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Analysis of Reducing Carbohydrates by Reductive Tryptamine Derivatization Prior to Micellar Electrokinetic Capillary Chromatography

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A micellar electrokinetic capillary chromatography method for determination of low molecular weight carbohydrates (dp 1−2) with an unbound carbonyl group as in aldoses or other reducing carbohydrates has been developed. Reductive amination of aldoses on the carbonyl group using tryptamine introduced a chromophor system to the carbohydrates enabling their sensitive UV detection at 220 nm and identification based on the indole group using diode array detection. Twelve carbohydrates including pentoses (D-ribose, L-arabinose, and D-xylose), hexoses (D-glucose, D-mannose, and D-galactose), deoxy sugars (L-rhamnose and L-fucose), uronic acids (D-glucuronic acid and D-galacturonic acid), and disaccharides (cellobiose and melibiose) are included in the study, using D-thyminose (2-deoxy-D-ribose) as the internal standard. Detection of all 12 carbohydrates is performed within 30 min. Linearity with correlation coefficients from 0.9864 to 0.9992 was found in the concentration range of 25−2500 μmol/L for all carbohydrates; the relative standard deviation on the migration times was between 0.27 and 0.80 min, and limits of quantification and limits of determination were in the picomole range.

KEYWORDS: Micellar electrokinetic capillary chromatography; reducing carbohydrates; reductive amination; tryptamine

INTRODUCTION

Carbohydrates are widespread in living organisms. They occur in DNA and RNA backbones, glycoproteins, proteoglycans, various glycosides, starch, plant storage organs, and plant cell wall β-glycans. Their role in the organisms varies as do their structure with function as carbon and energy sources as well as the different physiological and/or therapeutic effects of both digestible and indigestible carbohydrates. This makes carbohydrate chemistry and methods of analysis important in relation to various scientific disciplines such as, for example, plant and animal science, genetics, human nutrition, food technology, and pharmacology.

The diversity of carbohydrate species is greater than that of any other biochemical substance (*1*). Monosaccharides can be divided into aldoses, ketoses, alditols, aldonic acids, uronic acids, aldaric acids, etc., and each of these groups can be divided into subclasses based on number of carbons, configurational differences, and modifications such as deoxy and deoxyamino derivatives (*1*). Further classification of carbohydrates can be made according to the dp where dp 1 sugars are monosaccharides (e.g., glucose and fructose) and dp 2 sugars are disaccharides (e.g., sucrose, maltose, trehalose, and lactose). Oligosaccharides such as malto-, fructo-, and galactooligosaccharides have dp 3−10, and polysaccharides such as starch and NSP-like pectin, hemicellulose, and cellulose have dp > 10 (*2*). Other ways of carbohydrate classification can be done according to chemical properties caused by the presence of specific functional groups, type of glycosidic bonds, or by properties reflected in physiology or human health.

The great diversity and the various matrix systems from which the carbohydrates are obtained result in a great challenge for the analytical carbohydrate chemist. In fact, the multiplicity of carbohydrate structures is so wide that analytical methods allowing simultaneous analysis of all monosaccharides have still not been developed even as various high-resolution methods have been developed for carbohydrate analysis such as high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (*3*).

Analysis of mono- and disaccharides can as well be performed by various high-performance liquid chromatography (HPLC) and HPCE methods. Both HPLC and HPCE systems are commonly equipped with UV−vis or fluorescence detectors. The general lack of chromophoric and fluorophoric moieties in carbohydrates, as well as the lack of charge in neutral carbohydrates, thus gives difficulties when HPLC and HPCE systems based on such detectors are used. Appreciable efforts have therefore been devoted to overcome these problems. Introduction of charge can be obtained using strong alkaline conditions, but because UV−vis absorption or fluorescence is not raised by

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this, other detection systems such as PAD or indirect UV detection (*4*) must be used in these cases. Intensive examination of the polyol interactions with boric acid in aqueous systems was performed more than 50 years ago (*5*), and alkaline ionophoresis was used in studies of the interactions between carbohydrates and boric acid buffer (*6*). As compared to alkaline ionization (*7*−*12*), complex formation between carbohydrates and boric acid at weak alkaline conditions increases both the electrophoretic mobility and to a minor extent the UV extinction at 195 nm (*13*, *14*).

Low selectivity due to a large number of compounds with absorption in the lower UV area around 200 nm increases the importance of sample preparation when carbohydrate/boric acid complexes are detected at wavelengths near 200 nm (*13*). Derivatization of carbohydrates by use of chromophore or fluorophore compounds combined with alkaline (pH 9−10) complex formation between boric acid and the derivatives are possible solutions to overcome the problems, increasing both selectivity and sensitivity. Derivatization of reducing carbohydrates is often performed by reductive amination using amines with strong chromophors or fluorophors such as 4-aminobenzoic acid ethyl ester (*15*), 2-aminobenzoic acid (*16*), 4-aminobenzoic acid (*17*, *18*), 4-aminobenzonitrile (*19*), 2-aminopyridine (*20*, *21*), *S*-(−)-1-phenylethylamine (*22*, *23*), and *R*-(+)-1-phenylethylamine (*23*). Instead of complex formation with borate buffer at high pH, Chiesa and Horváth (*24*) used triethylammonium phosphate buffer at pH 2.5 for capillary zone electrophoresis of 8-aminonaphthalene-1,3,6-trisulfonic acid derivatives of maltooligosaccharides exploiting the negative charge of the three sulfonic acid groups at very low pH values. Reductive amination of reducing carbohydrates using ammonia followed by condensation with 3-(4-carboxybenzoyl)quinoline-2-carboxyaldehyde has also been performed (*25*, *26*), as well as condensation of carbohydrates with 3-methyl-1-phenyl-2-pyrazolin-5-one both as precolumn derivatization (*20*, *27*−*29*), and as on-column condensation (*30*).

In the present study, mono- and disaccharides occurring in various biological systems, e.g., as part of dietary fibers (DF), are derivatized by reductive tryptamination prior to analysis by MECC (*31*).

MATERIALS AND METHODS

Chemicals and Reference Compounds. CM-Sephadex C-25 (H^+), Dowex 50W×8, 200−400 mesh (H^+), and Dowex 1×8, 200−400 mesh (acetate), column materials for ion exchange chromatography, sodium cyanoborohydride ($NaBH_3CN$), cholic acid, SDS, sodium dihydrogenphosphate ($NaH_2PO_4 \cdot H_2O$), disodium hydrogenphosphate ($Na_2HPO_4 \cdot 2H_2O$), sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$), and L-fucose, D-galacturonic acid, D-glucuronic acid, and melibiose were obtained from Sigma Co. (St. Louis, MO). D-Galactose, D-glucose, and D-mannose were purchased from Merck (Darmstadt, Germany), L-arabinose was from BDH Biochemicals (Pool, U.K.), and D-thyminose 2-deoxy-D-ribose) was from Serva Finechemicals (New York, NY). Cellobiose, L-rhamnose, D-ribose, and D-xylose were obtained from the local collection of chemicals at the Chemistry Department, and tryptamine hydrochloride was purchased from Aldrich Chemical Co. (Milwaukee, WI). Water was purified in a Millipore Milli-Q system (Bedford, MA).

Plant Material. The lupine sample (*Lupinus angustifolius* cv. Zubra) was kindly provided by the plant breeding group at RVAU (Højbakkegaard, Tåstrup, DK).

Sample Preparation. The carbohydrate reference sample used for optimization of the HPCE procedure included the pentoses D-ribose and D-xylose, the hexoses L-arabinose, D-galactose, d-glucose, and D-mannose, the uronic acids D-galacturonic acid and D-glucuronic acid, the disaccharides cellobiose and melibiose, the deoxy sugars L-fucose and L-rhamnose, and an internal standard D-thyminose. The reference compounds were dissolved in milli-Q water (18.2 $\mu\Omega$) before derivatization with tryptamine.

Finely ground (<0.3 mm) seed material (0.2 g) was added 40 μL 12.5 mmol/L D-thyminose as internal standard. Extraction (2 × 2 min) was performed using 2 × 3 mL of 70% boiling methanol and Ultra Turrax T 25 (Janke & Kunkel, Staufen, Germany). The homogenates were centrifuged in a table centrifuge at 2000*g* for 2 min, and the supernatants thus obtained were pooled and evaporated to dryness by compressed air. The residues were then redissolved in 5 mL of milli-Q water, and part of these extracts (3.0 mL) was subjected to group separation by ion exchange chromatography according to the principles described by Sørensen et al. (*31*) and Bjerg et al. (*32*). Aqueous suspensions (1 mL; 1:1) of (A) CM Sephadex C-25 (H^+), (B) Dowex 50W×8, 200−400 mesh (H^+), and (C) Dowex 1×8, 200−400 mesh (Acetate) were packed into 1 mL plastic columns supplied with disks of silica material at the bottom. The columns were regenerated by use of 15 mL of 2 M acetic acid (A), 20 mL of 1 M HCl (B), and 15 mL of 2 M sodium acetate, respectively, followed by wash with water till neutral pH (31). Column A was placed at the top of column B, which was placed at the top of column C, and the collected columns were placed in a vacuum manifold (Supelco, Bellefonte, PA). After application of the sample (6 × 0.5 mL), the extracted material was allowed to pass into the column material and the columns were then washed with 2 × 5 mL water. The aqueous effluent containing neutral compounds including carbohydrates was evaporated to dryness and redissolved in 200 μL of water before derivatization and HPCE analysis.

Reductive Amination. The derivatization by reductive amination was carried out using a modification of the procedure described by Noe et al. (*23*) and Richter et al. (*33*). The sugar containing aqueous effluents from the group separation procedure (100 μL) was added to 12.5 μL of 0.15 M tryptamine dissolved in 10% propanol and heated at 90 °C for 10 min. To the reaction mixture containing Schiff bases was added 4.5 μL of aqueous sodium cyanoborohydride solution (0.3 g/mL), which then was mixed, and kept at 90 °C for 60 min to create stable amines. Dilutions with milli-Q water were performed before analysis by MECC in cases of high concentrations of aminated carbohydrates.

HPCE Analysis. The apparatus used was an ABI 270A-HT capillary electrophoresis system (Applied Biosystems) equipped with a UV detector. The capillary was a 760 mm × 0.05 mm i.d. coated fused silica capillary with a UV detection window placed on-column at a position of 530 mm from the injection end. The signal wavelength was set at 220 nm. Samples were introduced from the anodic end of the capillary by vacuum injection for 1 s at 5 kPa. The specific HPCE conditions were varied during optimization of the method. The separation buffer was basically composed of sodium tetraborate decahydrate, cholic acid, and 1-propanol adjusted to varying pH values (8.3−10.0). FZCE using sodium ortophosphate or sodium tetraborate buffers as well as MECC with SDS micelles also were tried. The separation buffer was filtered through a 0.20 μm membrane filter before use. The capillary was conditioned by flushing with 1 M NaOH for 45 min before use, and a standard procedure for capillary wash including flush with 1 M NaOH for 2 min, milli-Q water for 1 min, and separation buffer for 5 min was performed between the analyses.

Relative Response Factors (RRF). RRFs were used for quantification of the individual carbohydrates in the samples. RRFs (relative to the internal standard D-thyminose) were determined as $\alpha_{\text{D-thyminose}}/\alpha_x$ where α is the slope of the calibration curves and x refers to the individual sugars.

RESULTS AND DISCUSSION

All 12 reducing carbohydrates present in the reference sample are compounds commonly present in carbohydrate fractions, which form part of the various DF fractions. HPCE analysis of the standard sugar mixture derivatized with both phenyl ethylamine, 3,4-*di*-methoxyphenyl ethylamine, and tryptamine was tested in various buffer systems. FZCE using boric acid or sodium phosphate buffers and MECC using sodium phosphate buffer and SDS micelles (*31*) were tested on the carbohydrate

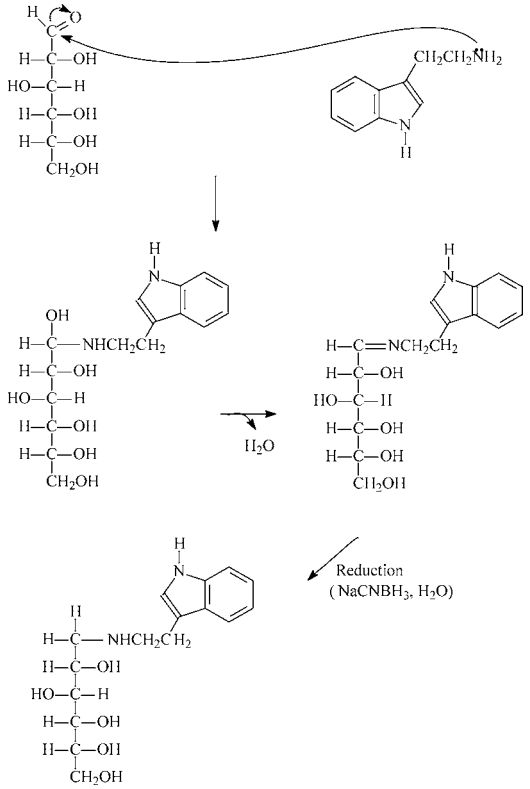

Figure 1. Reductive tryptamination of D-glucose.

mixture, but sufficient separation was not reached in these systems. Changing the system to borate buffer containing cholate micelles resulted in improved performance. Tryptamine derivatized sugars showed the most promising performance during the initial capillary electrophoresis runs, and MECC with aqueous buffer solutions based on sodium tetraborate and cholate micelles modified with 1-propanol gave the best separation of the standard mixture with mono- and disaccharides (data regarding buffer type optimization are not shown). Scheme of reductive tryptamination of D-glucose is shown in **Figure 1**.

Optimization of the separation buffer system was performed with respect to concentration of borate (5−150 mM) and pH (8.0−10.0), which was shown to be the major determinant of separation in the presented method. The borate concentration was of importance in relation to both migration time and signal response.

The influence of pH on migration time is illustrated in **Figure 2**. The migration time generally increased as the buffer pH increased, probably as a result of decreased boric acid protonization leading to decreased dissociation constant (K_D) of the carbohydrate−borate complex as described in Hofstetter-Kuhn et al. (*14*). Optimized separation conditions were reached at 30 °C and 30 kV using a buffer system consisting of 35 mM cholate, 100 mM borate, and 2% 1-propanol adjusted to pH 9.7 by 1 M sodium hydroxide as seen in the electropherogram of the standard carbohydrate mixture in **Figure 3**. Both variations in pK_a' values of the individual sugars and differences in the affinity toward borate may influence the net charge of the carbohydrate−borate complex. As the run buffer pH is 9.7, pK_a' variations of the sugars are expected only to have limited effects on the net charge of the borate complexes.

Comparison of the migration times of the individual carbohydrate derivatives clearly showed that the presence of a carboxylate group as in uronates increases the migration time. D-Glucuronate and D-galacturonate migrated thus approximately

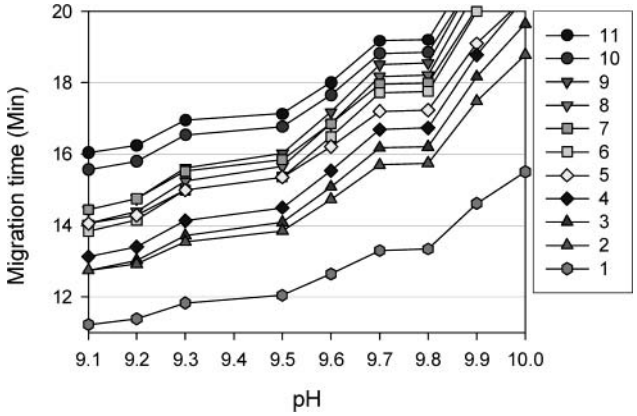

Figure 2. Influence of run buffer pH on migration time for tryptaminated carbohydrates. The capillary was a 760 mm × 0.05 mm i.d. coated fused silica capillary with an effective length of 530 mm. Signal UV wavelength is 220 nm. Samples are introduced from the anodic end of the capillary by vacuum injection for 1 s at 5 kPa. Separation conditions are 30 °C and 30 kV using a buffer system consisting of 35 mM cholic acid, 100 mM borate, and 2% 1-propanol. Peak number as in **Table 1**. D-Glucuronic and D-galacturonic acid are omitted as they show high resolution in the entire pH range.

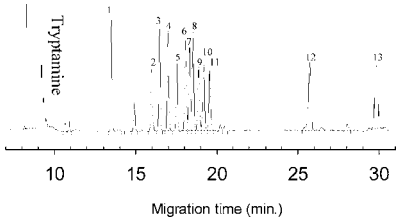

Figure 3. Standard electropherogram of tryptaminated carbohydrates using a pH 9.7 buffer. Run conditions as in **Figure 2** and peak numbers as in **Table 1**.

50% slower than D-glucose and D-galactose, respectively. It is well-known that the magnitude of charge for borate−carbohydrate complexes depends on the stability of the complex and that the stability of the complexes depends strongly on the configuration of hydroxyl groups in the carbohydrate (*14*). D-Mannose, D-glucose, and L-arabinose were not sufficiently separated using FZCE with borate buffer. Therefore, the separation of neutral carbohydrate derivatives in the optimized micellar system seems to arise from a combined effect of differences in K_D values of the sugar−borate complexes, as well as varying stability of the interactions between the individual carbohydrate derivatives and the cholate micelles, and the MECC method was preferred in the present work. Tryptamine has absorption peak maxima at λ = 197, 220, and 276 nm, and detection at 220 nm gives high sensitivity and minimizes interference from a large number of naturally occurring organic compounds. Proteins containing tryptophan and other compounds in the crude extracts with the same indole chromophor group as tryptamine may interfere with the developed method of carbohydrate analysis. It is, therefore, important to perform the described group separation prior to carbohydrate analysis to separate proteins and other indole-containing compounds such as alkaloids and indol-3-ylmethyl glucosinolates from the carbohydrate fraction.

The results of the linearity study shown in **Table 1** indicate a high precision with respect to normalized areas for the optimized method. As all carbohydrates have an identical chromophor after derivatization, the variations in RRF are worth noticing. RRF is determined relative to the internal standard

Table 1. Linearity and RRF of Standard Carbohydrate Solutions in the Concentration Range from 25 to 2500 μmol/L[a]

no.	carbohydrate	M_w	RMT	SDV	α	R^2	RRF
1	D-thyminose	134.1	1.00	0.38	0.0533	0.9990	1.00
2	L-rhamnose	164.2	1.19	0.27	0.0486	0.9864	1.10
3	cellobiose	342.3	1.23	0.27	0.0714	0.9947	0.75
4	D-xylose	150.1	1.27	0.28	0.0702	0.9921	0.76
5	D-ribose	150.1	1.31	0.30	0.0354	0.9981	1.51
6	melibiose	342.3	1.35	0.31	0.0709	0.9985	0.75
7	L-arabinose	150.1	1.36	0.33	0.0575	0.9982	0.93
8	D-glucose	180.2	1.38	0.34	0.0769	0.9985	0.69
9	D-mannose	180.2	1.40	0.39	0.0499	0.9946	1.07
10	L-fucose	164.2	1.43	0.41	0.0576	0.9971	0.93
11	D-galactose	180.2	1.45	0.42	0.0541	0.9976	0.98
12	D-glucuronic acid	194.2	1.89	0.65	0.0482	0.9992	1.11
13	D-galacturonic acid	194.2	2.20	0.80	0.0463	0.9992	1.15

[a] RRFs are calculated as the slope α of the internal standard D-thyminose relative to α of the carbohydrates. RMTs are the migration times relative to D-thyminose, and SDVs are the SD (min) of the migration times ($n = 25$).

D-thyminose from the slope (α) of the calibration curves for the individual carbohydrates (X) and the internal standard according to the equation: $RRF_x = \alpha_{D-thyminose}/\alpha_x$.

Variations in extinction coefficients are expected to be a sum of three factors. Hofstetter-Kuhn et al. (*14*) demonstrated thus that carbohydrate conformations may influence the UV_{195nm} signal as borate complex formation increased the extinction coefficient of the carbohydrates. This effect has not been investigated on tryptaminated carbohydrates, but limited conjugation electrons in the diol−borate complex reduce the effect at 220 nm. Cholate buffer micelle interactions with the analytes alter both the borate complex formation and most likely also the chromophor behaviors as the hydrophobic part of the micelles interacts with the indole part of the tryptaminated carbohydrates. Finally, dissimilar ratios of derivatized carbohydrates may arise during derivatization. Differences in K_D of the carbohydrate−tryptamine Schiff base complexes result in varying RRF values as Schiff bases are reduced to stable amines whereas free aldoses are reduced to alditols not detected at 220 nm, thus varying portions of carbohydrates ends as stabilized tryptaminated products.

The highest detector response was found in the tryptaminated D-glucose sample having 40% higher UV_{220} molar extinction coefficient than D-thyminose resulting in RRF = 0.69. The lowest response was found in the D-ribose samples having RRF values of 1.51, correlating to approximately 34% lower signal response than the internal standard.

The detector response was shown to be linear within the concentration range from 25 to 2500 μmol/L for all carbohydrate standard samples using 1 s of vacuum injection at 5 kPa. The LOQs (signal × noise^{-1} = 5) have been determined to a concentration of 25 μmol/L whereas the LODs (signal × noise^{-1} = 3) have been determined to be 10 μmol/L. The absolute LODs and LOQs obtained from the linearity studies were estimated to be 125 and 50 pmol, respectively.

Figure 4 shows the separation of reducing carbohydrates isolated from 9 day old seedlings of *L. angustifolius* L. cv. Zubra, purified and analyzed by the described MECC cholate procedure. Quantification of the carbohydrates in the sample is shown in **Table 2**.

During germination, various enzyme systems are activated degrading both storage as well as structural polysaccharides for utilization of the growing plant. D-Glucose and D-galactose were the major reducing monosaccharides present in the seedlings at concentrations of 27.3 and 7.2 μmol/g, corresponding to 4.9 and 1.3 0/00 (W/W), respectively. The high concentration of galactose was not seen in seeds, where large amounts of galactose were found as RFO (*4*). In the seedlings, the RFO content had decreased indicating activation of α-galactosidase during germination of lupine seeds. The high content of glucose (27.31 μmol/g) and the minor contents of L-rhamnose (1.3 μmol/g), D-xylose (2.0 μmol/g), D-mannose (2.6 μmol/g), and L-fucose (0.8 μmol/g), which were detected, may in part reflect the metabolism of DFs. The pentoses D-ribose and L-arabinose as well as the disaccharides cellobiose and melibiose were detected in concentrations lower than LOQ.

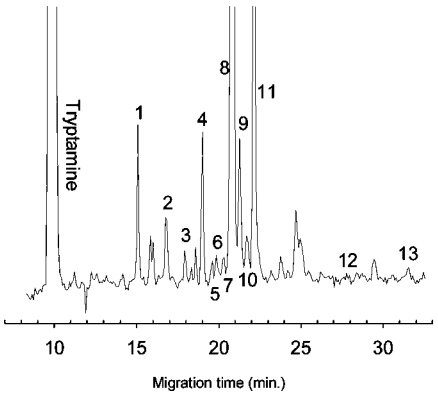

Figure 4. Electropherogram of reducing carbohydrates from seedlings of *L. angustifolius* L. cv. Zubra after 9 days of germination. Peak numbers as in **Table 1**.

Table 2. Concentration of Reducing Carbohydrates in Seedlings of *L. angustifolius* cv. Zubra after 9 Days of Germination

no.	carbohydrate	concn (μmol/g)	no.	carbohydrate	concn (μmol/g)
2	L-rhamnose	1.25	**8**	D-glucose	27.31
3	cellobiose	trace[a]	**9**	D-mannose	2.61
4	D-xylose	2.04	**10**	L-fucose	0.83
5	D-ribose	trace[a]	**11**	D-galactose	7.16
6	melibiose	trace[a]	**12**	D-glucuronic acid	ND[b]
7	L-arabinose	trace[a]	**13**	D-galacturonic acid	ND[b]

[a] Concentration < LOQ. [b] Concentration < LOD.

The uronic acids D-glucuronic acid and D-galacturonic acid were not detected in the sample, which may indicate either minimal degradation of pectic material or utilization of the uronic acids by the growing seedling. Degradation of the uronic acid during sample pretreatment does not seem likely as addition of galacturonic and glucuronic acid to plant materials prior to carbohydrate analysis was detected as expected (data not shown). Analyses for the content of uronic acids and other anions imply that it must be taken into consideration that the samples are prepurified by ion exchange chromatography. Low pH due to release of protons from the B column, as well as high content of acidic compounds in the extracts as compounds such as phytate and glucosinolates, which has a higher affinity for the C column material as compared to aliphatic compounds (*32*), may lower the binding of aliphatic carboxylic acids such as uronic acids to the C column. Therefore, both C column eluates and the water effluents from the ion exchangers have to be considered for reliable determination of uronic acids.

The method now developed is selective with respect to determination of carbohydrates with a free carbonyl group (hemiacetals or hemiketals) without including nonreducing carbohydrates of the aldose type. It is a high-resolution technique considered to be a supplement to methods such as HPAEC-

PAD and other HPLC and HPCE methods. Tryptamine as the chromophore group gives the method a relatively high sensitivity by detection at 220 nm ($\epsilon = 46\ 700\ M^{-1}\ cm^{-1}$), and combined with the typical indolyl absorption at 280 nm ($\epsilon = 5600\ M^{-1}\ cm^{-1}$), it gives basis for identification of tryptaminated carbohydrates when diode array detection is included in the procedure. The sample preparation procedure included as part of the method is relatively simple to perform and gives a basis for avoiding various potential interfering impurities, which occur in various crude extracts.

ABBREVIATIONS USED

1, D-thyminose; **2**, L-rhamnose; **3**, cellobiose; **4**, D-xylose; **5**, D-ribose; **6**, melibiose; **7**, L-arabinose; **8**, D-glucose; **9**, D-mannose; **10**, L-fucose; **11**, D-galactose; **12**, D-glucuronic acid; **13**, D-galacturonic acid; α, slope; λ, wavelength; dp, degree of polymerization; FZCE, free zone capillary electrophoresis; HPCE, high-performance capillary electrophoresis; i.d., internal diameter; LOD, limit of determination; LOQ, limit of quantification; MECC, micellar electrokinetic capillary electrophoresis; ND, not detected; NSP, nonstarch polysaccharides; RMT, relative migration time; RFO, raffinose family oligosaccharides; SDS, sodium dodecyl sulfate; SDV, standard deviation.

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