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CHARACTERIZATION OF BONDED-AMINE PACKING FOR LIQUID CHROMATOGRAPHY AND HIGH-SENSITIVITY DETERMINATION OF CARBOHYDRATES

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ABSTRACT

Characterization of bonded-amine packing was achieved with an organic–aqueous eluent by use of aromatic compounds as standard samples. The results indicate that the main interaction between the stationary phase and the solute is hydrogen bonding, hydrophobicity of the packing being negligible. The amine packing was also employed for an analysis of carbohydrates. A colorimetric detection of a reduced form of Tetrazolium Blue served for the determination (limit of detection: 10 ng of saccharide). The separation time was reduced by gradient elution. An oligosaccharide of d.p. 20 was eluted in less than 40 min.

INTRODUCTION

Numerous authors[1–7] have discussed the optimization of liquid chromatography for ionic compounds by use of distribution ratio concepts. In the case of non-ionic compounds, hydrophobicity has been shown to be very important, and the optimization has been realized for reversed-phase-mode chromatography[8,9]. In nonaqueous-solvent systems, the separation is mainly caused by hydrogen bonding and charge-transfer interaction[10–12]. A classification based on molecular interaction between the solute and the stationary phase[13] is useful to select the chromatographic mode of known, but less so to optimize the separation of unknown compounds. The ability to achieve a useful optimization requires some knowledge of the specificity of the packing material available. In the present work, we will illustrate the characterization of a primary amine-bonded packing and its application for optimizing the separation of various carbohydrates. High-sensitivity carbohydrate analysis is a very important tool in clinical chemistry and appropriate for the illustration.

The method reported here is based on a post-column reaction with 3,3′-[(3,3′-dimethoxy-1,1′-biphenyl-4,4′-diyl)]bis(2,5-diphenyl-2*H*-tetrazolium) dichloride, hereafter referred to as Tetrazolium Blue. Thus, a high-speed, high-sensitivity determination of oligosaccharides was achieved with gradient elution.

RESULTS AND DISCUSSION

Carbohydrates have been separated on silica gel[14,15] and on anion-exchange resins[16,17]. The separations were explained by hydrogen bonding or by complex formation with borate ion on ion-exchange resin. Comparison of the separation modes in unbuffered systems suggested the formation of hydrogen bonds between the carbohydrates and the surface of the packing material[15]. We made use of these findings to optimize a separation of carbohydrates on a bonded-primary amine packing.

Nature of the interaction with the primary amine packing. — As a first step, the separation mechanism on primary amine packing was studied with hexane–ethyl acetate as eluent, proton acceptors and donors, and polar and nonpolar solutes (see Fig. 1). As suggested previously[18], hydrogen bonding is the main interaction responsible for separation. Naphthalene was not adsorbed in 1 : 49 (v/v) ethyl acetate–hexane, log k′ being −0.87. Similar results were obtained with acetonitrile–water and methanol–water as eluent (see Fig. 2). Although hydrophobicity has been recognized as a predominant interaction in reversed-phase-mode chromatography, the polyaromatic hydrocarbons seemed to interact through ion-dipole forces when the acetonitrile content was low.

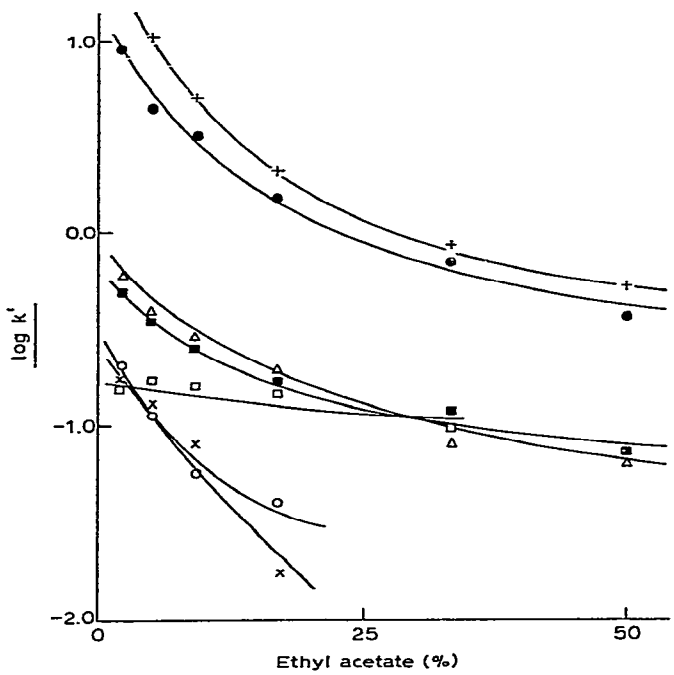

Fig. 1. Capacity ratio (log k′) as a function of the proportion of ethyl acetate in the eluent: (+) Phenol, (●) aniline, (△) isopropyl benzoate, (■) 1-butyl benzoate, (○) cumene, (□) napthalene, and (x) ethylbenzene. Column: Chromosorb-NH_2 (25 cm × 4.3 mm i.d.). Eluent: ethyl acetate in hexane; flowrate: 1 mL·min^{-1}. Detector: Hitachi, 254 nm.

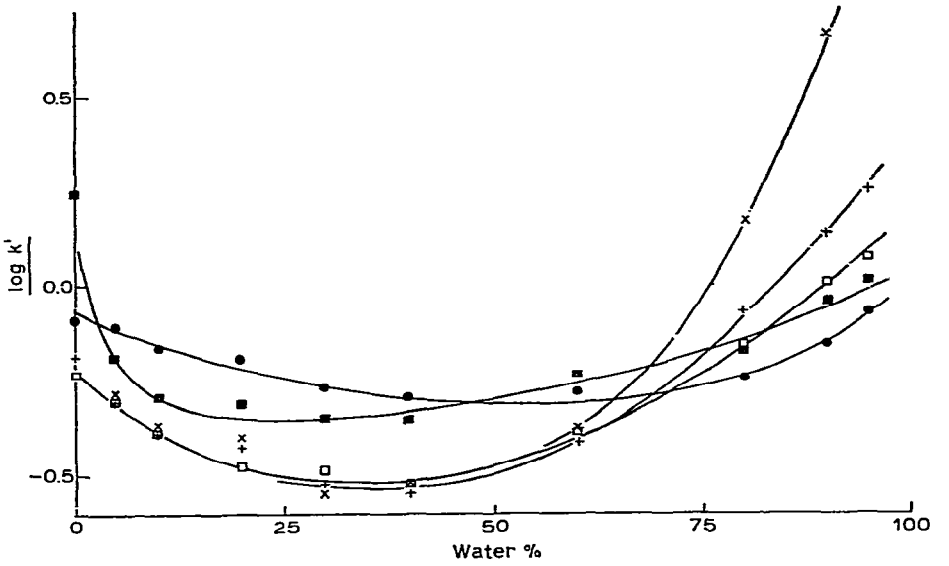

Fig. 2. Capacity ratio (log k') as a function of the proportion of water in the eluent: (x) anthracene, (+) naphthalene, (□) ethylbenzene, (■) phenol, and (●) caffeine. Column: Lichrosorb-NH_2 (25 cm × 4.3 mm i.d.). Eluent: acetonitrile–water; flowrate: 1 mL·min^{-1}. Detector: Perkin–Elmer LC-55, 254 nm.

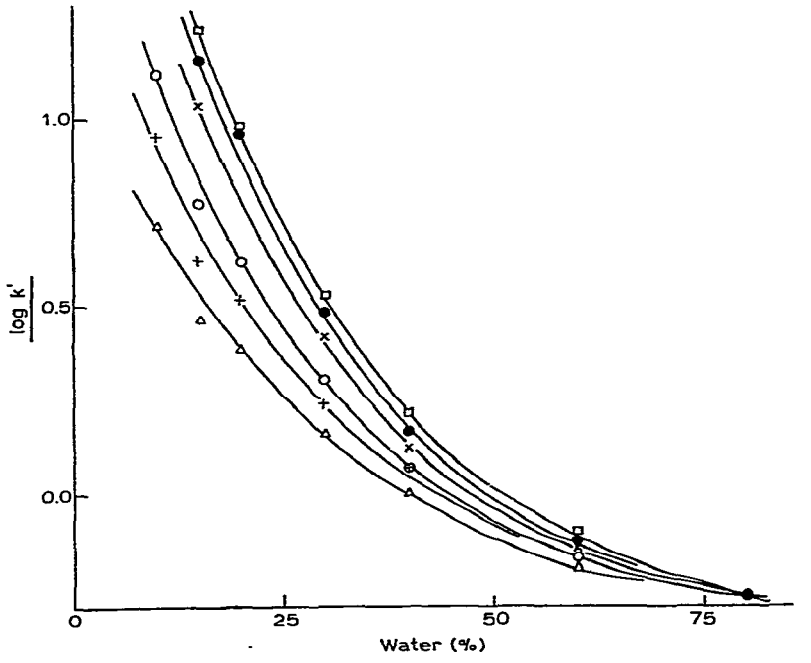

Fig. 3. Capacity ratio (log k') for various sugars as a function of the proportion of water in the eluent: (△) D-Xylose, (+) D-fructose, (○) D-glucose, (x) sucrose, (●) maltose, and (□) lactose. Column: Lichrosorb-NH_2 (25 cm × 4.3 mm i.d.). Eluent: acetonitrile–water; flowrate: 1 mL·min^{-1}. Detector: Perkin–Elmer LC-55, 195 nm.

TABLE I

CAPACITY RATIO OF CARBOHYDRATES[a]

Compound	*k'*
D-Fucose	1.43
D-Xylose	1.46
D-Fructose	1.76
D-Mannose	1.89
D-Glucitol	1.98
D-Glucose	1.99
D-Mannitol	2.03
D-Galactose	2.10
Sucrose	2.62
Maltose	2.99
Trehalose	3.11
Lactose	3.38
Gentiobiose	3.47

[a]Column of Lichrosorb-NH_2, 25-cm long, 4.3-mm i.d., with 7:3 (v/v) acetonitrile–water as eluent, at room temperature and a flowrate of 1 $mL \cdot min^{-1}$; detection by u.v. at 195 nm.

The log k' *vs.* composition curve shows a minimum capacity ratio for each solute studied. This may be explained by molecular adsorption in high organic-solvent content and by coulomb interaction in low organic-solvent concentration.

Separation of carbohydrates. — The capacity ratios for the various compounds studied are shown in Fig. 3 and the values obtained in 7:3 (v/v) acetonitrile–water reported in Table I. These results suggest that the total number of hydroxyl groups as well as their distribution on the molecule is the important factor for separation. Thus, capacity ratios were higher for compounds having hydroxyl groups on the same side of the molecule. A similar behavior was observed for methanol–water; however, in this case, the capacity ratios were too small for insuring a good separation. D-Fructose and D-galactose had a capacity ratio of 0.53, and lactose one of 9.78 in 9:1 (v/v) methanol–water on Lichrosorb-NH_2. On the other hand, in 9:1 (v/v) acetonitrile–water, D-fructose and D-glucose had capacity ratios of 9.0 and 13.3, respectively, for the same column. Unfortunately, these chromatographic conditions were not suitable for application to the separation of monosaccharides under biochemical conditions because the separation of D-fructose, D-mannose, D-glucose, and D-galactose was not satisfactory.

Another important problem of carbohydrate research is the separation of oligo- and poly-saccharides. Gel-permeation chromatography serves extensively for this purpose[19–21]. Oligosaccharides have been separated also on cation-exchange resin (Li^+) with 4:1 (v/v) ethanol–water as eluent[22]. Similarly, oligosaccharides have been separated on bonded-primary amine packing[23,24], a starch gel[25], and a glyconol gel[26] with acetonitrile–water as eluent system. A critical examination of these data support the hypothesis that the principal molecular interaction in carbo-

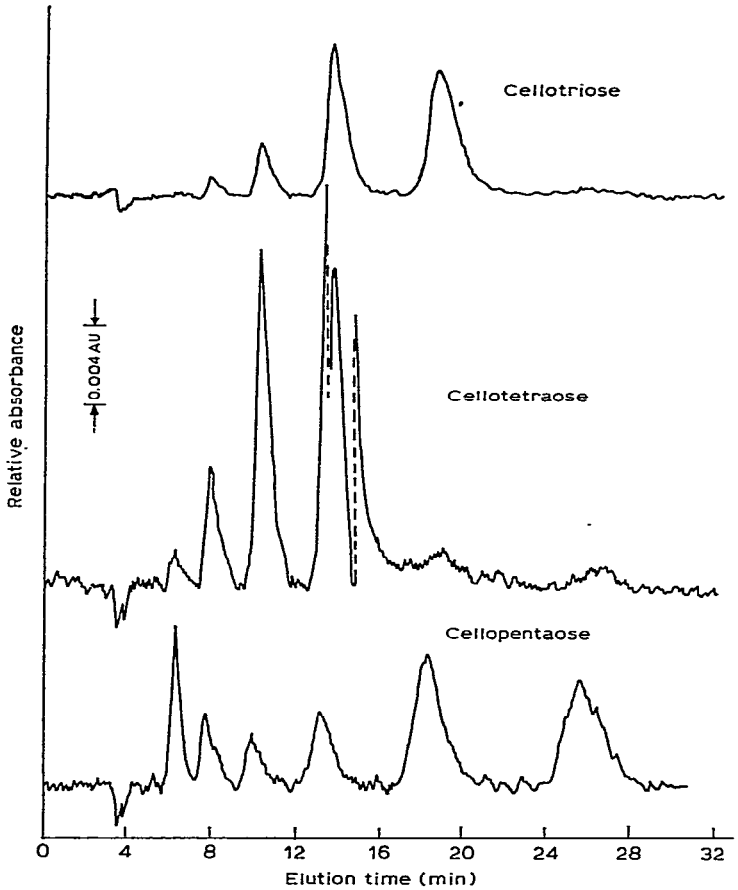

Fig. 4. Elution patterns of various oligosaccharides. Column: Chromosorb-NH_2 (25 cm × 4.3 mm i.d.). Eluent: 7:3 (v/v) acetonitrile–water; flowrate: 1 mL·min^{-1}. Tetrazolium Blue flowrate: 1.7 mL·min^{-1}. Reaction temperature: 85°. Detector: Perkin–Elmer LC-55, 530 nm.

hydrate separation is hydrogen bonding between compound and packing. The present study reports the high-speed separation of oligosaccharides on a bonded-primary amine packing with a gradient of acetonitrile and water as eluent (see Figs. 4 and 5).

Detection. — Carbohydrates are usually detected by formation of an absorbing derivative. Differential refractometry and coulometry have also been used (see Table II). The usual, refractive-index detector has a limit of detection of a few μg of carbohydrate, a value larger than that obtained with a flame-ionization detector[36].

The direct detection of carbohydrates at 195 nm was investigated for synthetic samples. The limit of detection observed for D-fructose was 0.6 μg, and for D-glucose 3.2 μg; other values are given in Table III. In an attempt to lower the limit of detection, the reduction of Tetrazolium Blue (TB) by various sugars was investigated. The reduction proceeds according to reactions[37] (*1*) and (*2*). The following various

TB (yellow) + $2e^-$ + $2H^+$ → TB-monoformazan (red, λ_{max} 525 nm) (*1*)

TB-monoformazan + $2e^-$ + $2H^+$ → TB diformazan (blue, λ_{max} 590 nm) (*2*)

parameters of the post column reduction were studied: reaction temperature and time,

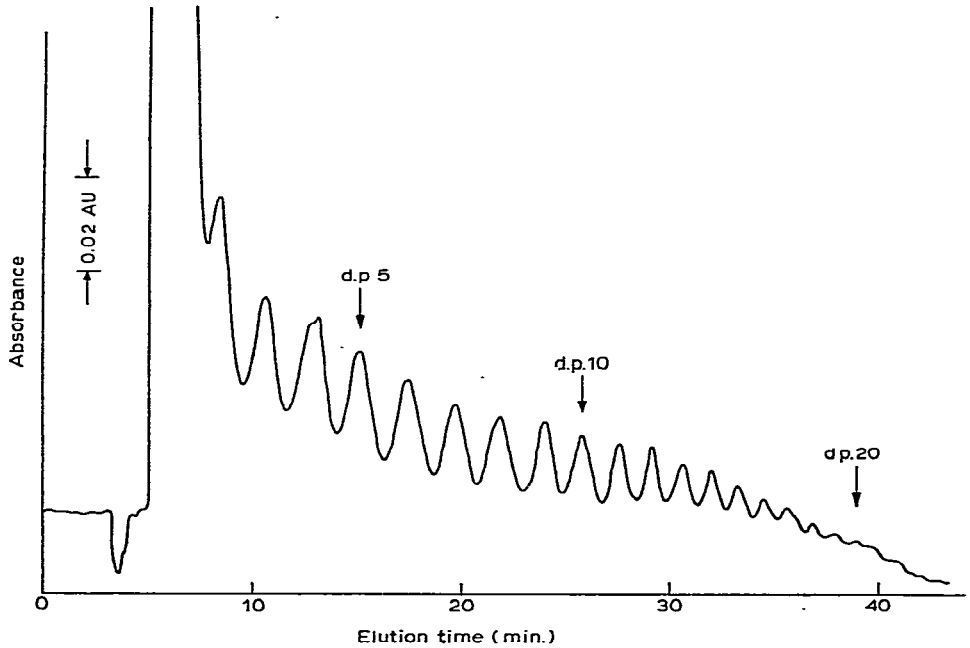

Fig. 5. Pattern of elution of hydrolyzed inulin. Column: Chromosorb-NH_2 (25 cm × 4.3 mm i.d.). Elution: linear gradient from 66:34 to 57:43 (v/v) acetonitrile–water in 40 min; flowrate: 1 mL·min^{-1}. Tetrazolium Blue flowrate: 1.7 mL·min^{-1}. Reaction temperature: 85°. Detector: Perkin–Elmer LC-55, 530 nm.

TABLE II

DETECTION OF CARBOHYDRATES

Method	*Reagent*	*Limit*[a] *(μg)*	*Ref.*
Colorimetric	Orcinol–H_2SO_4	0.1 (M)	27
Colorimetric	Anthrone–H_2SO_4	1.0 (M)	28
Colorimetric	Ferricyanide–alkali	0.2 (M)	29
Colorimetric	Neocuproine	1.0	30
Colorimetric	Mopper–Gindler reagent	0.2	31
Colorimetric	Alkali–TB	0.2	32
U.v. spectrum	Thermal decomp.	3.5 (D) 1.7 (M)	33
Refractive index		few	34
Flame-ionization		few	15
Coulometry		<20	35

[a]Abbreviations: D, disaccharide; M, monosaccharide.

TABLE III

LIMIT OF DETECTION[a] OF VARIOUS CARBOHYDRATES[b]

Compound	*k'*	*Method*	
		U.v. spectrum at 195 nm[c] (in μg)	*TB, absorbance at 530 nm[c] (in ng)*
Xylose	1.42	2.6	9
Fructose	1.70	0.6	10
Mannose	1.80	2.4	20
Glucose	1.80	3.2	9
Glucitol	1.90	1.5	
Mannitol	1.94	0.9	
Galactose	1.95	2.1	9
Sucrose	2.27	4.1	
Maltose	2.53	7.0	42
Lactose	2.82	7.0	42
Maltotriose	3.35	10.0	98
Stachyose	5.55	14.0	

[a]The limit of detection was calculated as twice the noise level. [b]The same experimental conditions as described in Table I were used except for a Chromosorb-NH_2 packing. [c]For the conditions of separation, see Table I. The colorimetric system had a flowrate of Tetrazolium Blue of 1.7 mL·min^{-1}, a reaction temperature of 85°, and the detector was set at 530 nm.

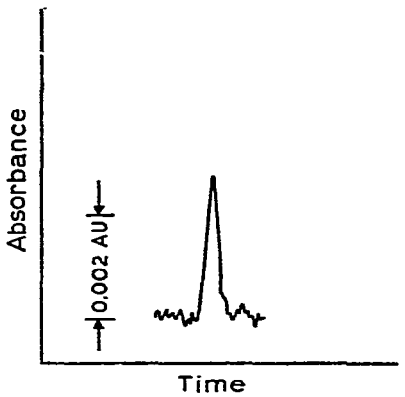

Fig. 6. Signal and noise in pattern of elution corresponding to 50 ng of D-glucose. Column: Chromosorb-NH_2 (25 cm × 4.3 mm i.d.). Eluent: 7:3 (v/v) acetonitrile–water; flowrate: 1 mL·min^{-1}. Detector: Perkin–Elmer LC-55, 530 nm.

concentration of Tetrazolium Blue, and pH in ethanolic solution. The optimum conditions were found to require a temperature of 85°, a 4-m long tubing of 0.5-mm i.d., and 2% Tetrazolium Blue in 0.18M sodium hydroxide in 1:1 (v/v) water–ethanol. Determination of oligosaccharide with this system allowed a limit of detection of ~10 ng; Table III reports the various capacity ratios and the limits of detection

observed. The signal-to-noise ratio for 50 ng of D-glucose is shown in Fig. 6. The calibration curves are linear from 0 to 20 μg of monosaccharide.

Although the method is not useful for nonreducing carbohydrates, it is quite effective for the analysis of hydrolyzed oligosaccharides (see Fig. 4 and 5). For example, an oligosaccharide of d.p. 20 was eluted in less than 40 min by gradient elution and detection at 530 nm.

The possibility of applying the same detection method to gel permeation chromatography was also examined. In this case, a column (25 cm-long, 10 mm i.d.) was filled with 5-μm starch gel (TSKLS170P5, Toyo Soda, Tokyo). The eluent was water, and Tetrazolium Blue was dissolved in 71% ethanol. The sensitivity was found to be $^1/_5$th of that observed with the analytical system. The limit was imposed by the solubility of the reaction products in water–ethanol. In the present survey, the limit of detection for the various carbohydrates was not established.

EXPERIMENTAL

Apparatus. — The chromatograph consisted of two pumps, Waters Associates, model 6000A (Water Associates Inc., Milford, MA 01757), with a solvent programmer, Waters Associates, model 660, coupled to a variable-wavelength detector, Perkin–Eimer, model LC-55 (Perkin–Elmer Corp., Norwalk, CT 06856). The injector was a universal injector, Altex, model 905–19 (Altex Scientific Inc., Berkeley, CA 94710). Chromatograms were recorded with a Brinkman recorder, model 2541 (Brinkman Instrumentals Inc., Westburg, NY 11590). A small peristaltic pump (Sodeco–Saia) or a Waters Associate pump, model 6000A, was used as an auxiliary pump for the preparation of derivatives.

A stainless-steel column (Altex, 4.3 mm i.d. × 250 mm) was packed in our laboratory by a balanced slurry method using a Haskel Fluid pump, model 28646 (Haskel Engineering & Supply Co., Burbank, CA 91502). The column packing material was 10-micron Chromosorb-NH_2 particles, lot 456483 (Johns-Manville, Denver, CO 80217) or 10-micron Lichrosorb-NH_2 from E. Merck (Darmstadt, Germany).

The reduction reaction was accomplished in a reactor maintained at a moderately high temperature with a water bath (Colora, model NB34431).

Chemicals. — High-quality standard chemicals were used without further purification. Stachyose tetrahydrate, maltotriose, and D-galactose were purchased from Aldrich Chemicals Co. (Milwaukee, WI 53233); amylose from J. T. Baker Chemical Co. (Phillipsburg, NJ 08865); D-glucose from Raylo Chemical Ltd. (Edmonton, Alb., Canada T6C 4A9); mannitol, glucitol, trehalose, fructose, lactose, gentiobiose, fucose, and inulin from ICN Nutritional Biochemicals (Cleveland, OH 44128); D-xylose (reagent grade) from Fisher Scientific Corp. (Pittsburgh, PA 15219); Dextran-T from Pharmacia Fine Chemicals (Piscataway, NJ 08854); Tetrazolium Blue from Sigma Chemical Co. (St. Louis, MO 63178), and acetonitrile (HPLC grade) from Fisher Scientific Corp. or from Burdick & Jackson Laboratories

Inc. (Muskegon, MI 49442). Water was first distilled in glass and then treated with the Milli-Q system of the Millipore Corp. (Bedford, MA 01730). Methyl α-maltotrioside, maltose, cellotriose, cellopentaose, and cellotetraose were kindly provided by Prof. R. H. Marchessault, Polymer Laboratory, University of Montreal.

Chromatographic procedures. — Characterization of the packing material was done isocratically at room temperature by use of a u.v. detector. The chromatographic conditions are given in the legends to Figs. 1, 2, and 3. The sample of aromatic (~10 μg) or carbohydrate compounds (300 μg) was injected through the loop injector. The volume injected was ~50 μL.

The carbohydrates were detected after reduction of Tetrazolium Blue in a 4-m long reactor at the outlet of the column. The first 3 m were kept at 85°, and the last meter was in a cold-water bath. The reactor collected the effluent from the column and the Tetrazolium Blue which was supplied by the auxiliary pump. A three-port Swagelok (1.6 mm i.d.) insured the connections between the column, the pump, and the reactor, which was made of polyfluorocarbon tubing (0.5 mm i.d.) rolled on itself to make a 5-cm coil. A variable-wavelength detector (530 nm) was connected to its outlet, and the total volume of the reactor was ~0.8 mL.

Amylose and inulin were hydrolyzed with perchloric acid prior to injection.

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