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ION-EXCHANGE CHROMATOGRAPHY OF LACTOSE–LACTULOSE ISOMERIZATION MIXTURES USING A BORIC ACID–BORATE ELUENT

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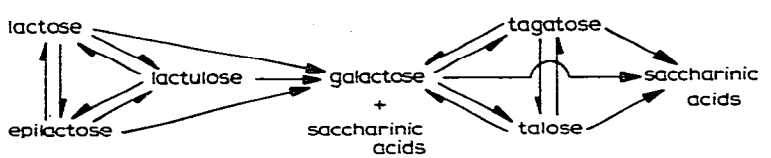
SUMMARY

Mixtures obtained by the isomerization of lactose on anion exchangers have been analysed by ion-exchange chromatography. With a 57-mm column of Aminex A-25 and 0.400 *M* H_3BO_3–0.005 *M* $Na_2B_4O_7$ as eluent, a complete separation of actose, epilactose (4-O-β-D-galactopyranosyl-D-mannose), lactulose, galactose and tagatose is obtained in about 50 min. The influence of various factors on the qualitative and quantitative results is given. The ratio of $Na_2B_4O_7$ to H_3BO_3 in the eluent has an important influence on the order of elution of ketoses and aldoses, especially at low ratios. A special device is described for removing excess of lactose during the analysis. The method described is also applicable to the analysis of lactulose syrups.

INTRODUCTION

The reactions in the isomerization of lactose on anion exchangers, which has been studied in our laboratory, are as follows:



The reaction conditions are such that the main isomerization products are lactulose and galactose, the amounts of epilactose (4-O-β-D-galactopyranosyl-D-mannose), tagatose and especially talose being relatively small. To study the kinetics of the reaction, a rapid and reliable method of analysis, particularly for the main products, had to be developed.

Many methods for the determination of lactose and galactose have been described. Vachek[1], Vachek *et al.*[2] and Zagrodzki *et al.*[3] used spectrophotometry

to determine lactulose in the presence of lactose and galactose with a specific colour reaction for ketoses. If necessary, lactulose was first isolated by using paper chromatography and extracted prior to colorimetric detection[2]. Zagrodzki *et al.*[3] removed excess of lactose from a lactose–lactulose mixture by crystallization in ethanol to avoid interferences. Müller *et al.*[4] used paper chromatography to separate lactulose in urine from the other components. The lactulose was extracted, concentrated, silylated and determined by gas–liquid chromatography. All of these methods are time consuming. Carulli *et al.*[5] used thin-layer chromatography for the determination of lactulose, galactose and glucose in urine and blood, a method which gives, however, less reliable quantitative results.

Ion-exchange chromatography in the presence of boric acid and borate ions is useful for the analysis of sugar mixtures, and has been reviewed by Jandera and Churáček[6]. Usually gradient elution is applied in order to separate a large number of sugars. In a similar manner, Voelter and Bauer[7] analysed sugar isomerization products and gave retention data for all of the sugars of interest to us, except epilactose. However, gradient elution causes many difficulties in the routine analysis of large series of samples.

We have developed a method without gradient elution, which enables us to separate and determine lactose, epilactose, galactose, lactulose and tagatose within 50 min. We made use of the fact that at low ratios of borate to boric acid in the eluent, retention times for ketoses increase considerably, whereas aldoses are relatively unaffected. The saccharinic acids formed did not hamper the determination of the sugar composition. The acidic compounds could be analysed by isotachophoresis as described by Dirkx *et al.*[8].

EXPERIMENTAL AND RESULTS

Apparatus

The system used is almost identical with that described earlier[9], and is based on the Technicon AutoAnalyzer. A different type of Orlita pump was used (DMP/AE-10-4.4), and the Chromatronix valve for sample introduction was exchanged for a home-made valve, with an injection volume of 10 μl. For detection we used orcinol reagent [70% (v/v) sulphuric acid, 1 g/l orcinol] and reaction at 90° for 13 min. The separation column (I.D. 4 mm) was packed with Aminex A-25 (Bio-Rad Labs., Richmond, Calif., U.S.A.) to a height of 57 mm.

Influence of eluent composition

In Table I, capacity factors (k') are given for some sugars, obtained using three different types of eluent:

$$k' = (\bar{v}/eX) - 1$$

where $\bar{v}$ is the volume of eluent passed through the column from the moment of injection to the attainment of the maximum concentration of a component at the column outlet, e is the interstitial fraction ($e = 0.4$) and X is the column volume. The k' values are independent of the eluent flow-rate and the height of column packing. Data are also given on sugars that do not occur in lactose isomerization

TABLE I

CAPACITY FACTORS FOR SEVERAL TYPES OF ELUENT

Parameter	*Eluent*		
	1	*2*	*3*
Boric acid concentration (*M*)	—	0.190	0.500
Sodium tetraborate concentration (*M*)	0.075	0.010	—
Sodium chloride concentration (*M*)	0.020	0.025	—
pH	9.25	7.6	4.1
Column temperature (°C)	65	75	75
k' values			
Cellobiose	2.5	1.0	1.0
Maltose	3.5	1.2	1.4
Lactose	3.9	1.6	1.9
Epilactose	9.1	5.3	8.6
Mannose	9.3	6.2	12.5
Galactose	13.6	9.8	19.2
Glucose	22.9	18.0	38.7
Maltulose	4.6	5.2	53.2
Lactulose	8.7	10.0	>75
Fructose	10.3	11.5	
Tagatose	14.9	16.6	
Sorbose	15.5	20.0	

mixtures. Talose could not be acquired, which was not a serious drawback because during isomerization reactions it is only formed in very small amounts.

It can be seen that there are large differences between the k' values of lactose, lactulose and galactose using eluent 1. However, epilactose and tagatose are eluted very close to lactulose and galactose, respectively. Eluent 2 does not separate lactulose and galactose, but it does separate all of the other sugars in the lactose isomerization mixtures. Eluent 3 results in a good separation of lactose, epilactose and galactose, but also in long retention times for lactulose and tagatose.

In order to decrease the retention times for lactulose and tagatose, we tested the influence of the addition of small amounts of tetraborate to eluent 3. The results (Fig. 1) indicate that with a decrease in tetraborate content all of the sugars under consideration can be separated in a reasonable time. The best results were obtained with an eluent containing 0.4 *M* boric acid and 0.005 *M* potassium tetraborate (pH 6.5), which we used in subsequent experiments. Using eluent 3 we measured a considerable increase in the retentions of all of the ketoses, whereas the increase in the retentions of the aldoses was relatively small. This seems to be a general and specific behaviour of ketoses, with possible applications in isolation experiments and in fixed and gradient elution systems in other sugar analysers.

Influence of column temperature

The column temperature was optimized by repeated analysis of mixtures of lactose, galactose and lactulose. The results are given in Figs. 2–4.

Increasing the column temperature causes the elution times to increase and the peak widths to decrease (Fig. 2), with a consequent large improvement in peak resolution. Fig. 3 shows peak resolutions according to Simpson[10], which must be

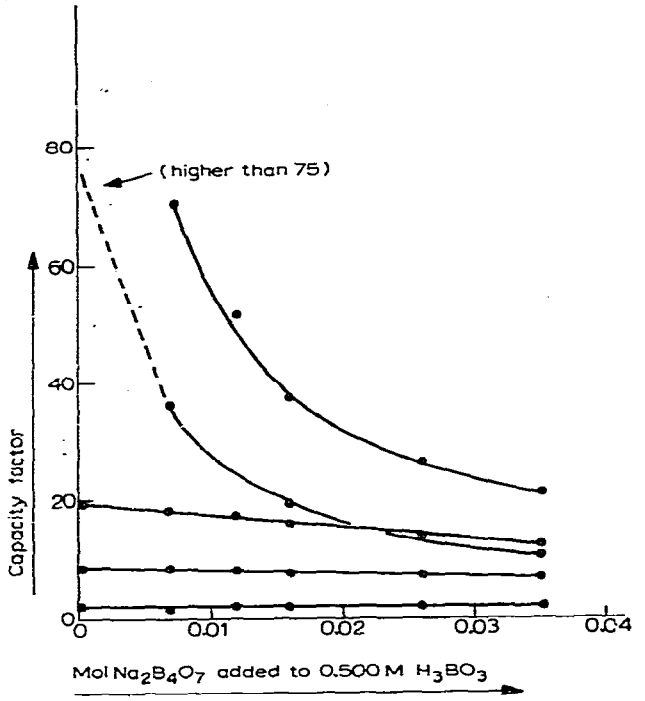


Fig. 1. Capacity factors for lactose, epilactose, galactose, lactulose and tagatose as a function of the amount of $Na_2B_4O_7$ added to 0.500 *M* H_3BO_3 in the eluent. Column: Aminex A-25, 75°.

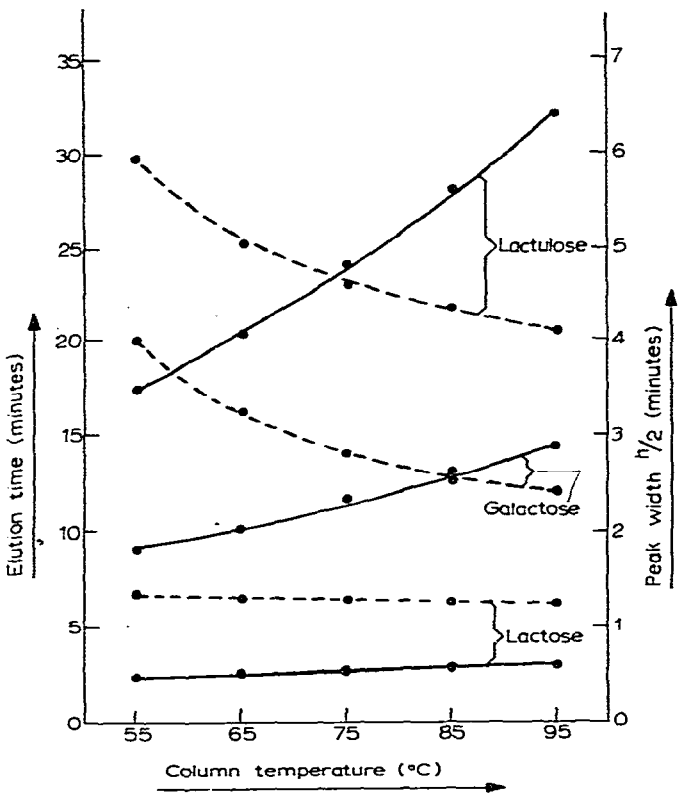


Fig. 2. Elution times (solid lines) and peak widths (broken lines) at half-height for lactose, galactose and lactulose as a function of column temperature. Column: 57 × 4 mm, Aminex A-25. Eluent: 0.400 *M* H_3BO_3 + 0.005 *M* $Na_2B_4O_7$; flow-rate, 0.80 ml/min.

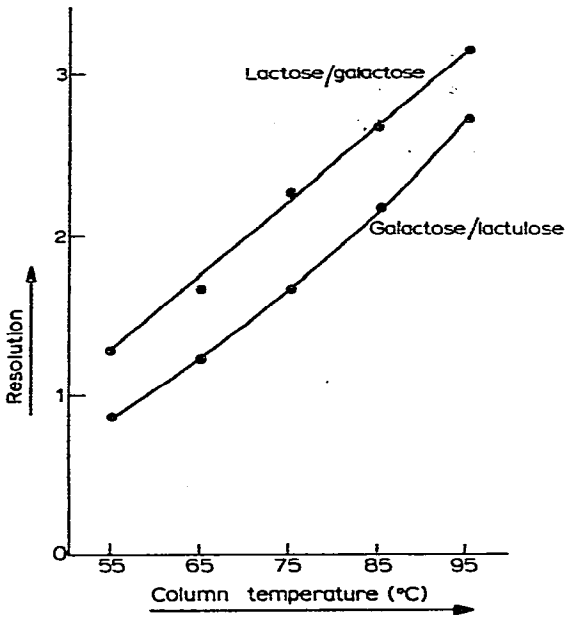


Fig. 3. Resolution for lactose–galactose and galactose–lactulose as a function of column temperature. Conditions as in Fig. 2.

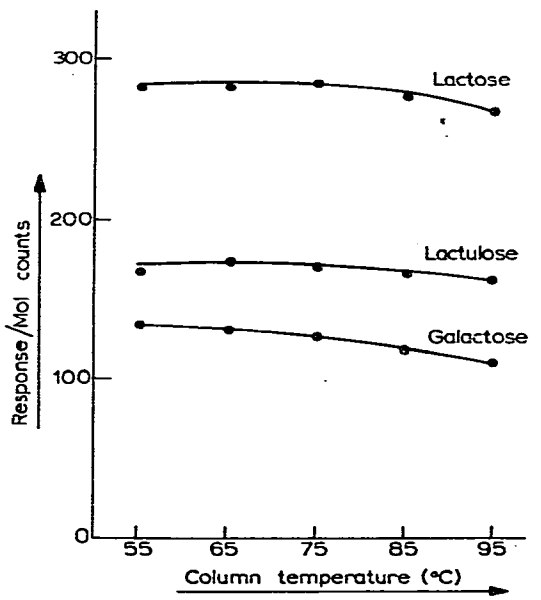


Fig. 4. Molar response of lactose, galactose and lactulose as a function of column temperature. Detection: orcinol reagent, 420 nm, 13 min reaction at 90°. Other conditions as in Fig. 2.

higher than 1.5 in order to have a degree of peak overlap less than 1%. Therefore, for a proper separation of galactose and lactulose the column temperature should be 75° or above. However, Fig. 4 shows that a small decrease in signal occurs at high temperatures for lactose and galactose, probably owing to degradation on the

column. As a proper separation of the sugars was obtained at 75°, and moreover at this temperature no loss of signal occured, we used 75° as the column temperature in subsequent experiments, together with an eluent flow-rate of 0.8 ml/min. We found no isomerization of lactose on the column as mentioned by Carubelli[11], probably owing to the lower pH and much shorter residence time in our system.

Quantitative determination

Analysis of samples with different concentrations of lactose, galactose and lactulose resulted in a linear relationship between the detector response and the amount injected. For epilactose and tagatose no calibration graphs were constructed, but from experimental values it could be estimated that the molar response of epilactose was 35% higher than that of lactose, and that of tagatose was 30% lower than that of galactose. For the three main products, the reproducibility was determined from ten replicate analyses, resulting in a standard deviation of lower than 2.5%. Fig. 5 shows a chromatogram of a mixture containing, in order of elution, lactose, epilactose, galactose, lactulose and tagatose. A good separation of all five sugars was obtained in approximately 50 min.

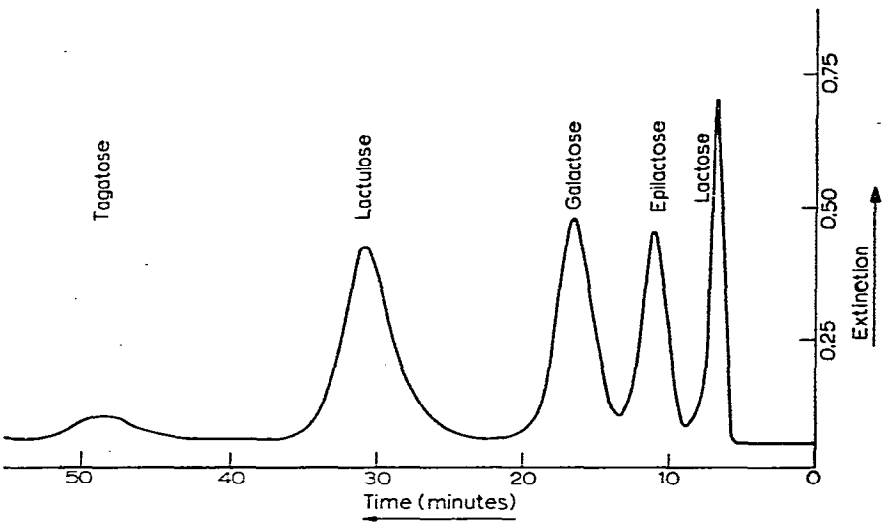


Fig. 5. Chromatogram of lactose (30 nmol), epilactose (30 nmol), galactose (110 nmol), lactulose (95 nmol) and tagatose (57 nmol). Column: 57 × 4 mm, Aminex A-25. Eluent: 0.400 *M* H_3BO_3 + 0.005 *M* $Na_2B_4O_7$; flow-rate, 0.80 ml/min. Detection: orcinol reagent, 420 nm, 4.6 min reaction at 95°.

Removal of excess of lactose

For a good understanding of the kinetics of the isomerization reaction, it is often necessary to determine the initial rates of formation of lactulose, galactose and epilactose from lactose. Therefore, small amounts of the sugars formed must be determined in the presence of a large excess of lactose. However, a high concentration of lactose causes the epilactose peak to be lost in the tail of the lactose

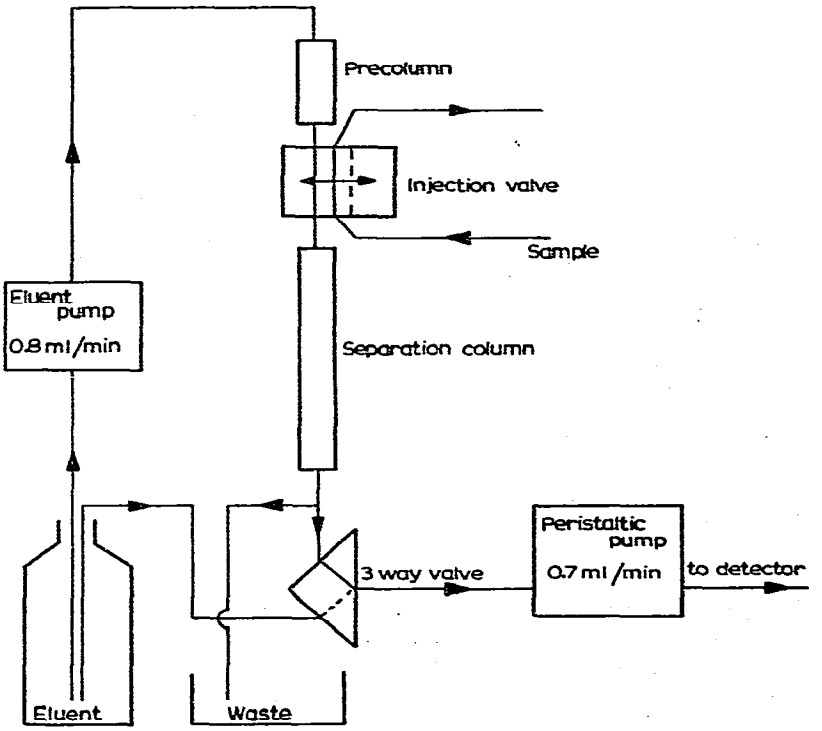


Fig. 6. Apparatus for ion-exchange chromatography with a three-way valve to remove excess of lactose.

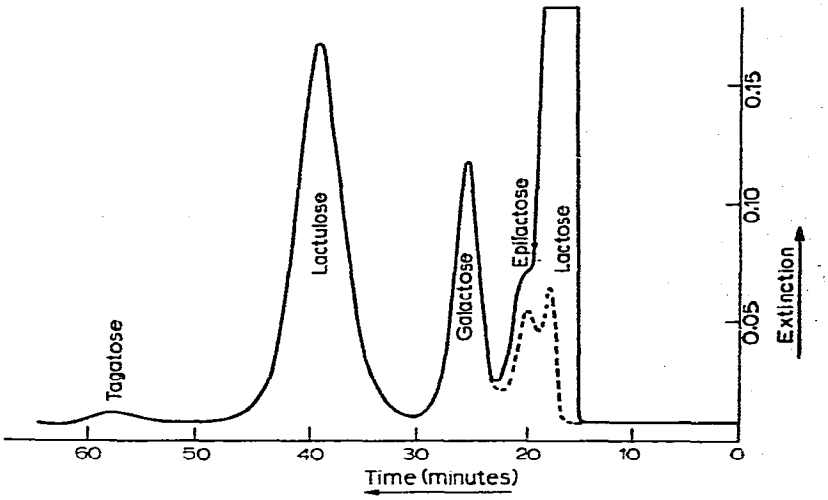


Fig. 7. Chromatogram of a lactose isomerization sample, with (broken line) and without (solid line) removal of excess of lactose. Detection: orcinol reagent, 420 nm, 13 min reaction at 90°. Other conditions as in Fig. 5.

peak, which is due mainly to overloading of the detection system. Also, black solids are formed in the colour reaction. To overcome this problem, a three-way valve was installed at the column outlet, as depicted in Fig. 6. Using an automatic timing

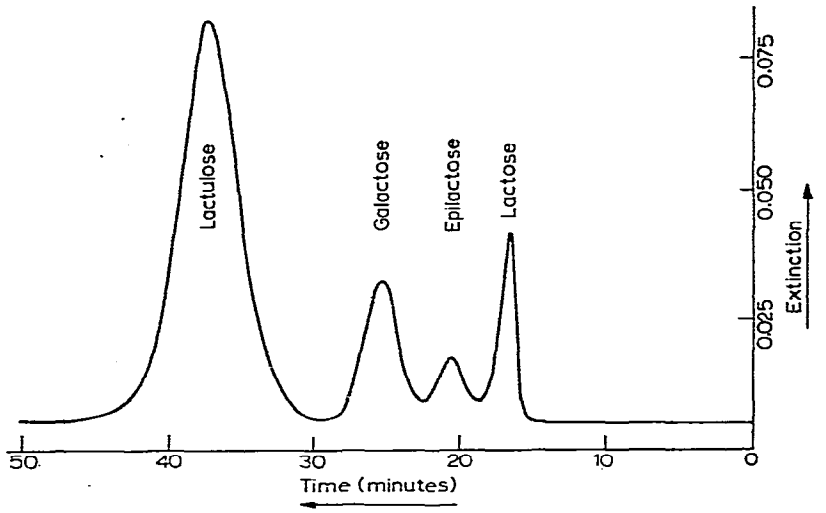


Fig. 8. Chromatogram of Dulphalac. Conditions as in Fig. 7.

device, the column eluent was exchanged for fresh eluent when lactose left the column during a 2.5-min interval starting from injection. The favourable effect on the chromatogram is shown in Fig. 7 and no formation of black solids occurred.

Fig. 8 shows a chromatogram obtained with Duphalac, a lactulose suryp from Philips-Duphar (Weesp, The Netherlands). It contains lactulose, galactose, lactose and epilactose, but no significant amount of tagatose. Routine analyses of these syrups, after dilution with water, could be performed with the system described at the rate of 35 min per sample.

REFERENCES

1 J. Vachek, *Cesk. Farm.*, 20 (1971) 96.
2 J. Vachek, B. Kakac and V. Rabek, *Cesk Farm.*, 23 (1974) 61.
3 S. Zagrodzki, A. Kulagowska and B. Król, *Qual. Plant. Mater. Veg.*, 16 (1968) 102.
4 M. Müller, J. Walker-Smith, D. H. Shmerling, H.-Ch. Curtius and A. Prader, *Clin. Chim. Acta*, 24 (1969) 45.
5 N. Carulli, G. F. Salvioli and F. Manenti, *Digestion*, 6 (1972) 139.
6 P. Jandera and J. Churáček, *J. Chromatogr.*, 98 (1974) 55.
7 W. Voelter and H. Bauer, *J. Chromatogr.*, 126 (1976) 693.
8 J. M. H. Dirkx, H. S. van der Baan and J. M. A. J. J. van den Broek, *Carbohydr. Res.*, 59 (1977) 63.
9 L. A. Th. Verhaar and J. M. H. Dirkx, *Carbohydr. Res.*, 59 (1977) 1.
10 C. F. Simpson, *Practical High Performance Liquid Chromatography*, Heyden, London, 1976, p. 10.
11 R. Carubelli, *Carbohydr. Res.*, 2 (1966) 480.