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Short communication

Simultaneous analysis of monosaccharides and oligosaccharides by high-performance liquid chromatography with postcolumn fluorescence derivatization

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Abstract

To develop a fluorimetric HPLC technique for the simultaneous microanalysis of reducing mono- and oligosaccharides, the technique of linear gradient elution was introduced into the postcolumn fluorimetric determination system of reducing saccharides with benzamidine. Fluorescence measurement was performed at 288 nm for excitation and 470 nm for emission and an optimization study for this postcolumn fluorescence derivatization carried out. Under optimum conditions, the detection limits of D-glucose and maltohexaose were 1.78 and 2.59 pmol, respectively. The present method was successfully applied to saccharide analysis and should prove useful for automated simultaneous microanalysis of reducing mono- and oligosaccharides in foods. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Saccharides; Monosaccharides; Oligosaccharides

1. Introduction

Recently several oligosaccharides were ascertained as having biological functions such as improvement of intestinal flora (stimulation of bifidobacteria) [1,2], and consequently, have begun to be utilized as ingredients in various food products. Therefore, simultaneous analysis of mono- and oligosaccharides in foods would be useful for manufacturer's quality control and improvement of these products, as well as inspection by public agencies.

High-performance liquid chromatography (HPLC) is one of the promising methods for the analysis of saccharides [3,4]. Among HPLC modes normal-phase partition chromatography (NPPC) produces higher resolution of mono- and disaccharides and rapid analysis of oligosaccharides [5,6], and has therefore been widely used for saccharide analysis. A gradient elution would seem best suited to the simultaneous analysis of mono- and oligosaccharides in NPPC. Most saccharide analysis in NPPC is carried out in combination with a refractive index detector. However, the refractive index detector is highly sensitive to column effluent temperature changes and to mobile phase composition, making it unsuitable for gradient elution [4]. In general, visualization of saccharides is necessary for the use of gradient elution, because most saccharides possess neither chromophores nor fluorophores, but Kai et al. [7] found that benzamidine gave off fluorescence when reducing saccharides are heated in an alkaline medium with benzamidine in a batchwise operation.

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Coquet et al. applied benzamidine to determination of reducing mono- and disaccharides in HPLC [8–10]. They also developed a method for determination of non-reducing disaccharides by HPLC followed by acidic hydrolysis and derivatization with benzamidine [11]. Furthermore, benzamidine has been used in several applications: the determination of monosaccharides in sugar beet [12], the determination of disaccharides [13], the simultaneous determination of vitamin C and its carbamylated derivatives [14], and the fucose screening of human airway secretions [15]. Such previous studies were successful, and indicate that benzamidine is a suitable derivatization reagent for saccharide microanalysis in HPLC. However, no benzamidine methods for the simultaneous determination of reducing mono- and oligosaccharides have been reported.

The aim of this work was to develop such a method in NPPC with postcolumn fluorescence derivatization. In this paper, we also report on optimum conditions suitable for postcolumn fluorescence derivatization with benzamidine and some applications of this method to saccharide analysis in foods.

2. Experimental

2.1. Chemicals and apparatus

Benzamidine hydrochloride monohydrate was obtained from Nacalai Tesque (Kyoto, Japan), and saccharides from Seikagaku Kogyo (Tokyo, Japan). Isomalto-oligosaccharide syrup and two transgalactosylated oligosaccharides were donated by Mr. M. Tanaka (Mitsui Sugar, Japan). All other chemicals were of reagent grade. TSKgel Amide-80 (250×4.6 mm I.D., 5 μm, 80 Å) were obtained from Tosoh (Tokyo, Japan). Chromatography was performed with two Model CCPM-II pumps equipped with two Model SD-8022 degassers, a Model MX-8010 mixer, a Model AS-8020 auto injector, a Model CO-8020 column oven, a Model RE-8020 reactor, a Model LCH-2000 cooler (Advantec Toyo, Japan), and a Model FS-8020 variable-wavelength fluorescence detector (all, except for the cooler, obtained from Tosoh).

2.2. Analytical procedure

Saccharides were separated at 80 °C in a TSKgel Amide-80 column packed with chemically bonded carbamoyl–silica gel. The flow rate of the eluent was 0.8 ml/min. The column eluent was mixed with 1.0 *M* potassium hydroxide solution and benzamidine solution delivered by separate reagent pumps, each at a flow rate of 0.6 ml/min. The mixture was led to a reactor and heated in the reaction coil (0.4 mm I.D., stainless steel tube). The eluent from the reaction coil was passed through a cooling coil (4 m×0.25 mm I.D.) in the cooler (maintained at 10 °C). Fluorescence intensities of the reaction mixtures were monitored at 288 nm for excitation and 470 nm for emission. The elution was performed using a 37.5-min linear gradient from Eluent 1 (acetonitrile–water, (80:20)) to Eluent 2 (acetonitrile–water, (60:40)).

3. Results and discussion

3.1. Optimum conditions suitable for postcolumn fluorescence derivatization with benzamidine

Effects of acetonitrile concentration in benzamidine solution, reaction time, reaction temperature, and benzamidine concentration on the fluorescence intensities of the derivatives obtained from reducing saccharides were investigated. Tested saccharides were D-ribose, D-fructose, D-glucose, maltose, maltotriose, maltotetraose, maltopentaose, and maltohexaose.

Although an increase of acetonitrile concentration in the benzamidine solution shifted the optimum reaction temperature for tested saccharides lower and increased the ratio of the signal-to-noise (S/N), 60% or more of acetonitrile in the solution increased the noise intensity (Fig. 1A). Thus, a concentration of 40% was used as the acetonitrile concentration in the benzamidine solution in the following study. The fluorescence intensities of D-glucose, maltose, and maltohexaose increased with increasing reaction time until 1.25 min at 95 °C, but then decreased with increasing reaction time from 1.50 min (Fig. 1B). Therefore, a time of 1.25 min was selected as the reaction time for this study. Although optimum

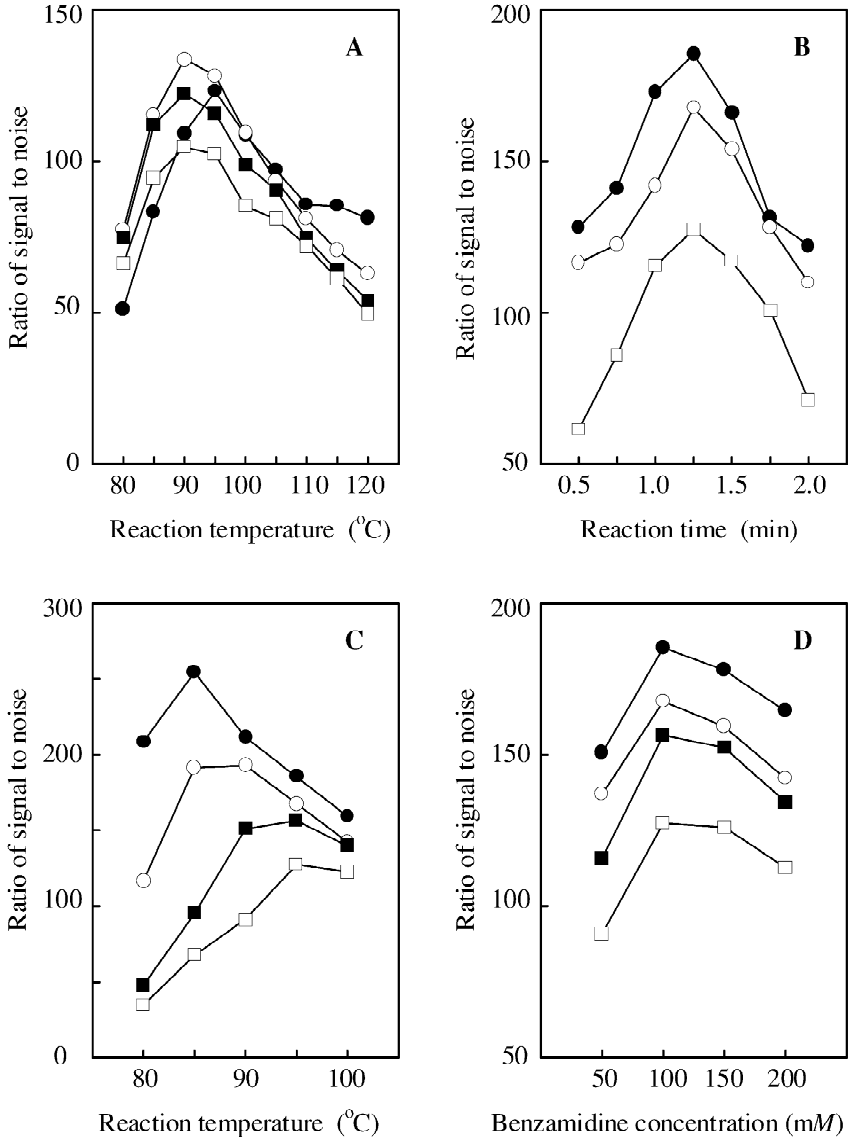

Fig. 1. Effects of reaction conditions on the ratios of signal-to-noise of the fluorescent derivatives of saccharides. Column: TSKgel Amide-80 (250×4.6 mm I.D.). Column temperature: 80 °C. Eluent: a 37.5-min linear gradient from Eluent 1 (acetonitrile–water, (80:20)) to Eluent 2 (acetonitrile–water, (60:40)) (0.8 ml/min). Reagent 1: 1.0 *M* potassium hydroxide (0.6 ml/min). Reagent 2: benzamidine solution containing acetonitrile (0.6 ml/min). Cooling temperature: 10 °C. Cooling time: 0.1 min. Detection wavelengths: excitation; 288 nm, emission; 470 nm. Injection sample: 110 pmol/injection (10 μl). (A) Effect of acetonitrile concentration in the benzamidine solution. Acetonitrile concentration: (●); 20%, (○); 40%, (■); 60%, and (□); 80%. Sample: D-glucose. (B). Effect of reaction time. Reaction temperature: 95 °C. Samples: (●); D-glucose, (○); maltose, and (□); maltohexaose. (C) Effect of reaction temperature. Samples: (●) D-glucose, (○) maltose, (■) maltotetraose, and (□) maltohexaose. (D) Effect of benzamidine concentration. Symbols as Fig. 1C.

temperatures of the saccharides tested varied from 85 to 95 °C (monosaccharides; 85 °C, maltose and maltotriose; 90 °C, the remaining saccharides tested; 95 °C), maltohexaose possessed the weakest fluorescence intensity among the glucose oligomers tested (Fig. 1C). For this reason, 95 °C was judged to be

the best reaction temperature. The maximum S/N values were achieved with 100 mM benzamidine (Fig. 1D).

The detection limits under the optimum conditions for reducing saccharides were as follows: D-ribose; 2.22, D-fructose; 1.53, D-glucose; 1.78, maltose; 1.97, maltotriose; 1.97, maltotetraose; 2.11, maltopentaose; 2.41, and maltohexaose; 2.59 pmol/injection. The fluorescence intensity was proportional to the saccharide quantity injected over a wide range (20–2000 pmol/injection) with a good correlation coefficient ($r=0.9998\sim0.9999$). Reproducibility was confirmed by repeated injections ($n=8$) of reducing saccharides (100 pmol/injection). The relative standard deviation (RSD) for the retention time for saccharides varied from 0.052 (maltotetraose) to 0.36% (D-ribose). The RSD values for the fluorescence intensity for saccharides varied from 1.14 (maltose) to 1.62% (maltopentaose). We consider that these RSD values for the retention time and fluorescence intensity were within acceptable limits for practical simultaneous microanalysis of mono- and oligosaccharides.

To demonstrate the effectiveness of the gradient elution program, a saccharide chromatogram obtained from a gradient elution program was compared with that from an isocratic elution program (Fig. 2). A mixture containing three monosaccharides (D-ribose, D-fructose, and D-glucose) and six laminarioligosaccharides (laminaribiose~laminariheptaose) was tested (each 2.0 μg/ml, 10 μl injection). The isocratic elution program suffered either incomplete elution of high molecular mass oligosaccharides or poor resolution of monosaccharides (Fig. 2A and B). On the other hand, a gradient elution program was capable of simultaneously accomplishing rapid analysis (less than 45 min) and high resolution (Fig. 2C). The introduction of postcolumn saccharide visualization enabled us to use a gradient elution program in NPPC, and consequently, determine rapidly and simultaneously the reducing mono- and oligosaccharides.

3.2. Applications

Fig. 3 shows examples of applications of the present method to the simultaneous microanalysis of mono- and oligosaccharides in saccharide mixtures

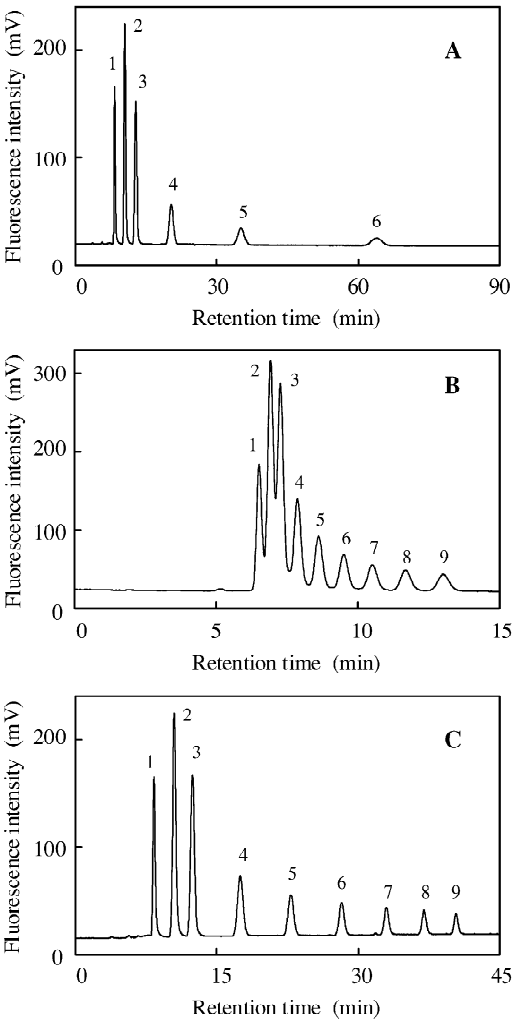

Fig. 2. Simultaneous analysis of three monosaccharides and six oligosaccharides. Column: TSKgel Amide-80 (250×4.6 mm I.D.). Column temperature: 80 °C. Reagent 1:1.0 M potassium hydroxide (0.6 ml/min). Reagent 2: 100 mM benzamidine solution containing 40% acetonitrile (0.6 ml/min). Reaction time: 1.25 min. Cooling temperature: 10 °C. Cooling time: 0.1 min. Detection wavelengths: excitation; 288 nm, emission; 470 nm. Injected sample: a mixture (each 2.0 μg/ml) containing D-ribose, D-fructose, D-glucose, and six laminarioligosaccharides (laminaribiose~laminariheptaose). Injection amount: 10 μl. Flow rate of eluent: 0.8 ml/min. Elution programs: (A) an isocratic elution with Eluent 1 (acetonitrile–water, (80:20)), (B) an isocratic elution with Eluent 2 (acetonitrile–water, (60:40)), and (C) a 37.5-min linear gradient from Eluent 1 to Eluent 2. Peak assignment: (1) D-ribose, (2) D-fructose, (3) D-glucose, (4) laminaribiose, (5) laminaritriose, (6) laminaritetraose, (7) laminaripentaose, (8) laminarihexaose, (9) laminariheptaose.

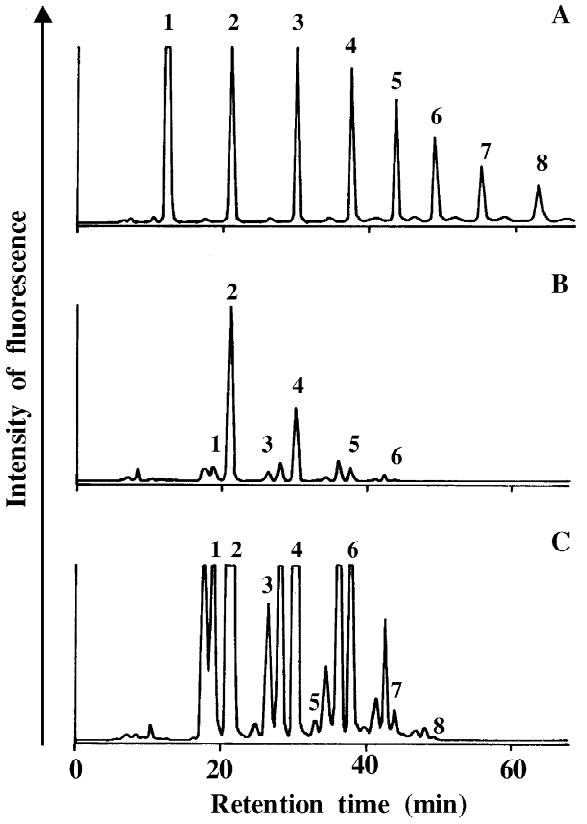

Fig. 3. Chromatograms of saccharide mixtures containing isomalto-oligosaccharides. Samples: (A) isomalto-oligosaccharide standard (30 μg/10 μl injection), (B) isomalto-oligosaccharide syrup (IMO syrup) (2 μg/10 μl injection), and (C) IMO syrup (100 μg/10 μl injection). Samples of (B) and (C) contained 100 pmol of D-ribose as an internal standard. Chromatographic conditions were the same as described in Fig. 2C. Peak assignment: (A1); glucose, (A2, B2, and C2); isomaltose, (A3, B4, and C4); isomaltotriose, (A4, B5, and C6); isomaltotetraose, (A5, B6, and C7); isomaltopentaose, (A6 and C8); isomaltohexaose, (A7) isomaltoheptaose, (A8); isomaltooctaose, (B1 and C1); maltose, (B3 and C3); maltotriose, (C5); maltotetraose.

containing isomalto-oligosaccharides. An isomalto-oligosaccharide standard was used as a retention time marker (Fig. 3A). Fig. 3B shows that isomalto-oligosaccharide syrup (IMO syrup) contained large amounts of isomalto-oligosaccharides (1101 ng) (isomaltose (611 ng), isomaltotriose (373 ng), isomaltotetraose (101 ng), and isomaltopentaose (16 ng)), together with small amounts of maltose (49 ng), maltotriose (47 ng). Glucose and maltotetraose were present in trace amounts. This composition was as expected and comparable to that in the literature [1]. Because the water content in IMO syrup is about 25%, saccharide recovery (the sum of the six saccharides described above) was 79.9% and the isomalto-oligosaccharide percentage (the sum of the four isomalto-oligosaccharides described above) to total dry weight was 73.4%. Because the manufacturing procedure of IMO syrup requires control of a complex enzymatic reaction, the saccharide composition monitoring of products is important. The present simultaneous analysis seems to be applicable to quality control of food ingredients containing mono- and oligosaccharides.

Recently, several saccharide mixtures containing galacto-oligosaccharides have been produced from highly concentrated lactose solution with β-galactosidase. Because galacto-oligosaccharides have a bifidobacteria growth promoting effect, their presence in a higher proportion to total saccharides in the mixtures is desirable for food ingredients. But in some cases undesirable large amounts of lactose as a residual starting material (also as a byproduct), and glucose and galactose as byproducts have been found in final commercial products [16]. The result of the comparative experiment utilizing the present method indicates that the lactose content in mixture A (Fig. 4A) was lower than that in mixture B (Fig. 4B). However, the present method could not determine whether the peak having a retention time of about 12.40 min (peak G) corresponded to glucose or not, because of the low resolution between glucose and galactose. Another analytical method such as ion-exchange chromatography is necessary for detailed peak identification. Fig. 4C and D indicate the differences in the patterns of reducing saccharides between mixtures A and B. Such pattern assays of reducing saccharides, similar to fingerprint and peptide mapping, will be useful for quality control and inspection of oligosaccharide products.

4. Conclusion

The present method, which is a combination of the technique of linear gradient elution in NPPC and postcolumn fluorimetric determination of reducing saccharides with benzamidine, has several advantages, such as high sensitivity, rapid analysis, good

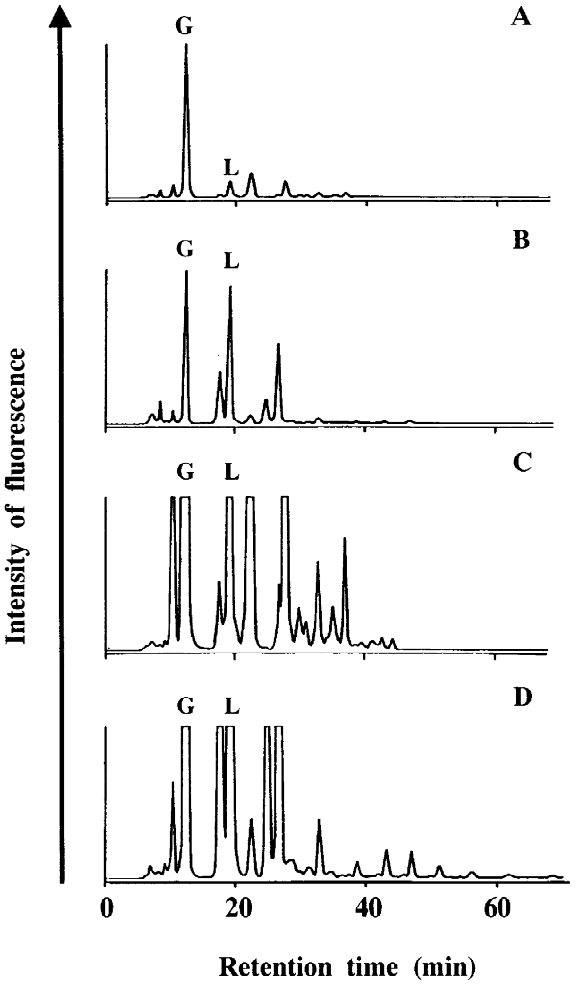

Fig. 4. Chromatograms of saccharide mixtures containing transgalactosylate oligosaccharides. Samples: (A) saccharide mixture A (2 μg/10 μl injection), (B) saccharide mixture B (2 μg/10 μl injection), (C) saccharide mixture A (100 μg/10 μl injection), and (D) saccharide mixture B (100 μg/10 μl injection). Samples contained 100 pmol of D-ribose as an internal standard. Chromatographic conditions were the same as described in Fig. 2C. Peak assignment: (L); lactose, (G); unknown.

linearity over a wide range, and good reproducibility. Thus the present method will prove useful for automated simultaneous microanalysis of reducing mono- and oligosaccharides.

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