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Identification of the fructo-oligosaccharides common to beet medium invert sugar and pyrolysed sucrose

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Chromatography and enzymolysis have been used to identify the primary oligosaccharides common to beet medium invert sugar and pyrolysed sucrose. Anion exchange and reversed phase HPLC showed the two materials to contain a similar spectrum of primary oligomers, although at a much higher level in pyrolysed sucrose. Confirmation of the similarity was obtained by reversed phase fractionation and anion exchange HPLC analysis of the individual fractions.

Gel filtration chromatography was used to estimate the size of the oligomers with gel filtration fractionation of the sample of beet medium invert sugar and subsequent anion exchange HPLC analysis of the fractions, confirming that the primary oligosaccharides elute in the same position as the starch trisaccharide maltotriose.

Enzymolysis, using invertase EC 3.2.1.26, of beet medium invert sugar and pyrolysed sucrose, and subsequent analysis of the digest using anion exchange HPLC, showed that four of the oligosaccharides were cleaved by the enzyme, indicative of a terminal β-*D*-fructofuranoside residue in each. Another two oligosaccharides were stable to the enzyme, and therefore do not possess the terminal β-*D*-fructofuranoside feature. One additional oligosaccharide appeared during the enzymolysis.

From the enzymolysis and chromatographic data and by comparison of the elution positions of the primary oligosaccharides with those of reference standards it was concluded that the primary oligosaccharides present in both beet medium invert sugar and pyrolysed sucrose are the kestoses, 1-kestose, 6-kestose, and neokestose and their α-anomers, *iso*-1-kestose, *iso*-6-kestose, and *iso*-neokestose. Although the kestoses may be the products of enzymic action on sucrose the *iso*-kestoses are not produced and therefore must originate through a thermal process.

These oligosaccharides used as indicators for the detection of beet medium invert sugar extension of orange juice are unlikely to occur naturally in oranges as orange invertase has α-*D*-glucosidase activity and therefore oligosaccharides produced *in situ* would have glucose linked to sucrose rather than the kestoses and *iso*-kestoses, produced by β-*D*-fructosidase or thermolysis, where fructose is linked to sucrose.

INTRODUCTION

The extension of orange juice and orange juice precursors has become a serious problem facing manufacturers of pure orange juice. The ability to sell cheaper material in place of pure fruit juice provides the profit of falsification. In practice, this means extending the juice or juice concentrate by the addition of low cost commodities such as water, sugar, and fruit-derived extenders, while maintaining the specification of the product. A constant battle exists between the adulterators and the

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authorities in their respective attempts to falsify and detect falsification of juices, respectively. Sugar is the main component of the soluble solids content of orange juice, and therefore any extension necessitates the addition of sugar. However, orange juice contains three primary sugars, *D*-glucose, *D*-fructose, and sucrose, in the approximate ratio 1:1:2 (Echeverria & Valich, 1988). Therefore this ratio must be retained in the extended product if it is not to be detected by simple carbohydrate ratio analysis (Brause *et al.*, 1984). One way of achieving this is by the addition of partially inverted sucrose. Such a commercially available product is beet medium invert sugar, which is acid and/or enzyme hydrolysed beet sucrose with a *D*-glucose, *D*-fructose, sucrose ratio of approximately 1:1:2.

The identification of the addition of beet medium invert sugar to orange juice or orange juice precursors relies on the anion exchange HPLC analysis of the oligosaccharide profile (Swallow *et al.*, 1991). Beet medium invert sugar shows a characteristic oligomer profile which has not been observed in any of the pure samples of orange juice analysed to date. The identification of the fingerprint oligosaccharides from the beet medium invert sugar profile in a sample of commercial orange juice or orange juice precursor has therefore been taken as an indication of product extension. The nature and origin of these oligosaccharides in beet medium invert sugar, and hence their likelihood of being components of pure orange juice, is as yet unclear.

Sugar beet contains hydrolase enzymes which exhibit β-*D*-fructofuranosidase activity. These enzymes catalyse the production of oligosaccharides by transfructosylation (Masuda *et al.*, 1987). The carbohydrate products of such action would accumulate in the sugar beet and, if they have pyranose structures which are more stable than furanose rings (Aspinall & Telfer, 1955), they may remain intact during the extraction and processing of the sucrose. Oligosaccharides are known to be present in sugar beets and sucrose preparations extracted from them, the trisaccharide raffinose being used to identify the source of commercial sugar, since it is present in beet but not detectably in corn or cane (Tsang *et al.*, 1990). Alternatively, the oligosaccharides could be produced during the production of beet medium invert sugar from sucrose. In addition to *D*-glucose and *D*-fructose, oligosaccharides are also produced during the acid hydrolysis of sucrose (Belitz & Grosch, 1987). The enzymic method for partial inversion could also produce oligosaccharides by the reverse action of the invertase. At low sucrose concentrations invertase exhibits hydrolase activity, but at high sucrose concentrations this group of enzymes may also exhibit a secondary transferase activity (Woodward & Wiseman, 1989). Invertase from yeasts, *Saccharomyces cerevisiae* or *Saccharomyces uvarum*, is used in the commercial production of beet medium invert sugar (Beuchat, 1987), and due to its β-*D*-fructosidase activity can therefore produce oligosaccharides where fructose is linked to sucrose. If this was indeed the source of the oligosaccharides, then they would differ from those which occur naturally in oranges, as the orange invertase has α-*D*-glucosidase activity and therefore would produce oligosaccharides where glucose was linked to sucrose (Huleme, 1970). The beet medium invert sugar oligomers could also be produced by thermal action during its production/concentration. Richards (1986) reported that the thermal degradation of sucrose under certain conditions produces trisaccharides. This confirmed the earlier work of Bollman & Schmidt-Berg (1965) who identified 13 products, including five non-reducing trisaccharides, when sucrose was heated at 170°C.

This work was undertaken in order to identify the oligosaccharides in beet medium invert sugar which are used as indicators of its addition to orange juice or orange juice precursors and to determine their likely source and the possibility of them occuring naturally in oranges.

MATERIALS AND METHODS

Materials

The beet medium invert sugar (BMIS) used in this study was a gift from Mr Alan Brooks (RHM Research, High Wycombe, UK); the reference samples of kestoses and *iso*-kestoses a gift from Dr Merilyn Manley-Harris (University of Montana, Missoula, USA); and the beet sucrose was produced by British Sugar Ltd (Norwich, UK). The invertase EC 3.2.1.26 was from bakers' yeast (Sigma Chemical Company, Poole, UK). The aqueous sodium hydroxide (NaOH) used for the preparation of the HPLC eluents was 50% w/w and all other reagents were of AR grade (FSA Laboratory Supplies, Loughborough, UK).

Anion exchange HPLC method

Anion exchange HPLC was performed using two CarboPac PA1 columns, 250 × 4·0 mm ID, operated in series (Dionex (UK) Ltd, Camberley, UK). A guard column of the same packing material was also used. The HPLC instrument consisted of a 625-LC solvent delivery system with a non-metallic flow path, a 464 pulsed amperometric detector (PAD) fitted with a gold working electrode and a base stable reference electrode and a Whisp 712 injector (Waters Chromatography Division of Millipore (UK) Ltd, Watford, UK). A single piston reciprocating pump (Scientific Systems Inc., State College, PA, USA) with two metres of polymer tubing was used to add 300 mM NaOH, at a flow rate of 0·7 ml/min, to the eluent stream between the columns and detector. For the analysis of carbohydrates the PAD was operated in the cathodic mode with the following

series of potentials: 50 mV for 200 ms; 800 mV for 200 ms and −600 mV for 500 ms. After equilibrating the system with 100 mM NaOH a sample aliquot (200 μl) was injected. The eluent composition was held constant for 7 min after which the NaOAc concentration was increased, linearly, to 3 mM over the next 16 min followed by a linear increase to 100 mM NaOAc over the next 30 min. The NaOAc concentration was held at this level for a further 10 min after which the columns were regenerated by flushing with 300 mM NaOH for 60 min followed by re-equilibration in 100 mM NaOH for 40 min before injecting the next sample. The eluent flow rate was 0·7 ml/min throughout.

Reversed phase HPLC

Reversed phase HPLC was carried out using a μ Bondapak C18 column, 300 × 3·9 mm ID (Waters Chromatography Division of Millipore (UK) Ltd., Watford, UK). The isocratic HPLC instrument consisted of a twin piston reciprocating pump, RI detector and a strip chart recorder (Knauer GmbH, Bad Homburg, Germany). Sample injection was achieved using a Rheodyne 7125 injection valve fitted with a 200 μl loop (Rheodyne Inc., Cotati, USA). The system was equilibrated with 18·2 MΩ water prior to sample injection, 20 μl for analysis or 200 μl for fractionation. Fractionation was achieved by collecting 1 ml portions as the eluent exited the RI detector cell.

Gel filtration chromatographic fractionation

The fractionation was performed using Biogel P2 <400 mesh gel (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) packed in a glass column 900 × 18 mm ID, which was thermostatted at 65°C. The column was equilibrated with 18·2 MΩ water at a flow rate of 13 ml/min prior to injecting 225-μl aliquots of beet medium invert sugar, prepared in 18·2 MΩ water at a concentration of 25% w/v, and fractions collected every 15 min.

Pyrolysis of beet sucrose

Beet sucrose (6 g) and citric acid (0·06 g) were dissolved in 18·2 MΩ water (4 ml). The solution was freeze-dried to produce an amorphous sponge. The sample was kept *in vacuo* (0·1 Torr) whilst being heated in a boiling water bath. The sample was heated for 60 min, during which time the sponge collapsed to give a viscous yellow liquid. After cooling to ambient temperature, during which it formed a hard transparent glass, it was dissolved in 18·2 MΩ water (30 ml).

Invertase treatment

A sample of either beet medium invert sugar or pyrolysed sucrose (300 mg) was dispersed in 1 mM NaOAc, pH 4·5 (4 ml). After equilibration at 50°C, a portion (500 μl) was removed and placed in a boiling water bath for 4 min. This sample was used to obtain the anion exchange HPLC oligosaccharide profile of the sample prior to the addition of invertase EC 3.2.1.26. Invertase EC 3.2.1.26 (500 μl containing 45 units of activity) was then added to the equilibrated substrate solution. After incubation at 50°C for 30 min the enzyme was deactivated by placing the sample in a boiling water bath for 4 min.

RESULTS AND DISCUSSION

Several neutral di- and tri-saccharide standards and an acid hydrolysed starch, cellulose and inulin sample containing a range of oligomers, DP1–DP10, have been analysed in our laboratory by anion exchange HPLC. Whilst the elution order is not based on molecular size, but on the charge density of the solute, the elution volumes for neutral di-, tri- and tetra-saccharides were in the range of 30–60 ml, with limited correlation to degree of polymerisation. This is similar to the range of elution volumes obtained with the beet medium invert sugar oligosaccharides, indicating that if they are indeed neutral oligosaccharides, then they are most likely di-, tri-, or tetra-mers.

A sample of beet sucrose was analysed using anion exchange HPLC in order to determine whether the oligosaccharides are present in the sucrose prior to partial hydrolysis or if they are produced during the manufacture of the medium invert. Although the sucrose solution was prepared at the same concentration as the total sugar content of beet medium invert sugar used for oligosaccharide fingerprinting, none of the fingerprint oligomer peaks could be seen in the anion exchange chromatogram. There was only one very small peak which eluted after 45·8 min which corresponded to the elution position of raffinose. These fingerprint oligosaccharides are therefore components of beet medium invert sugar produced during its manufacture, as they are not present in commercial beet sucrose.

As di-, tri-, and tetra-saccharides are known to be produced by the pyrolysis of sucrose (Bollman & Schmidt-Berg, 1965), such a sample would be a source of oligosaccharides for comparison with the beet medium invert sugar fingerprint. A sample of pyrolysed beet sucrose was therefore analysed under the same conditions as used for the analysis of beet medium invert sugar. The chromatograms of the pyrolysed beet sucrose and the beet medium invert sugar are very similar (Fig. 1). The primary products of the thermal degradation of beet sucrose, as determined by anion exchange HPLC, elute in the same positions as the fingerprint peaks, 7, 12, 16, and 18, in the beet medium invert sugar profile. The differences between the two

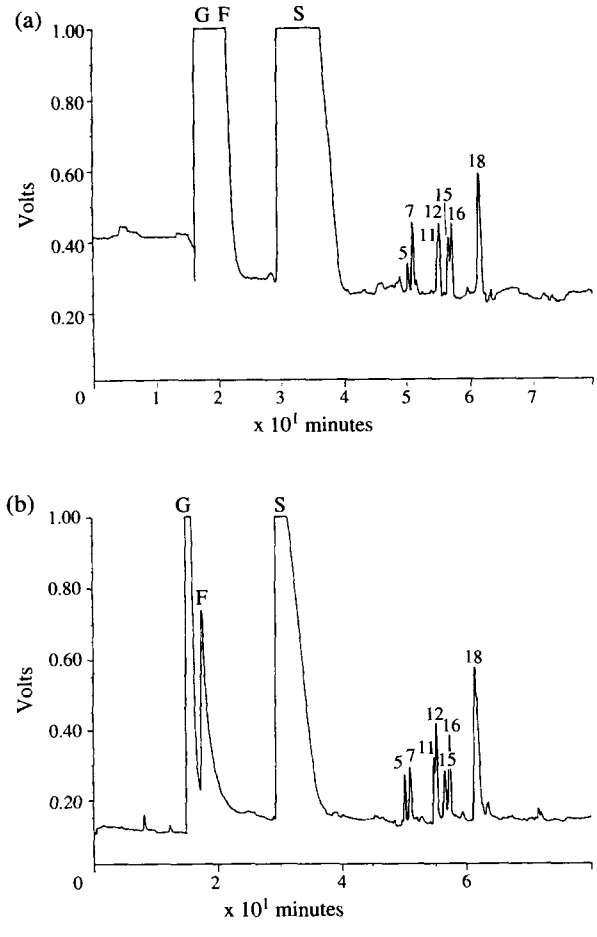

Fig. 1. Anion exchange HPLC chromatograms of a sample of beet medium invert sugar (a) and pyrolysed sucrose (b).

profiles are that in the sample of pyrolysed sucrose there is a shoulder on the front edge of peak 18 and the resolution between peaks 11 and 12 is improved.

Manley-Harris & Richards (1991) proposed a mechanism for the thermal decomposition of sucrose. Initially the glycosidic bond between the glucose and fructose moieties is broken to give a resonance-stabilised fructosyl cation which is then the subject of nucleophilic attack. Suitable nucleophiles are alcohols, water or other sugar molecules. In their studies using simple alcohols as the nucleophiles, Moody & Richards (1981) established that the fructosyl cation is most reactive towards primary alcohols. Therefore, in the thermal decomposition of sucrose, where another molecule of sucrose acts as the nucleophile, there would be a possibility of producing three oligosaccharides depending upon which of the three primary hydroxyls was used. Chemical transfructosylation is not stereospecific, and therefore the glycosidic linkage in the three oligosaccharides generated can adopt the α- or β-anomeric configuration. The trivial and systematic names of these three trisaccharides, where the new linkage is β, are: 1-kestose (*O*-β-*D*-fructofuranosyl-(2-1)-β-*D*-fructofuranosyl-(2-1)-α-*D*-glucopyranoside), 6-kestose (*O*-β-*D*-fructofuranosyl-(2-6)-β-*D*-fructofuranosyl-(2-1)-α-*D*-glucopyranoside) and neokestose (*O*-β-*D*-fructofuranosyl-(2-1)-α-*D*-glucopyranosyl-(6-2)-β-*D*-fructofuranoside and their α-anomers *iso*-1-kestose (*O*-α-*D*-fructofuranosyl-(2-1)-β-*D*-fructofuranosyl-(2-1)-α-*D*-glucopyranoside), *iso*-6-kestose (*O*-α-*D*-fructofuranosyl(2-6)-β-*D*-fructofuranosyl-(2-1)-α-*D*-glucopyranoside), and *iso*-neokestose (*O*-β-*D*-fructofuranosyl-(2-1)-α-*D*-glucopyranosyl-(6-2)-α-*D*-glucopyranoside). The structures of these trisaccharides are shown in Fig. 2. The presence of these trisaccharides and a number of disaccharides in a sample of pyrolysed sucrose has been confirmed by structural analysis (Manley-Harris & Richards, 1991).

Although the anion exchange HPLC chromatograms of a sample of pyrolysed sucrose and beet medium invert sugar are similar it can not be concluded that the oligosaccharide components of the two samples are the same. In the anion exchange HPLC technique, oligosaccharides are separated based on differences in their charge. Those solutes with a higher charge density are retained longer on the column. It is therefore possible to have two oligosaccharides which have the same charge density, and hence elution volume in the anion exchange separation, but which have different structures. To assist in the confirmation of the similarity of the oligosaccharide compositions, a second HPLC technique which has a different selectivity was required.

Reversed phase HPLC, using both an amine and C18 bonded phase, has been used for the analysis of oligosaccharides in enzyme digests (Ivin & Clarke, 1987). Solutes are separated based on differences between their affinities for the hydrophobic stationary phase. With the C18 bonded phase the eluent for oligosaccharide analysis is water, and therefore any possibility of alkaline degradation of *D*-glucose and *D*-fructose or production of the oligosaccharides by the eluent system, as with the NaOH used in anion exchange HPLC, is avoided. We have shown in our earlier work (Lloyd *et al.*, 1994) that these oligosaccharides present in beet medium invert sugar are not alkaline degradation products of *D*-glucose or *D*-fructose and are indeed base stable. From this it was concluded that they are not reducing sugars. Figure 3 shows the reversed phase chromatograms of the sample of beet medium invert sugar and pyrolysed sucrose. In both chromatograms, in addition to peak *a*, *D*-glucose + *D*-fructose, and peak *b*, sucrose, two other peaks can be seen, peaks *c* and *d*. Although in reversed phase HPLC, solute retention increases with increasing hydrophobicity, for a series of closely related oligosaccharides the elution order is partly related to size. It is therefore possible that peaks *c* and *d* could be the disaccharides and trisaccharides, including the kestoses, produced by the pyrolysis of sucrose. The profile of peaks is similar to that obtained by Smouter & Simpson (1993) in their reversed phase

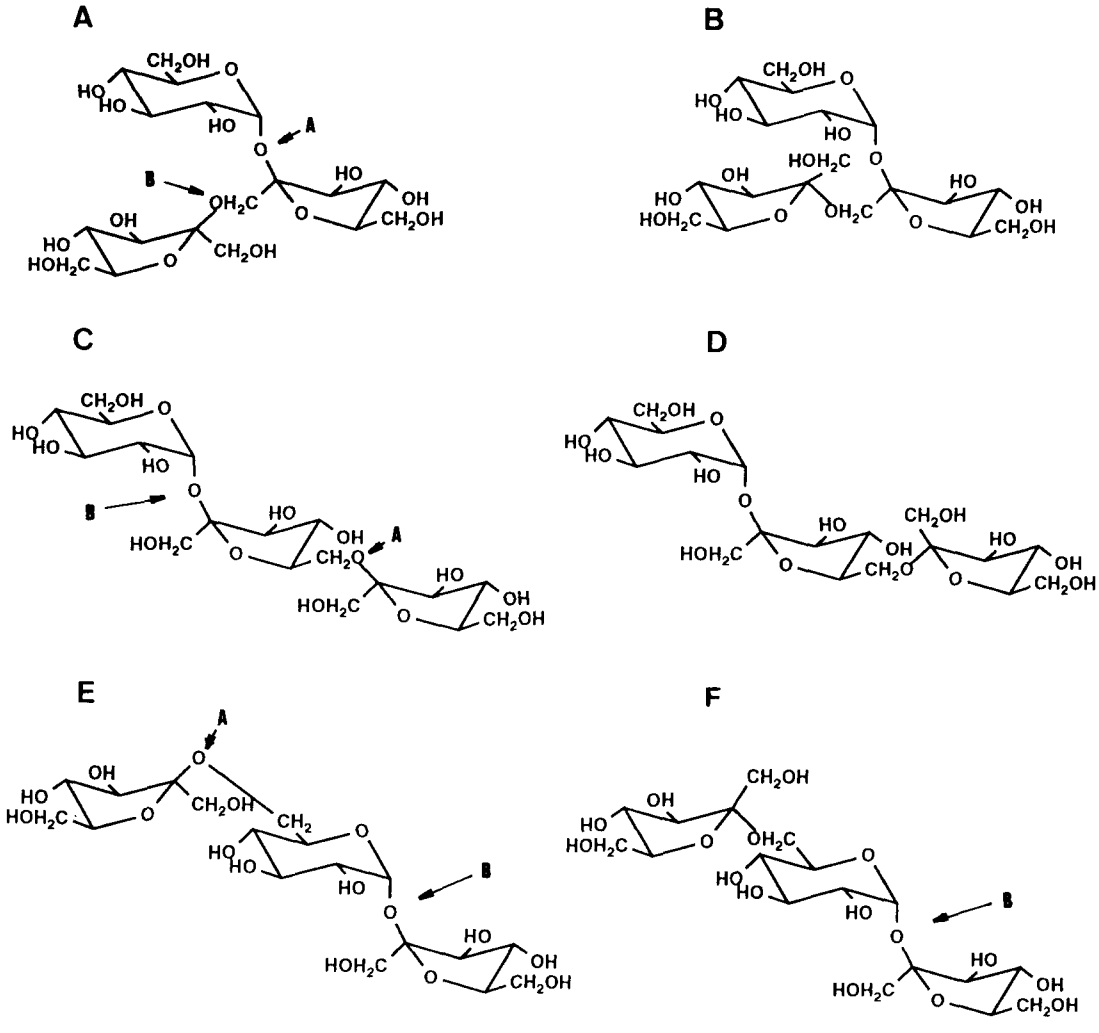

Fig. 2. Structure and invertase EC 3.2.1.26 cleavage points of the trisaccharides 1-kestose (A), *iso*-1-kestose (B), 6-kestose (C), *iso*-6-kestose (D), neokestose (E), and *iso*-kestose (F).

purification of inulin oligosaccharides, including 1-kestose, 6-kestose, and neokestose.

Both the anion exchange and reversed phase HPLC profiles of the samples of beet medium invert sugar and pyrolysed beet sucrose are similar (Figs 1 and 3). Confirmation that the two sets of oligomers are the same would be obtained by performing the two chromatographic techniques, which have different solute selectivities, in series. Reversed phase HPLC fractionation of the two samples and analysis of the fractions by anion exchange HPLC was therefore carried out. Figures 4 and 5 show the chromatogram of the reversed phase run used for the fractionation and the anion exchange chromatograms of selected fractions for the beet medium invert sugar and pyrolysed beet sucrose respectively.

The anion exchange HPLC chromatograms of the fractions obtained from the two samples are similar. Fraction 4 of the beet medium invert sugar samples contains three components, peaks 7, 11, and 15, but in the pyrolysed beet sucrose fraction only two peaks can be seen, 7 and 11. Fraction 5 of both samples contains four peaks, 7, 12, 15, and 18. The absence of peak 15 in fraction 4 of the pyrolysed beet sucrose sample is attributed to slight displacement of the peaks/fractions. In the fractionations, peak *c* was collected in fraction 6 of the beet medium invert sugar sample and in fraction 7 of the pyrolysed sucrose. These two fractions, peak *c*, contain peaks 7, 12, and 18 and beet medium invert sugar fraction 6 also contains peak 15. Peak 18 can be seen in fraction 9, which contains reversed phase peak *d*, of both the beet medium invert sugar and pyrolysed beet sucrose samples. Peak 16 is also present in fraction 9 of the pyrolysed sucrose sample. Although with the large sucrose peak in the reversed phase chromatogram of the beet medium invert sugar it is not possible to see the additional two peaks, one before and one after sucrose which appear as shoulders in the pyrolysed sucrose chromatogram, the same oligosaccharides are present in the two samples as shown by anion exchange HPLC analysis of the fractions.

The reversed phase HPLC fractionation of beet

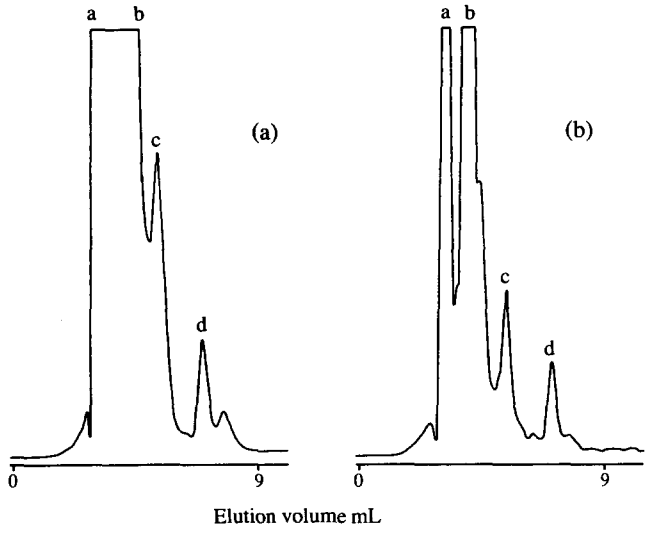

Fig. 3. Reversed phase HPLC chromatograms of an 11·9-mg sample of beet medium invert sugar (a) and 0·8 mg pyrolysed sucrose (b).

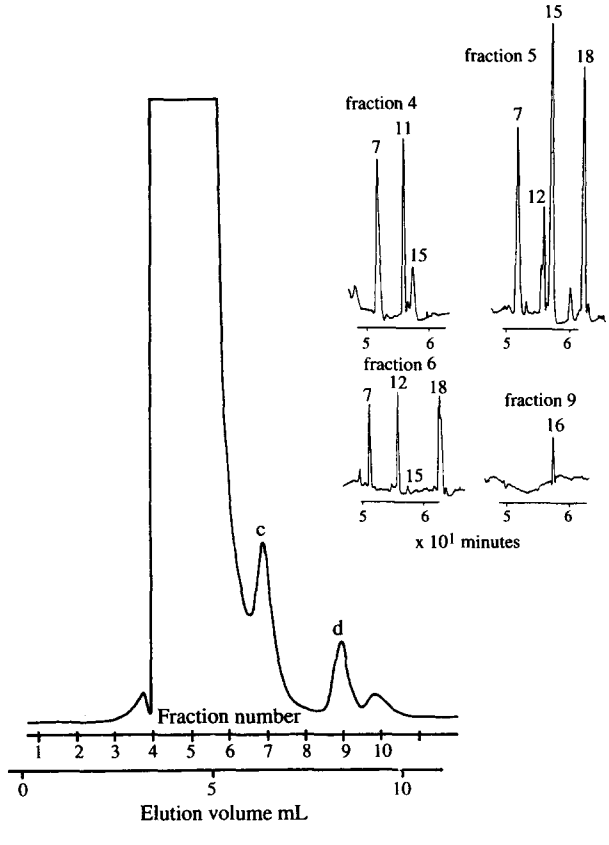

Fig. 4. Reversed phase HPLC fractionation of beet medium invert sugar and anion exchange HPLC analysis of fractions 4, 5, 6, and 9.

medium invert sugar and pyrolysed sucrose and subsequent analysis of the fractions by anion exchange HPLC have confirmed the similarity in the oligosaccharide profiles of the two samples. Although the oligomeric content is lower in the beet medium invert

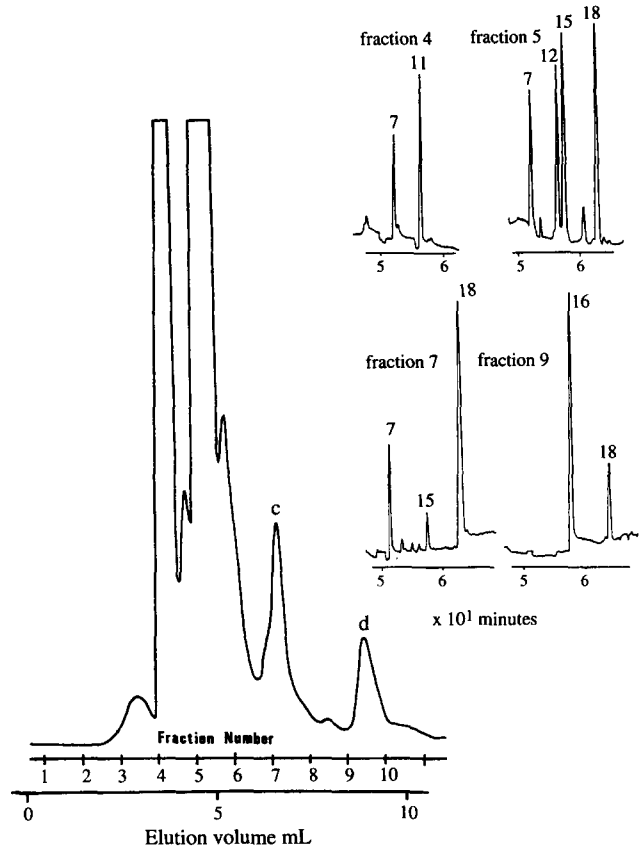

Fig. 5. Reversed phase HPLC fractionation of pyrolysed sucrose and anion exchange HPLC analysis of fractions 4, 5, 7, and 9.

sugar sample compared to the pyrolysed beet sucrose, the increased loading of the beet medium invert sugar, 11·9 mg compared with 0·8 mg, enabled identification of all the oligosaccharides to be achieved.

The chromatographic data so far obtained indicates that the fingerprint oligosaccharides in beet medium invert sugar are also present in a sample of pyrolysed beet sucrose. As the major products of sucrose pyrolysis are the trisaccharides, kestoses and *iso*-kestoses, then these are most likely the oligosaccharide components in beet medium invert sugar. If this is indeed the case then it should be possible to fractionate them from the three major carbohydrates present in beet medium invert sugar, *D*-glucose, *D*-fructose, and sucrose, by gel filtration chromatography, a technique in which solutes are separated based on differences in their size in solution—hydrodynamic volume.

Much work has been reported on the analysis of oligosaccharides using gel filtration chromatography (Kennedy *et al.*, 1988, 1989). With high resolution small pore packings such as the BioGel P2 material it is possible to resolve, *inter alia*, mono-, di-, and trisaccharides. Where the oligosaccharides are of the same chemical type and with the same glycosidic linkage, the elution order is related to the oligomer size. In a homologous series, the larger the oligomer the earlier it elutes

from the column. However, if the oligosaccharides have the same number of repeat units and are of the same chemical composition, but have different glycosidic linkages, then they may not co-elute. Therefore, unless the column is calibrated with oligosaccharide standards of the same chemical type and glycosidic linkage as the solutes to be characterised, it is not possible to determine the molecular weight or degree of polymerisation of an unknown. However, if the column is calibrated with a homologous series of oligomers, then it is at least possible to get an indication of the degree of polymerisation of an unknown oligosaccharide.

In order to attempt to obtain an indication of the size of oligosaccharides in beet medium invert sugar, i.e. if there is indeed a trisaccharide component which could be the kestoses and *iso*-kestoses, a sample was analysed by gel filtration chromatography. Figure 6 shows the gel filtration chromatogram of a sample of acid hydrolysed starch, where the peak numbers refer to the degree of polymerisation (DP), peak 1, 2, and 3 being the mono-, di-, and tri-saccharides, respectively, and superimposed is the trace of the beet medium invert sugar. Since the three main carbohydrates in the sample of beet medium invert sugar are *D*-glucose, *D*-fructose, and sucrose, peak *a* will be the two monosaccharides, *D*-glucose and *D*-fructose, and peak *b* the disaccharide sucrose, their elution positions being very similar to peaks 1 and 2 in the sample of hydrolysed starch. In the beet medium invert sugar chromatogram there is also a shoulder on the leading edge of peak *b*. This could be a trisaccharide component, the elution position being very similar to peak 3 in the hydrolysed starch chromatogram.

By fractionating the beet medium invert sugar sample using gel filtration chromatography and analysing the fraction using anion exchange HPLC it was possible to establish if the 'trisaccharide' component is composed of the fingerprint oligosaccharides. The gel filtration fractionation and anion exchange HPLC analysis of fractions 6 and 7 are shown in Fig. 7. All the fractions from the gel filtration separation were analysed, but the beet medium invert sugar fingerprint oligosaccharides

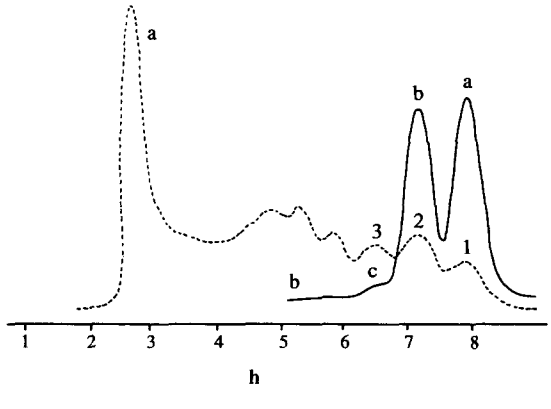

Fig. 6. Gel filtration chromatograms of a sample of acid hydrolysed starch - - - - (a) and beet medium invert sugar ——— (b).

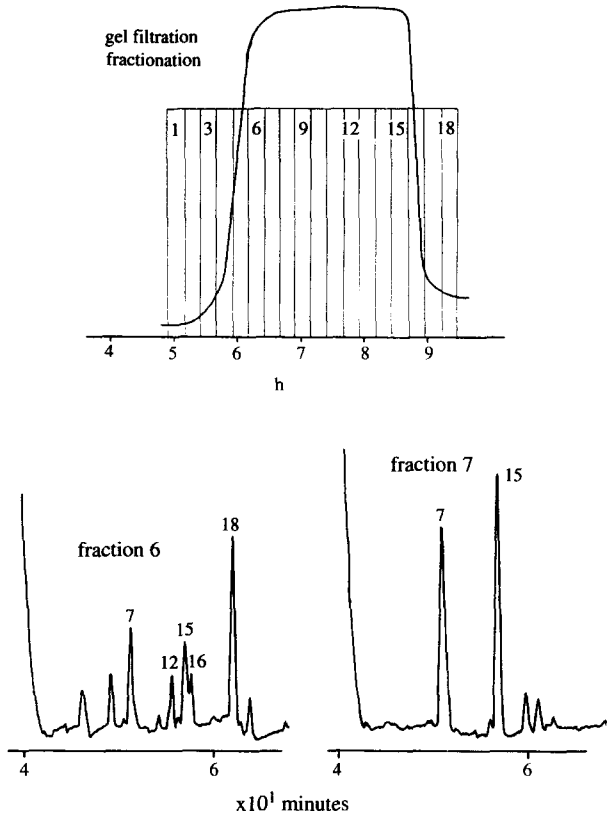

Fig. 7. Gel filtration fractionation of beet medium invert sugar and anion exchange HPLC analysis of fractions 6 and 7.

were only evident in fractions 6, 7, and to a lesser extent 8. All the fingerprint oligosaccharides are contained in the oligosaccharide component which elutes at the starch DP3 oligomer position when analysed by gel filtration chromatography.

The chromatographic data so far obtained indicate that the fingerprint oligosaccharides in the anion exchange HPLC chromatogram of beet medium invert sugar are non-reducing trisaccharides. They do not degrade in alkali to produce saccharinic acids and they elute at the starch DP3 position in gel filtration chromatography. Both the anion exchange and reversed phase HPLC profiles of beet medium invert sugar are similar to those of pyrolysed sucrose. These oligosaccharides could therefore be the kestoses and/or *iso*-kestoses produced by either enzymic or chemical transfructosylation.

If these oligosaccharides are the kestoses and if they are present in beet medium invert sugar due to the fructotransferase activity of the yeast invertase EC 3.2.1.26, which may be used to partially invert the beet sucrose, then they would be the *β-D*-isomers, the kestoses. The enzyme action is stereospecific, producing only the *β-D*-isomers and not the *α-D*-anomers, the *iso*-kestoses, but if the kestoses were produced by chemical means, acid inversion and/or pyrolysis of sucrose during the concentration of beet medium invert sugar after inversion, then

both the β-*D*- and α-*D*-isomers would be produced. Chemical transfructosylation is not stereospecific.

It is possible to differentiate between the two sets of anomeric isomers, β-*D*- and α-*D*-, by the action of the enzyme invertase (β-*D*-fructofuranoside) EC 3.2.1.26. This enzyme, in dilute substrate solutions, hydrolyses the terminal non-reducing β-*D*-fructofuranoside residue in a β-*D*-fructofuranoside. It does not hydrolyse α-*D*-fructofuranosides. It should therefore be possible to distinguish between the kestoses and *iso*-kestoses, the kestoses being trisaccharides with the *D*-fructose moiety, being β-linked to sucrose at one of the primary hydroxyl positions, and the *iso*-kestoses being their α-anomers.

The action of invertase EC 3.2.1.26 on both 1- and 6-kestose is sequential, cleaving first at position A and then at position B, Fig. 2, to give two molecules of *D*-fructose and one of *D*-glucose. The products are the same when neokestose is cleaved by invertase EC 3.2.1.26, but in this case the glycosidic linkages are simultaneously hydrolysed at positions A and B. The α-anomers, *iso*-1-kestose and *iso*-6-kestose, are not cleaved by invertase EC 3.2.1.26, but in the case of *iso*-neokestose invertase EC 3.2.1.26 will cleave the glycosidic link at position B, producing *D*-fructose and the disaccharide *O*-α-*D*-fructofuranosyl-(2-6)-α-*D*-glucose. In summary, if the sample contains six oligosaccharides, 1-kestose, 6-kestose, neokestose, *iso*-1-kestose, *iso*-6-kestose, and *iso*-neokestose, then the products of the action of invertase EC 3.2.1.26 would be *D*-fructose, *D*-glucose, *iso*-1-kestose, *iso*-6-kestose, and the disaccharide *O*-α-*D*-fructofuranosyl-(2-6)-α-*D*-glucose.

Samples of beet medium invert sugar and pyrolysed beet sucrose were incubated with invertase EC 3.2.1.26 to identify if any of the oligosaccharides had a terminal β-*D*-fructofuranoside residue. The anion exchange HPLC chromatograms of beet medium invert sugar before and after invertase EC 3.2.1.26 treatment are shown in Fig. 8. After invertase EC 3.2.1.26 treatment only three peaks remained, 11, 15, and 18. The height of peak 15 had increased. The oligosaccharides corresponding to peaks 7, 12, and 16 must therefore have a terminal β-*D*-fructofuranoside residue, as they are no longer evident after the enzymic incubation. Figure 9, the anion exchange HPLC chromatograms of pyrolysed sucrose before and after incubation with invertase EC 3.2.1.26, showed the same effects.

From this enzymic study it was concluded that the same oligosaccharides are present in beet medium invert sugar and pyrolysed sucrose. Peaks 11 and 18 are the oligosaccharides which do not have a terminal β-*D*-fructofuranosyl residue, *iso*-1-kestose and *iso*-6-kestose, and peaks 7, 12, 15, and 16 are the kestoses which are susceptible to enzymic action, 1-kestose, 6-kestose, neokestose and *iso*-neokestose. The increase in the height of peak 15 was tentatively attributed to this also being the elution position of the disaccharide product of invertase EC 3.2.1.26 action on *iso*-neokestose. The

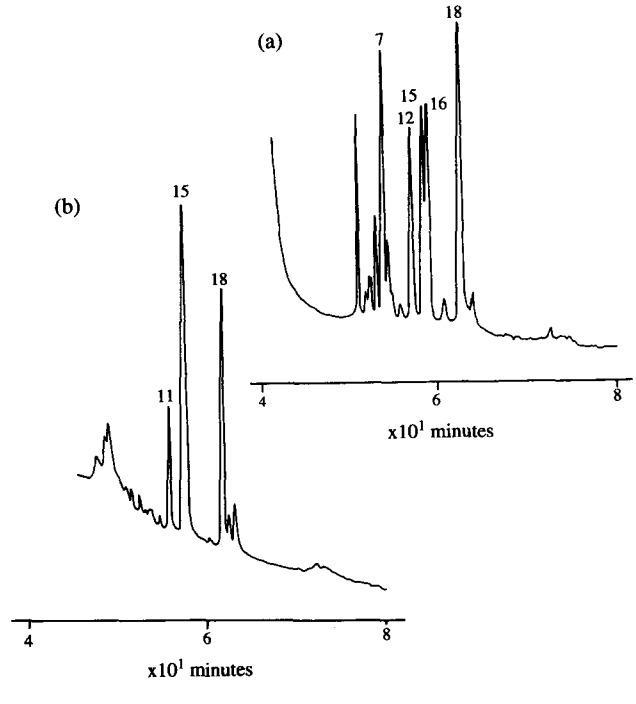

Fig. 8. Anion exchange HPLC chromatograms of a sample of beet medium invert sugar before (a) and after (b) incubation with invertase EC 3.2.1.26.

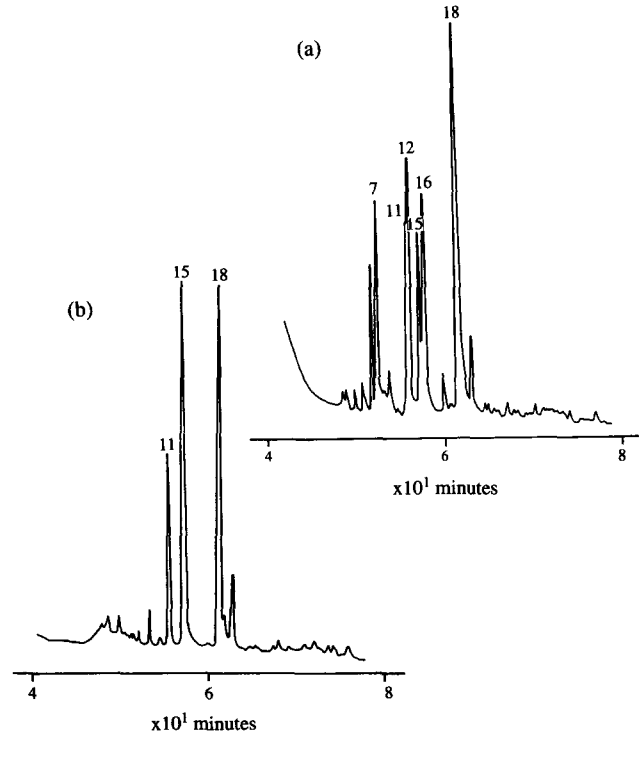

Fig. 9. Anion exchange HPLC chromatograms of a sample of pyrolysed sucrose before (a) and after (b) incubation with invertase EC 3.2.1.26.

generous gift of reference samples of the kestoses and *iso*-kestose enabled confirmation of the peak assignments to be made: peak 7 = 1-kestose, peak 11 = 6-kestose, peak 12 = *iso*-1-kestose, peak 15 = *iso*-neokestose and the disaccharide *O*-α-*D*-fructofuranosyl-(2-6)-α-*D*-glucose, peak 16 = neokestose, and peak 18 = *iso*-6-kestose.

Therefore the fingerprint oligosaccharides in the anion exchange HPLC chromatogram of beet medium invert sugar are trisaccharides, kestoses and *iso*-kestoses, formed by transfructosylation, i.e. the addition of a fructosyl residue to one of the three sucrose primary hydroxyls. As both the β-*D*- and α-*D*-isomers are present, this must be a chemical, rather than enzymic, process.

Information on the identity of the oligosaccharides in beet medium invert sugar has been published by Swallow, K.W. & Low, N.H., 1993. (*J. Agric. Food Chem.*, **41**, 1587) and correlates with the information in this paper.

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