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High-performance liquid chromatographic determination of sugars and polyols in extracts of lichens and sugarcane juice

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ABSTRACT

Several polyols and monosaccharides can be efficiently separated from standard mixtures by high-performance liquid chromatography. Separation is carried out under isocratic conditions using acetonitrile–water (80:20, v/v) as mobile phase. Detection is performed by measurement of UV absorbance at 195 nm, which improves the sensitivity of the method. Linearity for both polyols and sugars is obtained over a wide range of concentrations. The method was applied to the determination of the polyol content of two lichen extracts, obtained from *Evernia prunastri* and *Himantormia lugubris*. Xylose, arabinose and galactose were identified, for the first time, in crude juices from sugarcane, in addition to the well known fructose, glucose and sucrose. Two main fractions of soluble polysaccharides could also be separated from the same juice and analysed, after acidic hydrolysis, as being composed of fructose and galactitol.

INTRODUCTION

The assessment of the carbohydrate and polyhydric alcohol content of plants, and the biotrophic transport of these products, is important in the analysis of several physiological processes. Many C3 [1] and several C4 grasses [29], such as sugarcane, produce fructans when sucrose biosynthesis and transport exceed the physiological demand. Biological production of fructans involves two main enzyme systems, sucrose–sucrose–fructosyl transferase (SST) and fructan–fructan–fructosyl transferase (FFT) [3], the products of which have sometimes been determined by high-performance liquid chromatography (HPLC). In contrast, hydrolysis products of fructans, achieved by several fructanases, are commonly detected by thin-layer or gas–liquid chromatography (GLC) [4]. These procedures are often used to analyse the sugar composition of plant extracts, such as sugarcane juices, in which only glucose and fructose appear as free monosaccharides [5].

Polyols are produced by many plant species but are predominantly accumulated in lichens [6]. A high proportion of all the carbon fixed by the algal partner passes to the fungus as a single type of molecule. This molecule is ribitol in lichens containing a green alga as photobiont or glucose in cyanobiont-containing lichens [7]. Moreover, studies using intact thalli show that most of the mobile carbohydrates are immediately converted to fungal polyols, especially mannitol and arabitol [8].

Several methods have been developed for studying carbohydrates and polyols

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and applied to several plant preparations. HPLC was first applied to the study of lichen metabolites to elucidate the derivatives of the acetate–polymalonate pathway [9]. Gordy *et al.* [10] developed an HPLC method for application to carbohydrates and polyols from lichens, using Bondapak and Aminex Q-150 S columns. MacFarlane and Kershaw [11] separated carbohydrates from several lichen species by using a Bio-Rad Labs. Aminex HPX-87C column. Similar methods have been applied to identify sucrose, glucose and fructose in sugarcane juices [5,12] and sugars produced after polysaccharide hydrolysis in many grasses. A refractive index detector, compatible only with isocratic elution, was always used.

In this paper, we report the determination of polyols and sugars by HPLC using a UV detector at 195 nm instead of a refractive index detector, improving the sensitivity of sugar determination.

EXPERIMENTAL

Plant material

Saccharum officinarum L., field-grown, *Evernia prunastri* (L.) Ach., growing on branches of *Quercus pyrenaica* Willd., and *Himantormia lugubris* (Hue) Lamb, growing on soil in King George Island (Antarctica), were used.

Sample preparation from sugarcane stalks

Stems of 17 month-old plants were mechanically crushed and the crude juice produced was adjusted to pH 8.0 by adding saturated ammonium carbonate solution. The juice was then centrifuged at 20 000 *g* for 15 min at room temperature and the supernatant was filtered through Whatman No. 3 filter-paper. Sodium azide was added to the filtrate to reach a final concentration of 0.02% (w/v). A 10-ml aliquot of this clarified juice was then filtered through a Sephadex G-10 column (15 cm × 2.5 cm I.D.), equilibrated at pH 8.0 with aqueous ammonium carbonate solution containing 0.02% sodium azide.

Calibration of this column was performed by filtering through it 5.0 ml of 0.02% (w/v) blue dextran 2000 and 10% (w/v) cobalt(II) chloride solution. Elution was carried out with distilled water. Fractions (1.0 ml) 1–20 were discarded. Fractions 21–32 ml were collected and stored to prepare a heterogenous mixture of fructans [13]. Low-molecular-weight sugars, including sucrose, were eluted from the Sephadex G-10 column from 42 to 58 ml of filtrate [14] and detected by the method of Dubois *et al.* [15]. This fraction was lyophilized and stored at −38°C. When required, residues were dissolved in 2.5 ml of acetonitrile–water (80:20, v/v) and loaded onto the chromatographic column. Fractions 20–32 ml were filtered through a Sephadex G-50 column (30 cm × 2.5 cm I.D.), equilibrated as above. Fractions 40–70 ml from this last column contained soluble polysaccharides (SP preparation), whereas mid-molecular-weight carbohydrates (MMWC preparation) eluted in fractions 70–120 ml.

Extraction of sugars and polyols from thalli of E. prunastri and H. lugubris

Air-dried thallus samples (2.0 g) were rehydrated at room temperature for 5 min with distilled water, gently dried with filter-paper and macerated with 20 ml of acetone to remove lichen phenols [16]. The dry residues were then ground with 10 ml of cold 80% ethanol, filtered through a double cheese-cloth and the filtrates stored at

−13°C for 14 h. The precipitates were then discarded and the supernatants heated at 60°C for 20 min. To an aliquot (5 ml) of clear supernatant, 5 ml of 80% cold ethanol were added and then heated again to dryness. This procedure was repeated three times under the same conditions as above. The last residues were reconstituted with 5.0 ml of cold 80% ethanol and centrifuged at 3000 *g* for 15 min. The supernatants were evaporated to dryness under reduced pressure [17]. Each residue was dissolved in 2.5 ml of acetonitrile–water (80:20, v/v); and loaded into the chromatographic column.

Reagents

Polyols (ribitol, arabitol, galactitol and mannitol) and sugars (D-xylose, D-arabinose, D-ribose, D-fructose, D-galactose, D-glucose, D-mannose, D-rhamnose and sucrose) were provided from Sigma (St. Louis, MO, U.S.A.). Acetonitrile (HPLC grade) (Carlo Erba, Milan, Italy) was used as received and doubly distilled water (Carlo Erba) was filtered through Millipore GS filters (0.22-μm pore diameter) before use.

A Micropack NH_2 column (Varian, Palo Alto, CA, U.S.A.), supplied in hexane for use in normal-phase chromatography, was prepared for sugar analysis employing the following series of solvents: 20 ml of isopropanol at a flow-rate of 1.0 ml min^{-1}, 20 ml of distilled water at 1.0 ml min^{-1}, 60 ml of 0.1 *M* H_3PO_4 (pH 1.5) at 2.0 ml min^{-1}. The column was finally washed with pure acetonitrile and kept in this solvent until required.

HPLC separation of sugars and polyols

HPLC was performed on a Varian Model 5060 liquid chromatograph equipped with a Varichrom TM VUV/10 UV detector and a Vista CDS 401 computer. The chromatographic conditions were as follows: column, MicroPack NH_2 10 P/N (30 cm × 3 mm I.D.) from Varian; sample loading, 10 μ; mobile phase, acetonitrile–water (80:20, v/v) isocratically or with a gradient where indicated; flow-rate 1.3 ml min^{-1}; temperature, 20°C; detector, UV (195 nm), 0.005 a.u.f.s.; attenuation, 16; internal standard, 2.0 mg ml^{-1} D-ribose when polyols were analysed and 2.0 mg ml^{-1} ribitol when sugars were analysed.

RESULTS

Separation of polyols

The main problem in the separation and determination of polyols from lichens is the difficulty of a clear resolution between enantiomers. In this work, the complete separation of the enantiomers ribitol and arabitol, and also mannitol, was achieved using a MicroPak NH_2 10 P/N column, packed with 10-μm silica which has a chemically bonded phase containing aminopropyl groups. The polar alkylamine stationary phase, which has strong hydrogen-bonding properties, separates sugars by a partition mechanism. Reconversion of the normal-phase column by the use of the solvent series described above permits this column to be used in the reversed-phase mode. This allows the use of acetonitrile as a component of the mobile phase, which improves sugar separations by decreasing the polarity of the mobile phase and, as a consequence, increasing the sugar retention.

Complications arise from the use of the detector at 195 nm, as there is a slight baseline shift for several reasons. The use of a wavelength of 195 nm, 0.005 a.u.f.s. and acetonitrile–water as the mobile phase implies very extreme conditions of analysis. This also produces a large background absorbance. Therefore, even with an isocratic procedure, baseline correction must be applied. This is constructed from the start of the first peak to the lowest valley point at the end of the same peak and, in this way, after a baseline segment has been constructed, the area of the peak is corrected. The result is stored in a time and area file and then the next baseline segment is calculated for the included peaks. Each successive baseline segment starts at the end of the preceding one, to include all the peaks. A signal-to-noise ratio of 1.0 is included in the program for the method, which allows the detection of very small positive peaks representing sugar alcohols. Under these conditions, stability of the response is higher than that obtained by using only water as solvent for separating sugars in the normal-phase mode.

Analysis for ribitol, arabitol and mannitol is complete in less than 13 min, although the complete elution procedure for the next injection needs about 30 min (Fig. 1). The calibration graphs shown in Fig. 2 indicate that the goodness of fit is

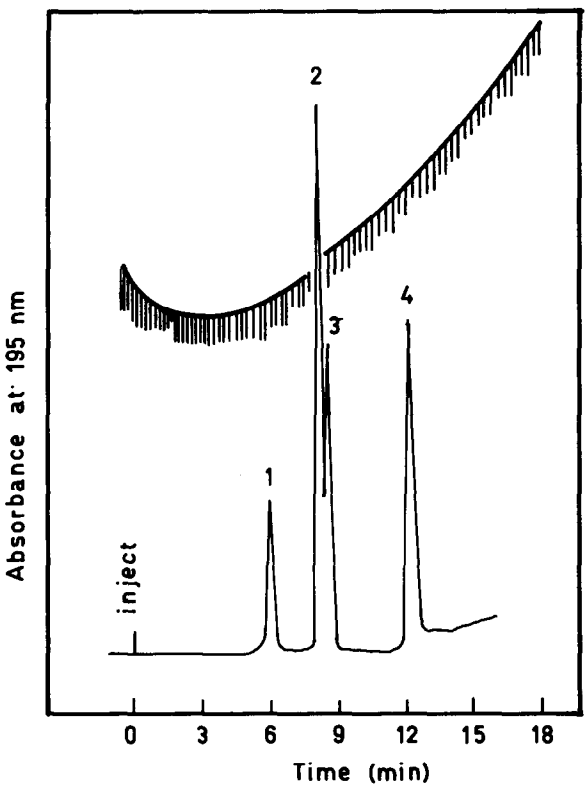

Fig. 1. HPLC separation of a standard mixture of 10 μg each of D-ribose, ribitol, arabitol and mannitol on a MicroPak NH_2-10 P/N column. Solvent, acetonitrile–water (80:20, v/v), isocratic. Flow-rate, 1.3 ml min^{-1}. Peaks: 1 = D-ribose as internal standard; 2 = ribitol; 3 = arabitol; 4 = mannitol. The line drawn across the chromatogram indicates baseline correction.

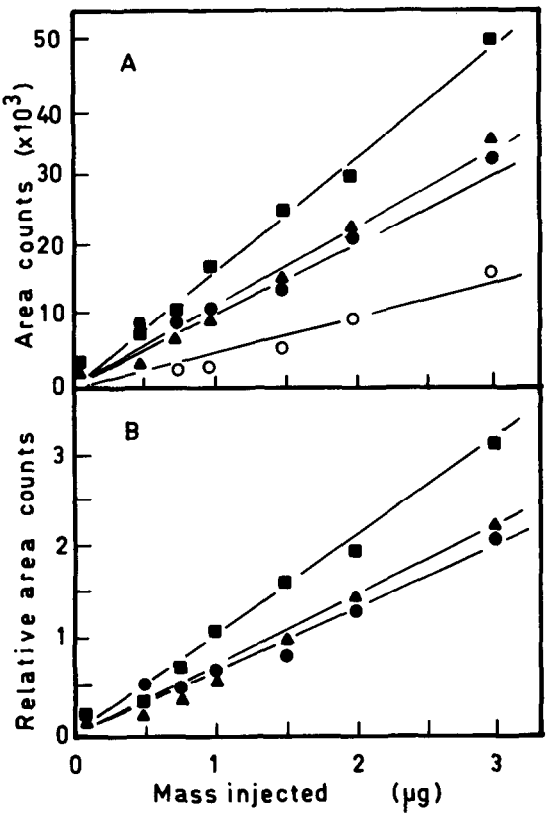

Fig. 2. Calibration lines of sugar alcohols by HPLC. Chromatographic conditions as in Fig. 1. (A) Direct calibration of (○) D-ribose, $y = 6.10x - 3.24$, $r^2 = 0.99$; (●) ribitol; $y = 10.36x + 0.15$, $r^2 = 0.99$; (▲) arabitol, $y = 11.59x - 1.04$; $r^2 = 0.99$; and (■) mannitol, $y = 11.85x - 0.006$, $r^2 = 0.99$. (B) Calibration with respect to an internal standard (2.0 mg ml^{-1} D-ribose) of (●) ribitol, $y = 0.67x + 0.008$, $r^2 = 0.99$, (▲) arabitol, $y = 0.75x - 0.07$, $r^2 = 0.99$ and (■) mannitol, $y = 1.03x - 0.005$, $r^2 = 0.99$. Data are the means of four replicates. The standard error was never larger than the symbols.

almost perfect and that the method is linear from at least 1.0 to 3.0 μg of each polyol injected. Equations fitted by linear regression have a determination coefficient of about 0.99 in all instances.

Gradient elution is often used to decrease the separation time and sharpen peaks, but adds complexity to the separation because of the column equilibration time and baseline offset, especially with low wavelength detection. Fig. 3 shows the chromatographic trace obtained by applying a linear gradient from 80:20 to 60:40 (v/v) acetonitrile–water in 30 min. The retention times of the three polyols decrease by *ca.* 0.4 min. Mannitol elutes 1.5 min earlier when gradient instead of isocratic elution is used.

The plate number (N) is *ca.* 4000. Maximum N values for ribitol (3850) and arabitol (4220) are obtained with an isocratic composition of the mobile phase, although the maximum plate number for mannitol (3823) is achieved with gradient

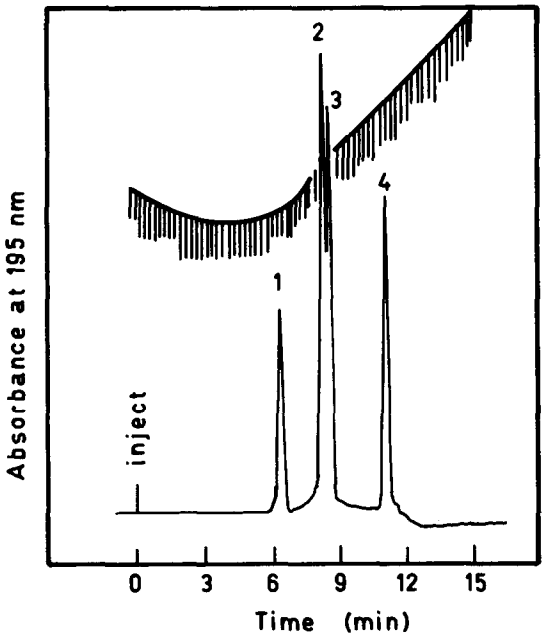

Fig. 3. HPLC separation of a mixture of 10 μg each of D-ribose, ribitol, arabitol and mannitol on the same column as in Fig. 1. The mixture was eluted from the column at 1.3 ml min^{-1} with a linear gradient from 80:20 to 60:40 (v/v) acetonitrile–water in 30 min. Peak assignments as in Fig. 1. The line drawn across the chromatogram indicates baseline correction in the gradient of acetonitrile–water.

elution. The resolution (R_s) is very high for mannitol. Values of R_s higher than 2 permit one to identify clearly the different components present in the mixture.

This HPLC method was applied to lichen samples by using thalli of *E. prunastri* and *H. lugubris* floated on distilled water for 1 h in the dark. Fig. 4 shows a chromatographic sequence to determine whether in fact the peak which eluted between 8 and 9 min was ribitol or arabitol when extracts from *E. prunastri* were analysed. When the sample was loaded with 2.0 mg of ribitol (Fig. 4B), the absorbance at 195 nm of peak a did not increase. It is clear that this peak is arabitol because, as is shown in Fig. 4C, its absorbance increased on loading the sample with 2.0 mg of arabitol. Fig. 4D shows the result of applying the same procedure but loading the sample with 2.0 mg of mannitol. In this instance the absorbance of peak m increased 1.5-fold with respect to that without any added mannitol, showing that this peak is mannitol.

Chromatographic separation of the different components of *H. lugubris* extracts revealed similar polyols to those found in *E. prunastri*. A mannitol peak was evident whereas ribitol hardly appeared and arabitol was absent from these extracts (data not shown).

The composition of polyols extracted from both lichen species is shown in Table I. The procedure was highly reproducible, with standard errors lower than 5.0%. Here, and also in the chromatograms from *E. prunastri* extracts, peaks corresponding to fructose and glucose appeared as the only representative hexoses.

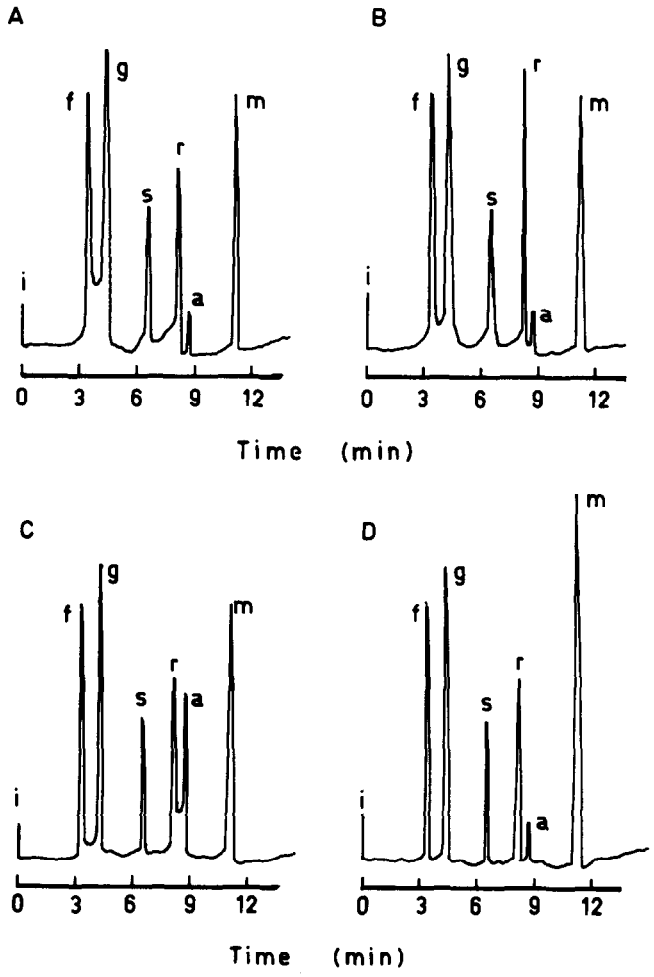

Fig. 4. (A) HPLC elution profile of an extract from the lichen *E. prunastri*. The column was eluted at 1.3 ml min^{-1} isocratically acetonitrile–water (80:20, v/v). Peaks: i = injection; f = fructose, g = glucose; s = D-ribose as internal standard; r = ribitol; a = arabitol; m = mannitol. (B), (C) and (D), similar profiles for extracts loaded with 2.0 mg of ribitol, arabitol and mannitol, respectively.

Separation of sugars

Unidentified peaks in both *Evernia* and *Himantormia* extracts were tested against chromatograms of several monosaccharides used as standards. By using the same procedure, and ribitol as internal standard, rhamnose, xylose, arabinose, fructose, mannose, glucose, galactose and sucrose were conveniently separated in less than 11 min (Fig. 5). The calibration lines, shown in Fig. 6 for four representative sugars, indicate that the method is linear over the range 1.0–100 μg injected.

Peaks with retention time of 3.2 and 4.3 min in the chromatographic trace of lichen extracts can be identified as fructose and glucose, respectively (Fig. 4A). On loading the lichen extracts with 2.0 mg ml^{-1} of fructose, only the peak with a reten-

TABLE I

QUANTITATIVE COMPOSITION OF POLYOL FRACTION ISOLATED FROM *EVERNIA PRUNASTRI* AND *HIMANTORMIA LUGUBRIS* THALLI AND ANALYSED BY HPLC

Polyol	Polyol content[a] (mg g^{-1} dry thallus)	
	Evernia prunastri	*Himantormia lugubris*
Ribitol	33.7 ± 1.2	1.3 ± 0.05
Arabitol	2.1 ± 0.08	n.d.[b]
Mannitol	1.6 ± 0.07	1.1 ± 0.04

[a] ± Standard error of four replicates.
[b] Not detected.

tion time of 3.24 min increases, whereas an increase in the second peak, with a retention time 4.3 min, only occurs when the sample is loaded with 2.0 mg ml^{-1} of glucose (data not shown).

It is to be expected that other plant extracts will show a more complex chromatographic behaviour on the basis of the well known contents of different sugars. The extract defined as the low-molecular-weight fraction obtained from sugarcane juices

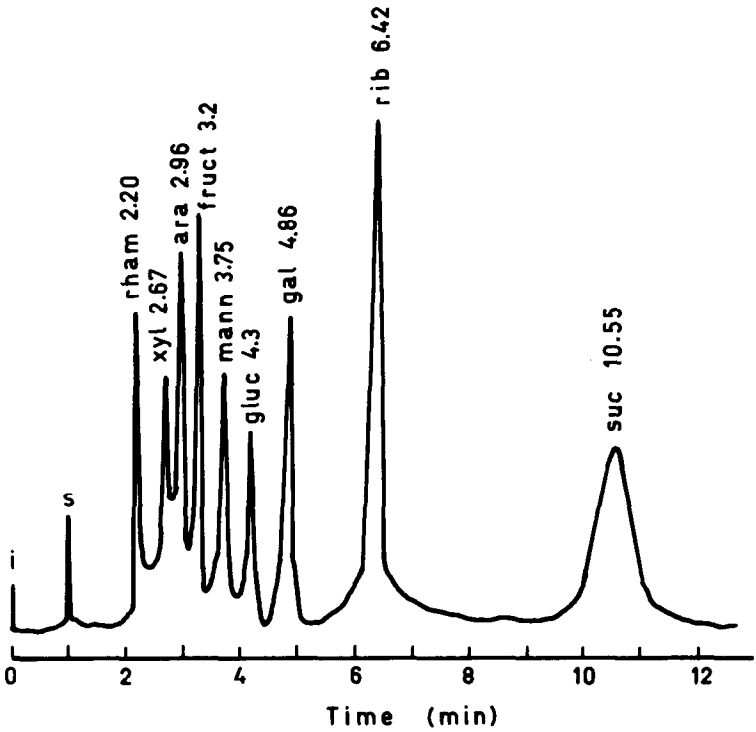

Fig. 5. HPLC separation of a standard mixture of D-rhamnose, D-xylose, D-arabinose, D-fructose, D-glucose, D-galactose, D-ribose and sucrose (2.0 mg of each in 1.0 ml of mobile phase). The column was eluted at 1.3 ml min^{-1} isocratically with acetonitrile–water (80:20, v/v).

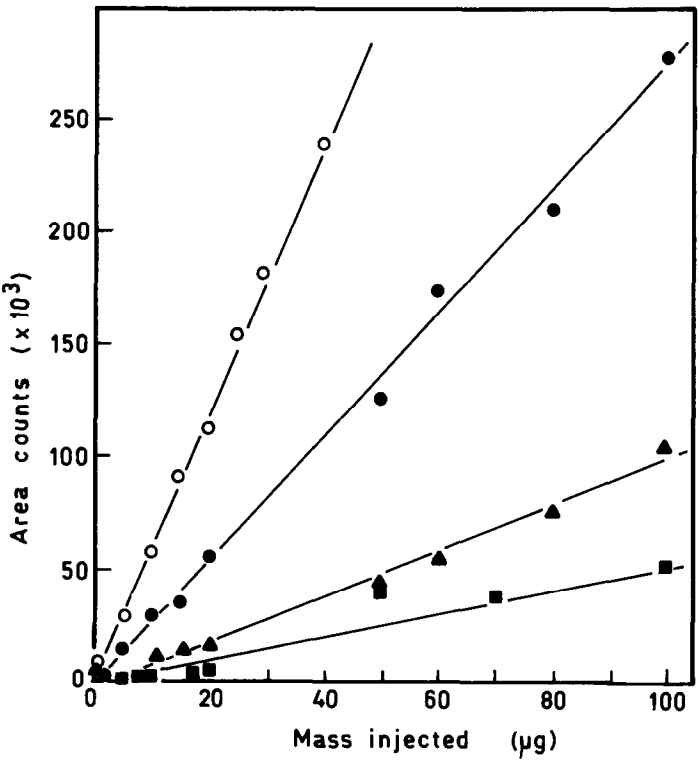

Fig. 6. Calibration lines for four representative monosaccharides from the standard mixture. Chromatographic conditions as in Fig. 1. Direct calibration of (○) ribose, $y = 5.9x - 4.88$, $r^2 = 0.99$, (●) fructose, $y = 2.73x - 3.12$, $r^2 = 0.99$, (▲) galactose, $y = 0.99x - 3.26$, $r^2 = 0.98$ and (■) glucose, $y = 0.56x - 3.66$, $r^2 = 0.98$. Data are the means of four replicates. The standard error was never larger than the symbols.

TABLE II

QUANTITATIVE COMPOSITION OF LOW-MOLECULAR-WEIGHT FRACTION ISOLATED FROM SUGARCANE JUICE BY FILTERING IT THROUGH SEPHADEX G-10 AND ANALYSED BY HPLC

Sugar	Concentration ($\mu g\ ml^{-1}$)	Percentage of the fraction	Percentage of the total juice
Xylose	4.87 ± 0.46	7.23	1.95
Arabinose	6.12 ± 0.54	9.08	2.45
Galactose	4.12 ± 0.39	6.11	1.65
Fructose	3.18 ± 0.42	4.71	1.27
Glucose	2.66 ± 0.19	3.95	1.06
Sucrose	18.51 ± 1.64	27.50	7.43
Totals	39.46	58.58	15.81

[a] ± Standard error of four replicates.

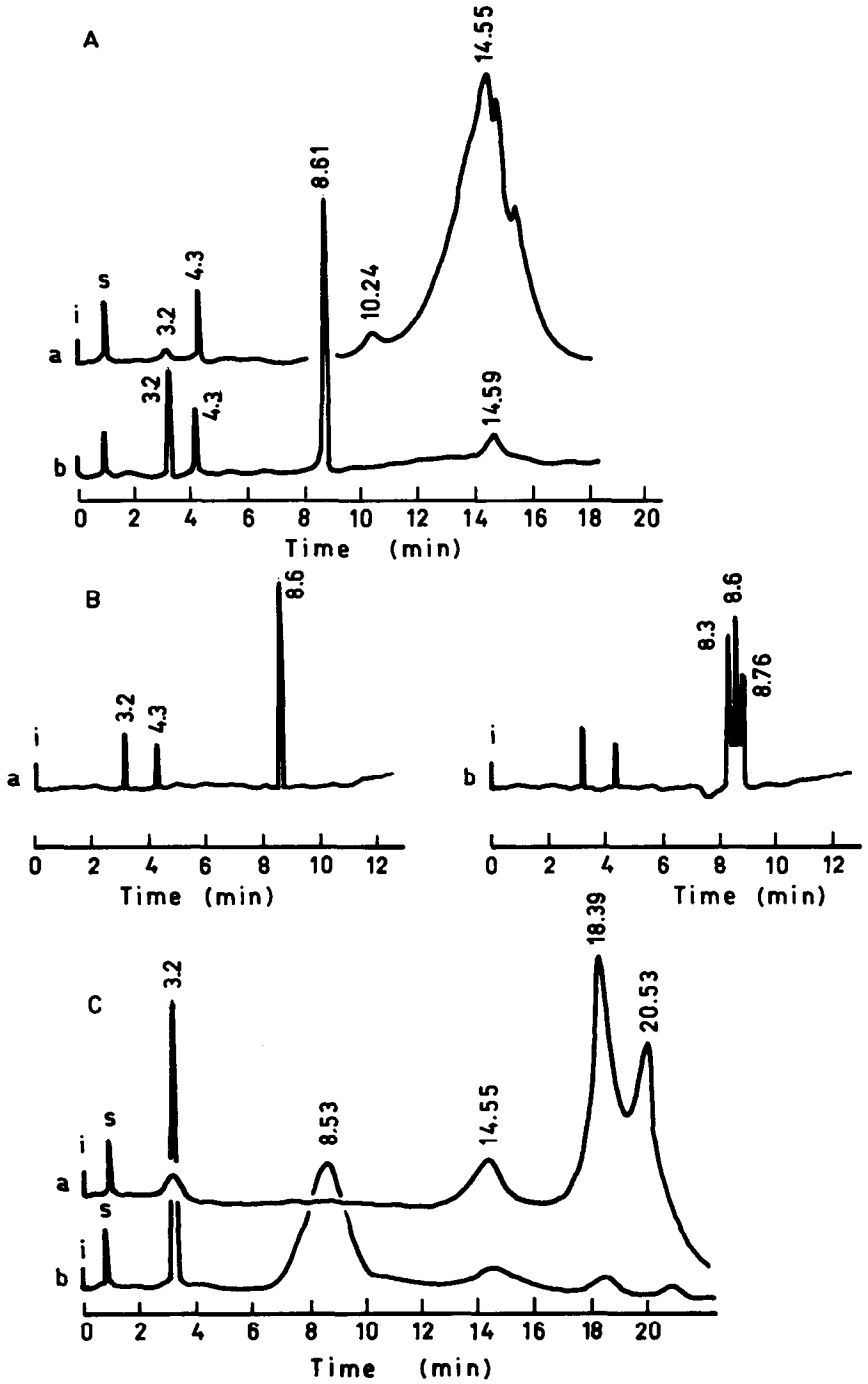

Fig. 7. (A) HPLC elution profiles of MMWC fraction isolated by Sephadex G-10 and G-50 filtration of clarified sugarcane juice (a) before and (b) after acidic hydrolysis. Peaks: i = injection; s = solvent. Numbers on the peak are retention times (min). (B) HPLC elution profiles of a hydrolysate of MMWC loaded with (a) 2.0 mg ml^{-1} of galactitol or (b) 2.0 mg ml^{-1} of ribitol or arabitol. (C) HPLC elution profiles of SP fraction isolated by Sephadex G-10 and G-50 filtration of clarified sugarcane juice (a) before and (b) after acidic hydrolysis.

as described under Experimental gave peaks with retention times of 2.20, 2.67, 3.2, 4.3, 4.86 and 10.55 min. On loading the samples with 2.0 mg ml^{-1} of the different standards, fructose [retention time (t_R) = 3.2 min], glucose (t_R = 4.3 min) and sucrose (t_R = 10.55 min) were identified. Xylose (t_R = 2.2 min), arabinose (t_R = 2.63 min) and galactose (t_R = 4.91 min) were identified by using the same procedure. Although sucrose represent about 27.5% (dry weight) in this fraction, xylose, arabinose and galactose are the main monosaccharide forms (Table II).

Nature of sugarcane juice polysaccharides

The filtrate from the Sephadex G-50 column defined as the MMWC fraction contains traces of glucose, fructose and sucrose [not more than 0.67% (dry weight) of each] and a large peak with a retention time of 14.55 min (Fig. 7A, trace a). This peak possibly represents the major polysaccharide form in this fraction. In contrast, the filtrate defined as SP seemed to be composed of a heterogeneous pool of polymeric forms, with retention times of about 14.55, 18.4 and 20.63 min (Fig. 7C, trace a). Both the SP and MMWC fractions were then dried in air and hydrolysed with 6 *M* hydrochloric acid for 2 h at 60°C. The hydrolysates were dried in air to remove the acid and extracted as described above. The residues were dissolved in a sufficient volume of acetonitrile–water (80:20, v/v) to give a final concentration of 1.0 mg ml^{-1} dry residue and analysed by HPLC.

The peak with a retention time of 14.55 min disappeared after acidic hydrolysis of MMWC and, simultaneously, the amount of fructose increased and a new peak appeared with a retention time of *ca.* 8.6 min (Fig. 7B, trace a), which was tentatively identified as galactitol. By loading the sample with 2.0 mg ml^{-1} of different polyols and sugars, this peak only increased when galactitol was added (Fig. 7B, trace b). The SP fraction, after acidic hydrolysis, was found to be composed only of galactitol and fructose (Fig. 7C, trace b).

DISCUSSION

Many techniques have been described previously for sugar and polyol analysis. However, HPLC allows extremely accurate detection and determination. The decision about the column to be used is complex and requires the evaluation of various factors. In general, non-destructive detection (UV), inexpensive solvents, short retention times and high sensitivity of the detector can be advantageous.

Over the last decade, a number of HPLC methods for separating reducing sugars have been reported, although there are few methods for separating polyols [18–20]. Even using HPLC procedures, MacFarlane and Kershaw [11] emphasized the difficulty in the separation of mannitol and arabitol, perhaps because the refractive index detector used did not permit gradient elution. In previous papers [17,21], we reported the separation of ribitol, arabitol and mannitol as trimethylsilyl derivatives by GLC, although ribitol and arabitol were eluted as a single peak.

We have reported here the complete separation of ribitol from mannitol and arabitol with R_s values of *ca.* 4 under isocratic conditions, and the use of a linear gradient decreased the R_s values by 1 unit. In addition, the use of a UV detector substantially improved the determination and the linearity of the detector response. A convenient separation in the isocratic mode was also achieved for sugars. Isocratic

separation of mono- and oligosaccharides by HPLC have also been reported previously for plant extracts [22], medical formulations [23] and several hexosamines [24]. Fructose and glucose, identified by this procedure in both *E. prunastri* and *H. lugubris* extracts, are the main components of the monosaccharide fraction of many lichen species [11].

Arabinose, xylose and galactose have been detected in sugarcane juice as normal constituents of low-molecular-weight sugars separated by filtration of clarified juice through a Sephadex G-10 column (Table II). Their concentrations are higher than those of both glucose and fructose but, obviously, lower than that of sucrose. Glucose and fructose have been described as normal components of fresh crushed juice [5,12] and raw sugar [25]. In the latter, xylose also appears to be a constant constituent. However, there is no report of the occurrence of both arabinose and galactose in crude juice as free monosaccharides, although they are the main products of hydrolysis (analysed by GLC) of high-molecular-weight polysaccharides, defined as arabinogalactans [26]. Hence it is possible that both monosaccharides were produced by hydrolysis during the processing of sugarcane stalks or juice. In spite of this, other polysaccharides have been detected without any significant hydrolysis. Therefore, it is unlikely that a single polysaccharide can be completely hydrolysed during juice storage whereas others are not. In addition, separation of monosaccharides by HPLC from sugarcane juices, reported previously, did not indicate components other than glucose and fructose because only water was used as the mobile phase at a high flow-rate (higher than 2.0 ml min^{-1}) to ensure a good resolution of sucrose [5]. Under these conditions, many peaks eluting before 6 min can be superimposed on that of glucose and fructose. Probably the use of acetonitrile as a component of the mobile phase improves the separation of sugars by increasing their retention.

The fractions defined here as MMWC contain a main polysaccharide which is composed of fructose and galactitol, as was revealed by acidic hydrolysis. A small amount of sucrose contaminates these fractions eluted from Sephadex G-50, and it is responsible for the formation of glucose after hydrolysis. However, the amount of fructose that appears in the hydrolysate cannot be derived only from sucrose. Hence a large amount of this fructose must be produced from polysaccharide hydrolysis. In any case, the quantitative decrease of the peak with a retention time of 14.55 min confirms that this hydrolysis has been achieved.

An unexpected result of this hydrolysis was the production of a compound that has been identified as a polyol. Production of polyols, such as mannitol, has previously been reported from deteriorated sugarcane juices [5], but the simultaneous production of both ethanol and lactic acid clearly indicates that the juices contain products of a fermentation process. These juices were completely different from those, freshly produced, used in this work. However, the peak of galactitol was perfectly identified as it was the only peak that increased after loading the sample with exogenous galactitol and, in addition, the identity of the products of hydrolysis of both MMWC and SP are very significant. It is possible that the resistance to enzymatic hydrolysis of a fraction of juice polysaccharides when bacterial dextranases or glucanases are used [27] can be achieved by including a polyol in the polymeric sequence.

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