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Evaluation of a Reversed Phase High Performance Liquid Chromatographic Column for Estimation of Legume Seed Oligosaccharides

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ABSTRACT

The retention characteristics of the major legume seed oligosaccharides (sucrose, raffinose, stachyose and verbascose), and also those of some minor oligosaccharide components of soybeans, lupin seeds and fermented soybean products, were investigated using an RP-18 reversed phase column. Excellent separation of sucrose, raffinose and stachyose from one another and from minor oligosaccharide components was achieved using distilled water as the mobile phase, but verbascose could not be determined separately as it coeluted with raffinose. Addition of salts to the mobile phase increased retention and improved selectivity of separation. Rapid and reasonably efficient separation of sucrose, raffinose, stachyose and verbascose was achieved when 0·3 M *ammonium sulphate was used as the mobile phase. These oligosaccharides were clearly resolved from minor oligosaccharide components. A comparison of the retention characteristics of the reversed phase column with those of a continuously modified plain silica column, using an acetonitrile/water/amine modifier mixture as mobile phase, revealed major differences in selectivity which may be of value for separation and identification purposes.*

INTRODUCTION

Oligosaccharides of the raffinose family occur widely in legumes. Soybeans contain appreciable quantities of the oligosaccharides sucrose, raffinose and stachyose (Bianchi *et al.*, 1984) but only traces of verbascose

Food Chemistry 0308-8146/86/$03·50

(Smith & Circle, 1972). The last oligosaccharide occurs in significant quantities in other legumes such as lupin seeds (Macrae & Zand-Moghaddam, 1978). These oligosaccharides cause flatulence in man when ingested (Cristofaro *et al.*, 1974; Rackis, 1981). For this reason, a number of processes—such as boiling and fermentation—have been applied to legumes to reduce the oligosaccharide content (Puwastien & King, 1984).

For part of our research programme aimed at monitoring antinutritional factors in soybeans, lupin seeds and other legumes, and also fermented and non-fermented legume products, a rapid and simple method for the determination of oligosaccharides in large numbers of samples was required.

A number of methods have been used for the separation and quantification of these oligosaccharides (Macrae & Zand-Moghaddam, 1978). High performance liquid chromatography (HPLC) offers the possibility of rapid and simple procedures for sample preparation and chromatography. Methods have been described involving the use of μ-Bondapak/Carbohydrate columns (Black & Bagley, 1978) and Spherisorb amino columns (Macrae & Zand-Moghaddam, 1978). Acetonitrile/water mixtures were used as the mobile phase. Methods of this type involve extraction of defatted meal with aqueous alcohol, and also concentration and clarification of extracts prior to chromatography.

A procedure which eliminates the need for sample concentration has been described very recently (Kennedy *et al.*, 1985). Oligosaccharides were extracted from defatted meal with distilled water and high molecular weight material was removed by ultrafiltration. Stachyose, raffinose, sucrose and hexoses were separated on a Dextropak reversed phase column using distilled water as the mobile phase.

An important advantage of reversed phase HPLC is that the use of toxic constituents in the mobile phase, such as acetonitrile and amine modifiers, is avoided. Rapid and efficient separations of sucrose, raffinose and stachyose can be achieved and baselines with the refractive index detector are generally more stable when water is used as the mobile phase instead of acetonitrile/water mixtures, resulting in lower detection limits and more accurate quantification.

The objectives of our own investigation were as follows.

(i) To evaluate a reversed phase HPLC column with regard to its suitability for the estimation of major oligosaccharide components (sucrose, raffinose and stachyose) in soybeans and

soybean products, bearing in mind the low oligosaccharide content of the latter.

(ii) To assess the feasibility of extending the method to include determination of verbascose.

(iii) To investigate possible interferences arising from some minor legume seed oligosaccharide components.

(iv) To compare the performance of the reversed phase column with that of a more commonly used type of column for oligosaccharide analysis, i.e. continuously modified plain silica (Aitzetmüller, 1978), using an acetonitrile/water/amine modifier mixture as the mobile phase. Separations on the latter column are similar to those obtainable using amino columns or μ-Bondapak/Carbohydrate columns.

EXPERIMENTAL

Materials

Oligosaccharides were obtained from commercial sources, with the exception of verbascose, which was isolated chromatographically (see sub-section 'Isolation of verbascose' for details) and manninotriose, which was prepared by treatment of stachyose with β-fructosidase. The hydrolysate was used for chromatography after clarification and deionisation (Wight & van Niekerk, 1983).

Locally grown soybeans and lupin seeds (variety unknown) were used as test materials. Tempeh, a fermented soybean product, was prepared in our own laboratory (van der Riet, 1985) and lyophilised.

Sample preparation

Samples were ground ($< 0{\cdot}5$ mm particle size) and mixed in a sample splitter prior to analysis. Ground material was defatted with petroleum ether.

Oligosaccharides were extracted from defatted material with hot water and the extract was clarified, deionised and filtered prior to chromatography according to the method of Wight & van Niekerk (1983) with the following modifications. The extraction period was 2 h, an increased quantity of resin (5 g per 15 ml extract) was used and the ethanol treatment after deionisation was omitted. Filtered samples were used for chromatography without further concentration.

Chromatography

Columns for reversed phase HPLC were packed in our own laboratory. The column (25 cm × 0·46 cm inside diameter) and guard column (6 cm × 0·46 cm inside diameter) were slurry packed with 5 μm LiChrosorb RP-18 (Merck) in methanol, and stored in methanol when not in use.

Chromatograms were also run on the following columns for comparison and identification purposes: HPX-42A (Bio-Rad, pre-packed column) and 5 μm LiChrosorb Si60 (Merck, column dimensions and packing procedure as for the RP-18 column).

The chromatographic system consisted of a Varian model 5000 isocratic pump fitted with a column heating attachment, a Valco injection valve fitted with a sample loop (38 μl), a Waters model R401 differential refractometer and a Hewlett-Packard model No. 3390A integrator.

Isolation of verbascose

Verbascose was isolated chromatographically in small quantities from a concentrated lupin seed extract, using the HPX-42A oligosaccharide column. The fraction (corresponding to the peak V eluting immediately before stachyose—Fig. 1) collected from a large number of replicate injections was concentrated and rechromatographed twice on the same column.

The retention time of the fraction V was consistent with that of a pentasaccharide and could be identified as verbascose with a reasonable degree of certainty on the basis of the following observations:

(i) The retention time on the Si60 column, relative to those of raffinose and stachyose (Table 1) was similar to that reported by other research workers using similar chromatographic systems (Macrae & Zand-Moghaddam, 1978; Quemener & Mercier, 1980).

(ii) Treatment of lupin seed extracts with β-fructosidase (Wight & van Niekerk, 1983) resulted in the disappearance of the 'verbascose' peak, as shown by chromatography on both the Si60 and RP-18 columns (see also 'Results and Discussion' section), indicating that the oligosaccharide contained fructose as a non-reducing end-group.

TABLE 1

Comparison of Retention Times (Relative to Raffinose) of Various Monosaccharides and Oligosaccharides on RP-18 and Si60 Columns

	RP-18 column			*Si60 column*
	Mobile phase: H_2O	*Mobile phase $(NH_4)_2SO_4$*		*Mobile phase: CH_3CN/H_2O/amine modifier (62/38/0·01%)*
		0·3 M	*1·5 M*	*1·5 M*	
Flow rate (ml/min)		1·0	1·3	1·6	2·5
Temperature (°C)		Ambient	Ambient	60	Ambient
Sugars					
Inositole	0·453		0·245		0·853
Galactose	0·478		0·261		0·670
Glucose	0·483	0·483	0·268	0·427	0·631
Fructose	0·498		0·277	0·438	0·575
Melibiose	0·521	0·516	0·326	0·500	0·900
Trehalose	0·551	0·554	0·369	0·556	0·787
Manninotriose	0·552	0·586		0·579	1·350
Verbascotetraose[a]	NR			0·60 (S)	2·082
Sucrose	0·696	0·691	0·514	0·642	0·701
Raffinose	1·000 (6·86)[b]	1·000 (7·25)	1·000	1·000	1·000
Stachyose	0·907	0·935	1·089	1·071	1·484
Verbascose	0·994	1·044	1·497 (14·43)[b]	1·348 (7·20)[b]	2·222 (9·51)[b]
Cellobiose	0·588		0·417	0·596	0·787
Maltose	0·563		0·358, 0·369[c]	0·543	0·794
Lactose	0·500, 0·529[c]		0·302, 0·334[c]	0·449, 0·488[c]	0·846
Methanol	0·614				
Ethanol	0·993				

[a] Tentative identification only. It is assumed that verbascotetraose is the most strongly retained oligosaccharide formed on treatment of the lupin seed extract with β-fructosidase. (NR = not resolved from manninotriose, S = shoulder on manninotriose peak together with estimated relative retention time.)

[b] The retention time (in minutes) of the most strongly retained oligosaccharide with each system is listed in parentheses after the relative retention time.

[c] Eluted as a double peak owing to partial separation of α- and β-anomers.

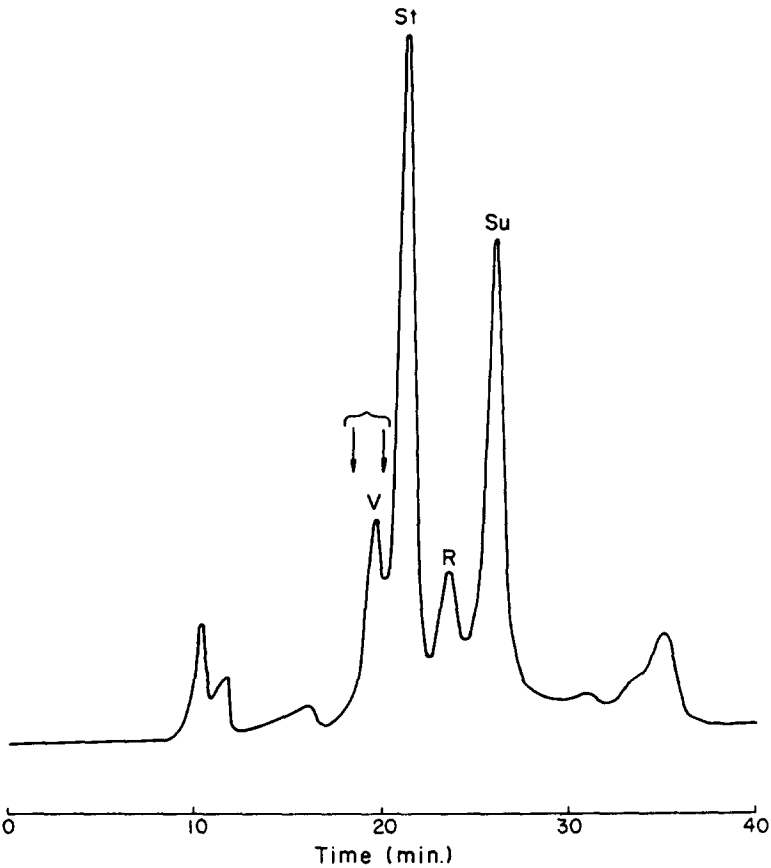

Fig. 1. Chromatogram of a lupin seed extract. Column: HPX-42A; Mobile phase: deionised distilled water; flow rate: 0·3 ml/min; column temperature: 60°C; verbascose (V), stachyose (St), raffinose (R), sucrose (Su). The fraction which was isolated is indicated by the arrows.

RESULTS AND DISCUSSION

Chromatography

A small proportion of high molecular weight material remained in the water extracts after sample cleanup, as indicated by the presence of an exclusion peak when samples were chromatographed on the HPX-42A column (Fig. 1). Although this material was apparently adsorbed onto

the RP-18 packing material during chromatography, column life was quite satisfactory. The guard column was repacked after injection of approximately 500 samples and standards, following a gradual loss in resolution and increase in back pressure, but no noticeable deterioration of the main column occurred during this period, apart from a slight loss of retention.

Chromatographic separation

In addition to sucrose, raffinose and stachyose, a number of other oligosaccharides have been reported as minor constituents in soybeans (Molnár-Perl *et al.*, 1984) and other legumes (Schweizer *et al.*, 1978; Quemener & Brillouet, 1983). The chromatographic behaviour of some of these oligosaccharides, for which standards were available or could be prepared, was investigated. These included melibiose, manninotriose, verbascotetraose and cellobiose. Melibiose has been reported as a product appearing at intermediate stages of tempeh manufacture (Shallenberger *et al.*, 1967). Trehalose was also investigated as a possible product of inoculation of soybeans with Rhizopus mould spores during tempeh manufacture. The relative retention times of these oligosaccharides on the RP-18 column are listed in Table 1.

Sucrose, raffinose and stachyose were more strongly retained than other oligosaccharides and were well separated from one another and from monosaccharides on the RP-18 column when water was used as the mobile phase. The separation of sucrose, raffinose and stachyose was similar to that reported by Kennedy *et al.* (1985) on Dextropak columns but, unlike the Dextropak column, the RP-18 column clearly resolved sucrose from other disaccharides such as melibiose (Fig. 2). The minor oligosaccharide components were poorly resolved from one another under these conditions, however. For this reason, this system was used only for the estimation of sucrose, raffinose and stachyose.

Estimation of sucrose, raffinose and stachyose

Regression analysis data (peak area versus concentration) and detection limits for these oligosaccharides are listed in Table 2. Responses for individual oligosaccharides were very similar and calibrations were linear throughout the concentration range 0·005% to 0·250%.

Comparison of a chromatogram of raw soybean oligosaccharides

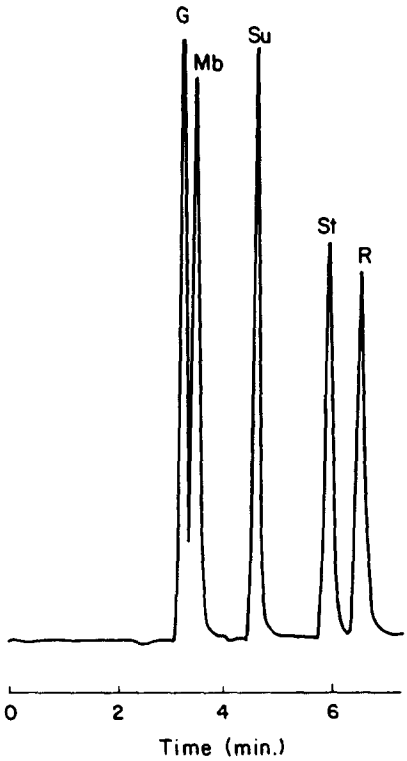

Fig. 2. Chromatogram of a standard. Column: RP-18 plus guard column; mobile phase: water; flow rate: 1·0 ml/min; column temperature: ambient; glucose (G), melibiose (Mb), sucrose (Su), stachyose (St), raffinose (R) (all *ca.* 0·05% w/v).

TABLE 2
Regression Equations.[a] Correlation Coefficients, Detection Limits[b]

Oligosaccharide	*A*	*B*	*Correlation coefficient*	*Detection limit (ng)*
Trehalose	0·063 274	539·77	0·999 99	75
Sucrose	0·144 16	541·80	0·999 99	90
Stachyose	−0·023 402	482·39	0·999 87	105
Raffinose	−0·041 375	480·42	0·999 98	105

[a] $Y = A + BX$, peak area $\times 10^{-3}$ versus concentration (g/100 ml).
[b] Values were estimated for signal:noise ratio of 2:1.

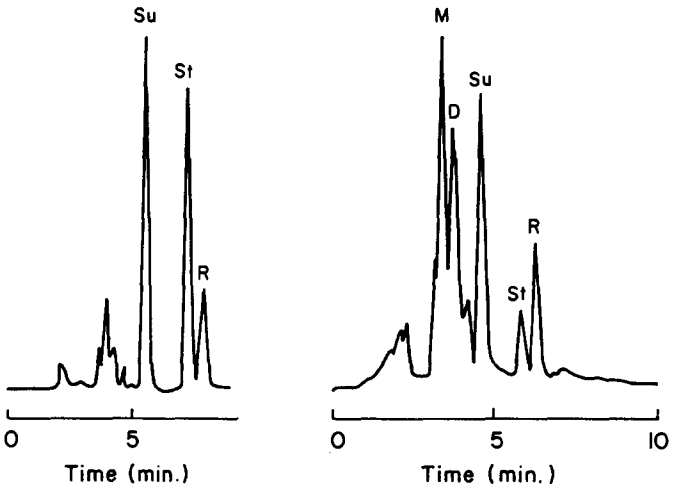

Fig. 3. Chromatograms of a raw soybean extract (left) and a soybean tempeh extract (right). Column and conditions as in Fig. 2. Monosaccharides (M); unidentified component (probably a mixture of disaccharides, D); sucrose (Su), stachyose (St), raffinose (R).

with that of soybean tempeh (48 h fermentation period, Fig. 3) indicated that a new component (D, probably a mixture of disaccharides) was formed in appreciable quantities on fermentation. The retention time of this component was intermediate between those of melibiose and trehalose.

Sucrose, raffinose and stachyose could be determined with a satisfactory degree of accuracy and precision even in the tempeh sample, where the oligosaccharide concentration was low and also more complex than that of the raw soybeans. Analysis data for sucrose, raffinose and stachyose for five tempeh sub-samples are listed in Table 3. These figures

TABLE 3
Analysis Data[a] (Tempeh)

Oligosaccharide	*Mean content (g/100 g)*[b]	*Coefficient of variation* (%)
Sucrose	0·820	1·00
Stachyose	0·246	2·23
Raffinose	0·546	2·09

[a] Mean content (g/100 g sample on natural basis).
[b] Mean values for five determinations on different sub-samples (duplicate injections of each sub-sample); mean value for four determinations in the case of sucrose.

exclude sucrose data for one sub-sample as sucrose was incompletely resolved from a minor component, resulting in an anomalously high peak area. In all other cases, sucrose, raffinose and stachyose were clearly resolved from other components.

Verbascose was not determined but only traces of this oligosaccharide were present in locally grown soybeans, as shown by chromatography on the HPX-42A column.

Chromatographic behaviour of verbascose

Comparison of chromatograms of soybean (Fig. 3) and lupin seed extracts (Fig. 4a), using the RP-18 column and water as mobile phase, failed to indicate any component which could be identified tentatively as verbascose on the basis of its higher content in the latter sample. It appears, therefore, that verbascose coelutes with another oligosaccharide under these conditions.

Isolation of verbascose, as described in the experimental section, followed by injection of the isolated material onto the RP-18 column, indicated that verbascose coelutes with raffinose (Table 1). It has been shown recently (Cheetham & Teng, 1984), however, that addition of salts to the mobile phase increases oligosaccharide retention, the degree of increase being greater for larger molecules. When ammonium sulphate was added to the mobile phase, an increase in degree of retention in the order sucrose < raffinose < stachyose < verbascose was, in fact, observed. At salt concentrations below 0·3 M, verbascose was incompletely resolved from raffinose.

Optimal separation of raffinose, stachyose and verbascose was achieved at a salt concentration of around 0·3 M (Fig. 4b). At higher salt concentrations, separation of raffinose from stachyose at first deteriorated with increase in salt concentration but subsequently improved as stachyose became more strongly retained than raffinose and baseline separation was achieved at a salt concentration of around 1·5 M (Fig. 4c). Analysis times were relatively long, but could be shortened with minimal loss in resolution by the use of elevated temperatures and increased flow rates (Fig. 4d). The use of high salt concentrations was inconvenient for routine use, however, and shortened useful column life, particularly if elevated temperatures were used. A salt concentration of 0·3 M provided the most rapid and efficient separation of oligosaccharides consistent with minimal deterioration of the column. Storage of columns

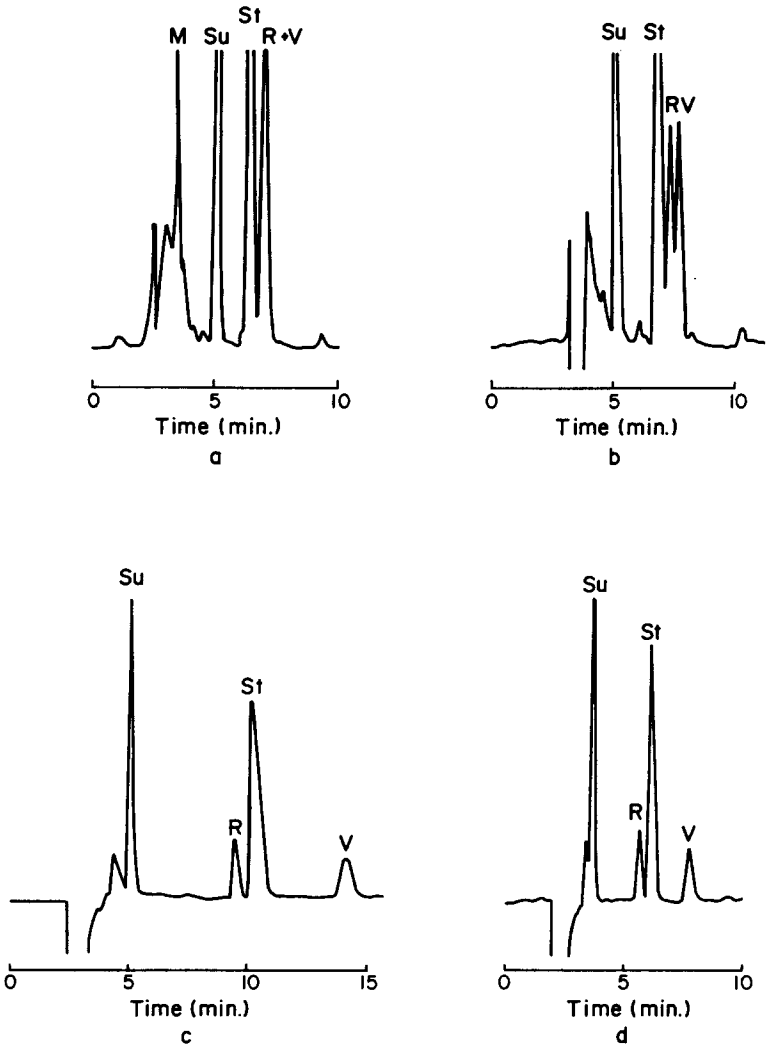

Fig. 4. Chromatograms of a lupin seed extract. Column: RP-18 plus guard column. (a) Mobile phase: water; flow rate: 1·0 ml/min; column temperature: ambient. (b) Mobile phase: 0·3M ammonium sulphate; flow rate: 1·0 ml/min; column temperature: ambient. (c) Mobile phase: 1·5M ammonium sulphate; flow rate: 1·3 ml/min; column temperature: ambient. (d) Mobile phase: 1·5M ammonium sulphate; flow rate: 1·6 ml/min; column temperature: 60°C. Designation of peaks as in Figs 1–3.

overnight in ammonium sulphate caused rapid loss of retention and selectivity and therefore the column was flushed daily with distilled water after use.

Comparison of chromatographic separations

A comparison of retention characteristics of the RP-18 column, with and without addition of salts to the mobile phase, and the Si60 column, is given in Table 1. An important difference in selectivity between the two columns is that oligosaccharides containing fructose as a non-reducing end group are generally retained much more strongly on the RP-18 column than oligosaccharides which do not contain fructose. This may be advantageous for the estimation of the raffinose family of oligosaccharides, particularly raffinose itself, as interference from minor components is eliminated. This is illustrated in Fig. 5 in which chromatograms of a lupin seed extract on the RP-18 and Si60 columns are compared. The oligosaccharides remaining after treatment of the extract with β-fructosidase are weakly retained on the RP-18 column.

Addition of salts to the mobile phase for the RP-18 column caused large negative peaks in samples and standards. These negative peaks did not interfere with the determination of sucrose and the raffinose family of oligosaccharides. If determination of weakly retained components such as melibiose is also required, salts can be added to samples and standards to match the mobile phase salt concentration. Separation of individual disaccharides, for example melibiose and trehalose, improved with increasing salt concentration.

In addition to legume oligosaccharides, the chromatographic behaviour of a number of other oligosaccharides and monosaccharides of importance in food analysis was also compared on the RP-18 and Si60 columns (Table 1). While differences in selectivity between individual monosaccharides were generally too small to be put to practical use, differences in selectivity between individual dissacharides were, in some cases, much greater.

Bonded phase silica columns, or continuously modified plain silica columns, using acetonitrile/water mixtures as mobile phase, are most commonly used for separation of disaccharides. A few combinations of disaccharides which were not resolved, or only poorly resolved, on the Si60 column, for example maltose and cellobiose, were more clearly resolved on the RP-18 column, the selectivity being improved by the

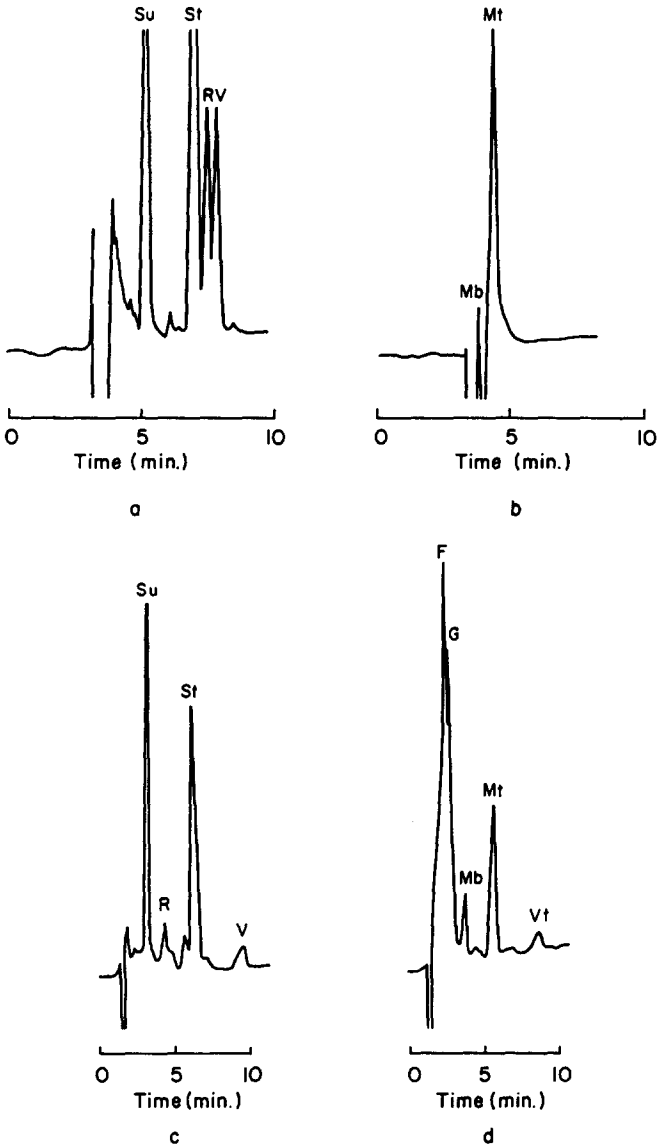

Fig. 5. Chromatograms of a lupin seed extract before and after treatment with β-fructosidase. (a) Untreated extract. Column: RP-18 plus guard column; mobile phase: 0·3M ammonium sulphate; flow rate: 1·0 ml/min; column temperature: ambient. (b) Extract treated with β-fructosidase; column and conditions as for chromatogram (a). (c) Untreated extract; column: Si60; mobile phase: acetonitrile/water/HPLC amine modifier (62/38/0·01%); flow rate: 2·5 ml/min; column temperature: ambient. (d) Extract treated with β-fructosidase; column and conditions as for chromatogram (c). Fructose (F), manninotriose (Mt), verbascotetraose (Vt, tentative identification), designation of other peaks as in Figs 1–3.

addition of salts to the mobile phase. Inositol, a minor constituent of soybeans, elutes before monosaccharides such as glucose and galactose on the RP-18 column and could be determined separately, if required. By contrast, inositol is more strongly retained than glucose and galactose on the Si60 column, and could be mistaken for a disaccharide.

One disadvantage of reversed phase HPLC for food carbohydrate analysis is that reducing oligosaccharides may elute as double peaks owing to separation of α- and β-anomers. This effect was not observed for any of the legume seed oligosaccharides investigated on the RP-18 column when water was used as the mobile phase and, of the common reducing disaccharides studied, only lactose exhibited this effect.

CONCLUSIONS

The main advantages of the reversed phase HPLC method are the use of non-toxic mobile phases and also speed and simplicity of sample preparation and chromatography, although the latter advantage is lost to some extent if verbascose must also be determined. The raffinose family of oligosaccharides were more clearly resolved from minor legume oligosaccharides on the reversed phase column than on other types of column. Reversed phase HPLC should provide a valuable alternative to more commonly used HPLC methods for oligosaccharide determination in view of its completely different selectivity.

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