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PREPARATION, OPTIMISATION AND SLURRY PACKING OF AN AMINO BONDED PHASE FOR THE ANALYSIS OF SUGARS IN FOOD BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Optimisation of the separation of fructose, glucose, sucrose, maltose and lactose on an amino bonded phase is described. The factors affecting support loading have been investigated with a view to simplifying and shortening the phase preparation procedure. Columns were packed using different slurry media to determine the effect on peak shape and column efficiency. It is shown that careful control of support loading improves sugar separations thus permitting a faster analysis time.

INTRODUCTION

The analysis of saccharides in food by high-performance liquid chromatography (HPLC) has become an attractive proposition owing to recent advances in bonded phase columns. Separation times are shorter than for gas–liquid chromatography[1]. which also suffers from the problems of derivatisation and extensive sample clean-up. Although several papers[2–5] have demonstrated the HPLC separation of a wide range of saccharides on bonded phases, only one author[6] has defined the nature of the phase and given a method of preparation. There is no information concerning investigation of phase synthesis and the effect of support loading on chromatography. As will be demonstrated, a full understanding of these factors is of fundamental importance if the full potential of the amino alkyl phase is to be realised for the analysis of saccharides in food.

EXPERIMENTAL

Apparatus

The detector was a differential refractometer model R401 from Waters Assoc. (Hertford, Great Britain) and was used in conjunction with a Model 750/03 reciprocating pump from Applied Chromatography Systems (Luton, Great Britain). Sample injection was achieved on a Specac 2,500-p.s.i. valve (Sidcup, Great Britain) fitted with a 30-μl loop. All columns were made from 316 grade seamless stainless-steel tube 6.35 mm O.D. $\times$ 4.6 mm I.D. obtained from Tubesales (Southampton, Great Britain).

An Orlita pump model DMP 1515 (A. J. G. Waters, Slough, Great Britain) was used for column packing. Stainless-steel unions were obtained from H. S. Chromatography Packings (Bourne End, Great Britain) and stainless-steel mesh from Sankey Green Wire Weaving (Warrington, Great Britain).

Chemicals and reagents

Acetonitrile and hexane were both HPLC grade from Rathburn (Walkerborn, Great Britain). All other solvents were analytical reagent grade. Solvents that were used for phase synthesis were stored over molecular sieve 4A in order to remove residual water. The chromatographic support was Partisil 5 (the same batch was used throughout) from Whatman (Maidstone, Great Britain) and was modified with γ-aminopropyltriethoxysilane from Aldrich (Wembley, Great Britain).

Evaluation of bonding procedure

General technique and determination of support loading. Silica used for bonding was stored over a saturated solution of lithium chloride (relative humidity 12%, 20–35°) for 24 h prior to its reaction with silane. The silica was then either shaken at room temperature or refluxed in a dry non-polar solvent with γ-aminopropyltrieth-oxysilane. After filtering through a No. 4 sintered glass funnel and washing with solvent, the bonded phase was dried in an oven at 120°.

Support loading was determined by combusting approximately 1 g of phase over a bunsen burner at 600°. The percentage support loading was then expressed as:

$$\% \text{ Loading} = \frac{\text{Weight loss after combustion} \times 100}{\text{Weight of phase before combustion}}$$

Factors affecting support loading. The effect of silica:silane ratio, reaction temperature and reaction time on support loading were investigated with a view to obtaining a simple rapid procedure for phase preparation.

Electron microscopy. All phases were examined under the electron microscope for evidence of silane polymerization. Since this has an important effect on chromatography, it was felt that the technique might provide valuable information on the interpretation of the chromatographic performance.

Column packing

General technique. The packing equipment used was very similar to that described by other authors[7,8] and consisted of a slurry reservoir and analytical column connected by a $^1/_4$-in. stainless-steel union. The analytical column was terminated in a zero dead volume $^1/_4$–$^1/_{16}$ in. reducing union fitted with a 8-μm mesh disc. Three grams of phase were dispersed in a slurry medium and poured into the packing assembly. The slurry was compressed to a compact bed by pumping solvent through the assembly at 5000 p.s.i. The column was then rapidly equilibrated to the conditions required for sugar analysis with 50 ml of acetonitrile–water (4:1, v/v). After disconnecting from the reservoir the top of the column was fitted with a 8-μm disc and a $^1/_4$–$^1/_{16}$ in. zero dead volume reducing union.

Slurry packing investigation. All columns used in the chromatographic evaluation were slurried in acetonitrile–water (4:1) and the slurry compressed by pumping

through with hexane. Subsequently however, a slurry packing investigation was undertaken to determine the effect of different slurry media on column efficiency, pressure and peak shape. The effect of using hexane and acetonitrile–water (4:1) to compress the slurry was investigated. Each column was evaluated in an identical manner using a 30-μl standard containing fructose, glucose and sucrose. Column efficiency was determined using the sucrose peak (3 separate determinations).

Chromatographic evaluation of phases and application to sugar analysis

Phases with a wide range of support loadings were evaluated chromatographically using a sugar standard containing fructose, glucose, sucrose, maltose, and lactose. Column efficiency (N) and capacity ratio's (k') were calculated and the optimum support loading for the separation of the above five sugars determined.

RESULTS AND DISCUSSION

Factors affecting support loading

Silica:silane ratio. The effect of silica:silane ratio on support loading is shown graphically in Fig. 1. At high silane concentrations a small decrease in concentration produced a large reduction in support loading. This trend is reversed at low concentrations, where for instance ratio's of 20:1 and 10:1 gave a difference of only 0.8%.

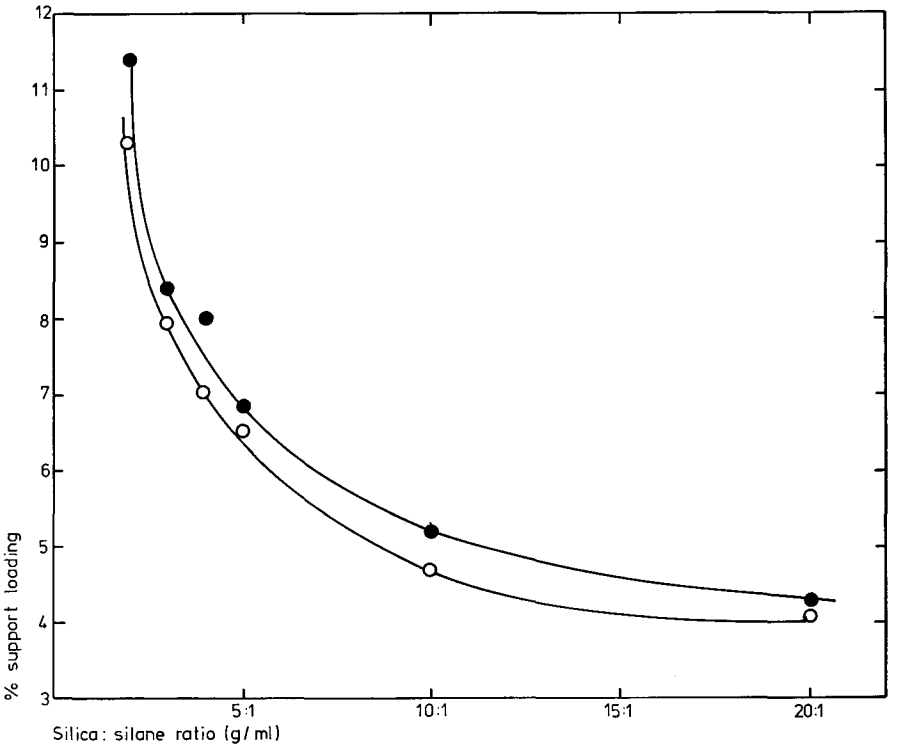


Fig. 1. Graph of percentage support loading against silica:silane ratio. ○, 5-min shake; ●, 2-h reflux.

TABLE I

EFFECT OF TEMPERATURE ON SUPPORT LOADING

Solvent	*Reaction temperature (°C)*	*Phase loading (%)*
Hexane	25	10.2
Hexane	68	11.4
Toluene	110	11.2
Xylene	142	12.4

TABLE II

EFFECT OF REACTION TIME AND TEMPERATURE ON SUPPORT LOADING

Reaction time (min)	*Support loading (%)*
90	8.0
60	8.0
30	8.0
15	8.0
5*	7.0

* At room temperature.

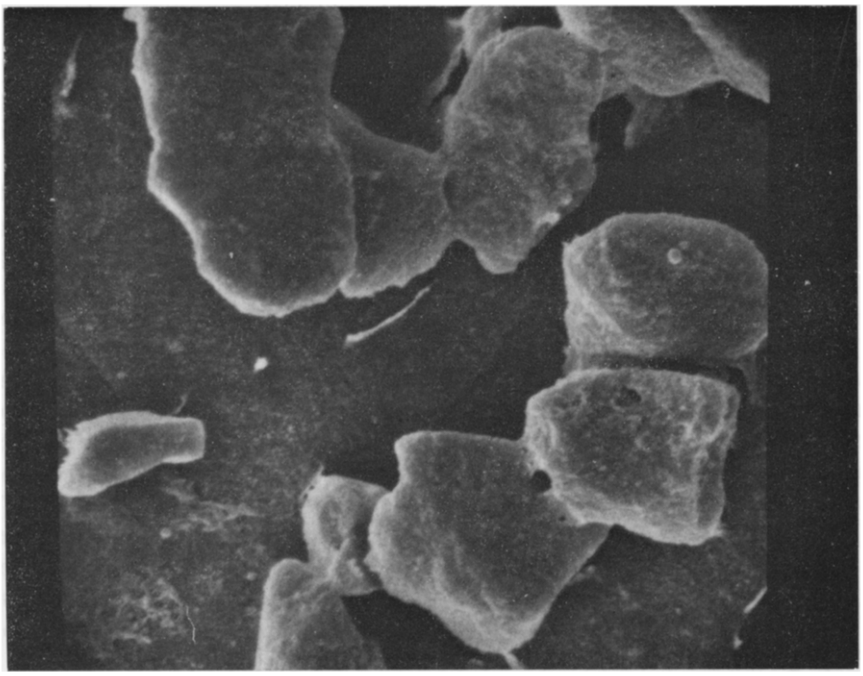


Fig. 2. "Webbing" effects on Partisil 5. Magnification, ×10,000; scale, 1 μm = 10 mm.

Reaction temperature and time. Table I shows that reaction temperature has very little effect on support loading. It required a 117° rise in temperature to effect an increase in loading from 10.2 to 12.4%. Further evidence of the ease with which the ethoxysilane reacts with the surface silanols came from the investigation of the effect of reaction time on loading (Table II). Identical loadings were recorded for silica–silane reactions taking place in refluxing hexane from 90 min down to 15 min. Indeed, shaking for 5 min at room temperature still resulted in a 7% loading. A graph of silica:silane ratio against loading using the "5-min shake" procedure gave a similar curve to that obtained for the 2-h reflux and is shown in Fig. 1. This curve was used to obtain specific support loadings for chromatographic evaluation.

Electron microscopy studies

Electron microscopy on the high amino phase loadings showed silica particles that were either covered in a "web" or connected by long "strands" (see Fig. 2 and 3). These effects were still apparent after the phase had been combusted at 600°. The strands in Fig. 3 are 500–1000 Å thick, although much of this is due to the gold film with which the slide was made up. It is highly improbable that a large number of

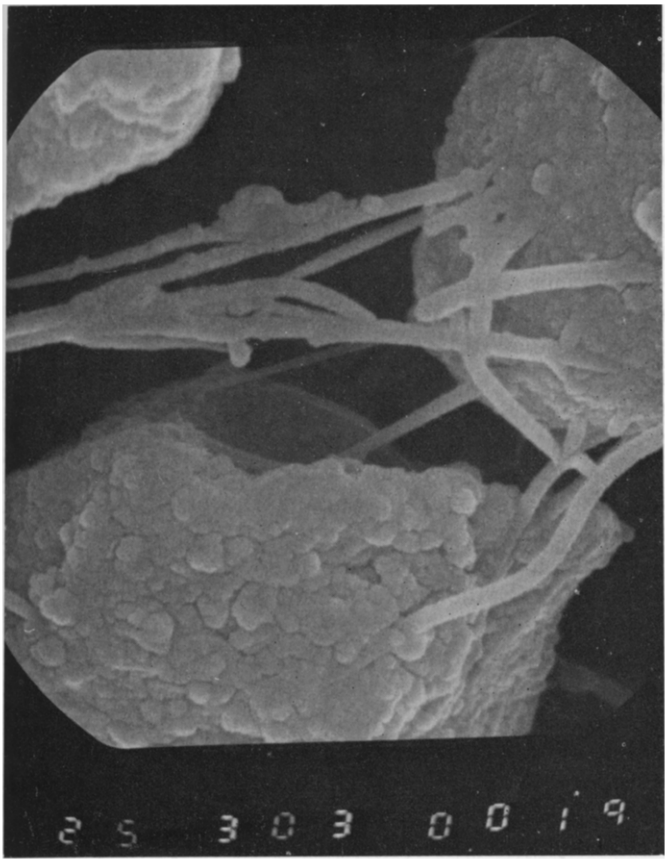


Fig. 3. High amino phase loading on 5μm silica. Magnification × 15,000.

small particles of silica are present prior to "bonding"[9]. Therefore the strand thickness cannot be represented by small silica particles bonded to a siloxane chain as in Fig. 4. This would therefore imply a considerable degree of polymerisation. The later batches of phase were devoid of any "strands" or "webbing" effects. It therefore seems likely that these were caused either by water entering the reaction mixture or impure silane.

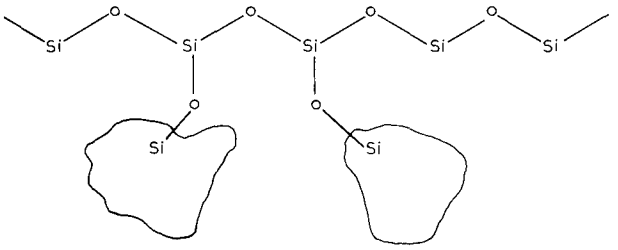


Fig. 4. Small silica particles bonded to a siloxane chain.

Phases that exhibited "strands" also proved very difficult to pack. Although the packing pressure was in excess of that used for routine analysis, the level of the phase always dropped by approximately $^1/_8$ in. below the top of the column after a short period of use. This is probably caused by the continual shearing of the strands under continuous mechanical pressure. The net result is a decrease in resolution as a consequence of the dead volume left at the top of the column. We have also observed "strands" with other bonded phases that exhibited "packing down".

Chromatographic evaluation of phases and implications for sugar analysis

The effect of support loading on sugar capacity ratio's is shown in Fig. 5. The graph may be divided into two specific regions. Above a support loading of 8.5%, k' is a constant for a particular sugar. Below this figure, k' decreases in an approximately linear manner. Since water on the silica surface can also cause silane condensation, the increase in loading without an accompanying increase in k' is interpreted as representing phase polymerisation. The fact that the more heavily loaded supports yielded columns with lower efficiencies (Table III) favours this argument, as the lower efficiencies would be anticipated on the grounds of poor mass transfer resulting from the polymeric matrix[10,11]. Initially, the predominant reaction probably occurs between the silica surface silanols and the silane monomers, with only a small degree of polymerisation. Once, however, all the available surface hydroxyls have reacted, *i.e.* a monolayer of aminopropyl groups exists, the polymerisation of the silane monomers undoubtedly becomes the predominant reaction. An 8.6% loading, therefore, probably represents an amino-phase monolayer.

Slurry packing investigation

A summary of the effect of different slurry media on column pressure, efficiency and peak shape is given in Table IV. Column number 145 was the only one where hexane was used to compress the slurry, acetonitrile–water (4:1) being used for the rest. The best column efficiency was obtained with a water slurry and pumping through with acetonitrile–water. Using a water slurry but pumping through with

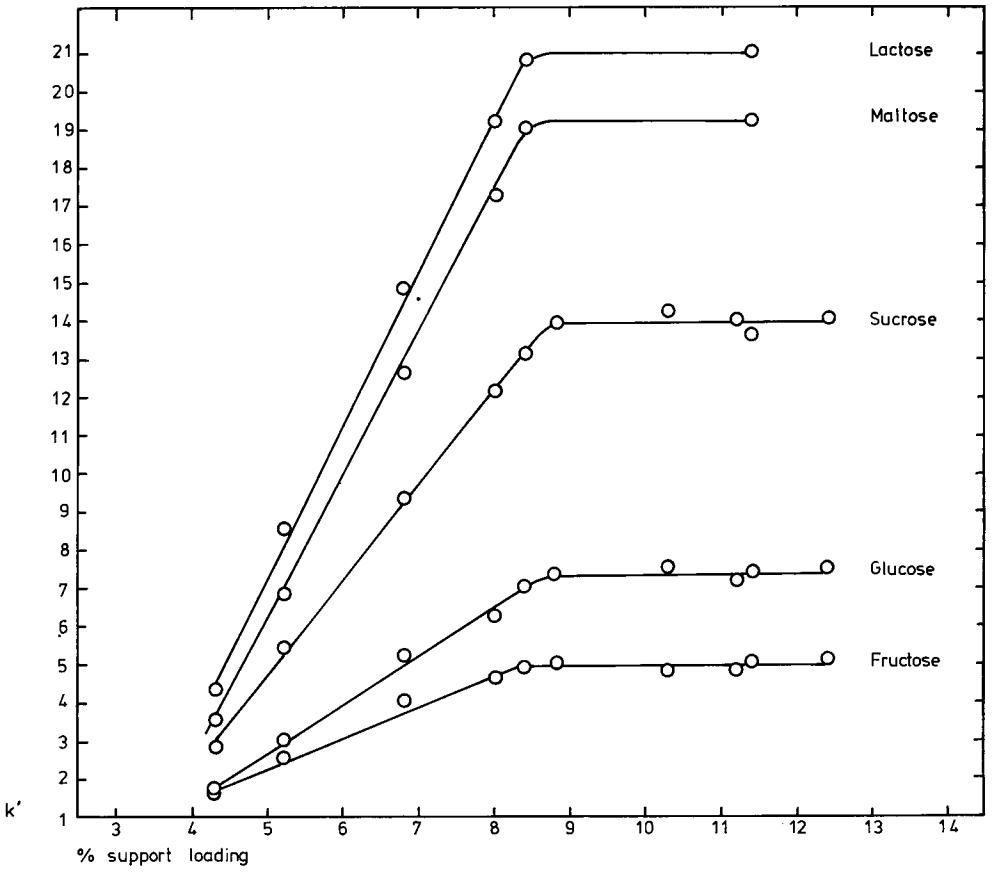


Fig. 5. Graph of k' against support loading for fructose, glucose, sucrose, maltose and lactose.

hexane gave a lower column pressure and poorer efficiency. The acetonitrile and acetonitrile–water slurries both gave columns with low back pressures and poor efficiencies. This could be interpreted as due to a less compact bed. Both columns exhibited badly tailing peaks. The tetrabromoethane slurry gave the column with the highest pressure, suggesting a compact bed, however, it also yielded the lowest efficiency. This might at first appear to present an anomaly. However, the sugar k' values

TABLE III

EFFECT OF SUPPORT LOADING ON COLUMN EFFICIENCY

Column efficiency (plates per metre)	*Support loading (%)*
4200	12.4
7500	11.2
6300	11.4
9200	10.2

TABLE IV

EFFECT OF SLURRY MEDIA ON COLUMN PRESSURE, EFFICIENCY AND PEAK SHAPE

Column no.	*Slurry media*	*Pressure (p.s.i.)*	*Column efficiency (plates per metre)*			*Average efficiency*	*Peak shape**
			1	*2*	*3*		
137	Acetonitrile–water (4:1)	1510	9600	10,700	11,300	10,500	T
138	Water	1720	12,400	12,100	11,300	12,100	G
146	Water	1720	13,500	12,300	11,800	12,500	G
140	Acetonitrile	1570	9300	9100	10,100	9500	T
141	Carbon tetrachloride	1760	9000	9100	8600	8900	VG
142	Tetrabromoethane	1980	9200	8600	8500	8800	L
145	Water	1620	10,000	9200	10,100	9800	G

* T = tail, G = good, VG = very good, L = leading edge.

on this system were considerably lower than expected and it is probable that this effect was caused by the retention of the tetrabromoethane within the phase. High column pressure could then be attributed to the increased viscosity and the lower k' values to the more hydrophobic nature of the phase. Peak shape on this system was characterised by a leading edge. The column packed with carbon tetrachloride slurry exhibited similar characteristics to the tetrabromoethane slurry.

Optimisation of analysis time for sugars

Resolution[12] is a function of α (separation factor), N (the column efficiency) and k' (the column capacity ratio):

$$R_s = \tfrac{1}{4}\left(\frac{\alpha - 1}{\alpha}\right)\sqrt{N}\left(\frac{k'}{1 + k'}\right)$$

The resolution between maltose and lactose ($R_{m/l}$) and between fructose and glucose ($R_{f/g}$) were plotted against support loading to determine the value for which $R_{m/l}$ and $R_{f/g}$ are at a maximum. Sucrose was omitted from this study as it is always well separated from the other four sugars and therefore not a limiting factor in the analysis time. The graph obtained is given in Fig. 6. As might be anticipated $R_{f/g}$ increased with increasing phase loading and consequently 8.5% represents the optimum for fructose–glucose separation. Surprisingly $R_{m/l}$ peaks at 6.3% which consequently represents the best value for maltose–lactose separation. The optimum loading for all five sugars was chosen as 7.0%. Although $R_{f/g} > R_{m/l}$ at this point the fructose peak tailed in practice and consequently 7.0% and not 6.3% was the loading at which equal resolution was achieved between the maltose–lactose and fructose–glucose peaks. If the resolution between adjacent peaks exceeds baseline separation, analysis time is wasted. Where this occurs the column efficiency is greater than required since, at a fixed percentage loading, resolution is a function of N. The efficiency may be reduced by an increase in the linear velocity or a reduction in column length. High linear velocities are commonly used with microparticulate silica owing to the relatively flat curve obtained for the plot of HETP (height equivalent to a theoretical plate) vs. u (linear velocity). Fig. 7 shows this plot for lactose using 6.2% loading and flow-rates from 1 to 7 ml/

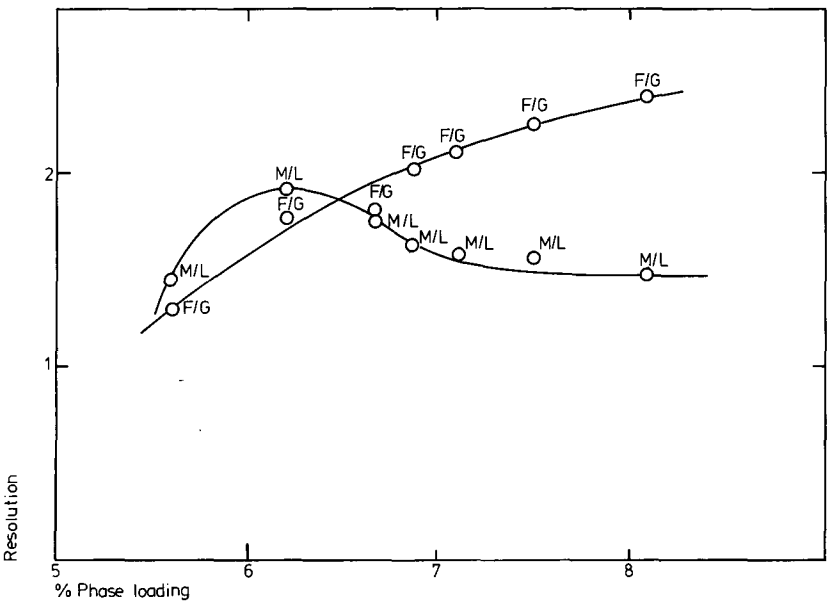


Fig. 6. Graph of resolution against percentage support loading for fructose–glucose (F/G) and maltose–lactose (M/L).

min. From this it can be seen that a six-fold increase in flow-rate, for lactose, produces only a two-fold increase in HETP. Unfortunately a high linear velocity generates a high column back pressure and consequently a compromise was reached by reducing the column length and increasing the flow-rate to give a rapid analysis time with a reasonable back pressure. Fig. 8 demonstrates the separation of all five sugars in 10 min on a 16 × 0.46 cm I.D. column at a flow-rate of 3 ml/min and a pressure of 1900 p.s.i.

Application to food analysis

We have been analysing sugars in a wide range of foodstuffs for several months. Over this period of time a general extraction procedure has evolved: (1) Sample maceration (2) cold water extraction, (3) protein precipitation, (4) filtration, (5) freeze drying of filtrate.

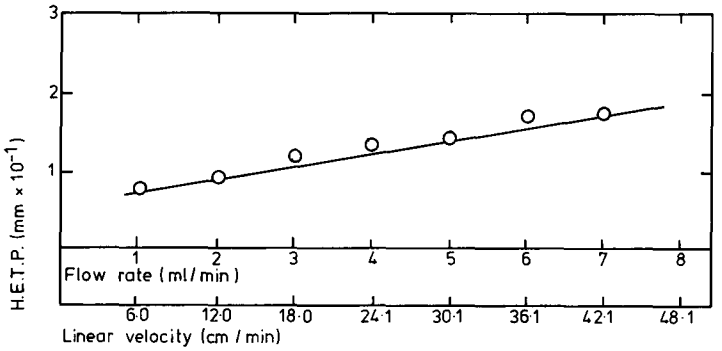


Fig. 7. Graph of HETP against flow-rate and linear velocity for lactose.

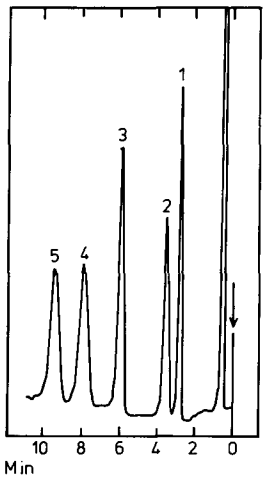
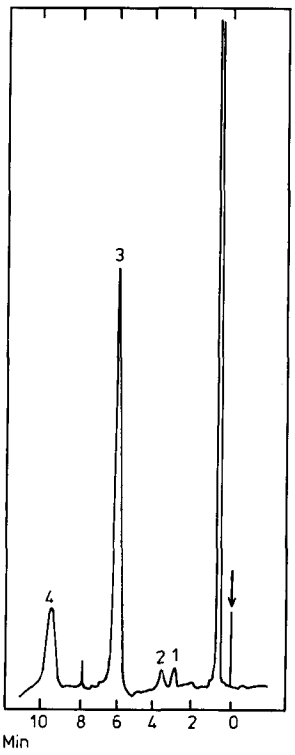


Fig. 8. HPLC separation of sugars. 1 = Fructose, 2 = glucose, 3 = sucrose, 4 = maltose, 5 = lactose. Solvent, acetonitrile–water (4:1). Column. Partisil 5, 7% NH_2 phase, 16 × 0.46 cm I.D. Flow-rate, 3 ml/min.



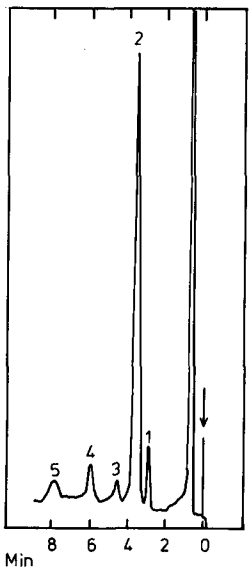


Fig. 9. Ice cream extract. 1 = Fructose, 2 = glucose, 3 = sucrose, 4 = lactose. Solvent, acetonitrile–water (4:1). Column, Partisil 5, 7% NH_2 phase, 16 × 0.46 cm I.D. Flow-rate, 3 ml/min.

Fig. 10. Enzyme-treated soy extract. 1 = Fructose, 2 = glucose, 3 = unknown, 4 = sucrose, 5 = maltose. Other conditions as in Fig. 9.

Obviously some food samples need not be taken through all the steps (*e.g.* sweets, fruit drinks), but conversely we have not as yet been presented with a food sample that we could not analyse by this procedure. Samples are taken through steps 1–5 (where necessary) as soon as they are received for analysis. In this manner the possibility of sample degradation is eliminated. The freeze dried extract is then dissolved in acetonitrile–water (3:7) just prior to chromatography. Three examples of food analyses are given in Fig. 9, 10 and 11.

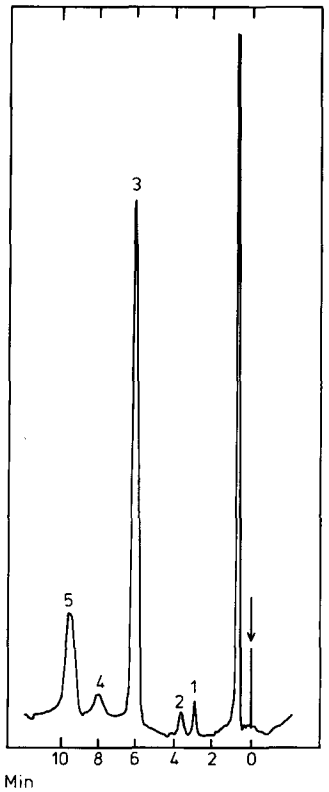


Fig. 11. Breakfast cereal extract. 1 = Fructose, 2 = glucose, 3 = sucrose, 4 = maltose, 5 = lactose. Other conditions as in Fig. 9.

CONCLUSIONS

A wide range of support loadings may be prepared in 5–10 min by shaking Partisil in varying concentrations of γ-aminopropyltriethoxysilane in hexane at room temperature.

The best column efficiencies were obtained using a water slurry and pumping acetonitrile–water (4:1) through the column to compress the phase to a compact bed. This also serves to equilibrate the column to the conditions required for sugar analysis. By using a 7% support loading the separation of fructose, glucose, sucrose, maltose, and lactose may be achieved in only 10 min on a 16 $\times$ 0.46 cm I.D. column at a pressure of 1900 p.s.i.

The application of the amino column to food analysis is not difficult. Some sample extracts may be chromatographed directly. However, large quantities of co-extractable material should, as far as possible, be removed in order to maintain a reasonable column lifetime.

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