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Borate Complex Ion Exchange Chromatography with Fluorimetric Detection for Determination of Saccharides

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An analytical technique is described for the detection of fluorescent products formed by the reaction of reducing and nonreducing saccharides with ethylenediamine. The saccharides are separated by anion exchange chromatography in borate media with the amine reagent already present in the mobile phase. Fluorescent products are formed on-line in a postcolumn reactor whose temperature may be varied to optimize the response toward reducing and/or nonreducing saccharides. The detection limit is under 1 nmol for most saccharides. Applications to environmental and natural product samples involving little or no sample pretreatment are given.

Borate complex anion exchange chromatography has been shown to be an extremely powerful tool in the rapid separation of complex mixtures of carbohydrates (*1*, *2*). Almost any combination of sugars can be resolved by adjustment of chromatographic conditions, e.g., column temperature, buffer molarity, and pH. Thus, mixtures of 15–25 sugar components have been successfully separated (*2*, *3*). Furthermore, a wide variety of sample materials, e.g., urine, blood plasma, acid hydrolysates, beverages, etc., can be analyzed either directly or after only a simple filtration step. This is advantageous since tedious and sometimes contaminating clean-up, desalting, and derivatization steps (as required by gas or partition liquid chromatography) are often superfluous.

Despite these positive attributes, automatic sugar analyzers have not received the widespread acceptance as, for example, automatic amino acid analyzers. The main hindrance appears to have been the lack of a safe and sensitive detection system applicable to all classes of sugar compounds. Reagents for postcolumn reaction systems may be divided into two broad categories: corrosive and noncorrosive. An example of the former is concentrated sulfuric acid–orcinol (*4*, *5*). This type of reagent reacts with most classes of sugars and is fairly sensitive (nanomole level); however, its corrosive nature generally detracts from its use in routine clinical and industrial analyses. Furthermore, precipitation problems leading to blocked reaction coils and leakage have been reported (*6*, *7*). Noncorrosive colorimetric reagents, such as copper(II)–aspartic acid–bicinchoninate (*7*, *8*), copper(II)–neocuprion (*9*), and *p*-hydroxybenzoic acid hydrazide (*10*), are also sensitive but react only with reducing sugars; nonreducing oligosaccharides, e.g., sucrose, raffinose, etc., are not detected.

Honda et al. (*11*, *12*) reported that reducing sugars form fluorescent products when reacted with ethylenediamine (EDA) in weakly basic phosphate buffer solutions. The present investigators have adapted this reagent to the on-line fluorimetric detection of sugars in borate complex anion exchange chromatography. The diluted reagent in buffer is safe to handle and, by proper control of the reaction conditions, can be made to react with nonreducing oligosaccharides as well as reducing sugars. Furthermore, EDA can be added directly to the mobile phase, the borate buffer, thereby eliminating the need for a separate reagent stream after the chromatographic column.

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Honda et al. (*13*) recently reported the use of 2-cyanoacetamide for the fluorimetric detection of sugars in borate complex ion-exchange chromatography. In contrast to the EDA reagent, the 2-cyanoacetamide reagent must be separately pumped into the column effluent prior to the reaction coil, thereby requiring a separate reagent stream. Furthermore, in common with the noncorrosive colorimetric reagents, 2-cyanoacetamide reacts only with reducing sugars (nonreducing saccharides are not detected) and, additionally, it reacts poorly with 2-deoxy sugars.

These drawbacks are overcome with the EDA reagent. In the present study we report our results on the optimization of the EDA reagent to the sugar analyzer system. The applicability of this analyzer to environmental and natural product samples is also illustrated.

EXPERIMENTAL SECTION

Instrumental Methods. A commercially available sugar analyzer, the Fluorimetric Sugar Module (Breda Scientific, The Netherlands), was used for the chromatographic separations as well as for tests of the EDA reagent. The column was packed with strongly basic anion exchange resin, DA X-4 20 μm (Durrum Chemical Co.) according to Mopper (*3*). The resin bed measured 250 × 6 mm. The column temperature was set at 78 °C during the chromatographic separations (*3*). Since the EDA reagent is mixed directly into the borate buffer mobile phase, the sugar analyzer is equipped with only a single piston pump. A flow rate of 0.7 mL min^{-1} was used, unless otherwise specified. The postcolumn reactor contains 30 m of 0.5 mm i.d. Teflon tubing, which provides a reaction time of about 9 min at the given flow rate. For chromatographic runs the reactor temperature was set to 145 °C, unless otherwise specified. Boiling in the reaction coil was suppressed with a restrictor ($\simeq$5 bar back-pressure) placed after the cuvet. After reaction, the fluorescence was continuously monitored with a filter fluorimeter (Gilson Medical Co.) containing a 45-μL flow-through cuvet. Broad band-pass filters centered about 360 and 455 nm were used for excitation and emission, respectively.

Static fluorescence measurements, including the determination of excitation and emission spectra, were performed with an Aminco-Bowman spectrophotofluorimeter (American Instrument Co.) equipped with a standard xenon lamp.

Reagents and Buffers. Reagent grade chemicals and deionized, doubly distilled water were employed. For routine analyses, the mobile phase consisted of 0.7 M boric acid adjusted to pH 8.6 with 8 N NaOH. Ethylenediamine (EDA) was purchased in a highly purified form from Breda Scientific (Holland). It is sold under the tradename of Nanochrome II and consists of a solution of triply distilled, aldehyde-free ethylenediamine in borate buffer, 100 μL EDA mL^{-1} buffer. For routine chromatographic separations Nanochrome II was added to the mobile phase at a concentration of 5 mL L^{-1}, which corresponds to a final concentration of 7.5 mmol EDA L^{-1} mobile phase; however, for clarity, the results are presented in terms of the final EDA concentration, millimoles EDA per liter buffer, instead of Nanochrome II concentrations. Individual sugar standards, procured from Merck (F.R.G.), were of biochemical grade. Stock solutions of 1 mmol L^{-1} were prepared, 10 mmol L^{-1} in the case of mannitol. The solutions were made 20% in 2-propanol to inhibit microbial growth.

RESULTS

Fluorescence Spectra. Glucose was added to the combined mobile phase/reagent (0.7 mol L^{-1} boric acid, pH 8.6, 7.5 mmol EDA L^{-1}) to yield a concentration of 10 μmol L^{-1}. This mixture (4 mL) was heated for 2 h at 120 °C in a sealed test tube. The tube was then quickly cooled, and the fluorescence spectra were recorded. The results (uncorrected) are shown in Figure 1a. Multiple maxima in both the excitation and emission spectra are observed. Additional heating at 120 °C (total 3 h) resulted in only a moderate increase in the fluorescence due to reaction with glucose. On the other hand, the fluorescence of the blank increased significantly,

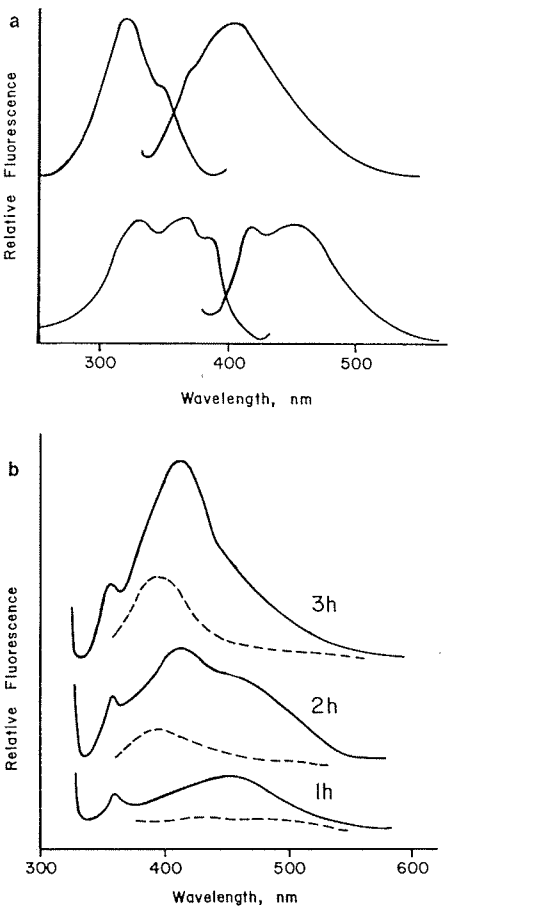


Figure 1. (a) Uncorrected excitation and emission spectra of the fluorescent product(s) produced on reaction of glucose with ethylenediamine in 0.7 M borate buffer, pH 8.6. The lower spectra depict the emission spectrum for excitation wavelength of 320 nm and excitation spectrum for emission wavelength of 460 nm. (b) Temporal changes in the emission spectrum of the glucose–EDA product(s) after reaction at 120 °C; the dashed line is the blank.

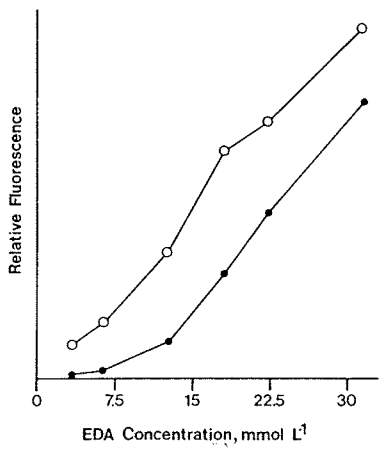


Figure 2. Effect of EDA concentration on the fluorescence response of the glucose–EDA product(s): 0.7 M borate buffer, pH 8.6; reaction time 1 h at 120 °C; Fl. 365/460; (O) glucose, (●) blank.

especially in the region 380–400 nm when excited at 320 nm, as depicted in Figure 1b.

The major excitation maxima for glucose–EDA products lie around 320 and 365 nm with emission maxima at about 410 and 460 nm. Similar results were obtained with other sugars, notably lactose, xylose, 2-deoxyribose, fructose, and glucuronic acid.

Effect of EDA Concentration. Sugars were reacted in sealed test tubes at 120 °C for 1 h with increasing concen-

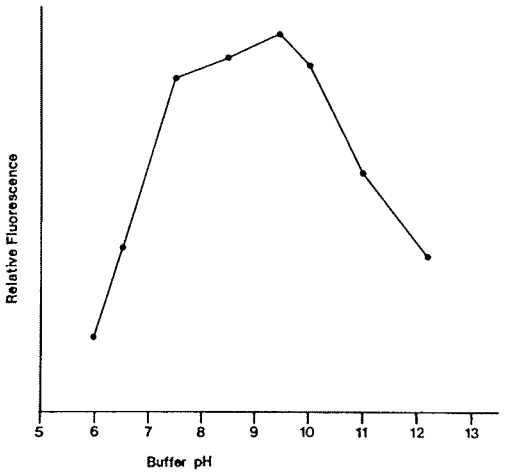


Figure 3. Effect of borate buffer pH on the fluorescence response of the glucose–EDA product(s): 7.5 mM EDA in 0.7 M borate buffer; reaction time 6 min at 136 °C.

trations of EDA in 0.7 mol L^{-1} borate buffer, pH 8.6. The results for glucose are shown in Figure 2. The fluorescence response increases linearly with increasing EDA concentration up to ≃30 mmol L^{-1}. However, the fluorescence of the blank also increases, especially at the higher EDA concentrations. As the detection limit of any method is ultimately determined by the level of background noise, the optimal EDA concentration is that which yields the highest signal to blank ratio. From Figure 2, this optimal concentration range is 3–9 mmol L^{-1}. Similar results were obtained with all other sugars tested.

Effect of pH. The pH of a 0.7 mol L^{-1} boric acid solution containing 7.5 mmol of EDA was varied from about 6.0 to 12.2 by additioin of 8 N NaOH. The fluorescence responses of different sugars were measured after reaction at 136 °C for 6 min in the sugar analyzer without separatory column. The results for glucose are depicted in Figure 3. The optimal pH range for most sugars lies between 7.0 and 10.5. Hexoses and deoxy sugars exhibit relatively constant fluorescence responses over this range, while other sugars showed stronger responses at the upper end of the pH range (uronic acids and di- and trisaccharides) due to alkaline cleavage of lactones and glycosidic bonds. Pentoses, on the other hand, showed better fluorescence responses at the lower end.

In borate complex anion exchange chromatography of sugars, the buffer pH range most frequently employed by various investigators lies between 7.5 and 10.0. Fortuitously, this range coincides perfectly with the optimal pH range of the EDA–sugar reaction.

Effect of Buffer Concentration. The borate concentration was varied from 0.05 to 0.8 mol L^{-1}. A pH of 8.60 and an EDA concentration of 7.5 mmol L^{-1} were employed. The fluorescence responses of various sugars were then measured after reaction at 136 °C for 4 min in the flow system. Most sugars exhibit similar and uniform fluorescence responses over the concentration range (≃0.05–0.8 mol L^{-1}) investigated with somewhat increased responses (≃30–50%) at higher molarities, which is in agreement with the results of Honda et al. (*12*).

In chromatographic sugar systems, the buffer concentrations most commonly employed are in the range of 0.2–0.8 M.

Reaction Temperature and Time. The effect of temperature on the reaction of EDA with a variety of sugars was examined in the flow system at a constant reaction time of 9 min. The buffer used was 0.7 mol L^{-1}, pH 9.0, and the EDA concentration was 7.5 mmol L^{-1}. The temperature of the reactor was increased in steps from 105 to 170 °C. The results are depicted in Figure 4. The fluorescence responses of most of the reducing sugars go through a maximum in the interval

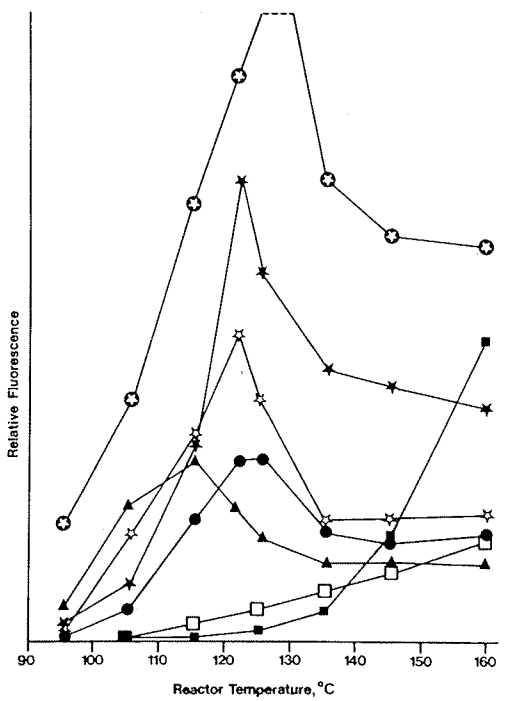


Figure 4. Effect of reaction temperature on the fluorescence responses of seven sugars (7.5 mM EDA in 0.7 M borate buffer, pH 9.0, reaction time 9 min): (☆) lactose, (★) galactose, (☆) fructose, (●) glucose, (▲) xylose, (□) mannitol, (■) sucrose; injected quantity = 10 nmol of each sugar, 100 nmol of mannitol.

120–130 °C and for the pentoses, 110–120 °C. With further increases in reaction temperature, the responses decrease until a plateau is reached. The high response of reducing disaccharides supports the hypothesis of alkaline hydrolysis prior to reaction with EDA. For the nonreducing di- and trisaccharides and sugar alcohols, the maxima appear to be shifted toward considerably higher reaction temperatures; however, for detection purposes, adequate responses can be obtained at reaction temperatures around 140–145 °C.

The effect of reaction time at various temperatures was also examined and, as expected, it was found that these two parameters are interdependent. Thus, a longer reaction time shifted the maximum toward lower temperatures and vice versa. For example, a 6-min reaction time shifted the maxima in the fluorescence responses up to 140–150 °C for most reducing sugars. Good responses for nonreducing oligosaccharides were obtained at reaction temperatures >155 °C.

Although simple overall relationships were not obtained with either reaction time or temperature (Figure 4), the first part of the curve (increasing slope) for most sugars approximately followed a first-order rate equation. For glucose, a forward rate constant, k_1, of 0.44 min^{-1} was calculated for 120 °C and 0.65 min^{-1} for 145 °C. The activation energy was calculated to be 5.1 kcal.

Response Factors, Linear Range, and Detection Limits. From Figure 4, it can be seen that the response factors of the different sugars at a fixed reaction time vary with the reaction temperature. According to the chromatographic problem at hand, a reaction temperature can be selected which favors the detection of, for example, either pentoses, hexoses, or nonreducing oligosaccharides. Alternatively, a compromise reaction temperature can be used which gives reasonably good fluorescence responses from nearly all types of sugars, e.g., 145 °C, 9 min. Response factors for various sugars relative to glucose at two reaction temperatures are given in Table I.

The linearity of the fluorescence responses of seven sugars (fructose, lactose, xylose, 2-deoxyribose, glucuronic acid, glucose, and sucrose) up to 100 nmol (500 nmol in the case

Table I. Response Factors Relative to Glucose (100%) at Two Reactor Temperatures[a]

sugar	124 °C	145 °C
glucose[b]	100	100
galactose	125	180
mannose	135	95
fructose	169	98
sorbose	441	405
tagatose	297	284
arabinose	86	67
lyxose	351	88
ribose	53	85
xylose	250	92
digitoxose	125	76
2-deoxygalactose	153	240
fucose	59	76
2-deoxyribose	66	98
rhamnose	71	57
2-deoxyglucose	33	43
3-*O*-methylglucose	200	191
gluco-heptose	133	421
manno-heptulose	76	146
galacturonic acid	438	143
glucuronic acid	500	163
cellobiose	205	565
gentiobose	117	147
lactose	125	1153
maltose	164	542
melibiose	133	205
raffinose	14	35
sucrose	10	24
trehalose	5	34
stachyose	3	16
mannitol	0	≃0.3

[a] Reaction time, 6 min; 7.5 mmol of EDA L^{-1} in 0.7 M buffer, pH 8.6. [b] $Glucose_{124°}$:$glucose_{145°}$ = 1:2.28.

of glucose) per injected sugar were examined in the chromatographic system. Reactor temperatures of 125 and 145 °C were used. Linear responses were obtained for all sugars at both temperatures, which is in agreement with Honda et al. (*12*). The detection limit (signal-to-noise ratio of about 3) for most sugars was in the range of 100–400 pmol per injected sugar. For reducing disaccharides, e.g., lactose, the detection limit was <50 pmol. The detection limits were governed chiefly by the reaction temperature and the response factors. Thus, at low reaction temperatures, 120–130 °C, more stable base lines were obtained due to the lower background; however, some sugars exhibited low response factors in this temperature range (Table I).

Precision, Accuracy, and Recovery. These parameters have been examined by Georgi (*14*) and can be summarized as follows. The coefficient of variation for five sugars was about 3% at the 50-nmol level. The accuracy and recovery were determined by employing standard addition techniques with filtered urine samples, whereby the accuracy was found to be >99% and the recovery about 95%.

DISCUSSION

Behavior of EDA in the Chromatographic System. As EDA is added directly to the mobile phase prior to being pumped through the column, the extent of reaction of EDA with the sugars in the chromatographic column was examined. With a column temperature of 75 °C, increasing increments in the EDA concentration had no noticeable effect on the sugar separation factors up to an EDA concentration of 15 mmol L^{-1}, above which peak broadening and a large increase in the fluorescence background were observed. The reasons for these effects were not explored, as this EDA concentration is considerably above the optimal range (cf. Results).

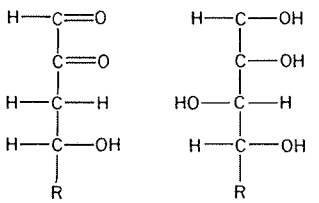
Reaction of EDA in the chromatographic column with sugars and traces of aldehyde contaminants in the buffer may add to the fluorescence background which in turn affects the detection limit. The contributions to the fluorescence background were, therefore, examined. It was found that reaction in the column adds only a minor contribution to the fluorescence background (generally <5%, column temperature 75 °C).

The long-range effects of pumping EDA-containing buffer over the anion exchange column were also studied. A column in continuous use for 6 months showed no significant variations in retention times, separation factors, and fluorescence response factors over the entire period. However, when a column had not been used for a week (stored at room temperature) was reconnected to the system, significantly lower fluorescence response factors were observed. The reasons for this effect were not explored. The normal response factors could only be restored after emptying, regenerating, and repacking the column. To avoid this loss in sensitivity, we have routinely adopted the following methods. When the system is not in use for a few days, the column temperature is reduced to about 50 °C and a slow flow of EDA-containing buffer, ≃0.2 mL/min is maintained. When the system is shut down for a longer period, the EDA-containing buffer is displaced from the column with buffer of lower strength containing no EDA.

The use of EDA in buffer gradient or multistep elutions has not been examined. The results of the studies presented here indicate, however, that the presence of EDA in the buffers should not offer any major problems for these types of elutions since the buffer strengths generally employed are well within the optimal range of the EDA–sugar reaction. It is expected, however, that the detection limit would be somewhat higher than for isocratic runs since the fluorescence background and, as a result, the base line would vary throughout the gradient.

Reaction Mechanism. The fluorescent products of the EDA–sugar reaction have not as yet been isolated and identified. However, from examination of the literature and from the present results some conjectures as to the nature of the products and the reaction mechanism can be made.

It is well-known that sugars are unstable in dilute basic solutions and undergo isomerization and fragmentation reactions. These rearrangements are related to the Lobry de Bruyn–Alberda van Ekenstein reaction. A number of highly reactive intermediates are formed, such as



diulose enediol

These unstable intermediates may react with excess amine to form fluorescent pyrazine, imidazole, and imidazoline derivatives (*15–17*). Nonreducing oligosaccharides are probably split to monosaccharides by alkaline hydrolysis prior to undergoing rearrangement.

Indications for the formation of several fluorescent products can be seen from the fluorescence spectra, Figure 1, where multiple maxima are observed in both excitation and emission spectra. The maxima increase at different rates on heating the sample. Information on the isolation and identification of some of the major fluorescent products will be published at a later date.

Interferences and Applications of the Sugar Analyzer. In static fluorescence measurements, strong quenching was

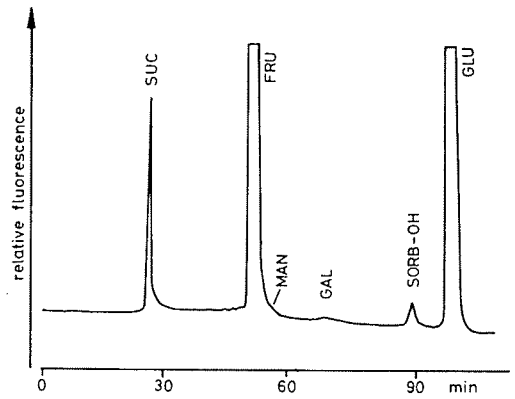


Figure 5. Direct injection of 20 μL of filtered apple juice: Suc = sucrose, Fru = fructose, Man = mannose, Gal = galactose.

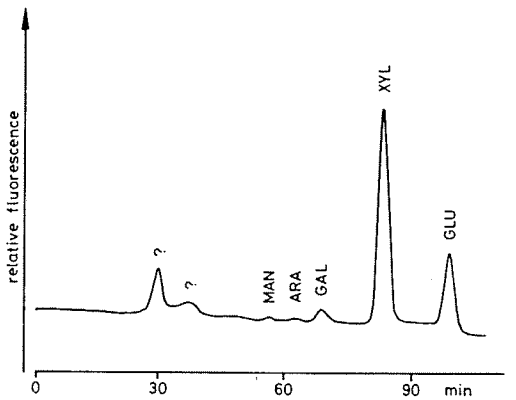


Figure 6. Direct injection of the neutralized hydrolysate of the calcified tissue of *Halimeda incrassata* (chlorophycease): Man = mannose, Ara = arabinose, Gal = galactose, Xyl = xylose, Glu = glucose.

caused by presence of silver, mercury, copper, and iodine ions; moderate quenching was observed with lead and calcium ions. The presence of a few percent of methanol, ethanol, or acetonitrile also caused moderate quenching, which implies that the EDA reagent is not compatible with chromatographic systems where organic mobile phases are employed. Mercaptoethanol and potassium cyanide also caused strong quenching. No quenching or interference was observed by amino acids or urea.

The presence of heavy metals and low molecular weight organic solvents in samples presented no problem in the chromatographic system, as these substances eluted in the vicinity of the system dead volume and resulted in only a momentary depression in the base line.

Examples of chromatograms of natural samples are given in Figures 5 and 6. In Figure 5, 20 μL of filtered, undiluted apple juice was injected with no further sample pretreatment. It should be noted that, in addition to the reducing sugars, nonreducing sugars such as sucrose and sugar alcohols such as sorbitol are also detected. Sugar alcohols, however, can only be determined at high concentrations (Table I). Previously published sugar detection systems employing noncorrosive reagents (*3*, *7–9*, *13*) are capable of detecting only reducing sugars and, therefore, are of little use in food and beverage industries where detection of nonreducing sugars, especially sucrose, is often required. Underivatized reducing and nonreducing sugars can be determined with nonspecific detectors, such as refractive index (*18*) and UV absorption at 192 nm (*19*). However, in comparison to the present system, these detectors have considerably lower sensitivities and are subject to interferences by nonsaccharidic compounds, such as amino acids and carboxylic acids, which sometimes make quantification difficult.

Sugar detection systems employing noncorrosive reagents have been used in the analysis of acid hydrolysates of various materials by direct injection (*3*, *7*). Unfortunately, the high pH (>10) of these reagents results in the precipitation of some inorganic salts, such as calcium, when present in the samples and may result in clogging of the reaction coil. This problem is not encountered in the present detection system since the pH is not raised in the reaction coil. Figure 6 shows a chromatogram obtained by direct injection of the neutralized hydrolysate of the calcified tissue of a coral.

The present fluorimetric detection system offers several important and unique advantages over all other previously described sugar detection systems, such as simplicity (a one-pump system), noncorrosive reagent, ability to detect both reducing and nonreducing saccharides, good sensitivity and specificity, and compatibility with direct injection of many types of sample materials. In addition, the system can be readily automated and, therefore, should be of considerable value to workers in the field of carbohydrate research and monitoring.

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