Journal of Chromatography, 449 (1988) 207–215
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 663

OPTIMIZING COPPER–BICINCHONINATE CARBOHYDRATE DETECTION FOR USE WITH WATER-ELUTION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: A TECHNIQUE TO MEASURE THE MAJOR MONO- AND OLIGOSACCHARIDES IN SMALL PIECES OF WHEAT ENDOSPERM

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(First received February 17th, 1988; revised manuscript received May 16th, 1988)

SUMMARY

Modifications are described to the copper–bicinchoninate detection of reducing sugars to increase sensitivity when used in conjunction with water-elution high-performance liquid chromatography and post-column catalytic hydrolysis of some oligosaccharides to a reducing form. The lowest limit of detection, taken to be the amount of substance that produces a peak height twice the noise level, was about 1 ng for a number of reducing and non-reducing sugars. Colour formation was linear (< 5% deviation) and reproducible (S.D. < 10% at extremes) for detection response equivalent to between 10 ng and 2.5 μg glucose. Use of this technique to measure the major monosaccharides and oligosaccharides in very small pieces of wheat endosperm is described.

INTRODUCTION

Detection of reducing sugars using post-column chemical reactions is simple, highly sensitive and uses reagents that are not highly corrosive. The copper–bicinchoninate technique is based on the reduction of Cu(II) by reducing sugars and the subsequent formation of a deep lavender complex between Cu(I) and 2,2′-bicinchoninate. It was designed originally to detect sugars eluted with 89% ethanol[1], then was developed further for use with borate buffer[2,3].

The copper–bicinchoninate technique is well-suited to sugar analysis by high-performance liquid chromatography (HPLC) using the new generations of columns that use water only as the mobile phase[4] and with strongly acidic cation exchangers that hydrolyse some oligosaccharides to reducing form[5]. Without further development, however, the copper–bicinchoninate technique is not as sensitive as a similar detection technique based on 4-aminobenzoic acid hydrazide[6]. Nevertheless,

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0021-9673/88/$03.50

the copper–bicinchoninate technique is attractive on a number of accounts. For instance, the chemical solutions a and b are more stable than the corresponding solutions of the 4-aminobenzoic acid hydrazide technique, indeed may be kept for months at room temperature if kept in the dark[7,8], and the mixture of solutions a and b, in contrast, does not require refrigeration before use[8].

This paper reports modifications to the copper–bicinchoninate detection system to improve sensitivity when used in conjunction with water-elution separation of sugars and on-column hydrolytic conversion of some oligosaccharides to reducing form. The use of this modified technique to measure the major monosaccharides and oligosaccharides in very small pieces of wheat endosperm is described.

EXPERIMENTAL

The HPLC system comprised two pumps (Waters 510 for mobile phase and Milton-Roy 396-31 for detection reagent, both fitted with high sensitivity pulse dampeners), autoinjector (Waters Wisp 710B), two column heater blocks (controlled by the Waters temperature control module), variable-wavelength spectrophotometer (Waters 481, set at 562 nm), refractive index detector (Varian RI-3) and the Waters 840 control station.

Sugars were separated by Waters SugarPak column (30 cm × 6.5 mm I.D., polystyrene cation-exchange resin in calcium form, 10 μm particle size) or Waters DextroPak column (radial compression cartridge, 10 cm × 8 mm I.D., C_{18}, 10 μm particle size) using water only as the mobile phase (0.5 ml/min). A hydrolytic column (12 cm × 4.6 mm I.D.) consisting of Dowex 50W, 16% cross-linked, 200–400 mesh (regenerated periodically with 2 ml 1 *M* nitric acid) was placed either before or after the analytical column depending on experimental requirements. This resin material is rigid and open which gave long column life and allowed the regeneration solution to be introduced by syringe. Also it is cheap and readily available.

The copper–bicinchoninate solution was essentially as described by Churms[7]. The surfactant Brij 35 was added to solution b, however, to prevent possible deposition of calcium carbonate in the reaction coil which may result from the slow release of calcium from the SugarPak column[4]. Composition of the copper–bicinchoninate solution: Solution a was $CuSO_4 \cdot 5H_2O$ (1.0 g) and aspartic acid (3.7 g) dissolved in 1 l of high-purity water. Solution b was 2,2′-bicinchoninic acid (Sigma, 4,4′-dicarboxy 2,2′-biquinoline, 2.0 g), $Na_2CO_3 \cdot 10H_2O$ (38.0 g) and Brij 35 [Pierce, 30% (w/w), 3 ml] dissolved in 1 l of high-purity water. Colour reagent was a mixture (1:1) of a and b, degassed before use, protected from light.

The rate of calcium release from the SugarPak column is a function of temperature, hence the column was held at the minimum temperature that still provided sufficient isomerization of sugars within the column to prevent anomer separation (*i.e.* 75°C, as determined with glucose, results not shown in detail). The DextroPak column was operated at laboratory temperature.

On-column hydrolysis of sucrose, induced by calcium loss from the SugarPak column, developed to 1% after 6 h of continual use at 75°C (results not shown in detail). Accordingly, this column was regenerated frequently [calcium-EDTA (Sigma, disodium calcium salt), 500 mg/l, 90°C, 2 h, *e.g.* after 6 h use].

The reaction coil consisted of 3, 6, 9 or 12 m × 0.5 mm I.D. stainless-steel tubing

located in a separate heater block. Boiling and outgassing in the hydrolytic column and reaction coil was prevented by inserting a restrictor and cooling coil (1.5 m × 0.23 mm I.D. stainless-steel tubing) before the spectrophotometer and a backpressure valve (50 p.s.i.) after it.

Grains of wheat (*Triticum turgidum* var. durum cv. Fransawi), grown under controlled environment conditions (21°C/16°C, 14 h light, photon flux density 560 μmol/m^2/s at 400–700 nm), were harvested after 7 h of the day cycle twenty days after anthesis. All other tissues were peeled away from the endosperm, and the endosperm cavity was flushed with water (1 ml over 15 s). As soon as each endosperm was prepared (always within 1 min of harvest), it was plunged into hot (75°C) 90% ethanol, blended and extracted. Pooled supernatants (from six grains) were dried under vacuum and redissolved in pentachlorophenol solution (300 μg/l).

RESULTS AND DISCUSSION

Flow-rate of the copper–bicinchoninate solution

The volume ratio of copper–bicinchoninate solution to column mobile phase ranges between 0.7:1[9] and 4.0:1[3]. With water-elution HPLC, the volume ratio can be reduced substantially (Fig. 1). A ratio of 0.3:1 was chosen for all subsequent work.

Interaction of temperature and reaction time on the formation of colour

Colour yield for glucose and fructose, as related to temperature and reaction time is shown in Fig. 2. Reaction time was varied by using different lengths of reaction coil. The optimum balance between colour formation and decay occurred at 110°C with a reaction time of 1.8 min (6 m reaction coil). These conditions produced maximum signal-to-noise ratio and near-minimum band spreading (not shown).

Performance of the hydrolytic column

Equal amounts of sucrose, glucose and fructose were injected with the hydrolytic column placed between the analytical column and the copper–bicinchoninate

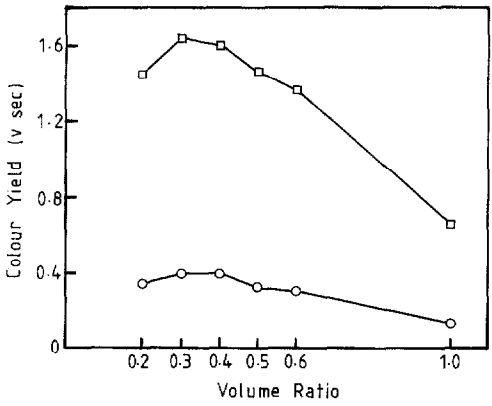

Fig. 1. Colour yield as a function of the volume ratio of copper–bicinchoninate reagent to mobile phase for 500 ng of glucose (○) and fructose (□). Flow-rate of mobile phase = 0.5 ml/min, length of reaction coil = 6 m, reaction temperature = 90°C.

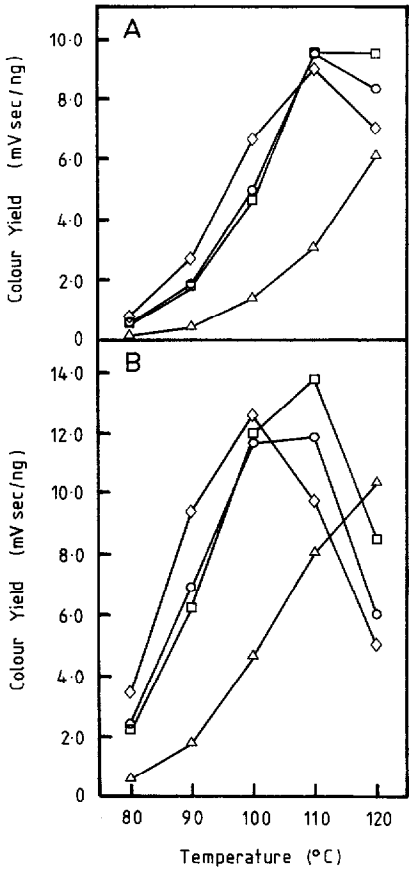

Fig. 2. Colour yield as a function of temperature and reaction time for glucose (A) and fructose (B); ◇ = 3.6 min, ○ = 2.7 min, □ = 1.8 min and △ = 0.9 min.

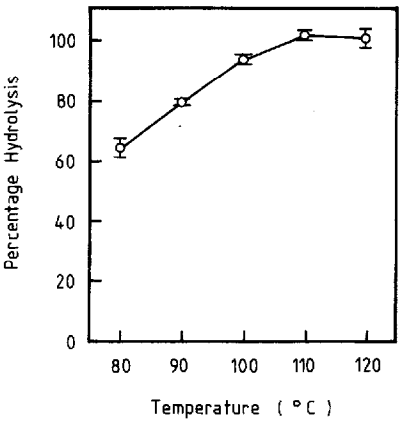

Fig. 3. Hydrolysis of sucrose (100 ng on-column) as a function of temperature; mean ± 1 S.D. of three replicates.

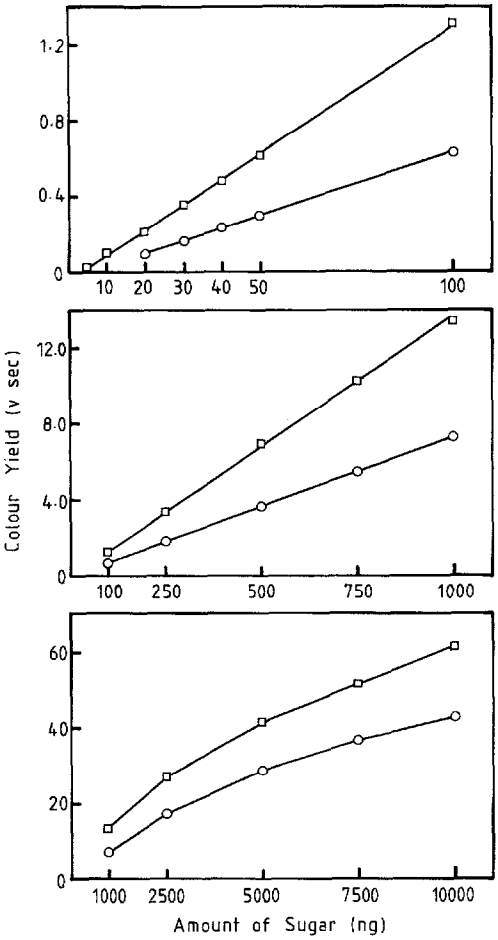

Fig. 4. Colour yield of glucose (○) and fructose (□) as a function of amount on column. (Mean of two replicates shown).

TABLE I

LOWEST LIMIT OF DETECTION AND MINIMUM ANALYSABLE AMOUNT OF A NUMBER OF SUGARS

See text for definitions. Mobile phase, water at 0.5 ml/min; copper–bicinchoninate solution, 0.15 ml/min; reaction time, 1.8 min; temperature of hydrolytic column and reaction coil, 110°C.

Sugar	*Lowest limit of detection (ng)*	*Minimum analysable amount (ng)*
Glucose	1.0	10
Fructose	0.5	5
Sucrose	1.0	7
Maltose	2.0	20
Raffinose	3.0	25
Stachyose	5.0	50

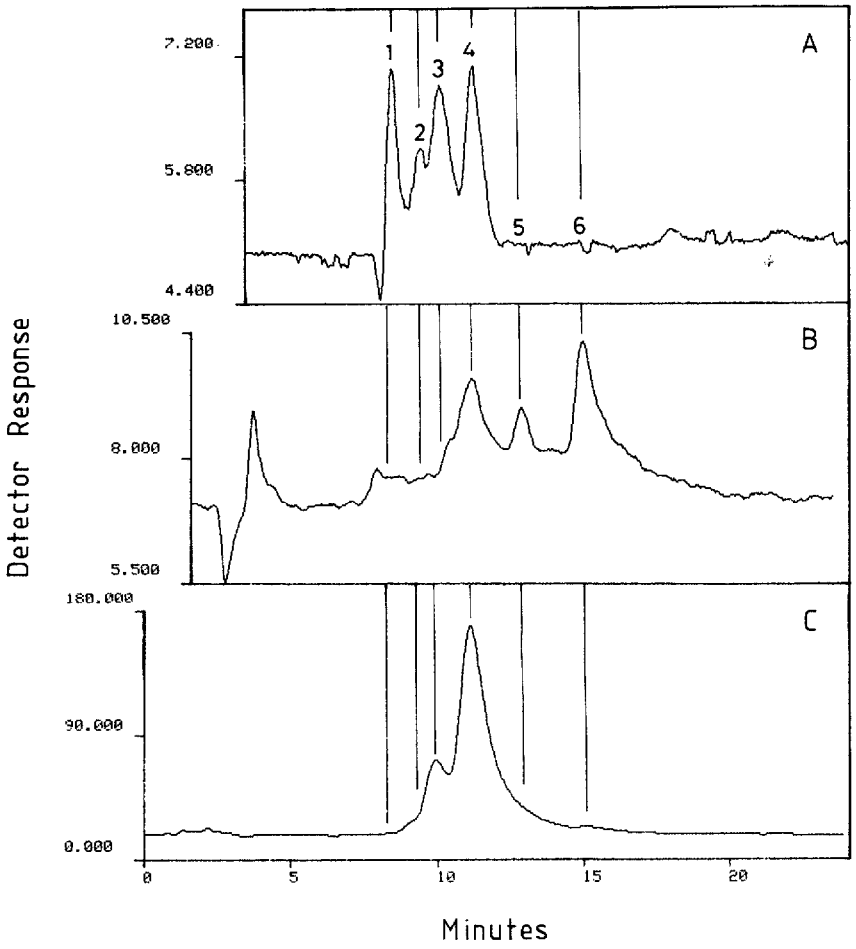

Fig. 5. Analysis of the ethanolic extract of wheat endosperm using the SugarPak column and three methods of detection: differential refractometry (A), the copper–bicinchoninate system (B), and the copper–bicinchoninate system with the hydrolytic column (C). Peaks: 1 = position of amino acids, salts; 2 and 3 = oligosaccharides with DP >2; 4 = sucrose, maltose; 5 = glucose; 6 = fructose.

detection system. The degree of sucrose hydrolysis was determined by colour yield at the retention time of sucrose relative to the mean colour yield of glucose and fructose. Hydrolysis was complete at 110°C (Fig. 3) and independent of amount on column between 20 ng and 5 μg (not shown).

In a second test, the hydrolytic column was placed in front of the analytical column and differential refractometry was used for detection. In this way, the appearance of the products of hydrolysis was measured concurrent with the decrease in the original compound. At 110°C, sucrose was hydrolyzed completely, producing equal amounts of glucose and fructose. Raffinose was hydrolyzed to fructose and melibiose, and stachyose to fructose and manninotriose. Maltose was unaffected. Evidently, under the conditions described α-D-(1–2) linkages were hydrolyzed while α-D-(1–4) and α-D-(1–6) linkages remained essentially intact.

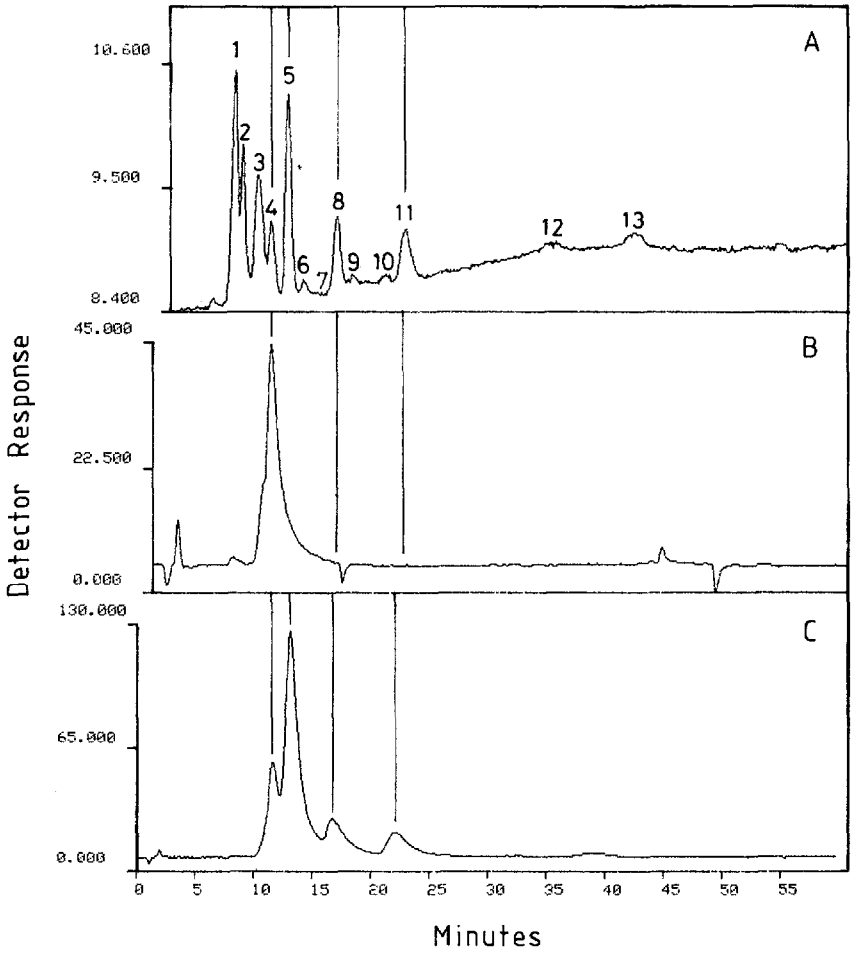

Fig. 6. As Fig. 5. Analysis using DextroPak column. Peaks: 1 and 2 = position of salts, amino acids, organic acids; 3 = hexoses, amino acids; 4 = maltose; 5 = sucrose; 6, 10 = unknown; 7 = stachyose; 8, 11, 13 = fructans; 9, 12 = artifacts with differential refractometry.

Linearity and range

Colour yield was linear (< 5% deviation) for glucose between 10 ng and 2.5 μg and for fructose between 5 ng and 1.5 μg (Fig. 4). Table I shows the lowest limit of detection (defined as the amount of substance injected onto the column that produces a peak height twice the noise level[10] and the minimum analysable amount (S.D. < 10% from six injections) for a number of carbohydrates.

Routine use

Our experimental programme called for a technique to measure the major soluble carbohydrates in very small pieces of wheat endosperm (100–150 ng dry weight). While it is difficult to compare sensitivities of different techniques without a direct side-by-side test and possibly further development, the copper–bicinchoninate

TABLE II

RETENTION TIMES AND RELATIVE RESPONSE FACTORS OF SOME STANDARD SUGARS AND SOME SUGARS EXTRACTED FROM WHEAT ENDOSPERM AFTER SEPARATION ON THE DEXTROPAK COLUMN

Peak numbers refer to peaks described in Fig. 6. Values for standard sugars are the mean of two replicates and the values for extracted sugars are the mean of four replicates. Mobile phase, water at 0.5 ml/min; copper–bicinchoninate solution, 0.15 ml/min; reaction time, 1.8 min; temperature of hydrolytic column and reaction coil, 110°C. ND = not detected.

	Standard sugars			*Extracted sugars*				
	Maltose	*Sucrose*	*Raffinose*	*Peak 4*	*Peak 5*	*Peak 6*	*Peak 11*	*Peak 13*
Detection by differential refractometry								
Retention time (min)	8.7	10.1	14.4	8.7	10.1	14.5	20.2	42.0
Detection using copper bicinchoninate system								
Retention time (min)	10.2			10.1				41.5
Relative response factor*	464	NIL	NIL	547	ND	ND	ND	13
Detection using hydrolytic column plus copper bicinchoninate system								
Retention time (min)	11.8	13.2	16.7	11.7	13.1	16.8	22.1	39.0
Relative response factor*	507	1205	190	569	1357	777	492	149

* Relative response factor = the ratio of the response factor value for detection using the described method to the response factor value for differential refractometry. In effect, it is a measure of the colour yield per unit of refractive index.

technique, as described, seemed at least as sensitive as the similar detection system based on 4-aminobenzoic acid hydrazide (Table I[6,8]).

The copper–bicinchoninate technique, with the hydrolytic column and the reaction coil placed in the one heater block at 110°C, has been used routinely for several years now, although this high temperature does accelerate performance decline of the hydrolytic column and the packing must be changed periodically.

Extracts from whole wheat endosperms were analyzed using the SugarPak and DextroPak columns with three methods of detection: differential refractometry, the copper–bicinchoninate system, and the copper–bicinchoninate system with the hydrolytic column (Figs. 5 and 6). Detection response was used as the first step in solute identification. For instance the retention time of peak number 8 after separation using the DextroPak column (see Fig. 6) suggested raffinose, but its detection response did not (Table II). Component analysis of the oligosaccharide(s) of peak number 8 showed later that no galactose was present, only glucose and fructose.

For routine determinations, the extracts of the small pieces of wheat endosperm were analyzed using the copper–bicinchoninate system in two ways: the SugarPak column without the hydrolytic column (Fig. 5b) and the DextroPak with the hydrolytic column (Fig. 6c). This allowed analysis of all the major monosaccharides and oligosaccharides in wheat endosperm with a high degree of sensitivity and precision.

A more detailed account of the component analysis of the oligosaccharides and of the distribution of soluble sugars within the wheat grain is to be published elsewhere.

ACKNOWLEDGEMENTS

We are grateful to B. Walker and M. Dowling of Waters Chromatography Division, Australia, who coached us in the art of HPLC usage. The work was assisted by grants to C.F.J. from Wheat Research Council.

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