Simultaneous Quantitative Determination of Mono-, Di-, Tri-, Tetra-, and Penta-Saccharides in Yogurt Products by a Simple HPLC System with a Refractive Index Detector

Joung Ho Ko and Won Jo Cheong*

Department of Chemistry and Institute for Basic Science, Inha University, Incheon 402-751, Korea
Received September 23, 2000

Keywords: Saccharides, Yogurts, Determination by classes, HPLC, Refractive index detector.

Some lactic acid bacteria such as bifidobacteria are known to show some therapeutic effects such as anti-cancer action as well as health-enhancing effects.[1] Polysaccharides and oligosaccharides produced by them are suspected to cause such effects. Sanford[2] classified such polysaccharides into intracellular, cell-wall, and extracellular polysaccharides. The pH of the culture solution decreases by the acids produced and slime-type extracellular polysaccharides are secreted to make the culture solution viscous as the fermentation proceeds.[3] Lactic acid bacteria produce various extracellular polysaccharides from the carbohydrate nutriments in the culture. The polysaccharides produced by lactic acid bacteria have drawn much interest owing to their use as a replacement of blood plasma, anti-cancer activity, and their ability of enhancing physiological activities.[4-6] The structures and compositions of polysaccharides have not been entirely determined and the mechanisms of the therapeutic effects including the anti-cancer action are not well known yet although many reports support that lactic acid bacteria and their various polysaccharide products show activities of preventing and curing cancers.[7-14]

Qualitative and quantitative determination of polysaccharides demands sophisticated analytical measures. Gel filtration chromatography (GFC) and gel permeation chromatography (GPC) are used for determining molecular weight distribution, and vacuum UV circular dichroism, Raman spectrometry, and small angle neutron and X-ray scattering for understanding molecuar size and conformation.[15,16] The contents of some main building monosaccharides can be determined by colorimetric methods, and the contents of uronic acids and proteins, by enzymatic methods.[16] In order to obtain complete compositions of monosaccharides, the polysaccharides should be acid-hydrolyzed and analyzed by HPLC or GC/MS.[16-21] Modifying the monosaccharides to more volatile derivatives is essential to GC/MS analysis.

Oligosaccharides are hard to analyze by GC/MS owing to difficulties in derivatization. Some studies of analyzing oligosaccharides by SFC/MS have been reported.[22-27] Qualitative analyses such as structural determination of oligosaccharides by LC/MS were introduced some time ago,[28-30] and a lot of studies using LC/MS for analysis of oligosaccharides have been reported thereafter. Quantitative application of LC/MS suffers from variation of response factors among different saccharide isomers and/or depending upon operational conditions.

Quantitative determination of oligosaccharides and/or smaller saccharides by a HPLC method with a refractive index detector (RID) has been hardly reported. Low sensitivity and inapplicability to gradient elution of refractive index detector retarded such approaches. Instead, the ion exchange chromatography system with a pulsed amperometric detector[31-33] has been known to show excellent performance in determination of saccharides and is prevalent in such analyses.

In this study, we have shown that a rather simple HPLC system with a refractive index detector is good enough to quantitatively analyze oligosaccharides and smaller saccharides by class based on molecular weight in commercial yogurt products. The merit of this method is simplicity and convenience in preparing standards. We found that the RID signals of the saccharides of the same molecular weight were virtually the same and that only one standard material was required for the saccharide isomers of the same molecular weight.

Experimental Section

Chemicals. Acetonitrile and water were of HPLC grade and purchased from Fisher (Pittsburgh, PA, USA) and used without purification. Fructose, galactose, glucose, lactose, maltose, sucrose, maltotriose, melezitose, maltotetraose, and maltopentaose were obtained from Sigma (St. Louis, Mo, USA) and used as received.

Chromatographic system. The chromatographic system we used was composed of a Shimadzu (Tokyo, Japan) LC-10AD VP pump, a Rheodyne (Cotati, USA) injector with a 5 μL loop, Shimadzu RID-6A refractive index detector, and a Kromasil NH_2 column (2.1 mm I.D. $\times$ 300 mm, 5 μm). We used a computer and the Chromate 3.0 version software made by Youlin-Gisul (Sung Nam, Korea) to obtain the raw chromatographic data. The eluent was 70/30 (v/v %) acetonitrile/water, and the flow rate was fixed at 0.5 mL/min.

Preparation of standard mixtures and calibration curves. The first standard solution was prepared by dissolving 75 mg fructose and 75 mg galactose (mono-saccharides), 40.0 mg lactose (di-saccharide), 12.0 mg maltotriose (tri-saccharide), 5.00 mg maltotetraose (tetra-saccharide), and 2.00 mg maltopentaose (penta-saccharide), respectively, in water and by adjusting the total volume of the solution to 1 mL. Such concentrations of standard solution were chosen to be similar to the concetrations of saccharides in real yogurt products.

The second standard solution was prepared in such a way

that the concentration of each component was about three times the concentration of the first standard. On the other hand, the concentration of each component of the third standard was chosen to be about one third of the concentration of the first standard. The calibration curves (signal intensity *vs*. concentration) of individual saccharides classified by molecular weight were made based on the chromatograms of the three standards. All the saccharide concentrations in real yogurt products were found within the ranges of the calibration curves, and the curves were nicely linear.

Determination of saccharides in yogurt samples by classes depending upon molecular weight. A 25 mL aliquot of a suspension type yogurt sample was taken, centrifuged at 20,000 rpm for 30 min, filtered with a 0.45 μm membrane filter to get a transparent solution. The solution was injected through the 5 μL injector to the Kromasil NH_2 column. The concentrations of individual saccharide classes (classified by molecular weight) in yogurt products were determined based on direct comparison of chromatographic measurements (peak areas) between a standard and a yogurt sample. The sample treatment followed by the chromatographic measurement was repeated four times on different days (mostly different weeks) for each yogurt product. Four commercial yogurt products in Korea were examined as follows: Pasteur, Bulgaris, Dr. Capsule, and GG.

Results and Discussion

Response factors of saccharides. The quantitative determination of individual saccharides by classes depending upon molecular weight in this study is based on the assumption that the response factors (area count divided by mass concentration) of saccharides of the same molecular weight are virtually the same. Complete separation of isomers of the same molecular weight and identification of individual isomers by a chromatographic method are difficult owing to limitation of chromatographic resolution and unavailability of all the isomeric standards. Some isomers of the same molecular weight were used to examine the validity of such assumption. The extent of variation of response factors among saccharide classes was also examined. The solutes were fructose, galactose, glucose (mono-saccharides), lactose, maltose, sucrose (di-saccharides), maltotriose, melezitose (tri-saccharides), maltotetraose (tetra-saccharide), and maltopentaose (penta-saccharide). The concentration of each saccharide was fixed 10 mg/mL, and each solution was chromatographed 5 times to obtain the average response factor and relative standard deviation. The response factors for the saccharides were summarized in Table 1. We can note that the response factors for monosaccharides are significantly lower than those of other saccharides but that the response factors are virtually the same for di-saccharides and higher oligosaccharides. In addition, the response factors of all the mono-saccharide isomers are very close, too. Thus we were convinced that the response factors of isomers of the same molecular weight are virtually the same, which supports validity of our method of determining saccharides by classes based on molecular weights.

Table 1. The RID response factors of individual saccharides including isomers of the same molecular weight and their standard deviations (in parentheses) for five measurements. The response factor was defined as the area count of the component divided by its mass concentration (mg/mL)

Carbohydrate	Molecular weight	Response Factor
Fructose	180	2358 (± 13)
Galactose	180	2355 (± 24)
Glucose	180	2303 (± 18)
Lactose	342	3311 (± 62)
Maltose	342	3051 (± 20)
Sucrose	342	3502 (± 11)
Maltotriose	504	3427 (± 5)
Melezitose	504	3413 (± 49)
Maltotetraose	666	3387 (± 75)
Maltopentaose	828	3142 (± 71)

Calibration curves. The calibration curves for standards for mono- and di-saccharides are shown in Figure 1, and those for tri-, tetra-, and penta-saccharides, in Figure 2. The signal intensity is nicely linear with the saccharide concentration in the concentration ranges. The concentrations of individual saccharide classes in yogurt samples were found within the ranges of the calibration curves. The regression results for individual saccharide classes are given below (y = area count, x = saccharide concentration, r = correlation coefficient).

Fructose + galactose	$y = 2400\ x,\ r^2 = 0.9994$
Lactose	$y = 3320\ x,\ r^2 = 0.9980$
Maltotriose	$y = 3370\ x,\ r^2 = 0.9990$
Maltotetraose	$y = 3450\ x,\ r^2 = 0.9996$
Maltopentaose	$y = 3160\ x,\ r^2 = 0.9965$

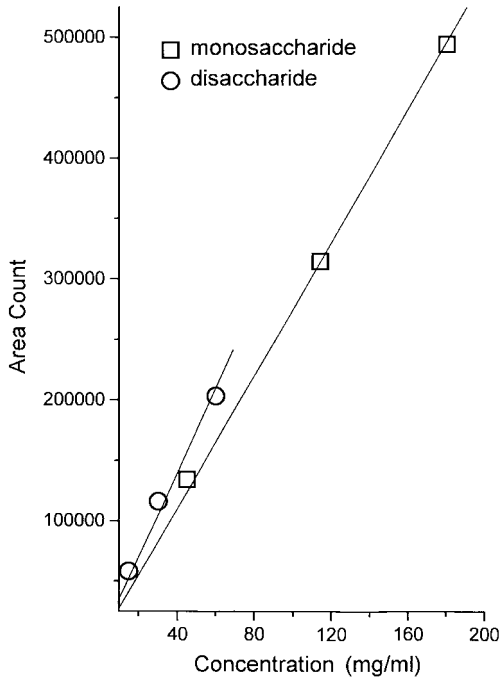

Figure 1. The calibration curves for mono-saccharide (fructose + galactose) and di-saccharide (lactose) based on the three standards.

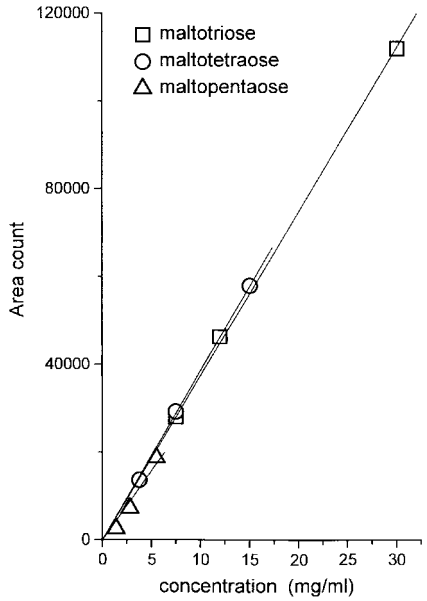

Figure 2. The calibration curves for tri-saccharide (maltotriose), tetra-saccharide (maltotetraose), and penta-saccharide (maltopentaose) based on the three standards.

Quantitative determination of each saccharide class for real yogurt samples. The chromatogram of the first standard is shown in Figure 3. The chromatogram of a yogurt sample, Pasteur, for example, is shown in Figure 4. Confirmation of the saccharide class (molecular weight) of each peak was carried out by a LC/MS run. Quantitative determination of saccharides based on molecular weights by LC/MS was difficult since the response factors of isomeric saccharides of the same molecular weight were quite different one another, thus we have come to develop the method of this study. Determination of structures of individual isomers is out of the scope of this study and such research is under way. The determined concentrations of individual saccharide classes (based on molecular weight) and their standard deviations for measurements of 4 batches are summarized in Table 2. We can see some fluctuation of saccharide contents among different batches since fermentation processes are not very reproducible. The Pasteur yogurt has proven to include more oligosaccharides (tri-saccharides or heavier) than others.

Uncertainty of quantitative determination of this method. Validation of our method may be carried out by analysis of certified reference materials and by comparison of the measured concentrations with the certified values. We have never heard about certified reference yogurt products, however. Another approach is to measure the yogurt products by an approved standard method and to compare the results with those determined by our method. Unfortunately, no approved standard method exists so far. The major problems are incomplete chromatographic separation of isomeric saccharides of the same molecular weight, different response factors among isomers, and unavailability of individual standard materials for so many saccharide isomers. This study is actually the first try to determine quantitatively concentrations of individual saccharide classes based on molecular weights for real yogurt

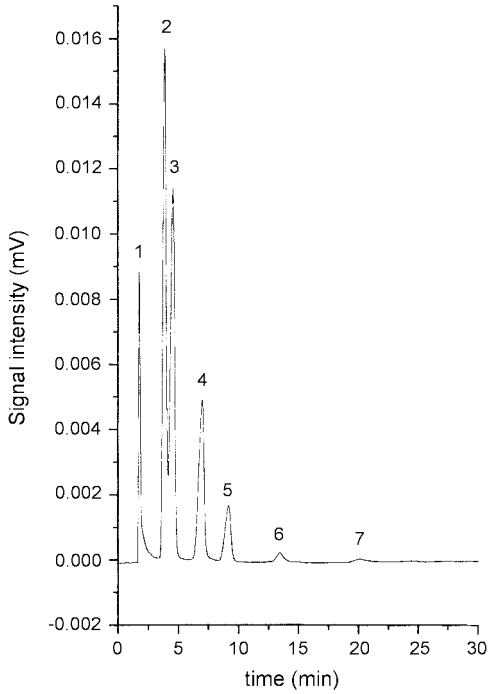

Figure 3. The chromatogram of the standard composed of 75 mg fructose, 75mg galactose, 40 mg lactose, 12 mg maltotriose, 5 mg maltotetraose, and 2 mg maltopentaose. The eluent was 70/30 (v/v %) acetonitrile/water with a Kromasil NH_2 column (2.1 mm I.D. × 300 mm, 5 μm), and the flow rate was 0.5 mL/min. 1; water, 2; fructose, 3; galactose, 4; lactose, 5; maltotriose, 6; maltotetraose, 7; maltopentaose.

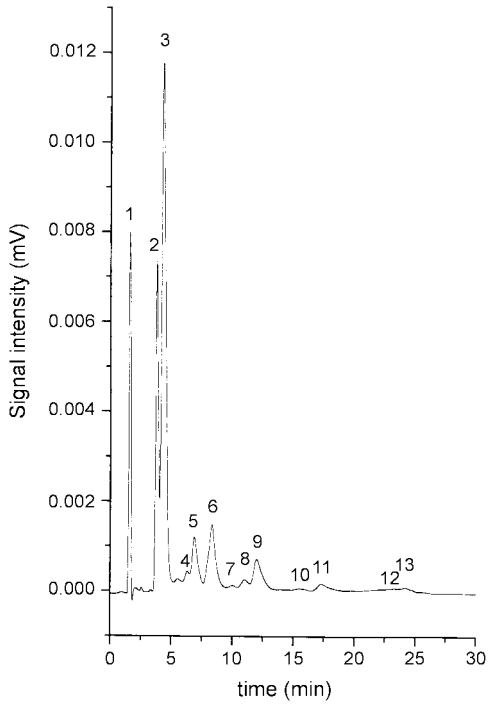

Figure 4. The chromatogram of a yogurt sample (Pasteur). The chromatographic conditions are the same as those in Figure 3. The molecular weight of each peak was determined by LC/MS. 1; water, 2,3; mono-saccharides, 4,5; di-saccharides, 6,7,8,9; tri-saccharides, 10,11; tetra-saccharides, 12,13; penta-saccharides.

Table 2. The determined concentrations and standard deviations (in parentheses, n=4) of individual saccharide classes (based on molecular weights) in four commercial yogurt products made in Korea. Unit: mg/ml

class \ product	Pasteur	Bulgaris	Dr. Capsule	GG
mono-saccharides	101.6(±2.9)	83.7(±3.2)	106.3(±26.9)	112.6(±4.9)
di-saccharides	11.12(±4.3)	12.8(±2.9)	47.0(±4.05)	17.6(±2.1)
tri-saccharides	24.2(±3.3)	8.63(±1.02)	5.85(±1.66)	5.09(±0.20)
tetra-saccharides	5.71(±0.83)	3.50(±0.40)	2.66(±0.41)	3.08(±1.01)
penta-saccharides	2.76(±0.62)	1.40(±0.58)	0	1.27(±0.61)

samples. Therefore, we are not able to give sound data for uncertainty of our method at the moment. We will instead give some general comments on uncertainty of our method.

We should note that the response factors of three di-saccharides are quite variant (Table 1), and that a considerable error occurs in quantitative determination of the di-saccharide class since our method is based on the assumption that the response factors (RF) of isomeric saccharides of the same molecular weight are virtually the same. The reason why a rather high variation exists only for the RID response factors of di-saccharides is not clear, but we guess it is related to the structural differences among the isomers. Anyway, we selected lactose as the standard material for di-saccharides since its response factor is close to the mean value of the three response factors. The highest error is *ca* 7% if we assume that there is no lactose in the yogurt sample and that only one of the rest components exists. The error will be much smaller since it is likely that a few di-sacchrides exist in real samples. Nevertheless, we should admit the possibility that a higher error can occur if another di-saccharide with a deviated RF value exists in the yogurt sample since not all the di-saccharides were identified yet.

The reproducibility of determination for repeated injections was better than 5% (not shown in Table 2) for the worst case. The reproducibility among different batches of the same product was much worse. Such reproducibility depends upon saccharide class and product maker. It is summarized in Table 2. We can also note in Table 2 that the relative standard deviations for heavier saccharides with low and broad chromatographic peaks are greater than those of smaller saccharides.

Thus, we have successfully determined saccharides in commercial yogurt products by classes based on molecular weights by using a rather simple HPLC system with a NH_2 column and a RID detector. The response factors of isomers of the same molecular weight have proven to be virtually the same, thus we were able to use a convenient commercially available standard for each saccharide class. The calibration curves were nicely linear for the composition ranges.

Acknowledgment. This work was supported by Korea Science and Engineering Foundation (981-0305-027-2).

References

1. Hughes, D. B.; Hoover, D. G. *Food Technol.* **1991**, *1991*, 74.
2. Sanford, P. A. *Adv. Carbohyd. Chem. Biochem.* **1979**, *36*, 265.
3. Pace, G. W.; Righelato, R. C. *Adv. Biochem. Eng.* **1984**, *15*, 41.
4. Whistler, R. L.; Bushway, A. A.; Singh, P. P.; Nakahara, W.; Tokuzen, R. *Adv. Carbohyd. Chem. Biochem.* **1976**, *32*, 235.
5. Knabe, M. *Jap. J. Diary and Food Sci*. **1981**, *30*, 219.
6. Keating, K. *Cultured Diary Products Journal* **1985**, *20*, 13.
7. Sanford, P. A. *Adv. Carbohyd. Chem. Biochem.* **1976**, *32*, 257.
8. Fernandes, C. F.; Shahani, K. M. *J. Food Protect* **1990**, *53*, 704.
9. Hosono, A.; Wardijo, R.; Otani, H. *Agric. Biol. Chem.* **1990**, *54*, 1639.
10. Mitsuoka, T. *A Profile of Intestinal Bacteria*; Yakurt Honsha Co., Ltd., Tokyo 1989.
11. Tanaka, R.; Takayama, H.; Morotomi, M.; Kuroshima, T.; Ueyama, S.; Matsumoto, K.; Kuroda, A.; Mutai, M. *Bifidobacteria Microflora* **1983**, *2*, 17.
12. Sekine, K.; Toida, T.; Saito, M.; Kuboyama, M.; Kawashima, T.; Hashimoto, Y. *Cancer Research* **1985**, *45*, 1300.
13. Toida, T.; Sekine, K.; Tatsuki, T.; Saito, M.; Kawashima, T.; Hashimoto, Y.; Sakurai, Y. *J. Can. Res. Clin. Oncol*. **1990**, *116*, 342.
14. Modler, H. W.; Mckellar, R. C.; Yaguchi, M. *Can. Inst. Food Sci. Technol. J.* **1990**, *23*, 29.
15. *Industrial Polysaccharides: Gordon and Breach Science Publishers*: Stivala, S. S.; Crescenzi, V.; Dea, I. C. M., Eds.; New York, London, Paris, Montreux, Tokyo, Melbourne, 1987.
16. Roberts, C. M.; Fett, W. F.; Osman, S. F.; Wiley, C.; O'Connor, J. V.; Hoober, D. G. *J. Appl. Bacteriol.* **1995**, *78*, 463.
17. McFeeters, R. F.; Thomson, R. L.; Fleming, H. P. *J. Food Sci.* **1993**, *58*, 832.
18. Tisza, S.; Sass, P.; Molnar-Perl, I. *J. Chromatogr.* **1994**, *676*, 461.
19. Lamb, J. D.; Myers, G. S.; Edge, N. *J. Chromatogr. Sci.* **1993**, *31*, 353.
20. MaFeeters, R. F. *J. Agric. Food Chem.* **1993**, *41*, 1439.
21. Morvai-Vitanyi, M.; Molnar-Perl, I.; Knausz, D.; Sass, P. *Chromatographia* **1993**, *36*, 204.
22. Pinkston, J. D.; Owens, G. D.; Burkes, L. G.; Delaney, T. E.; Millington, D. S.; Maltby, D. A. *Anal. Chem.* **1988**, *60*, 962.
23. Reinhold, V. N.; Sheeley, D. M.; Kuei, J.; Her, G. R. *Anal. Chem.* **1988**, *60*, 2719.
24. Sheeley, D. M.; Reinhold, V. N. *J. Chromatogr.* **1989**, *474*, 83.
25. Caesar, Jr., J. P.; Sheeley, D. M.; Reinhold, V. N. *Anal. Biochem.* **1990**, *191*, 247.
26. Merritt, M. V.; Sheeley, D. M.; Reinhold, V. N. *Anal. Biochem.* **1991**, *193*, 24.
27. Sheeley, D. M.; Reinhold, V. N. *Anal. Biochem.* **1991**, *193*, 240.
28. Sakairi, M.; Kambara, H. *Anal. Chem.* **1989**, *61*, 1159.
29. Reinhold, V. N.; Carr, S. A.; Green, B. N.; Petiton, M.; Choay, J.; Sinay, P. *Carbohyd. Res.* **1987**, *161*, 305.
30. Schols, H. A.; Mutter, M.; Voragen, A. G. J.; Niessen, W. M. A.; Van der Hoeven, R. A. M.; Van der Greef, J.; Bruggink, C. *Carbohydr. Res.* **1994**, *261*, 335.
31. Sullivan, J.; Douek, M. *J. Chromatogr. A* **1994**, *671*, 339.
32. McGuire, J. M.; Elliott, M. A.; Elliott, H. G.; Smith, K. D. *Carbohyd. Res.* **1995**, *270*, 63.
33. Farine, S.; Villard, C.; Moulin, A.; Mouren, G. M.; Puigserver, A. *Intern. J. Biol. Macromol.* **1997**, *21*, 109.