

## Analysis of Lactulose Preparations by Spectrophotometric and High Performance Liquid Chromatographic Methods

F. W. PARRISH,<sup>1</sup> K. HICKS,<sup>2</sup> and L. DONER  
Eastern Regional Research Center<sup>3</sup>  
Philadelphia, PA 19118

### ABSTRACT

Spectrophotometric methods of analysis were applied to sugar mixtures produced during isomerization of lactose to lactulose. A combination of four established spectrophotometric methods was used to quantitate galactose, tagatose, lactose, and lactulose, the four major sugars present. In addition, a method originally developed for determining lactose with methylamine at 65°C and pH 12.7 was suitable for combined measurement of lactose and lactulose. After a specific determination of lactose with  $\beta$ -galactosidase and glucose oxidase, lactulose was measured by difference. A high pressure liquid chromatographic separation of galactose, tagatose, lactose, and lactulose was achieved on a commercial carbohydrate analysis column with a mixture of water and acetonitrile used as eluent. Analysis of lactulose syrups by the new high pressure liquid chromatographic method yielded results that agreed with those by the spectrophotometric methods and required much less time.

### INTRODUCTION

As part of a program aimed at increased utilization of lactose and its derivatives in food applications, we have examined methods for isomerizing lactose to lactulose [4-O- $\beta$ -D-

galactopyranosyl- $\beta$ -D-fructofuranose] (12, 15, 21). The greater sweetness (19) and solubility (17) of lactulose compared to lactose indicate that lactulose has potential as a partial replacement for sucrose in baking applications where lactose has been unsatisfactory (1).

This paper describes application of established spectrophotometric methods and presents a more convenient high performance liquid chromatographic technique for analysis of mixtures containing lactulose and other sugars.

### MATERIALS AND METHODS

#### Sugar Standards

Reagent grade anhydrous glucose, fructose, tagatose, galactose,  $\alpha$ -lactose monohydrate, and lactulose were dried under vacuum at 65°C for 16 h and then used to prepare 100 mg/ml stock solutions.

Lactulose syrups were either purchased (Cephulac, Merrell-National Laboratories<sup>4</sup>) or prepared by isomerization of lactose with calcium hydroxide (15), triethylamine (18), or sodium aluminate (11). The syrups, stored as 70 to 80% solutions, were diluted to the appropriate concentrations prior to analysis.

#### Spectrophotometric Analyses

Total reducing sugars were measured by the 3,5-dinitrosalicylic acid method (14) with  $\alpha$ -lactose monohydrate solution (0 to 1 mg/ml) as standard; absorbance was measured at 540 nm. Ketose sugars were determined by the thiobarbituric acid method (20) with fructose solution (0 to 40  $\mu$ g/ml) as standard. Aldose sugars were measured by a hypiodite oxidation procedure (13) with  $\alpha$ -lactose monohydrate solution (0 to 2 mg/ml) as standard. Monosaccharides were determined in the presence of disaccharides by the method of Tauber and Kleiner (23) with fructose (0 to .4 mg/ml) as standard. Galactose (0 to 1.25 mg/ml) was assayed with galactose oxidase (9), and lactose was measured, after hydrolysis with  $\beta$ -galactosi-

Received February 4, 1980.

<sup>1</sup> Southern Regional Research Center, Agricultural Research, Science and Education Administration, US Department of Agriculture, New Orleans, LA 70179.

<sup>2</sup> Postdoctoral Fellow supported by Dairy Research Foundation.

<sup>3</sup> Agricultural Research, Science and Education Administration, US Department of Agriculture.

<sup>4</sup> Reference to brand or firm name does not constitute endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

dase (22), with a glucose oxidase assay procedure (2). The methylamine procedure for lactose (16) was applied also to lactulose (.5 to 2 mg/ml). All standard curves gave a linear relationship ( $r > .997$ ) between absorbance and sugar concentration. Absorbance was measured in 1-cm cuvettes with a Zeiss Model PMQII spectrophotometer. Analyses of samples solutions were in triplicate.

#### High Pressure Liquid Chromatographic Analysis

A standard mixture containing galactose, tagatose, lactose, and lactulose was prepared in a mixture of water and acetonitrile (1 + 1, by volume) as solvent. All sugars were at a known and equal concentration that was less than 20 mg/ml each. In all cases, the sugars were dissolved in water before the acetonitrile was added. Sugar mixtures from isomerization of lactose were deionized with Amberlite IR-120 (H) and Duolite A-561 (free base), and diluted so as to contain not more than 20 mg/ml of any component. A sample (20  $\mu$ l) of sugar mixture was applied by loop injection to an analytical (3.9 mm  $\times$  30 cm) Carbohydrate Analysis Column (Waters, Milford, MA). Separation of all four sugars was achieved by elution with a mixture of water and acetonitrile (77/23, wt/wt) at 2 ml/min with a modular chromatographic system including an Instrumentation Specialties Co. (ISCO) metering pump model 314, series 1240-003, a pressure monitor (ISCO model 1590), and a Waters Associates model R401 refractive-index detector. Sugars were quantified by comparison of peak heights with those of corresponding sugars in standard solutions.

#### RESULTS AND DISCUSSION

Spectrophotometric methods are available

for determining total sugar (7), total reducing sugars (10, 14), aldoses (13), and ketoses (20) in sugar mixtures. In addition, enzymatic methods enable specific sugars to be determined, e.g., glucose and galactose with oxidative enzymes (9). Application of two of these methods (9, 10) enabled quantitative determination of sucrose and its component monosaccharides, glucose and fructose, in mixtures of all three sugars (6). The complexity of spectrophotometric analyses of mono and oligosaccharides in mixtures, however, increases with number of sugars and number of methods that have to be combined to achieve the desired result. Accuracy also is affected by differential responses of individual sugars to a reagent as compared to that of the sugar used for a calibration standard. In this study, the lactulose syrups contained galactose and tagatose (4, 5) as well as lactose and lactulose. The presence of these four different sugars added to the complexity of the analysis.

Our studies involving several isomerization reagents previously described (11, 15, 18) with varying conditions such as time, temperature, and pH, have shown 3,5-dinitrosalicylic acid reagent (14) is accurate for measuring total reducing sugar. Equal absorbance was given by .875 mg galactose, .912 mg glucose, .985 mg tagatose, .993 mg lactulose, and 1.000 mg lactose. Lactose was used as the calibration standard because conditions for optimal yield of lactulose resulted in less than 10% monosaccharide.

To ascertain amounts of each of the four sugars in lactulose syrups, the approach in Table 1 was used. Separate analyses were to determine aldoses (13), ketoses (20), monosaccharides (23), and lactose (2, 22). Subtraction of monosaccharide from the sum of aldose and

TABLE 1. Spectrophotometric analysis scheme for lactulose in mixtures from isomerization of lactose.

Type of sugar	Method	Sugars determined	Reference
Aldoses	Hypoiodite	Lactose, galactose	13
Ketoses	Thiobarbituric acid	Lactulose, tagatose	20
Monosaccharides	Acid $\text{Cu}^{++}$ , molybdate	Galactose, tagatose	23
Lactose	$\beta$ -Galactosidase + glucose oxidase	Lactose	2, 22

TABLE 2. Spectrophotometric analysis of standard sugar mixtures as in the scheme of Table 1.

Mixture	Sugar (mg/100 ml)							
	Lactulose		Lactose		Galactose		Fructose <sup>a</sup>	
	Added	Found <sup>b</sup>	Added	Found	Added	Found	Added	Found
1	40	43(±2)	140	136(±4)	0	1(±1)	20	24(±2)
2	80	85(±4)	80	77(±2)	20	22(±1)	20	24(±2)
3	160	171(±7)	20	23(±2)	20	22(±1)	0	2(±1)

<sup>a</sup>The more readily available ketose, fructose, was substituted for tagatose because each gave identical responses in spectrophotometric tests.

<sup>b</sup>Average of three measurements with observed deviation.

ketose analyses gave a sum of lactose and lactulose concentrations. Lactose was measured by enzymatic hydrolysis (22) to galactose and glucose followed by estimation of glucose with glucose oxidase (2). The amount of lactulose was obtained by subtraction of lactose from the sum of lactose and lactulose concentrations. Application of these analytical procedures enables the concentrations of lactulose, lactose, galactose, and tagatose to be calculated (Table 2). Results are in Table 3 for mixtures containing optimal yields of lactulose by the three isomerization methods (11, 15, 18).

An alternative spectrophotometric procedure for measure of lactulose (Table 3) was developed for assays in which monosaccharide quantitation was not of interest. This was based on a methylamine procedure for lactose (16). Galactose produced negligible color under the same conditions. The absorbance for .867 mg

lactulose and 1.000 mg lactose was identical. The methylamine procedure together with the enzymatic procedure for lactose measurement permitted determination of lactulose by difference with more convenience and greater precision than analysis involving the combination of four assays (Table 3).

Because spectrophotometric analysis of multicomponent mixtures can be complex and time consuming, we examined the potential of high pressure liquid chromatography (HPLC) for this purpose. The use of HPLC in sugar analysis has been reviewed (3), and official methods for the quantitation of specific sugars by this chromatographic method exist (8). Verhaar and coworkers (24) developed a liquid chromatographic method for separation and quantitation of sugars in lactulose syrups. While their method produces baseline separation of the various sugars, it requires a complex detec-

TABLE 3. Spectrophotometric analysis of sugars in mixtures from isomerization of lactose.

Isomerization reagent	Sugars % (by wt) <sup>a</sup>				
	Procedures of Table 1			Methylamine procedure	
	Monosaccharides	Lactulose	Lactose	Lactulose	Lactose
Calcium hydroxide	4.5(±.5)	17(±2)	80(±3)	16(±1)	76(±3)
Triethylamine	2.5(±.5)	38.5(±3)	61(±2)	36(±2)	62(±2)
Sodium aluminate	4.5(±.5)	77(±4)	23(±1)	73(±3)	22(±1)

<sup>a</sup>Average of three measurements with observed deviation.

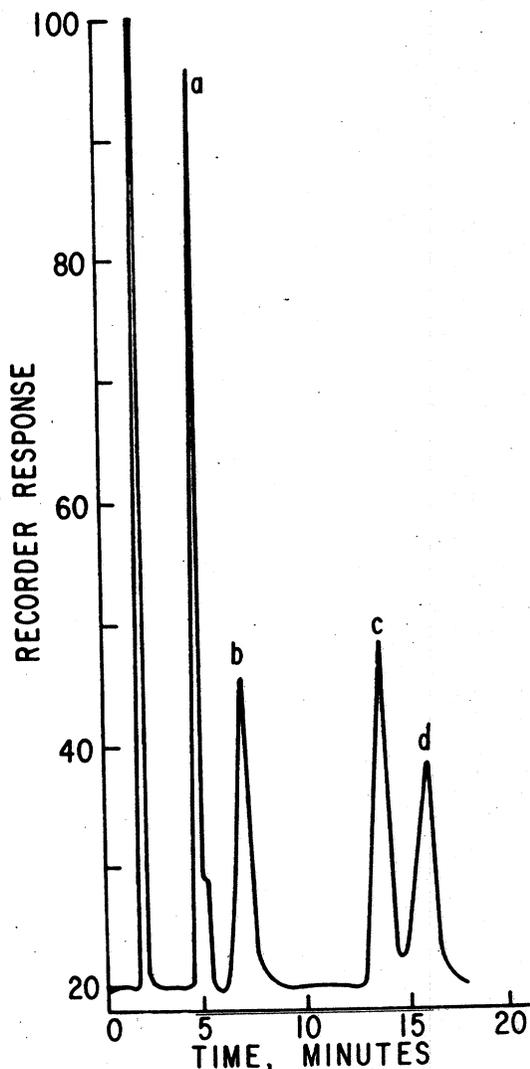


Figure 1. High performance liquid chromatogram of a standard mixture of tagatose (a), galactose (b), lactulose (c), and lactose (d), all at 11.4 mg/ml. Column was a Waters Associates Carbohydrate Analysis model eluted at 2 ml/min with 77/23 acetonitrile/ $H_2O$  (wt/wt).

TABLE 4. Liquid-chromatographic evaluation of standard sugars.

Sugar	Retention time ( $t_r$ , min)	Capacity factor ( $\kappa'$ )	Relative response
Tagatose	4.50	1.409	3.887
Galactose	6.72	2.540	1.387
Lactulose	13.40	6.074	1.539
Lactose	15.50	7.308	1.000

tion system, accurately prepared elution buffers, and extended times for each assay.

We now have developed a more rapid, simpler chromatographic system based on commercially available HPLC equipment and simple binary solvent mixtures. Separation of a standard mixture of the four major sugars in lactulose syrups is in Figure 1. Retention times, capacity factors, and relative responses to the refractive index detector for each sugar are in Table 4. Baseline separation is approached for all sugars in less than 16 min. Quantitation of each sugar in an unknown mixture is accomplished easily by peak height comparison to the appropriate sugar in the standard mixture. Peak height was proportional to sugar concentration over the range that in all cases was less than 20 mg/ml of each sugar. When the lactulose syrups analyzed by the spectrophotometric techniques in Table 3 were analyzed by the HPLC procedure (Table 5), the measures agreed with those by the former methods.

The sugar composition of a commercial pharmaceutical grade lactulose syrup, Cephulac (Merrell-National Laboratories), was examined by the HPLC procedure. The syrup was deionized and chromatographed as described in Materials and Methods. The chromatogram in

TABLE 5. High pressure liquid chromatographic analysis of sugars in mixtures from isomerization of lactose.

Isomerization reagent	Sugars % (by wt)			
	Tagatose	Galactose	Lactulose	Lactose
Calcium hydroxide	2.3	2.5	15.1	80.1
Triethylamine	3.7	2.2	32.8	61.3
Sodium aluminate	5.4	4.3	72.7	17.6

TABLE 6. Analysis of major sugars in Cephulac.<sup>a</sup>

Sugar	Trial					Statistic		
	1	2	3	4	5	$\bar{x}$	SD	CV (%)
Tagatose	1.12	.98	1.08	1.10	.94	1.04	.08	7.60
Galactose	12.58	12.32	12.35	12.63	12.31	12.44	.15	1.24
Lactulose	79.45	80.03	79.95	79.10	80.03	79.71	.42	.52
Lactose	6.86	6.66	6.61	7.17	6.72	6.80	.22	3.31

<sup>a</sup>In relative weight percent.

Figure 2 indicates several minor unknown compounds. The identified sugars, tagatose, galactose, lactulose, and lactose, were quantitated and are reported in relative weight percent in Table 6. The precision of the analysis also is given for standard deviations and coefficients of variation for five successive analyses.

The HPLC method described here is a rapid, convenient, precise, and specific alternative to existing spectrophotometric and chromatographic techniques for quantitative determination of sugars in lactulose preparations.

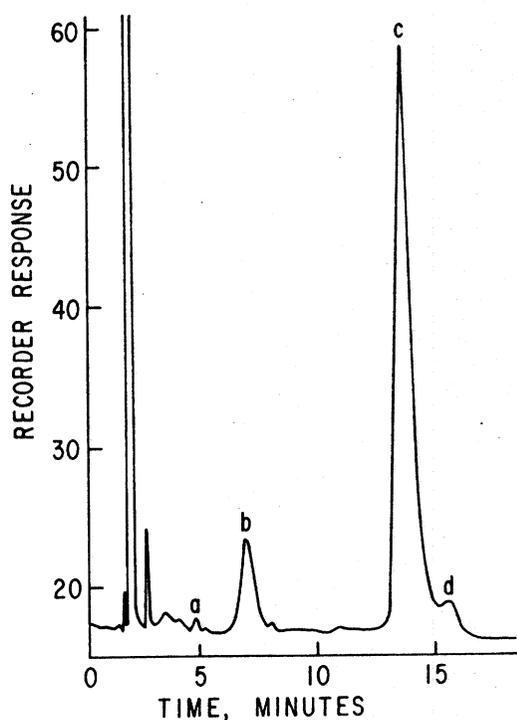


Figure 2. High performance liquid chromatogram of Cephulac: tagatose (a), galactose (b), lactulose (c), lactose (d).

#### REFERENCES

- Ash, D. J. 1976. Research on lactose indicates uses, limitations as a substitute for sucrose in bakery goods. *Food. Prod. Dev.* 10(6):85.
- Bergmeyer, H. U., and E. Bernt. 1963. D-Glucose. Determination with glucose oxidase and peroxidase. Page 123 in *Methods of enzymatic analysis*. H. U. Bergmeyer, ed. Academic Press, New York, NY.
- Conrad, E. C., and J. K. Palmer. 1976. Rapid analysis of carbohydrates by high-pressure liquid chromatography. *Food Technol.* 30:84.
- Corbett, W. M., and J. Kenner. 1953. The degradation of carbohydrates by alkali. Part 2. Lactose. *J. Chem. Soc.* 2245.
- Corbett, W. M., and J. Kenner. 1954. The degradation of carbohydrates by alkali. Part 5. Lactulose, maltose, and maltulose. *J. Chem. Soc.* 1789.
- DellaMonica, E. S., M. J. Calhoun, and P. E. McDowell. 1974. The quantitative determination of glucose, fructose, and sucrose in fruits and potatoes. *J. Food Sci.* 39:1062.
- Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28:350.
- Engle, C. E., and P. M. Olinger. 1979. High pressure liquid chromatographic determination of saccharides in corn sirups: Collaborative study, *J. Assoc. Off. Anal. Chem.* 62:527.
- Finch, P. R., R. Yuen, H. Schachter, and M. A. Moscarello. 1969. Enzymic methods for the micro assay of D-mannose, D-glucose, D-galactose, and L-fucose from acid hydrolyzates of glycoproteins. *Anal. Biochem.* 31:296.
- Furuholmen, A. M., J. D. Winefordner, F. W. Knapp, and R. A. Dennison. 1964. The quantitative analysis of glucose and fructose in potatoes. *J. Agric. Food Chem.* 12:109.
- Guth, J. H., and L. Tumerman. 1970. Method of making lactulose. US Pat. 3,546,206.
- Isbell, H. S., and W. W. Pigman. 1938. Pyranose-furanose interconversions with reference to the mutarotations of galactose, levulose, lactulose, and turanose. *J. Res. Nat. Bur. Standards* 20:773.
- Miller, G. L., and A. L. Burton. 1959. Spectro-

- photometric determination of aldoses by an iodometric procedure. *Anal. Chem.* 31:1790.
- 14 Miller, G. L., R. Slater, R. Birzgalis, and R. Blum. 1961. Application of different colorimetric tests to celodextrins. *Anal. Biochem.* 2:521.
- 15 Montgomery, E. M., and C. S. Hudson. 1930. Relations between rotating power and structure in the sugar group. Part 27. Synthesis of a new disaccharide ketose (lactulose) from lactose. *J. Am. Chem. Soc.* 52:2101.
- 16 Nickerson, T. A., I. F. Vujicic, and A. Y. Lin. 1976. Colorimetric estimation of lactose and its hydrolytic products. *J. Dairy Sci.* 59:386.
- 17 Oosten, B. J. 1967. Solubility diagram of lactose and lactulose in water. *Rec. Trav. Chim.* 86:675.
- 18 Parrish, F. W. 1970. Isomerization of glucose, maltose, and lactose with amino compounds. US Pat. 3,514,327.
- 19 Parrish, F. W., F. B. Talley, K. D. Ross, J. Clark, and J. G. Phillips. 1979. Sweetness of lactulose relative to sucrose. *J. Food Sci.* 44:813.
- 20 Percheron, F. 1962. Colorimetric determination of fructose and fructofuranosides by thiobarbituric reaction. *Compt. Rend.* 255:2521.
- 21 Perlin, A. S., P. Herve du Penhoat, and H. S. Isbell. 1973. Carbon-13 and hydroxyl proton NMR spectra of ketoses. Page 39 in *Carbohydrates in solution*. Adv. Chem. Series No. 117. Am. Chem. Soc., Washington, DC.
- 22 Reithel, F. J. 1963. Lactose. Page 103 in *Methods of enzymatic analysis*. H. U. Bergmeyer, ed. Academic Press, New York, NY.
- 23 Tauber, H., and I. S. Kleiner. 1932. A method for the determination of monosaccharides in the presence of disaccharides. *J. Biol. Chem.* 99:249.
- 24 Verhaar, L.A.Th., M.J.M. Van Der Aaist, J.A.W.M. Beenackers, and B.F.M. Kuster. 1979. Ion-exchange chromatography of lactose-lactulose isomerization mixtures using a boric acid-borate eluent. *J. Chromatogr.* 170:363.