}, of dilute solute in multicomponent mixtures, is given by the modified Wilke–Chang equation [25,26,27]:

$$D_{Am} = 7.4 \cdot 10^{-8}\,\frac{\left[\sum_{j=1}^{n} x_j \phi_j M_j\right]^{0.5} T}{\eta_m V_A^{0.6}} \cdot 60 \tag{8}$$

where T is the absolute temperature, V_A is the molar volume of the solute, and η_m is the viscosity of the solvent mixture (in this case, aqueous ethanol) and where x_j, ϕ_j and M_j

are the mole fraction, association factor and molecular weight, respectively, of solvent component j where $j \neq A$. For the 10% (v/v) ethanol, the parameters for water ($j = 1$) and ethanol ($j = 2$) are, respectively, $x_1 = 0.9584$, $\varnothing_1 = 2.6$ and $m_1 = 18$; and $x_2 = 0.0416$, $\varnothing_2 = 1.5$ and $M_2 = 46$. The molar volumes of Aspartame and phenylalanine were estimated to be 320 and 200 cm^3/gmol, respectively, by the method of LeBas (described in ref. 27). The viscosity of 10% (v/v) ethanol decreases with increasing temperature [28] while the diffusion constant for Aspartame in 10% ethanol, calculated from eqn. 8, increases from 2.5 to $6.0 \cdot 10^{-4}$ cm^2/min over the range 30–70°C. For phenylalanine, the estimated diffusion constant ranges from 3.4 to $8.0 \cdot 10^{-4}$ cm^2/min over the same temperature range.

Plate counts for Aspartame and phenylalanine from columns packed with 60-, 117- and 165-μm particle sizes are shown in Fig. 8a and b. The columns were run at 30 and 70°C with 10% ethanol as the eluent at interstitial velocities, u, of 5.4–55 cm/min. Values of v were in the range 50–700.

Unlike the strongly adsorbed Aspartame, a discernible temperature effect is lacking for weakly retained phenylalanine (compare Fig. 8a and b). Tables V and VI summarize results for the regression analysis of the data for Aspartame and phenylalanine fitted to eqn. 6 for $n = 0.5$ and $n = 1.0$. While both the mass-transfer ($n = 0.5$) and pore-diffusion ($n = 1.0$) forms give reasonable fits, the case for $n = 1$ is better. On this basis, pore diffusion is indicated to be the more significant factor although both mass transfer and pore diffusion are likely to contribute to the observed plate counts.

The values of m are calculated from the slopes ($= D_{Am}/m$) in Fig. 8, with the values of D_{Am} estimated by eqn. 8. This gives $m = 0.11$–0.12 at 30°C and 0.22–0.29 at 70°C (Tables V and VI), which is close to the expected $m \approx 0.1$ [23,24]. The values of m calculated for our data are probably high, since values of D_{Am} represent free solution

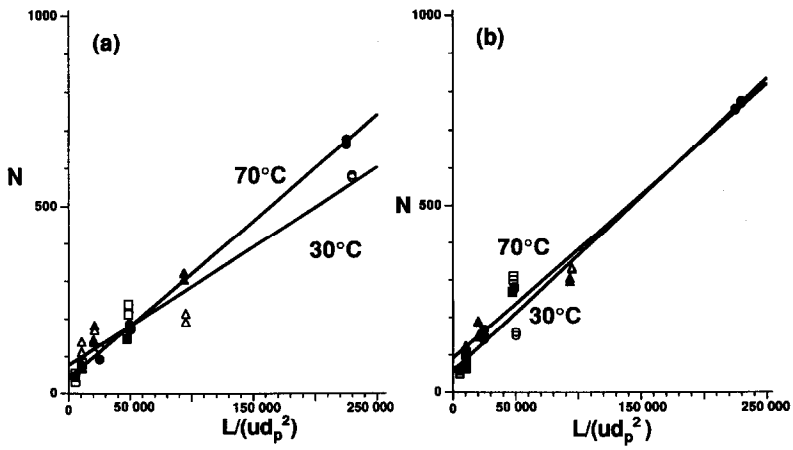

Fig. 8. Relation of plate count to particle size, eluent superficial velocity, and column length by eqn. 6 for $n = 1$ for (a) Aspartame and (b) phenylalanine. Eluent, 10% ethanol. Values of L, u and d_p have dimensions of cm, cm/min and cm, respectively. Open symbols for temperature 30°C; dark symbols for temperature 70°C. ○, ● = 60.3 μm; △, ▲ = 117.0 μm; □, ■ = 163.5 μm.

TABLE V

COMPARISON OF MASS TRANSFER ($n=0.5$) AND PORE DIFFUSION ($n=1.0$) CASES FOR ASPARTAME

Expression	$T=30°C$		$T=70°C$	
	$n=0.5$	$n=1.0$	$n=0.5$	$n=1.0$
Number of measurements	18[a]	18[a]	18[a]	18[a]
r^2	0.88	0.93	0.94	0.99
Standard error of estimate	54.0	42.7	46.4	21.0
y-Intercept	−7.6	76.8	−67.8	42.1
D^n_{Am}/m value	$12.2 \cdot 10^{-3}$	$2.1 \cdot 10^{-3}$	$16.0 \cdot 10^{-3}$	$2.79 \cdot 10^{-3}$
Standard error	$1.1 \cdot 10^{-3}$	$0.15 \cdot 10^{-3}$	$1.0 \cdot 10^{-3}$	$0.008 \cdot 10^{-3}$
Calculated D^n_{Am}[b]	$15.9 \cdot 10^{-3}$	$2.5 \cdot 10^{-4}$	$24.5 \cdot 10^{-3}$	$6.0 \cdot 10^{-4}$
Calculated m	1.3	0.12	1.5	0.22

[a] Based on 3 velocities (*ca.* 5.5, 25 and 50 cm/min, each run in duplicate).
[b] From eqn. 8 for diffusion in free solution. D_{Am} given in cm²/min.

diffusion, rather than an effective hindered diffusion inside the pores. The effective diffusion, D_{eff} is given by:

$$D_{eff} = \frac{\Theta \; D_{Am}}{\tau} \tag{9}$$

where τ is a tortuosity factor for the pores and Θ is the fraction of free cross secton for diffusion [29]. Tortuosity varies inversely with porosity, and generally has values ranging from 2 to 6 [30]. Since this stationary phase has a high porosity in the range 58–63%, a value of $\Theta = 0.6$ and $\tau = 2$ was used. Eqn. 9 thus gives $D_{eff} = 0.3\, D_{Am}$. On

TABLE VI

COMPARISON OF MASS TRANSFER ($n=0.5$) AND PORE DIFFUSION ($n=1.0$) CASES FOR PHENYLALANINE

	$T=30°C$		$T=70°C$	
	$n=0.5$	$n=1.0$	$n=0.5$	$n=1.0$
Number of measurements	18[a]	18[a]	18[a]	18[a]
r^2	0.90	0.96	0.95	0.97
Standard error of estimate	71.6	44.4	45.7	38.7
y-Intercept	−66.4	55.9	−27.7	91.0
D^n_{Am}/m	$18.0 \cdot 10^{-3}$	$3.1 \cdot 10^{-3}$	$17.0 \cdot 10^{-3}$	$2.9 \cdot 10^{-3}$
Standard error	$1.5 \cdot 10^{-3}$	$0.16 \cdot 10^{-3}$	$0.97 \cdot 10^{-3}$	$0.14 \cdot 10^{-3}$
Calculated D^n_{Am}[b]	$18.4 \cdot 10^{-3}$	$0.34 \cdot 10^{-3}$	$28.3 \cdot 10^{-3}$	$0.80 \cdot 10^{-3}$
Calculated m	1.0	0.11	1.7	0.28

[a] Based on 3 velocities (*ca.* 5.5, 25 and 50 cm/min, each run in duplicate).
[b] Calculated from eqn. 8 for diffusion in free solution. D_{Am} given in cm²/min.

this basis, the free solution diffusivities and values of m in Tables V and VI would be multiplied by 0.3 to give $m \approx 0.04$ and $m \approx 0.08$ at 30 and 70°C, respectively. Consequently, comparison between the values of m presented here with those given by general theory should be appropriately interpreted given the dependence of m on the definition of the diffusion constant.

The data show that $n = 1$ in eqn. 6 gives the best fit of N as a function of velocity, u, and particle size, d_p, for the particle sizes used here. Therefore, for a given temperature, changes in column length, due to changes in particle size, can be anticipated by the relation

$$L_1 = \left(\frac{N_1}{N_2}\right)\left(\frac{u_1}{u_2}\right)\left(\frac{d_{p,1}}{d_{p,2}}\right)^2 L_2 \tag{10}$$

where the subscripts 1 and 2 represent columns 1 and 2 which are packed with particle sizes 1 and 2, respectively. Eqn. 10 is useful in providing an order of magnitude estimate of column length, since particle size, plate count and eluent velocity usually change in scaling up a given separation [23,31,32]. Column length is a key specification in estimating run time, solvent usage and volume of stationary phase needed for a preparative separation.

CONCLUSIONS

Elution times and plate counts of phenylalanine and Aspartame for different LC columns are predicted using the distribution coefficient from batch equilibrium data and the framework of Knox and Pyper [23]. Other columns packed with stationary phase of average wet particle diameters of 38.7, 60.3, 117 or 165 μm demonstrated that particle size has little effect on retention time, while the plate count, N, varies inversely with the particle diameter, d_p^2. Retention behavior data show that this new stationary phase could be used in other amino acid separations. A detailed study on the separation of L-Phe and Aspartame also shows that this new macroporous polymeric sorbent has attractive loading characteristics, and facilitates transition from analytical-scale separations, usng a small particle size packing material, to preparative-scale separation, using a larger particle size of the same stationary phase. Solute retention is readily predicted over a range of temperatures, sample volumes and flow-rates.

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