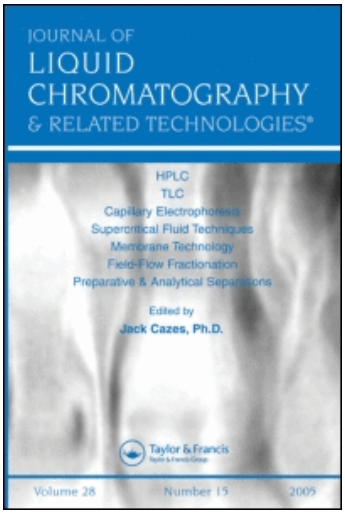


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# ULTRAMICRO ANALYSIS OF REDUCING AND NON-REDUCING SUGARS BY LIQUID CHROMATOGRAPHY

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## ABSTRACT

A post-column detection system for ultramicro amount of sugars has been developed using taurocyamine as the labelling reagent. Less than 10 pmol of reducing sugars were determined by this HPLC system. Non-reducing sugars were detected by the addition of periodate to the reagent.
Modification of the reaction reagent components made this detection system feasible to apply to various methods for separation of carbohydrates using pure water, acetonitrile/water mixtures, borate buffer or aqueous sodium hydroxide as the eluent.

## INTRODUCTION

Biological functions of carbohydrates are now being elucidated, and their microanalysis is required in the various branches of study.

Liquid chromatographic methods coupled with refractive index detectors [1,2] or a near-ultraviolet detectors [3,4] are not sensitive enough for biochemical analysis. Various reagents have therefore been devised for the fluorometric detection of carbohydrates in high-performance liquid chromatography (HPLC) such as ethylenediamine [5], malonamide [6], ethanolamine [7], taurine [8], 2-cyanoacetamide [9,10] and meso 1,2-bis(4-methoxyphenyl)ethylenediamine [11]. Particularly, arginine gave the sensitivity of reducing sugars down to 25 pmol [12]. However, these reagents gave little fluorescence when applied to sugar alcohols or non-reducing oligosaccharides. Non-reducing sugars were detected by the chemical reaction of formaldehyde produced by periodate oxidation [13,14]. Kato and Kinoshita have reported that taurine gave fluorescence when heated with reducing sugars, and this reagent could give fluorescence when it was allowed to react with non-reducing sugars in the presence of periodate [8, 15, 16], since it was stable to the periodate oxidation. This discovery facilitated the simultaneous detection and determination of reducing and non-reducing sugars by HPLC.

Kinoshita and co-workers further searched for such reagents having higher sensitivity and found that guanidino compounds were candidate materials. Among the guanidino compounds, glycocyamine (guanidinoacetic acid) was proved to give excellent fluorescence intensity in the detection and determination of myo-inositol in the presence of periodate, and this reagent was applied to the liquid chromatographic measurement of this sugar alcohol in the blood of patients suffering from renal failure [17]. However, glycocyamine decomposed on storage in aqueous solution to give high background. The reagent mixture should therefore be prepared daily.

In the present study, taurocyamine (N-amidinodtaurine, NATA) was found to be stable in solution and to give low background and high signal fluorescence. This paper describes the development of highly sensitive liquid chromatographic detection system for reducing and non-reducing sugars using taurocyamine.

## EXPERIMENTAL

The liquid chromatograph was assembled with three LC-9A pumps, a DGU-3A degassing unit, a CTO-6A column oven, an RF-535 fluorescence detector, a CRB-6A reaction oven, a SLC-6B system controller, a C-R6A integrator and a Rheodyne model 7125 injector from Shimadzu Corp (Kyoto Japan). The stainless steel reaction tube was 10 m x 0.5 mm i.d., and the cooling tube was 1.5 m x 0.5 mm i.d.
Taurocyamine was obtained from Wako Pure Chemical Industries Ltd. Osaka Japan and HPLC grade acetonitrile, standard sugars, buffer components were obtained from several sources.
A 5 μm of macro porous anion-exchange resin, Hitachi 3013N (Hitachi, Tokyo, Japan), was packed into a 25 cm x 4.6 mm i.d. stainless steel tube. A polyamine bonded vinylalcohol polymer column, Asahipak $NH_2P$-50 (25 cm x 4.6 mm i.d.), was kindly gifted by Dr. K. Noguchi from Asahi Chemical Ind. (Kawasaki, Japan).



## RESULTS AND DISCUSSION

The liquid chromatographic system is shown in Fig. 1 and the standard sugars are given in Table I. The optimization of the post-column reaction was studied in a flow injection method where the column and the column oven were removed from the liquid chromatographic system shown in the figure.
Aldehyde groups of the saccharides react with the guanidyl group of taurocyamine with the help of borate at high temperature in alkaline solution, and form UV absorbing and fluorescent compounds.
The reaction speed and yield depend upon the stability of the sugars in alkaline solution, further the concentration of periodate.
As the preliminary experiment, the excitation and fluorescence spectra were measured from glucose-taurocyamine reaction product. The excitation and fluorescence wavelengths were 325 and 420 nm,

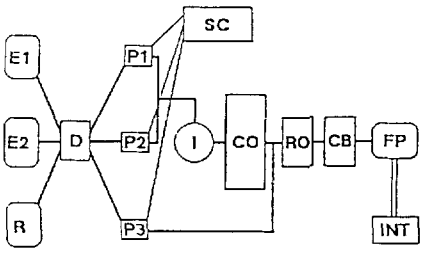


Fig. 1 Schematic diagram of liquid chromatograph
E1 and E2: eluent reservoirs, R: reagent solution reservoir, D: degassing unit, P1,P2 and P3: HPLC pumps, SC: system controller, I: injector, CO: column oven, RO: reaction oven including 10 m reaction coil, CB: cooling bath with 1.5 m cooling coil, FP: fluorescence detector and INT: integrator.



respectively. These wavelengths were therefore used for further study. First, the effect of taurocyamine concentration was examined in 0.2M potassium tetraborate (pH 8.5) solution. 10mM of taurocyamine was seemed to be enough for monosaccharides, and oligosaccharides required the higher concentration. Therefore, 20 mM of taurocyamine was used for the further study of the optimization of reaction conditions.

Borate was also required in this reaction and the higher concentration of borate did not significantly improve the sensitivity and precipitated in acetonitrile/water solutions required for certain separation as the eluent. Therefore, 0.2M potassium borate was used for the further experiment.

Although increasing the reaction temperature seemed to increase the sensitivity and the sensitivity of monosaccharides decreased over 150 °C. Therefore 150°C was selected as the reaction oven temperature.

The most important parameter was the pH. Some examples of pH effect are shown in Fig. 2. The higher pH increased the sensitivity of oligosaccharides and decreased that of monosaccharides. This

TABLE I Sugars and the Relative Sensitivity
The detail of experimental conditions is given in text. *Relative sensitivity to glucose is given as standard at each experiment, and (-) and (+) mean without and with periodate ion in the reagent.

| No. | | Sugars | Relative sensitivity* pH 8.5 $IO_4$ (-) | pH 8.5 (+) | pH 10.5 (-) | pH 10.5 (+) |
|---|---|---|---|---|---|---|
| 1 | Aldohexose | D-Glucose | 100 | 100 | 100 | 100 |
| 2 | | D-Galactose | 90 | 97 | 130 | 110 |
| 3 | | D-Mannose | 75 | 57 | 100 | 39 |
| 4 | | L-Fucose | 77 | 59 | 240 | 110 |
| 5 | Keto-hexose | D-Fructose | 89 | 93 | 100 | 110 |
| 6 | Aldopentose | D-Ribose | 54 | 34 | 170 | 43 |
| 7 | | D-Arabinose | 71 | 48 | 175 | 87 |
| 8 | | D-Xylose | 110 | 86 | 230 | 110 |
| 9 | | 2-Deoxy-D-Ribose | - | 340 | 44 | 610 |
| 10 | Oligo- | Maltose | 160 | 1100 | 220 | 390 |
| 11 | saccharides | Lactose | 190 | 1800 | 250 | 400 |
| 12 | | Gentiobiose | 93 | 17 | 180 | 190 |
| 13 | | Sucrose | - | 17 | 2 | 74 |
| 14 | | Trehalose | - | - | 2 | 39 |
| 15 | | Maltoriose | 110 | 1000 | 200 | 390 |
| 16 | | Raffinose | - | 48 | - | 65 |
| 17 | | Stachyose | - | 34 | - | 113 |
| 18 | | Maltopentaose | 120 | 97 | 220 | 510 |
| 19 | Sugar- | Sorbitol | - | 55 | 2 | 70 |
| 20 | alcohols | Galactitol | - | 41 | 2 | 43 |
| 21 | | Mannitol | - | 45 | 2 | 78 |
| 22 | | Myo-inositol | - | 21 | 2 | 39 |
| 23 | | Arabinitol | - | 24 | 2 | 35 |
| 24 | Others | D-Glucosamine | - | 52 | 120 | 230 |
| 25 | | Glucuronic acid | - | 38 | 13 | 17 |
| 26 | | Glucuronic acid lactone | - | 59 | 46 | 70 |

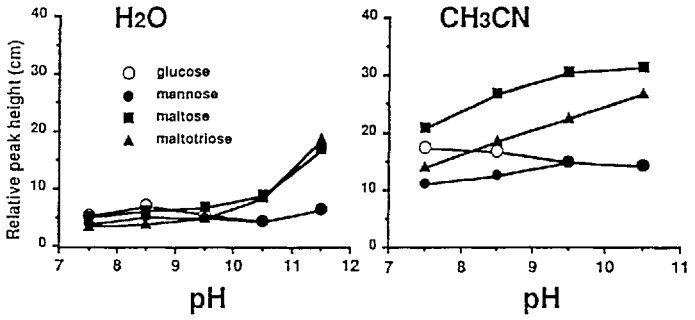


Fig. 2 pH effect on the fluorescence intensity of various sugars measured by a flow injection method.
A: measured in water as the carrier and B: measured in 70% aqueous acetonitrile as the carrier, flow rate: 0.8 mL/min. Reagent: 20 mM NATA, 0.2M tetraborate, flow rate: 0.4 mL/min. Reaction temperature: 150 °C, Sugars: 10 nmol each.



indicates the stability of saccharides in high pH solutions where oligosaccharides could be degraded to monosaccharides, especially disaccharides were easily degraded. The sensitivity of maltotriose was lower than that of glucose in low pH and higher than that of glucose in high pH solution. Monosaccharides can be detected under mild condition, pH 8.5, with high sensitivity. The stability and reactivity of saccharides were further investigated in sodium periodate solution which was required to analyze non-reducing sugars as shown in Fig. 3.

The sensitivity of reducing sugars, mono and oligosaccharides decreased in high concentration of sodium periodate, while that of non-reducing sugars and sugar alcohols increased up to certain periodate concentration. These results can be understood from the periodate reaction mechanism where hydroxyl groups are converted to aldehyde groups, and the ketone and aldehyde groups are converted to carboxyl groups.

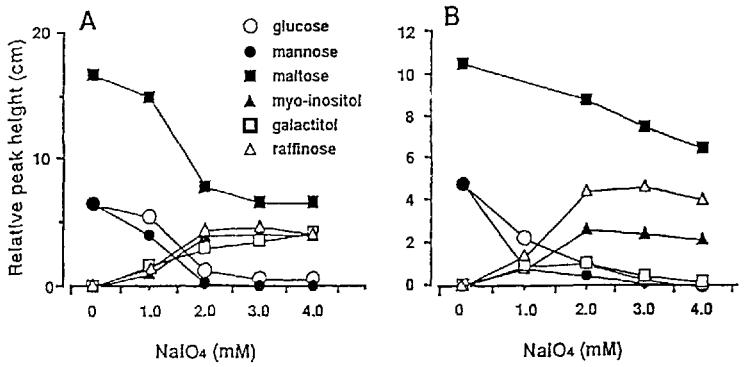


Fig. 3 Sodium metaperiodate concentration effect on the fluorescence intensity of various sugars measured by a flow injection method Reaction condition: see Fig. 2, Reagent solution pH: A=11.5, B=10.5.



The relative fluorescence intensity of various sugars was measured by a flow injection method where a separation column was removed from the system shown in Fig. 1. The amount of injected sugars was 10 nmol/10 μL each. The relative fluorescence intensity was given in Table I as that of D-glucose is 100. The intensity of pentoses was generally higher than that of hexoses except L-fucose. Sugar alcohols did not give fluorescence in pH 8.5 without periodate. This fact suggests that the α-ketol structure is essential for the development of fluorescence.

The calibration curves of D-glucose, L-fucose and maltose were linear from a few pmol to 10 nmol. The detection limit of D-glucose was 3.5 pmol (S/N = 3) measured on a chromatogram using Asahipak NH2-50 column in 70% aqueous acetonitrile as the eluent. A chromatogram of each 50 pmol of L-fucose (peak 1), D-glucose (peak 2), D-fructose (peak 3) and maltose (peak 4) obtained on the Asahipak NH2-50 column is shown in Fig. 4.

The examples of the analysis of sugars using an anion-exchange resin as the column packing and an aqueous sodium hydroxide solution as

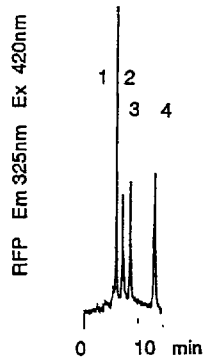


Fig. 4 Chromatogram of sugars obtained on Asahipak NH2-50. Column size: 25 cm x 4.6 mm i.d., Eluent: 75% aq. acetonitrile 1mL/min, Reagent: 20 mM NATA, 0.2M borate, pH 10.5 without periodate, 1 mL/min. Column and reaction temperature: 30 and 150 °C, Exitation and emission wavelengths: 325 and 420 nm, Peaks 1: fucose, 2: glucose, 3: fructose, 4: maltose



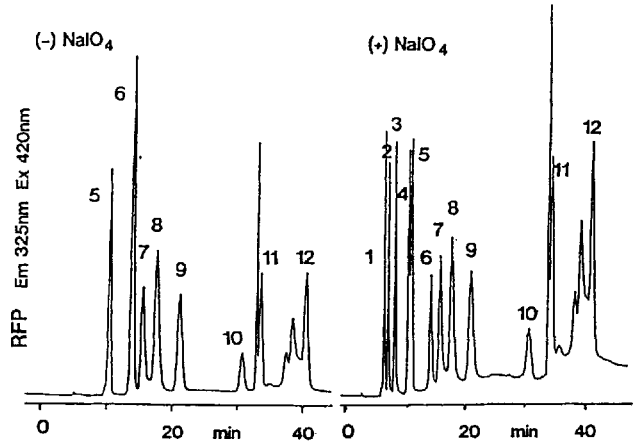


Fig. 5 Chromatograms of sugars obtained on an anion-exchange resin Column: 25cm x 4.6mm i.d., packed Hitachi 3013N, Eluent A: 0.1M NaOH, Eluent B: 0.1M NaOH + 0.5M NaOAc, Gradient: 0-13min, 0% B, 13-20min linear gradient from 0 to 15% B, 20-45min, 15% B, flow rate: 1.0 mL/min, Reagent: 20mM NATA, 0.2M borate, pH 11.5, flow rate: 1.0 mL/min, Column and reaction temperature: 40 and 150°C, 5a: detected without periodate, 5b: detected with 1mM sodium periodate in reagent.

the eluent are shown in Fig. 5. A mixture of each 5-30 nmol of mono- and oligosaccharides and sugar alcohols was chromatographed. The reagent contained 20 mM taurocyamine and 0.2 M potassium tetraborate. Addition of 1 mM sodium metaperiodate to the reagent solution made it possible to detect sugar alcohols (peaks 1-3, myoionisitol, arabitol and mannitol, respectively in Fig. 5b). Peaks 4-12 are 2-deoxy-ribose, fucose, arabinose, glucose, fructose, lactose, maltose, maltotriose and maltopentaose, respectively. The detection limit for glucose was 17 pmol in Fig. 5a and 85 pmol in Fig. 5b. The relative sensitivity of 26 sugars is given in Table I at various condition.

In conclusion, the basic components of the post-label reagent are 20 mM taurocyamine and 0.2M potassium tetraborate in liquid chromatography which use pure water or acetonitrile/water mixture or aqueous sodium hydroxide as the eluent. The pH 8.5 solution is required for reducing mono-saccharides, and higher pH is required for highly sensitive detection of oligosaccharides. The pH is 11.5 for water and 10.5 for acetonitrile/water mixture and aqueous sodium hydroxide as the eluent. 1 mM sodium periodate should be added in the solution for the detection of non-reducing sugars. When borate buffer is used as the eluent in ion-exchange liquid chromatography, the component is 20 mM taurocyamine. If it is necessary, 1 mM sodium periodate is added. The suitable pH of the reagent solution is 8.5 for reducing mono-saccharides and 10.5 for oligosaccharides.

This flexible post-column reaction detector can be applied in various fields which require high sensitive analysis of sugars.



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