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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR SUGAR ANALYSIS OF CRUDE DEPROTEINIZED EXTRACTS OF NEEDLES OF AIR-POLLUTED HEALTHY AND DAMAGED *PICEA* TREES

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SUMMARY

An automated sugar analyser is described that involves anion-exchange chromatography and fluorimetric detection. It alows the determination of reducing sugars and neutral polysaccharides after on-line post-column hydrolysis with *p*-toluenesulphonic acid. The method was developed for the analysis of unpurified deproteinized *Picea* needles from air polluted areas. The methodology and preliminary results are presented.

INTRODUCTION

During the past 20 years, considerable advances have been made in the automatic analysis of sugars by column chromatography with ion-exchange resins, thanks particularly to the fundamental methodological work of Jonsson and Samuelson[1]. At present the two most favoured methods for sugar analysis are the column chromatography of sugar–borate complexes[2] on anion-exchange resins and the analysis of free sugars on both cation- and anion-exchange resins by partition chromatography. Sharp separations of complex mixtures have become possible in a relatively short time, owing especially to considerable improvements in the quality of anion- and cation-exchange resins that are now commercially available.

Refractive index measurements have been utilized for the detection of carbohydrates, particularly in high-performance liquid chromatographic (HPLC) methods. However, owing to the large amounts of sample required, several post-column derivatization methods have been proposed[3–8], but most employ strong acids, so the equipment used should be acid resistant. Moreover, strongly acidic wastes may cause laboratory pollution. Nevertheless, particular mention must be made of a recent study[9] based on solid-state post-column hydrolysis on a strong cation exchanger, thereby avoiding the use of an extra reagent pump and permitting the detection of non-reducing and reducing oligo- and monosaccharides.

A method using ethanolamine in boric acid has been published[10] which allows fluorimetric detection at the nanomole level. The method is appicable only to the determination of reducing sugars. We have now adapted this method to allow also

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the detection of carbohydrates that do not give positive reactions but that can liberate reducing sugars after hydrolysis. In this paper we present details of a new sugar analyser system and the methodology for the on-line hydrolysis with *p*-toluenesulphonic acid of di-, tri- and polysaccarides to allow post-column fluorimetric detection and its application to the analysis of crude deproteinized samples of *Picea* needles from air-polluted areas.

EXPERIMENTAL

Chemicals

p-Toluene sulphonic acid was obtained from Fluka (Buchs, Switzerland). All other chemicals used for the preparation of buffers and reagents were obtained in the highest purity from Merck (Darmstadt, F.R.G.). Sugars were obtained from Fluka and Merck.

Sample preparation

Needles of seemingly healthy and damaged *Picea* trees were collected from an air-polluted area in the Vosges region (Massif du Donon, France). They were processed as described elsewhere[11].

Instrumentation and chromatographic conditions

The instrumentation used in the separation of sugars on the anion-exchange chromatographic system is depicted in Fig. 1. The system parameters are listed below:

Resin. Aminex A-25 anion exchanger, 17.5 μm (Bio-Rad Labs., Richmond, CA, U.S.A.), borate form.

Column. 350 × 4 mm I.D., glass, with water-jacket (68 ± 0.1°C).

Buffer. Degassed 0.4 *M* boric acid adjusted to pH 9.35 with 45% potassium hydroxide solution containing 2% of acetic acid.

Reagents. (1) Degassed 2 *N* *p*-toluenesulphonic acid. (2) To a mixture of 20 g of boric acid and 20 g of ethanolamine was added doubly glass distilled water to make 1 l of fluorescence reagent (stable for 1 week at room temperature).

Pumps. Precision piston pumps (Dosapro minipump, Milton Roy, Philadelphia, PA, U.S.A.).

Flow-rates. Buffer, 18 ml/h; *p*-toluenesulphonic acid, 7 ml/h; fluorescence reagent, 20 ml/h.

Reaction bath. 125°C (ethylene glycol monomethyl ether).

Tubing. PTFE, 0.3 mm I.D. and 1.6 mm O.D., was used for all the chromatographic system. The hydrolysis coil was 10 m long and the fluorescence developing coil was 20 m long. Both were immersed in the reaction bath (125°C).

Sample injector. An automatic sample injector (APE-1, Kontron, Zurich, Switzerland) for eight samples was used. Each of the eight sample coils was calibrated to hold 0.1 ml.

Detector. An RF-530 fluorimeter (Shimadzu, Kyoto, Japan) with a 12-μl volume square quartz flow cell was used. The monochromators were fixed at 342 nm (excitation) and 422 nm (emission).

Column back-pressure. 20 kg/cm^2.

Quantitation and automation. An SP 4200 integrator (Spectra-Physics, Les Ulis,

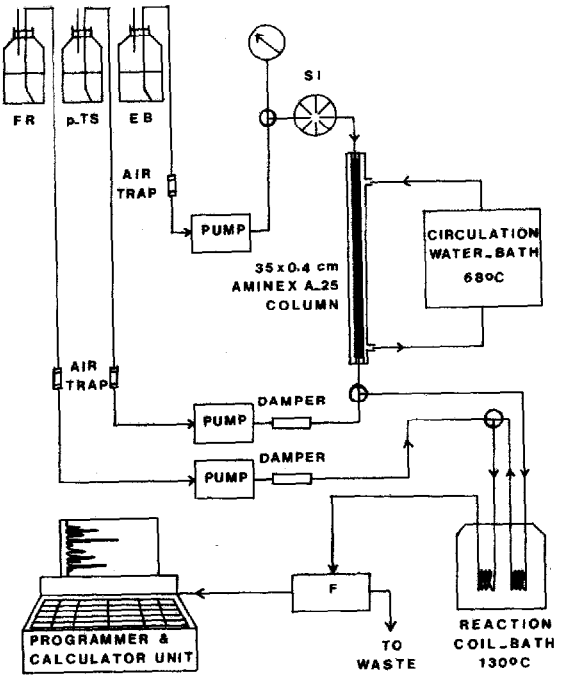

Fig. 1. Diagram of the automatic sugar analyser. Milton Roy precision piston pumps were employed. EB = elution buffer; *p*-TS = 2 N *p*-toluenesulphonic acid solution; FR = fluorescence reagent; SI = sample injector; F = fluorimeter.

France) was coupled to the fluorimeter for quantification by the internal standard method using xylose, which is not present in *Picea*. The six timed event external control cable of the integrator was used to automate the analytical system.

RESULTS AND DISCUSSION

Two series of chromatographic analyses were carried out: in the first, direct post-column fluorimetric detection of the eluates was applied, and in the second, the *p*-toluenesulphonic acid hydrolysis step was introduced before the fluorimetric detection step.

The chromatograms obtained with a standard mixture containing raffinose (2.5 nmol), saccharose (2.5 nmol), maltose, ribose, fructose and glucose (5 nmol each), and xylose (10 nmol, internal standard) are shown in Fig. 2A (without *p*-toluenesulphonic acid) and Fig. 2B (using the *p*-toluenesulphonic acid hydrolysis step). Table I shows the retention times and the surface areas of the peaks of the different sugars analysed.

The chromatograms obtained with crude, deproteinized extracts of needles of seemingly healthy and damaged *Picea* trees from air-polluted areas are shown in Fig. 3 (without the *p*-toluenesulphonic acid step) and Fig. 4 (using *p*-toluenesulphonic acid).

The chromatographic separation method presented here resolves well the mix-

TABLE I

RETENTION TIME AND EFFECT OF *p*-TOLUENESULPHONIC ACID ON FLUORESCENCE RESPONSE OF THE SUGARS ANALYSED

Values given are the average of ten determinations of a standard mixture of sugars. The system is quite stable and small fluctuations in the flow-rates of the reagents hardly affect the fluorescence intensity. Consequently, the assay is highly reproducible and the accuracy and precision are comparable to that obtained with sugar analysers (*i.e.* less than 3%). Surface areas are in arbitrary units.

Compound	*Abbreviation*	*Direct analysis without p-toluenesulphonic acid step*		*Analysis using p-toluene-sulphonic acid step*		*Action of p-toluene-sulphonic acid on fluorescence reactions: increase (%)*
		Retention time (min)	*Surface area of peaks*	*Retention time (min)*	*Surface area of peaks*	
Raffinoose	Raf	–	–	15.2	111 620	–
Saccharose	Sac	–	–	16.4	120 383	–
Maltose	Mal	16.3	39 520	17.8	85 316	116
Ribose	Rib	22.1	32 507	23.8	60 130	85
Fructose	Fru	25.5	91 529	27.2	104 813	14
Xylose	Xyl	28.5	126 583	30.3	181 487	43
Glucose	Glc	34.0	70 878	35.8	87 443	23

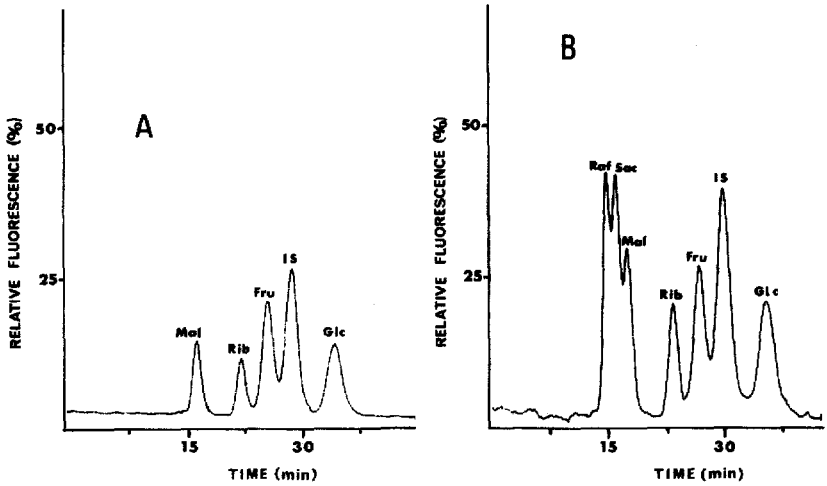

Fig. 2. Chromatographic analysis of a standard mixture of sugars. (A) Without *p*-toluenesulphonic acid step; (B) with *p*-toluenesulphonic acid step. Note the increase in the fluorescence response of sugars in the presence of *p*-toluenesulphonic acid. Abbreviations as in Table I.

ture of sugars used in this assay. Ion-exchange chromatography of sugars, based on sugar–borate complexation, has been employed extensively in industrial, biomedical and biochemical research and a considerable literature exists on this subject[12–16]. As the method has a high tolerance to salts and organic contaminants, raw samples can be injected directly after deproteinization without further treatment[17–19]. This is a significant advantage over other chromatographic techniques such as partition chromatography in ethanol–water, gas chromatography and classical HPLC, where considerable efforts are expended in sample purification, desalting and derivative formation[20–22]. The method presented here has retained all the advantages described above and has been considerably, simplified by the use of only one column temperature and one buffer for the chromatographic separation of seven sugars. This is largely satisfactory for the analysis of *Picea* samples that contain only saccharose, fructose and glucose. The addition of acetic acid to the borate buffer increases the symmetry of the peaks and makes them sharper. Complete analysis is achieved in 40 min.

However, the most interesting aspect of our work is the development of a post-column chromatographic detection system that allows not only the quantification of reducing sugars but also that of di-, tri- and polysaccharides, which are not able to react directly with the reagent used. Of course, the polysaccharide must contain in its structure at least one reducing monosaccharide. As can be seen in Fig. 1, *p*-toluenesulphonic acid is added to the column eluate using a pump. The mixture is then heated to hydrolyse the polysaccharide by passing it through a 10 m × 0.3 mm I.D. PTFE tube immersed in the reaction bath thermostated at 125°C. Thereafter, the fluorescence reagent, delivered by another pump, was added and the total mixture was again heated to develop the fluorophore by passing it through a 20 m × 0.3 mm I.D. PTFE tube immersed in the same reaction bath. Preliminary assays have shown positive results with saccharose, raffinose and trehalose. Reducing sugars, when heat-

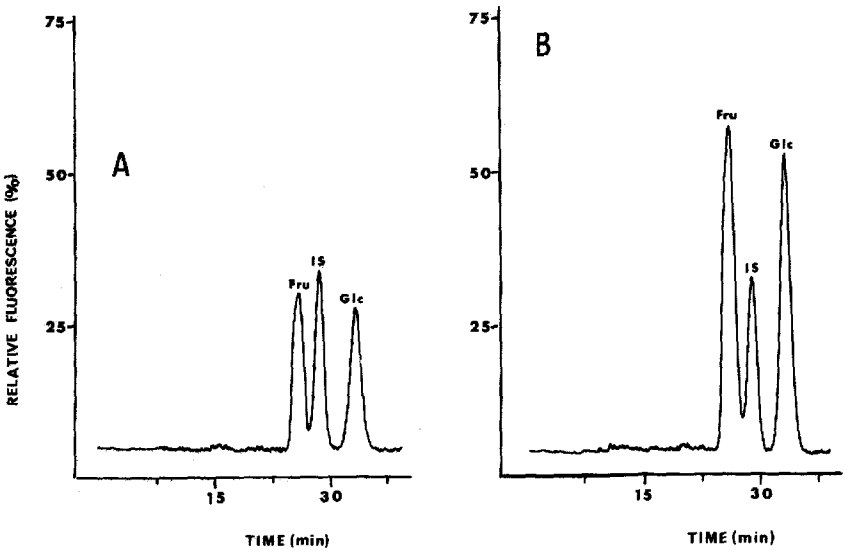

Fig. 3. Chromatographic analysis of crude extracts of *Picea* needles. The *p*-toluenesulphonic acid hydrolysis step was omitted. (A) Extracts of needles of healthy *Picea*; (B) extracts of needles of damaged *Picea*. Abbreviations as in Table I.

ed in the presence of *p*-toluenesulphonic acid, increased its fluorimetric response, as shown in Table I. Compare also Fig. 2A and B; no band broadening was observed and the high reproducibility of the total system as reported earlier[10] was maintained.

The use of ethanolamine–boric acid as a fluorogenic reagent for sugars has

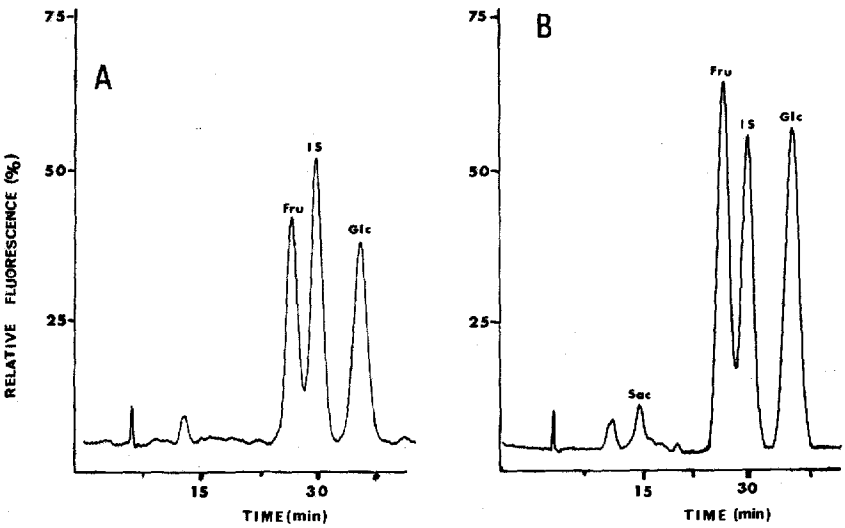

Fig. 4. Chromatographic analysis of crude extracts of *Picea* needles using the *p*-toluenesulphonic acid hydrolysis step. (A) Extracts of needles of healthy *Picea*; (B) extracts of needles of damaged *Picea*. Abbreviations as in Table I.

already been extensively studied[10]. The reaction is mild and rapid and in addition the reagents have little hazardous effect. Concerning the *p*-toluenesulphonic acid, a final concentration between 0.3 and 0.4 *M* seems to give the best results both for the hydrolysis of polysaccharides and for increasing the fluorimetric response effect of the reducing monosaccharides. Work is in progress in our laboratory to complete the optimization of all parameters of the total reaction sequence and to increase the number of compounds that can be analysed by this method. At present this method is being used for the comparative analysis of the sugar contents of healthy and damaged polluted *Picea* trees. Preliminary results showed that damaged trees accumulate free fructose and glucose, whereas the contrary was noted in healthy trees (see Figs. 3 and 4). This could indicate an abnormal situation in the biosynthesis and metabolism of polysaccharides caused by the physiological effects of air pollution. A similar situation has been reported for Scots pine subjected to fluoride and sulphur dioxide pollution[23].

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