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GAS–LIQUID CHROMATOGRAPHIC DETERMINATION OF THE RAFFINOSE FAMILY OF OLIGOSACCHARIDES AND THEIR METABOLITES PRESENT IN SOY BEANS

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SUMMARY

The relative retention times and detector responses of trimethylsilyl-oxime derivatives of the components of soluble soy saccharides, up to pentasaccharide, on SP-2250 liquid phase are reported. Arabinose, rhamnose, fructose, galactose, glucose, sucrose, cellobiose, galactobiose, melibiose, raffinose, cellotriose, galactotriose, manninotriose, stachyose, verbascotetraose and verbascose were well resolved. A gas–liquid chromatographic method for rapid separation and quantitation of the constituents of every member of the raffinose oligosaccharides, present in soy beans, is described.

INTRODUCTION

Raffinose[1] is second only to sucrose in abundance in the plant kingdom, and plays a prominent part in the biosynthesis of related compounds. A knowledge of the composition of soluble soy saccharides and their hydrolytic and thermal products is essential in the examination of the Maillard reactions[2] (carbonyl–amino group interactions) in soy samples.

The possible components of the raffinose family in soy bean are shown in Table I. In addition, the decomposition products of pectines and cellulose may also be present: as saccharides with various degrees of polymerization (D.P.), but mainly originating from chain ends, arabinose, rhamnose, as well as cellotriose, cellobiose and glucose.

No chromatographic method of analysis is available for the simultaneous determination of raffinose oligosaccharides including soy saccharides. Procedures principally employed for determination of the main components in soy samples are paper chromatography[3–5], carbon column chromatography[6], centrifugal chromatography[7], thin-layer chromatography[8], liquid chromatography[8–12], as well as gas chromato-

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TABLE I

STRUCTURAL RELATIONSHIPS OF THE RAFFINOSE OLIGOSACCHARIDES

Galactobiose

Galactotriose

Verbascotetraose

Manninotriose

Melibiose

Galactosyl–Galactosyl–Galactosyl–Glucosyl–Fructose

Sucrose

Raffinose

Stachyose

Verbascose

Galactosyl = α-D-galactopyranosyl-(1→6); glucosyl = α-D-glucopyranosyl-(→2); fructose = β-D-fructofuranoside.

graphy (GC)[13–15]. Among these procedures, most information has been obtained by the methods of Kawamura[3–5]. This author identified and measured sucrose, raffinose and stachyose in amounts of 6.6, 1.4 and 5.3%, respectively, as the main components among the soluble saccharides of soy bean, and arabinose, fructose, glucose and verbascose as traces. The shortcomings of the liquid chromatographic procedures[9–12] are that either the two main components, raffinose and stachyose, are separated only in the top of their peaks[9,12], or the separation of monosaccharides and disaccharides from sucrose is not complete[11]. Consequently, the decomposition products cannot be measured.

In our opinion, the most efficient method for this purpose is GC, if optimum derivatization and separation conditions are ensured. These requirements were only partly fulfilled in earlier methods[12–14] and only the main components could be determined. Our work was initiated to provide a method for rapid measurement of the main components and their decomposition products from a single chromatogram.

Based on earlier experience[15–23]: (i) the most suitable derivatives for separation are the silyl ether oximes[16–18]; (ii) for optimum separation the combination of a shorter and a longer column should be applied[23]; (iii) the separation of saccharides with D.P. of 1–5 should be done by programming over a wide temperature range and up to a high final temperature[23].

MATERIALS AND METHODS

Materials and reagents

D-Arabinose and L-rhamnose were products of Reanal (Budapest, Hungary),

D-fructose, α-D-glucose and sucrose, D-cellobiose were from Serva (Heidelberg, F.R.G.) and α-D-melibiose monohydrate from Fluka (Switzerland). Raffinose was purchased from E. Merck (Darmstadt, F.R.G.) and stachyose hydrate from Sigma (St. Louis, MO, U.S.A.).

The soy bean samples were untreated commercial products (1) or research intermediates (2, 3). Sample 2 was a heat-treated (100°C, 5 min) derivative of sample 1; sample 3 was an acid-treated (HCl, pH ≈ 2) derivative of sample 1.

Pyridine, trifluoroacetic acid (TFA), isopropanol and hydroxylamine hydrochloride were products from Reanal. Hexamethyldisilazane (HMDS) was from Applied Science Labs. (State College, PA, U.S.A.).

Apparatus

The gas chromatograph was a Model G.C.H.F. 18.3 instrument (Chromatron, Berlin, G.D.R.) equipped with a flame ionization detector. Chromatographic peak area determinations were made with a Chinoin Model Digint-34 μ computing integrator. Stainless-steel columns of 50 cm × 3 mm I.D. and 2 m × 3 mm I.D. were used. The packing materials were 3% silicone SE-30, 3% OV-17 both supported on 100–120 mesh Chromosorb W AW DMCS (Applied Science Labs.), and 3% SP-2250 supported on Supelcoport 80/100 (Supelco, Bellefonte, PA, U.S.A.).

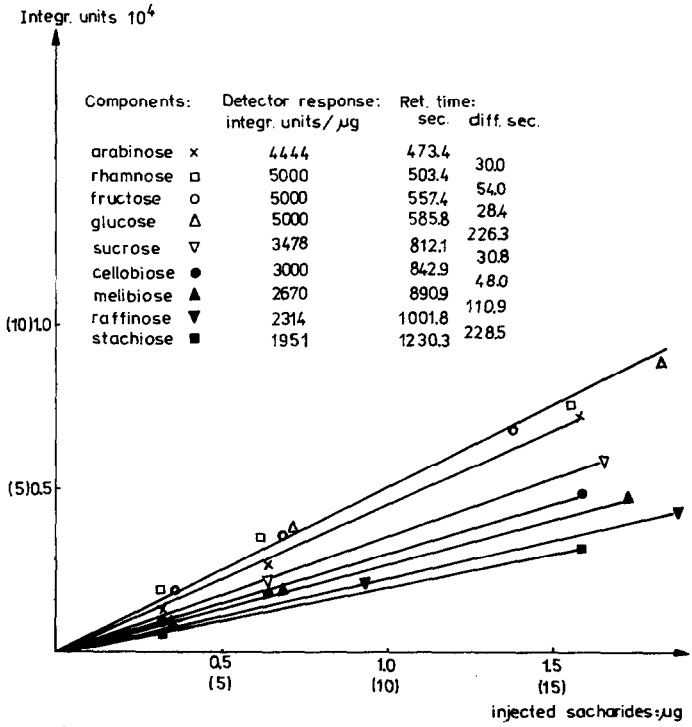

Fig. 1. Detector response of TMS-oxime derivatives on a 2-m long column of 3% SP-2250 on Supelcoport 80/100.

Separation of trimethyl silyl (TMS)-oxime derivatives

The temperatures of the injection and detector ports were 410 and 430°C respectively. With a temperature-programmed analysis from 80 to 380°C at a rate of 16°C/min, 20 min were required to elute the TMS-oxime derivatives. The nitrogen flow-rate was 60 cm^3/min.

Preparation of TMS-oxime derivatives

The model solutions as well as the extracts of soy bean samples containing 0.002–0.01 g of fructose, sucrose, raffinose, stachyose and different amounts of the possible metabolites of soy saccharides (see Fig. 1 and Table II) were evaporated to dryness in a rotary evaporator at 50–60°C. After this the residue was dried by evaporating it twice with 0.5 cm^3 of isopropanol.

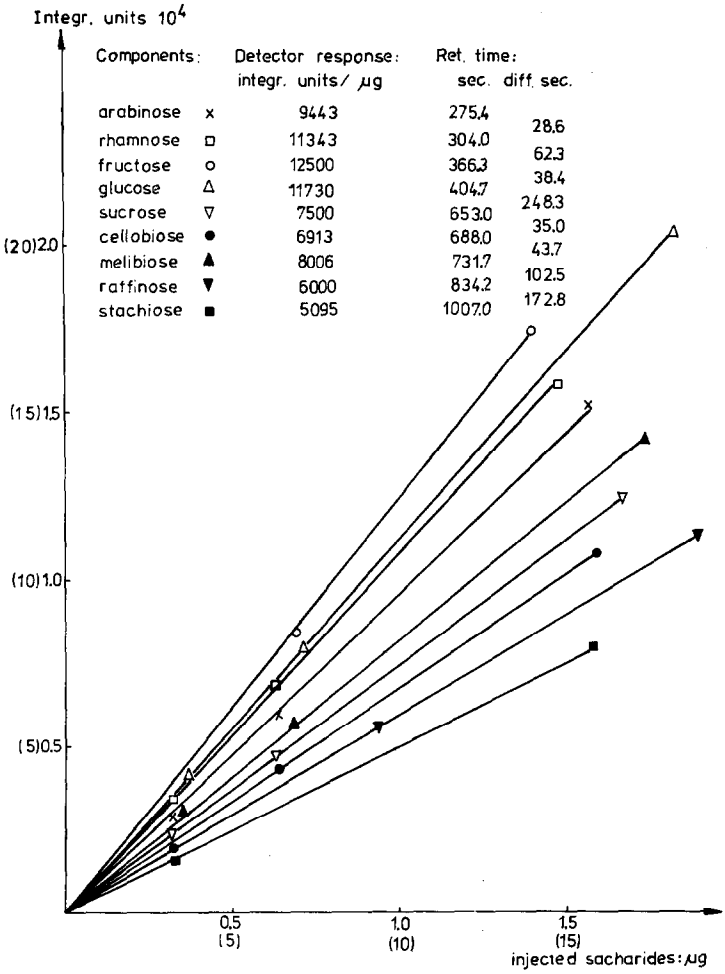

Fig. 2. Detector response of TMS-oxime derivatives on a 50-cm long column of 3% SP-2250 on Supelcoport 80/100.

The dehydrated residue was oximated with 500 μl of pyridine containing 1.25 g hydroxylamine hydrochloride 50 ml for 30 min at 70–72°C. The oxime derivatives were trimethylsilylated with a mixture of 900 μl HMDS and 100 μl TFA for 60 min at 70–72°C. Thereafter, 5 μl of the clear supernatant were injected into the gas chromatograph.

RESULTS AND DISCUSSION

Our investigations performed with authentic saccharides (see Materials and Methods) and with the characteristic representatives of soy bean samples, *i.e.*, samples 1–3, provided the following results.

(1) Column packings with 3% SE-30, 3% OV-17 and 3% SP-2250 were tested in parallel on 50 cm and 2 m long columns. The experiments showed that, in the case of packings SE-30 and OV-17, parallel measurements on the 50-cm and 2-m columns are necessary for the elution of the pentasaccharide verbascose and for the separation of saccharides with identical D.P.s, whereas with SP-2250 a single chromatogram on the 50-cm column is sufficient for optimum separation and measurement. The reasons for this are: (i) verbascose can only be eluted on the shorter column with each packing; (ii) the qualitative and quantitative evaluation of saccharides with the same D.P.s can be done successfully on 50-cm columns only with the packing SP-2250. Among the stationary phases studied, the best separation on columns with the same lengths was achieved with the packing SP-2250, the next best with OV-17.

(2) Comparing the capacities of the 50-cm and 2-m columns (Figs. 1 and 2), it is clear that the shorter column is preferable to the longer one, since: (i) the 50-cm column is suitable for the separation of all TMS-oximes with D.P. of 1–5; (ii) the elution of the saccharides from the same solution is complete in a time shorter by about 20%; (iii) the detector response of the TMS-oxime of a certain saccharide is more than twice as large on the 50-cm column as on the 2-m column; (iv) differences in the retention times of fructose, glucose, sucrose and cellobiose, in the most critical separations, are greater on the shorter column.

(3) As to derivatization, it has been proven, in accord with the literature[16–18], that in order to obtain reducing saccharides in a single peak the samples should be dehydrated by distillation with isopropanol before oxime formation. This makes possible the elution of reducing saccharides as a single peak, *i.e.*, the measurement of different amounts of saccharides with the same D.P. in the presence of each other. It should be noted that this is still controversial at present[22].

(4) Under our optimum conditions, the main components of soy saccharides and their decomposition products can be measured reproducibly even when present in a ratio of approximately 300:1 to the main component sucrose (Fig. 3, Table II).

(5) The qualitative and quantitative distribution of the soy saccharides in samples 1–3 shows (Figs. 4–6, Table III) that almost every theoretically possible component (Table I) is identified and measured in comparison with authentic saccharides and on the basis of logical considerations.

(6) As no authentic samples were available for galactobiose, galactotriose, manninotriose, verbascotetraose and verbascose, for their identification we assumed that (i) the elution sequence of monosaccharides, *i.e.*, fructose, galactose and glucose, is also valid for oligosaccharides containing these saccharides as terminal members;

TABLE II

REPRODUCIBILITY OF DETERMINATION OF POSSIBLE METABOLITES OF SOY SACCHARIDES IN THE PRESENCE OF THE MAIN COMPONENTS

Detailed data of Fig. 3. Amounts (μg) of components in samples A–F: fructose, 6.94; sucrose, 33.13; raffinose, 9.38; stachyose, 15.75. *a* = Amount injected (μg); *b* and *c* = integrator units obtained (*b*, total, *c*, equivalent to 1 μg). $\bar{x}$ = Average; S.D. = standard deviation.

Sample	*Arabinose (1)**			*Rhamnose (2)*			*Glucose (4)*			*Cellobiose (5)*			*Melibiose (6)*		
	a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
A	0.0			0.0			0.0	4290**		0.0			0.0	734**	
B	0.095	851	8957	0.093	1023	11,000	0.109	***		0.095	***		0.104	***	
C	0.190	1792	9431	0.186	2150	11,559	0.218	6723	11,160	0.190	1190	6263	0.208	2297	7514
D	0.316	2907	9199	0.310	3222	10,394	0.362	8816	12,502	0.318	2264	7119	0.346	3716	8618
E	0.632	6130	9699	0.620	6901	11,131	0.724	12,547	11,407	0.636	4476	7034	0.692	6333	8091
F	1.58	1569	9931	1.55	19,580	12,632	1.81	25,747	11,850	1.59	11,581	7239	1.73	14,229	7800
$\bar{x}$			9443			11,343			11,730			6913			8006
S.D.			3.87			832			587			765			471
S.D. (%)			4.1			7.3			5.0			11.1			5.9

* Peak number in Fig. 3.

** Impurities of fructose, sucrose (Serva) and of raffinose (Merck) and stachyose (Sigma).

*** Peak areas resulting from low concentrations of saccharides were not measured reproducibly with the electronic integrator. The areas of these peaks were determined by triangulation.

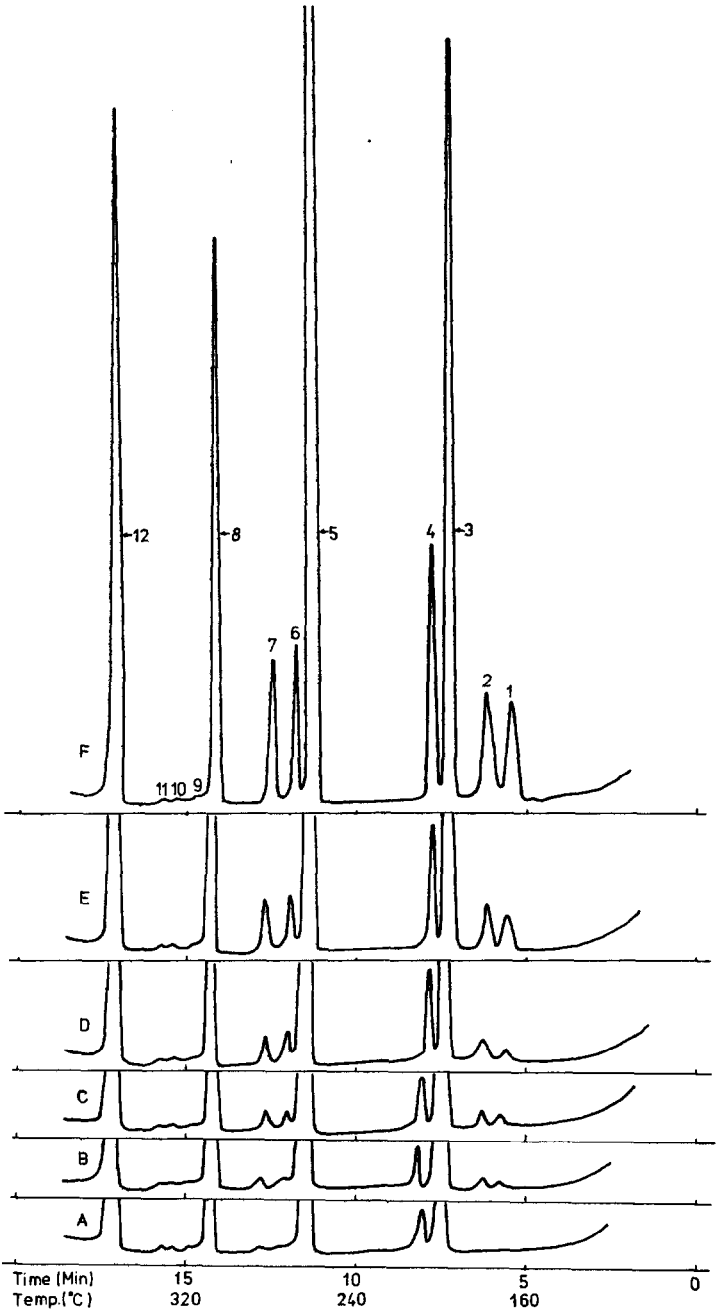

Fig. 3. Gas chromatograms of different amounts of possible metabolites of soy saccharides in the presence of the main components on a: 50 cm long column of 3% SP-2250 on Supelcoport 80/100. Peaks: 1 = arabinose; 2 = rhamnose; 3 = fructose; 4 = glucose*; 5 = sucrose; 6 = cellobiose*; 7 = melibiose*; 8 = raffinose; 9 = cellotriose*; 10 = galactotriose*; 11 = manninotriose*; 12 = stachyose*. On chromatogram A: impurities in Serva, Merck and Sigma products. Detailed data in Table II.

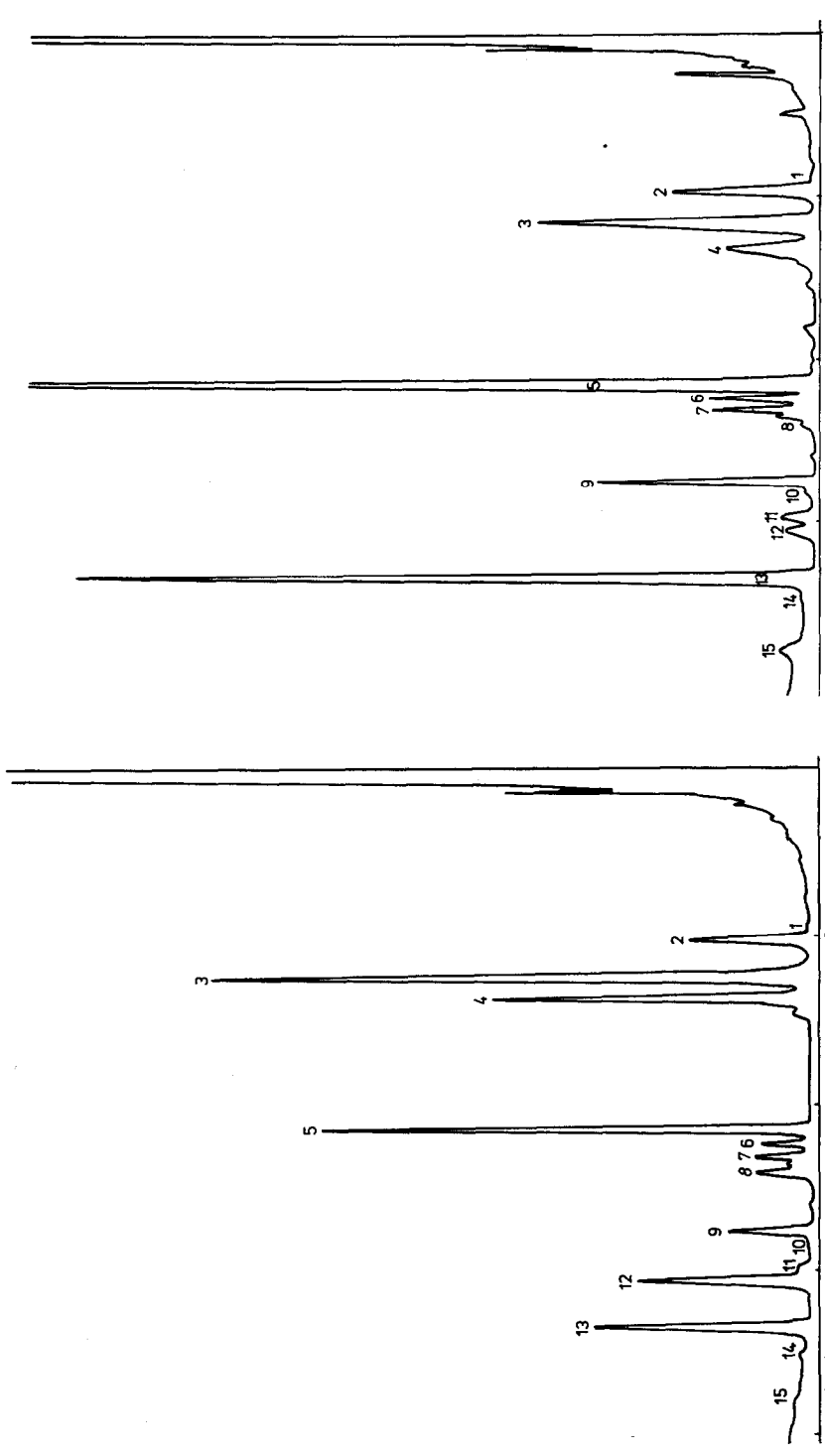

Fig. 4. Gas chromatogram of the soluble saccharides of an untreated sample (1) of soy bean on a 50 cm long column of 3% SP-2250 on Supelcoport 80/100. Peaks: 1 = arabinose; 2 = rhamnose; 3 = fructose; 4 = glucose; 5 = sucrose; 6 = cellobiose; 7 = galactobiose; 8 = melibiose; 9 = raffinose; 10 = cellotriose; 11 = galactotriose; 12 = manninotriose; 13 = stachyose; 14 = verbascotetraose; 15 = verbascose. Detailed data in Table III.

Fig. 5. Gas chromatogram of the soluble saccharides of a heat-treated sample (2) of soy bean. Details as in Fig. 4.

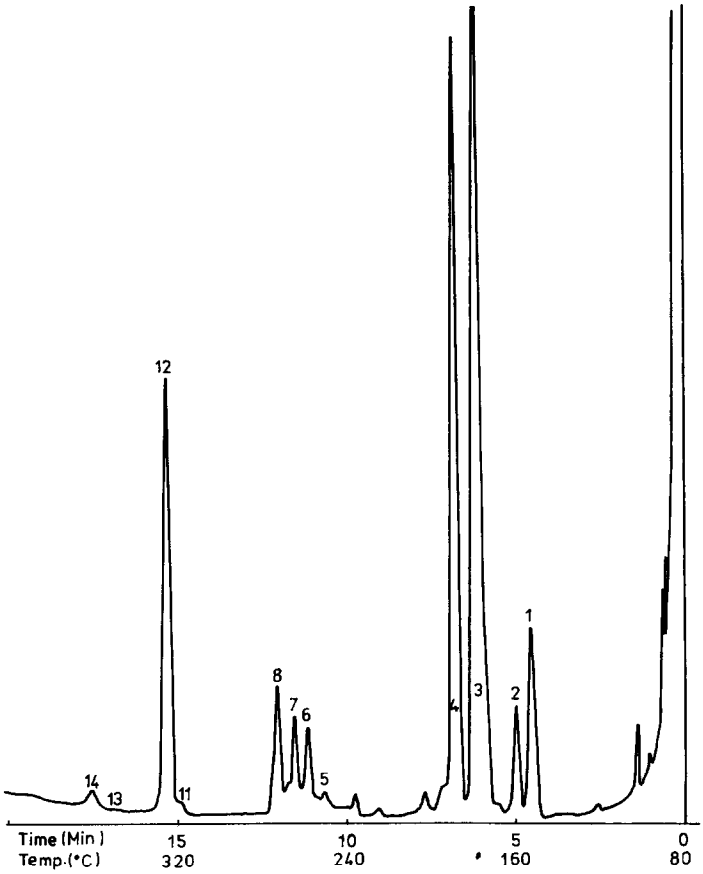

Fig. 6. Gas chromatogram of the soluble saccharides of an acid-treated sample (3) of soy bean. Details as in Fig. 4.

(ii) the authentic saccharides commercially available can be fitted into the above sequence, without exception. The order is: sucrose, galactobiose, melibiose, raffinose, galactotriose, manninotriose, stachyose, verbascotetraose, verbascose.

(7) We have not measured galactose: under our experimental conditions, galactose and glucose were eluted with a difference of 8 sec in the retention times, and they were separated only in the top of their peaks. According to our studies, a galactose:glucose ratio of 1:10 could have been measured. Accordingly, galactose must be present in an amount less than 10%, which is insignificant according to the literature[24], *i.e.*, immediately after its formation it is transformed into a derivative susceptible to biosynthesis.

(8) As a result of our method, it has been proven that —contrary to data obtained by earlier procedures— in addition to the main components (sucrose, raffinose, stachyose), saccharides should also be regarded as basic ones which are formed in larger quantities following treatments indispensable to obtain digestible soy products. With the exception of fructose, these saccharides have reducing properties: the

TABLE III

DISTRIBUTION OF THE SOLUBLE SACCHARIDES OF UNTREATED (1), HEAT-TREATED (2) AND ACID TREATED (3) SOY BEAN; IMPURITIES OF AUTHENTIC SUGARS

Components, expressed as % of the total dry material. –, Not present in measurable amount.

Sample	*Arabinose*	*Rhamnose*	*Fructose*	*Glucose*	*Sucrose*	*Cellobiose*	*Galactobiose*	*Melibiose*	*Raffinose*	*Cellotriose*	*Galactotriose*	*Manninotriose*	*Stachyose*	*Verbascotetraose*	*Verbascose*	*Total*
1.	0.03	0.40	0.55	0.07	5.20	0.46	0.42	0.10	0.86	0.06	0.28	0.23	3.79	0.08	0.25	12.78
2.	0.01	0.32	1.81	0.87	1.56	0.22	0.22	0.26	0.40	0.03	0.14	1.23	1.37	0.06	0.06	8.56
3.	0.71	0.31	3.20	2.38	0.19	0.37	0.39	0.57	–	–	0.18	2.86	0.02	0.43	–	11.61
Fructose (Serva)	–	–		2.69	0.72	–	–	–	–	–	–	–	–	–	–	3.41
Sucrose (Serva)	0.15	0.18	0.47	0.51		–	–	–	0.20	–	–	–	–	–	–	1.51
Melibiose (Fluka)	–	–	0.10	–	0.17	–	–		–	–	–	–	–	–	–	0.27
Raffinose (Merck)	0.05	0.20	0.24	0.10	0.40	–	–	1.51		–	–	–	–	–	–	2.50
Stachyose (Sigma)	–	0.09	0.19	–	0.11	–	–	–	0.78	–	2.77	1.45		–	1.30	6.97

qualitative-quantitative knowledge of reducing saccharides is therefore of great importance concerning the Maillard reactions which are undesirable from the point of view of the optimum nutritional value of the soy products.

(9) Concerning the purity of the authentic saccharides tested, (i) none proved to be pure (GC), the variation in quality and quantity of impurities determined in products from different sources (Table III) being remarkable; (ii) raffinose and stachyose contain the characteristic member of raffinose oligosaccharides; (iii) according to the sum of the extraneous components, only melibiose may be regarded as a disaccharide of high purity.

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