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Note

Liquid chromatography of non-reducing oligosaccharides: a new detection principle

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In the liquid chromatography (LC) of natural saccharide-containing samples, detection systems based on a chemical reaction between carbohydrates leaving the column and an appropriate detection reagent are more specific than conventional LC detectors (differential refractometer or UV photometer operated at 190 nm) and often have greater sensitivity[1–3].

Universal carbohydrate detection reagents[4] capable of detecting non-reducing as well as reducing sugars are unfortunately highly corrosive (H_2SO_4 content) and cannot be delivered by most commercially available precise laboratory micropumps. The detection of reducing saccharides can be performed under milder reaction conditions using a wider choice of detection reagents[1,2,5,6].

We have therefore devised a new saccharide detection technique which is compatible with the chromatographic method of Scobell *et al.*[7]. In our system, non-reducing oligosaccharides are hydrolyzed by passing the effluent from the chromatographic column through a small reaction column packed with a strongly acidic cation exchanger (H^+) and subsequently detected as reducing saccharides. Reaction with *p*-hydroxybenzoic acid hydrazide (*p*-HBAH)[8] is used in this final detection step.

EXPERIMENTAL

Strongly acidic spherical cation-exchange resins, Ostion LG KS 0802 (14.6 ± 2.0 μm) and Ostion LG KS 0803 (particle size 17.1 ± 1.9 μm), designed especially for the amino acid analysis, were obtained from the United Chemical and Metallurgical Works (Ústí nad Labem, Czechoslovakia). Each resin was converted into the Ca^{2+} form on a glass sinter disc by a 20-fold excess of a 2 *N* solution of $CaCl_2$, washed with the same quantity of deionized water, suspended in a two-fold excess of deionized water and packed into the column as a slurry in water. The reaction column was filled with resin (H^+), prepared similarly using 2 *N* HCl instead of $CaCl_2$.

The detection reagent was prepared by mixing 10 parts of a 5% solution of *p*-HBAH in 0.5 *M* HCl with 57 parts of 0.75 *N* NaOH. The reagent was stored in a brown glass container under a slight pressure of nitrogen.

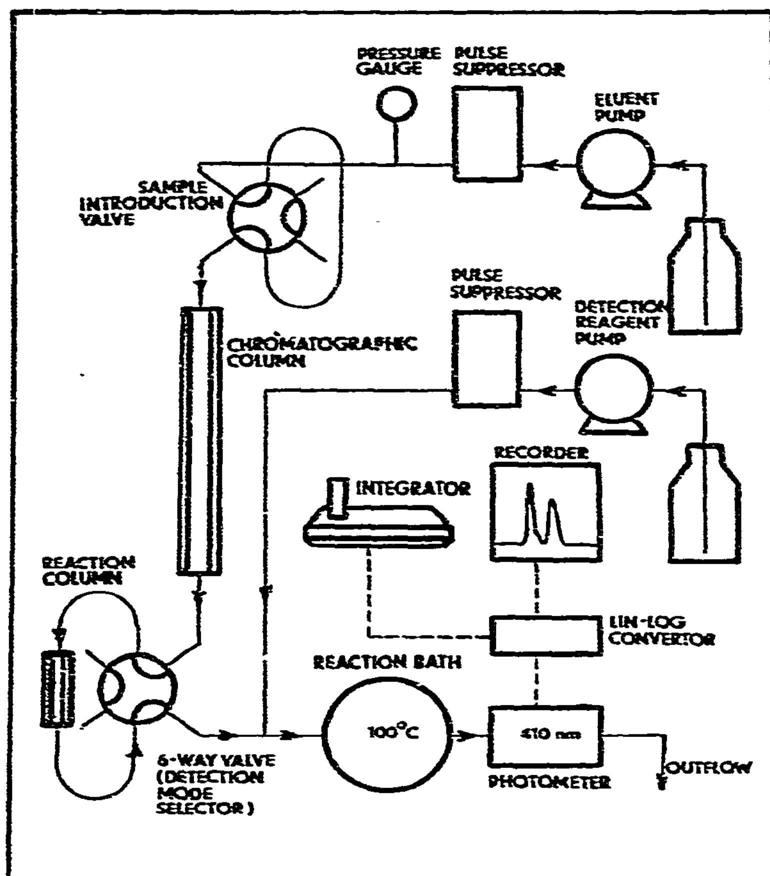

Fig. 1. Schematic diagram of the chromatographic system. Details are given under Experimental.

LC of sugars was carried out on a laboratory-assembled LC system shown in Fig. 1. A MC-300 micropump (Mikrotechna, Prague, Czechoslovakia) delivers deionized distilled water, used as column eluent, at a flow-rate of 35 ml/h via a pulse damper (Závody SNP, Žiar nad Hronom, Czechoslovakia) and a sample application valve (Mikrotechna) to a chromatographic column. The chromatographic column [63.0 × 0.8 cm, packed with Ostion LG KS 0803 (Ca^{2+})] was obtained from Mikrotechna as a standard accessory of a AAA 881 amino acid analyzer. The reaction column [5.0 × 0.6 cm, packed with Ostion LG KS 0802 (H^+)] was obtained from the same source and was originally used for the separation of basic amino acids. Both columns are jacketed and operated at 80° with a water-bath circulation system (not shown in Fig. 1).

The effluent from the chromatographic column may be either led into the reaction column or, by changing the position of the 6-port valve, may be directly mixed with the detection reagent. The effluent: reagent mixing ratio is 1:2. The intensity of colour developed in the flow-through capillary reactor (Mikrotechna),

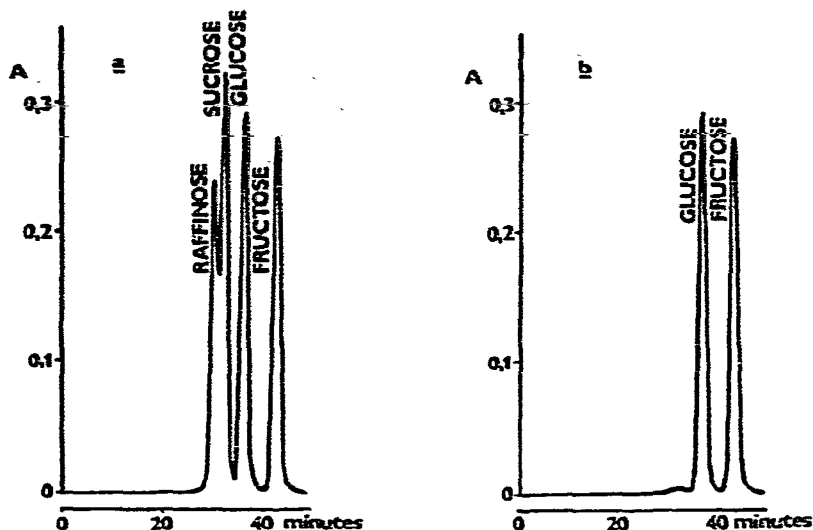

Fig. 2. Separation of a standard mixture of pure sugars in 24-μg quantities. a, Reaction column used; b, reaction column excluded. Operational conditions are given under Experimental and in Table I.

immersed in a bath of boiling water is measured by a filter photometer (Development Workshops, Czechoslovak Academy of Sciences, Prague, Czechoslovakia). The output of the photometer is amplified, linearized and integrated by a Minigrator integrator (Spectra-Physics, Palo Alto, Calif., U.S.A.).

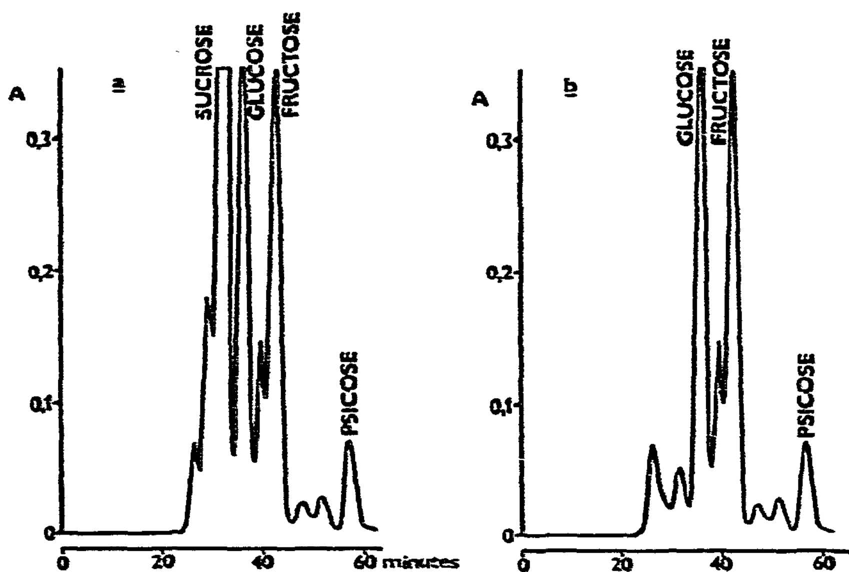

Fig. 3. Separation of a deionized sample of sugar cane molasses. a, Reaction column used; b, reaction column excluded. Operational conditions are given under Experimental and in Table I.

TABLE I

CHROMATOGRAPHIC EFFICIENCY OF THE SYSTEM

Chromatographic conditions: column (63 × 0.8 cm) filled with Ostion LG KS 0803 (Ca^{2+}); column temperature, 80°; flow-rate, 35 ml/h. Detection conditions: reaction column (5 × 0.6 cm) packed with Ostion LG KS 0802 (H^+); column temperature, 80°; residence time for unretained compounds, 48 sec. Capillary reaction coil: diameter, 0.7 mm; residence time, 1136 sec; temperature, 100°. k' = Capacity factor; H_c = plate height corrected for the peak spreading in the reaction coil; h_r = reduced plate height; n = efficiency of the column (plates); Δn = peak spreading in the reaction coil expressed as the loss of column efficiency.

Saccharide	k'	H_c (μm)	h_r	n	Δn (%)
Sucrose	0.16	194	11.3	3250	54.2
Glucose	0.37	214	12.5	2940	43.7
Fructose	0.67	174	10.2	3620	37.6

RESULTS AND DISCUSSION

The resolution attained using the described system is shown in Figs. 2 and 3. The separation is not significantly influenced by the reaction column (see Fig. 2), but the reaction coil of the flow-through reactor acts as an important source of peak spreading. Its contributions to the peak width were measured as described elsewhere[9] and are given in Table I.

The applied chromatographic method does not afford the separation of individual oligosaccharides characterized by the same degree of polymerization. The resolution of monosaccharides is more satisfactory. It was found that not one of fifteen tested monosaccharides interferes with glucose and only fucose and arabinose coelute with fructose. Figs. 1 and 3 illustrate both modes of the detector operation. This feature can be used to solve analytical problems which may otherwise require higher resolution, *e.g.*, the separation of glucose and fructose in the presence of a large excess of sucrose (Fig. 3) or the separation of a reducing disaccharide (maltose) in the presence of sucrose.

A comparison of the relative response of the detection for some common plant saccharides is presented in Table II. It is apparent that the reaction column is not equally effective for all tested non-reducing oligosaccharides. This may be attributed to the different degrees of penetration of oligosaccharide molecules into the resin and to the different resistances of the glycoside bonds of individual saccha-

TABLE II

RESPONSE OF THE DETECTION SYSTEM FOR VARIOUS SUGARS

Conditions as given in Table I.

Saccharide	*Relative peak area per one monosaccharide unit as (%) of the glucose peak area*	
	Reaction column excluded	*Reaction column used*
Fructose	101.9	103.4
Sucrose	0.0	97.4
Raffinose	0.0	67.4
Stachyose	0.0	40.4
Trehalose	0.0	*ca.* 1.2

rides to hydrolysis. In order to prevent the loss of the hydrolytic effect of the reaction column and taking into account the sensitivity of the reaction of *p*-HBAH with sugars to Ca^{2+} (ref. 8), it is advisable to run only deionized samples. When this precaution was taken and deionized water was used as the mobile phase, no loss of the hydrolytic effect was observed after 50 h of continuous use. Two identical reaction columns should be prepared, so that the exhausted one can be replaced without interrupting the operation of the chromatograph.

The relationship between the peak area and the amount of saccharide was linear providing the peak height did not exceed $A = 0.35$. The described detection system is characterized by a very smooth baseline, which facilitates the automated integration of peaks. With our present equipment the detection limit is *ca.* 0.5 μg of glucose.

To the best at our knowledge the proposed detection procedure is probably the only LC detection method employing a particle-filled column which plays an active role in the detection reaction. The method extends the applicability of present LC systems with post-column derivatization capabilities (especially of amino acid analysers) to the analysis of simple mixtures of non-reducing and reducing sugars, found in most samples of plant origin.

The presented column–detector combination lacks the advantage of some other methods suggested for sugar chromatography in that its selectivity cannot be improved by changing the mobile phase composition. However, the selectivity and the resolution can be influenced by changing the ionic form of the chromatographic column packing[10] or the temperature of the column. For example, a partial resolution of glucose anomers is possible at a lower column temperature (25°).

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