Journal of Chromatography, 553 (1991) 255–263
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2086

Selective post-column fluorigenic reaction with benzamidine for trace level detection of reducing saccharides in liquid chromatography

A. COQUET*, J.-L. VEUTHEY and W. HAERDI

Department of Inorganic, Analytical and Applied Chemistry, Sciences II, University of Geneva, 30 Quai E. Ansermet, 1211 Geneva 4 (Switzerland)

ABSTRACT

A liquid chromatographic post-column fluorigenic reaction with benzamidine for the trace level detection of reducing saccharides is described. The system consists of a Sugar Pak-1 gel column (300 mm × 6.5 mm I.D.) packed with a microparticulate resin in the calcium form and heated at 70°C. Elution is carried out with water containing 10^{-4} *M* CaEDTA at 0.4 ml min^{-1}. The post-column reaction is carried out with a mixture of 30 m*M* benzamidine and 1 *M* potassium hydroxide solution at 1 ml min^{-1} using a 530-μl reaction coil thermostated at 100°C. Fluorescence was monitored at 470 nm (emission) with an excitation wavelength of 360 nm. The system had good reproducibility (relative standard deviation 1–3%) for an injected amount of 0.9 μg (5 nmol). Linear calibration graphs were obtained between 16 and 500 nmol ml^{-1} ($r = 0.999$) and detection limits of 16–63 pmol at a signal-to-noise ratio of 3 were achieved. The detection of saccharides in plants is also described.

The separation and determination of saccharides is of considerable importance in diverse fields such as biology, biochemistry, medicine and food science. Liquid chromatography (LC) is the most commonly used technique for the analysis and purification [1] of simple monosaccharide mixtures and oligosaccharides. Different chromatographic techniques such as weak anion-exchange, reversed-phase and ligand-exchange modes have been used for this purpose and have been reviewed recently [2–5].

Detection of saccharides in the column effluent is commonly effected using either refractive index (RI) or ultraviolet (UV) detection. These types of detection are used when the analyte is present at a high concentration (micromole range) and if relatively little interfering material is present. RI detection is adequate for many classes of samples but the lack of selectivity and the low sensitivity of this detection system often require extensive sample clean-up. Further, strict control of the solvent composition (no elution gradients are possible) and of physical parameters (pressure, temperature and flow-rate) is necessary in order to avoid background noise. UV detection is generally carried out at 190 nm, but saccharides absorb only weakly at this wavelength and any impurities in the sample or the solvent with a strong chromophore may interfere. Consequently, UV detection has many of the same limitations as RI detection.

Another type of universal detector, based on mass detection, has been developed for carbohydrate analysis. This detector measures light scattering from a nebulized solute after solvent evaporation [6]. It can, in contrast to an RI detector, be used with solvent gradients but is not yet commonly found in most laboratories. Finally, in addition to these methods for direct detection, high-performance anion-exchange chromatography coupled with pulsed amperometric detection was developed in order to detect carbohydrates by electrochemical oxidation using a gold working electrode [7]. This method is more precise, sensitive (picomole range) and selective than RI detection.

The usefulness and sensitivity of conventional detectors based on UV absorption or fluorescence can be greatly increased by using pre- or post-column derivatization. For instance, pre-column derivatization with fluorigenic reagents (dansylhydrazine [8] or aminopyridine [9]) allows sensitive detection (10 pmol range) and is very useful for high-performance liquid chromatography (HPLC) on reversed-phase C_{18} or unmodified silica columns.

However, most studies have been performed with post-column reactors and many procedures have been published. Reducing sugars may be determined by post-column derivatization using a number of different fluorigenic reagents: 2-cyanoacetamide [10,11], ethanolamine [12], 2-aminoethylsulphonic acid (taurine) [13], 2-aminopropionitrile [14], arginine [15], ethylenediamine [16] and *meso*-1,2-bis(4-methoxyphenyl)ethylenediamine [17]. These reagents react with reducing carbohydrates in an alkaline or neutral medium at 100–150°C to produce fluorescent derivatives; the advantages of these reagents have been described elsewhere [18]. With similar view, Kai *et al.* [19] recently developed a fluorimetric determination of reducing sugars by HPLC using *p*-methoxybenzamidine as a fluorigenic reagent in the post-column derivatization system.

Another aromatic amidine, benzamidine, can be used to determine reducing carbohydrates under alkaline conditions [20]. This compound has good selectivity for reducing saccharides in complex mixtures as it does not react with seventeen L-α-amino acids, aldehydes, keto-acids, carboxylic acids and nucleosides. Further, benzamidine reacts more rapidly with carbohydrates than other fluorigenic reagents, gives a higher fluorescence yield and is now commercially available.

The aims of this work were to study the use of benzamidine as a fluorigenic reagent for the detection of reducing saccharides such as D-glucose, D-galactose, D-fructose and lactose in a post-column derivatization system coupled to LC and the applicability of the proposed method to the determination of trace levels of sugars in plant extracts. In these natural matrices, monosaccharides are found at very low concentrations and interfering materials are present. Thus, sample preparation is necessary prior to the analysis in order to enrich and to clean up the sample.

EXPERIMENTAL

Chemicals

Benzamidine hydrochloride was obtained from Fluka (Buchs, Switzerland). All saccharides were obtained from Sigma (St. Louis, MO, U.S.A.). EDTA calcium disodium salt was obtained from Fluka and potassium hydroxide from Merck (Darmstadt, Germany). All aqueous solutions were prepared using doubly distilled, deionized water. The eluent was filtered and degassed in a Millipore filtration system (0.22 μm) before chromatographic analysis.

Apparatus

Chromatographic studies were performed with an LC-5000 liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) equipped with a Rheodyne 7125 six-way switching valve (50-μl loop). The column temperature was thermostated in a Varian CC1V Croco-cil column oven (Varian, Paris, France).

A Kontron (Zurich, Switzerland) Model 414-T pump was used for the post-column derivatization step.

Detection was performed by means of a Hitachi (Tokyo, Japan) Model F-1050 spectrofluorimeter. The fluorescence was monitored at excitation and emission maxima of 360 and 470 nm, respectively. Peak areas were calculated with a Hewlett-Packard (Palo Alto, CA, U.S.A.) Integrator Model 3380-A.

Procedure

Separations were carried out using a 300 × 6.5 mm I.D. gel column packed with a microparticulate resin in the calcium form (Waters–Millipore, Milford, MA, U.S.A.) and thermostated at 70°C. The aqueous mobile phase contained 10^{-4} *M* CaEDTA to regenerate calcium in the resin and was pumped at 0.4 ml min^{-1}. The separation of saccharides was achieved by a ligand-exchange mechanism on the column packed with a polymer-based cation-exchange material (sulphonated polystyrene–divinylbenzene). Unlike the elution order on silica-based columns, saccharides elute as a function of decreasing degree of polymerization. This effect is ascribed to a size-exclusion mechanism, which is assumed to take place in addition to a ligand-exchange effect. Calcium-loaded columns allow the separation of mono- and disaccharides and sugar alcohols with good selectivity. Such columns are also compatible with the presence of inorganic anions in the eluent or in the sample, and may be readily regenerated.

Post-column reaction conditions

The reagent was prepared by adding a 30 m*M* aqueous benzamidine solution to a 1.0 *M* solution of potassium hydroxide. A 530-μl reaction coil (PCR1; Varian) thermostated at 100°C was used for the post-column reaction. The post-column reagent flow-rate was 1.0 ml min^{-1}.

Sample pretreatment

Adult vegetative plants of *Sinapis alba* L. (mustard) were used. This material was used as the environmental conditions that induce flowering of the plant also change the concentration of the reducing carbohydrates present [21]. The tissues were sealed in glass tubes containing 10 ml of 80% ethanol in water per gram fresh weight. The tubes were then heated at 100°C for 15–30 min in a water-bath. Preliminary experiments showed that this method allows a complete extraction of glucose and fructose [21]. The extract was evaporated to dryness and the residue dissolved in distilled water.

Because of the presence of interfering materials in the aqueous extract, the following sample clean-up procedure was used. Solid-phase extraction columns (Analytichem International; trimethylaminopropylsilica SAX and cyclohexylsilica CH) were activated by washing with 4 ml of methanol and 4 ml of water. As carbohydrates are neither anionic nor non-polar, they pass through both sorbents unretained. Organic acids are too polar to be retained on the CH sorbent and so they pass onto the SAX, where they are retained. The phenols are retained on the non-polar CH sorbent. Following this clean-up procedure, a 50-μl aliquot of the carbohydrate extract was injected into the chromatograph.

RESULTS AND DISCUSSION

Optimization of the post-column reaction system

The conditions used for the separation and post-column derivatization were investigated by using four reducing saccharides: D-glucose, D-fructose, lactose and D-galactose. Reducing sugars were separated on a gel column packed with a microparticulate resin in the calcium form with water as the mobile phase. CaEDTA, used in the eluent to regenerate calcium on the resin, does not interfere with the post-column derivatization. The use of ligand exchange is an advantage in this separation system including a chemical post-column reaction as an aqueous effluent can be used.

It has been suggested [22] that the post-column derivatization reaction between benzamidine and reducing sugars involves the coupling of the keto alcoholic moiety of carbohydrates to the amidino group of benzamidine. This reaction is carried out in concentrated potassium hydroxide and yields a fluorescent imidazole derivative [22].

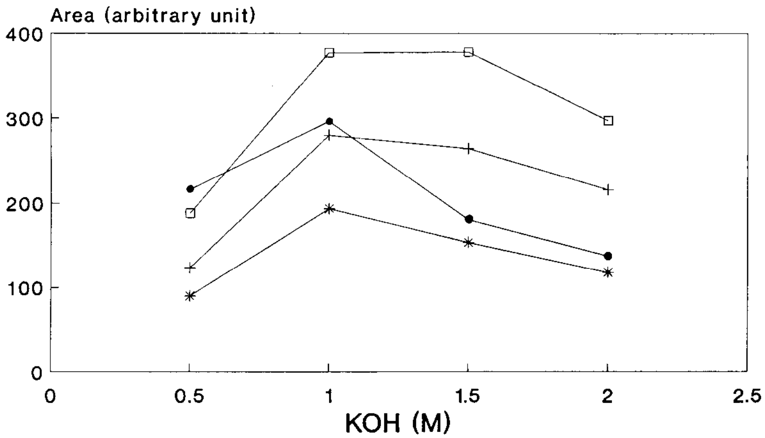

Fig. 1. Influence of potassium hydroxide concentration on the peak areas of four reducing carbohydrates. ● = Lactose; + = D-glucose; * = D-galactose; □ = D-fructose.

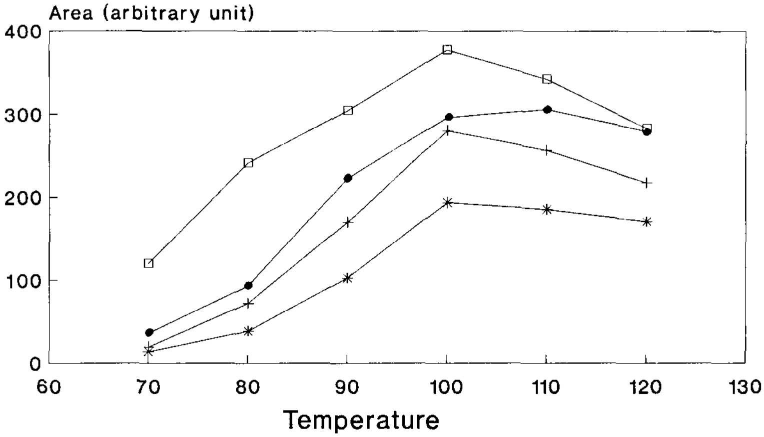

Fig. 2. Influence of reaction temperature (in °C) on the peak areas of four reducing carbohydrates. Symbols as in Fig. 1.

As potassium hydroxide is mixed with the column effluent, an important parameter to optimize is the concentration of this reagent in order to obtain the maximum fluorescence intensity. In Fig. 1, the influence of the concentration of potassium hydroxide is shown for four different saccharides. Maximum intensity was obtained using 1.0 *M* potassium hydroxide for all the reducing carbohydrates tested. D-Fructose was found to give the highest and D-galactose the lowest fluorescence intensity.

We also tested the influence of reaction temperature, as shown in Fig. 2. Optimum fluorescence was obtained at 100°C. Again, D-galactose shows the weakest response. It is important to note that even at lower temperatures, a good response can be obtained with ketohexoses such as D-fructose whereas only a very weak signal was obtained with aldohexoses.

Based on the above considerations, post-column derivatization of reducing sugars with benzamidine is best carried out at 100°C and in 1.0 *M* potassium hydroxide solution. These conditions were therefore used for all the experiments reported in this paper. The reaction time in chemical reaction detectors is determined by the flow-rates of the column effluent and the fluorigenic reagent and by the dimensions of the reactor. In our experience, a reaction time of *ca.* 20 s is sufficient.

Chromatographic analysis

Many reducing carbohydrates other than D-glucose, D-galactose, D-fructose and lactose were tested under the recommended conditions. The retention times for these saccharides and their respective detection limits are given in Table I. The retention times decreased with increasing column temperature up to 90°C, the recommended temperature. The column was kept at 70°C in this system in order to obtain a better separation of the different carbohydrates. As can be seen, possible coelution problems could exist between L-rhamnose, D-xylose, D-galactose and D-mannose (12.16–12.53 min) and between D-fructose and L-fucose (13.48–13.69 min). If separation of these compounds is necessary (which was not the case in our study), it would be necessary to modify the composition of the mobile phase.

TABLE I

RETENTION TIMES AND LIMITS OF DETECTION (LOD) FOR VARIOUS REDUCING CARBOHYDRATES

LOD is defined as the amount in a 50-μl injection volume to give a signal-to-noise ratio of 3.

Carbohydrate	Retention time (min)	LOD (pmol)
Lactose	8.94	20.8
D-Glucose	10.92	15.8
L-Rhamnose	12.16	62.5
D-Xylose	12.16	41.7
D-Galactose	12.25	20.8
D-Mannose	12.53	62.5
D-Fructose	13.48	15.8
L-Fucose	13.69	62.5
L-Arabinose	13.99	41.7

Detection limits, based on a 3-fold signal-to-noise ratio at the baseline, ranged from 16 to 63 pmol for a 50-μl injection. The precision was established by repeated determinations ($n=6$) of a standard mixture of the nine reducing carbohydrates for an injected amount of 5 nmol. The relative standard deviations (R.S.D.s) were D-glucose 1.3, lactose 1.9, D-galactose 2.8, D-fructose 2.0, L-rhamnose 2.4, L-arabinose 1.7, D-xylose 2.7, L-fucose 1.4 and D-mannose 3.2%.

A typical chromatogram of a standard mixture of four reducing carbohydrates, obtained by ligand-exchange HPLC with post-column derivatization using benzamidine, is shown in Fig. 3. The fluorescence excitation and emission maxima of the final reaction mixtures of all the carbohydrates were 360 and 470 nm, respectively, and the fluorescence spectra were almost identical with those obtained in the manual spectrofluorimetric method [20].

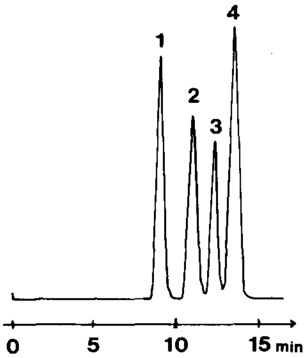

Fig. 3. Chromatogram of a standard mixture of four reducing carbohydrates. Peaks: 1 = lactose; 2 = D-glucose; 3 = D-galactose; 4 = D-fructose. Concentration, 10^{-4} M; column, 300 × 6.5 mm I.D.; stationary phase, Ca^{2+} ligand-exchange; column temperature, 70°C; mobile phase, 10^{-4} M aqueous CaEDTA; fluorimetric detection, λ_{ex} = 360 nm, λ_{em} = 470 nm; sensitivity, 0.012 a.u.f.s.; flow-rate, 1.4 ml min^{-1}.

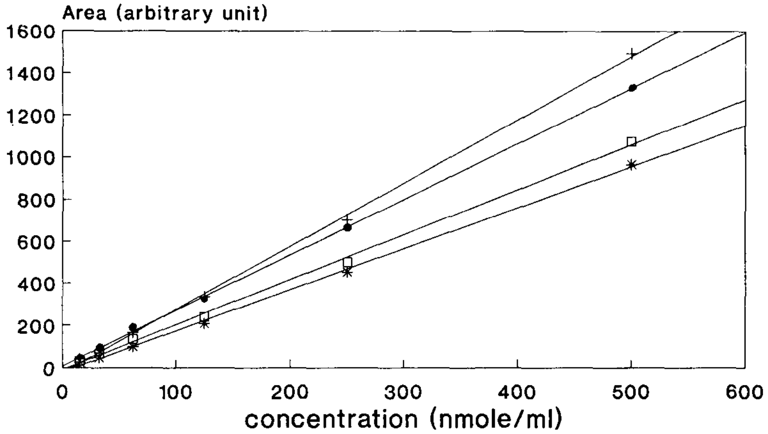

Fig. 4. Calibration graphs for HPLC of reducing carbohydrates. Aliquots (50 µl) of sugar standards were analysed as described in the text. Each point in the mean of six determinations. ● = D-Glucose; + = D-fructose; * = lactose; □ = D-galactose.

Calibration graphs, obtained by plotting peak area *versus* the concentration of four reducing carbohydrates, are displayed in Fig. 4. They were linear over the concentration range 16–500 nmol ml^{-1} ($r=0.999$) and passed through the origin.

Peak broadening due to the post-column derivatization system was tested by injecting D-fructose with and without the post-column reactor being installed. It was calculated by means of the Foley and Dorsey [23] experimental approximation of the standard deviation (σ), based on the measurement of peak width and peak asymmetry at 10% of the peak height. The 530-µl reactor caused an additional peak broadening

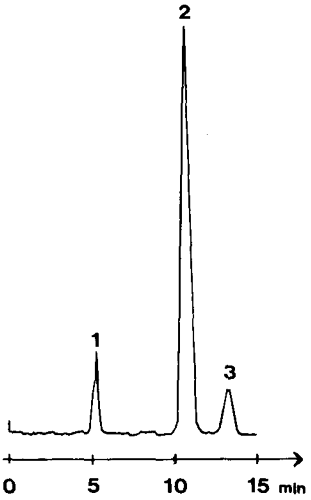

Fig. 5. Chromatogram of plant sample No. 2 (see Table II). Peaks: 1 = unidentified compound; 2 = D-glucose; 3 = D-fructose. Conditions as in Fig. 4. Sensitivity: 0.05 a.u.f.s.

TABLE II

DETERMINATION OF D-FRUCTOSE AND D-GLUCOSE IN MUSTARD EXTRACTS

Sample[a]	n	D-Fructose		D-Glucose	
		Mean concentration (μM)	R.S.D. (%)	Mean concentration (μM)	R.S.D. (%)
1	3	47	2.4	210	3.2
2	5	26	1.0	192	3.9
3	5	28	1.2	242	3.1

[a] 1, 2 and 3 represent different samples.

of σ = 4.0 s, which is relatively small compared with the peak broadening of the total chromatographic system (without reactor) of σ = 15.5 s.

Following these results, benzamidine proved to be easy to use as a fluorigenic reagent and to be compatible with on-line coupling to LC analysis of reducing carbohydrates. Indeed, the reaction time, the temperature and the addition of 1 *M* potassium hydroxide can be performed easily in a post-column reacting coil. Further, benzamidine is commercially available. Other fluorigenic derivatization agents have been developed by different workers (see Introduction); the aim of this work was not to compare these reagents but to develop a simple, sensitive and selective method for reducing carbohydrates. Benzamidine satisfies all these requirements.

Application to carbohydrate determination in plant samples

In order to apply the above method to a natural sample, the post-column derivatization system was used to determine monosaccharides in plant extracts. The complexity of the biological matrices and the low concentrations of monosaccharides led us to develop a very selective and sensitive method. Fig. 5 shows a typical chromatogram obtained with a plant extract. Aqueous sugar standards were pretreated in the same way as the samples and were used to prepare a calibration graph.

The sugar contents of plant extracts are summarized in Table II. The results were comparable to those given in the literature [21] and the differences fell within experimental error. This suggests that possible matrix effects were not significant and that the benzamidine post-derivatization technique has a good selectivity for reducing sugars in complex biological samples. The R.S.D.s of this post-column method for D-glucose and D-fructose are given in Table II and varied between 1 and 4%. The results show that this method is very selective for the study of reducing carbohydrates in plant samples. Such studies are of growing importance for process technology.

CONCLUSION

The post-column derivatization procedure using benzamidine as a fluorigenic reagent allows the LC determination of reducing sugars at the picomole level. This combination uses a ligand-exchange LC column for the separation of reducing carbohydrates and is more rapid, precise, sensitive and selective than LC with RI detection. The system described demonstrated high selectivity for the determination of several carbohydrates in plant materials. Consequently, the method could be readily used for screening purposes, *e.g.*, to check disorders of carbohydrate metabolism, to study plant metabolism or for the characterization of wines through their fermentable sugar fingerprint. Future research will include some of these aspects and validation of the proposed method for these different media. In particular, the analytical results for reducing sugars in different plant materials will be compared by both a batch enzymatic test and the proposed post-column derivatization method.

ACKNOWLEDGEMENTS

The authors thank Dr. D. Barcelo for helpful discussions concerning this work and Dr. R. degli Agosti for supplying plant extract samples.

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