Food Chemistry **43** (1992) 399–402
Analytical Methods Section

An improved colorimetric method for the estimation of lactulose in lactose–lactulose mixtures

R. Nagendra & S. Venkat Rao

Department of Nutrition and Food Safety, Central Food Technological Research Institute, Mysore–570 013, India

(Received 17 February 1991; accepted 13 March 1991)

An improved colorimetric method is described for the estimation of lactulose in mixtures of lactose and lactulose. The anthrone method for the estimation of lactulose was modified by altering the concentrations of alcohol and acetic acid added to the reaction mixture. This resulted in a two-fold increase in the sensitivity of the method and also facilitated increase in sample volume. Values for lactulose estimated in lactose–lactulose mixtures using this method, compared well with those obtained using a chromatographic method involving a prior separation of lactulose.

INTRODUCTION

The nutritional and physiological significance of lactulose (β-D-galactopyranosyl-D-fructofuranose) have received considerable attention in recent years, because of observation on the growth promoting effect of lactulose for Bifidobacterium (Adachi, 1965). Although lactulose has been detected in raw milk, it is found extensively as a secondary product in milk and milk products subjected to heat-treatment. It has been prepared on an industrial scale in the form of a syrup by alkaline isomerisation of lactose (β-D-galactopyranosyl-D-glucopyranose).

Since lactose, also a reducing disaccharide, is present along with lactulose in milk based products it is necessary to apply ketospecific methods for estimating it. Vachek (1971) used 60% sulphuric acid to estimate lactulose in lactulose syrups. Vachek (1974) further improved the method using a cysteine–HCl–H_2SO_4 reagent. These methods are applicable to pure lactulose syrups only due to interference of lactose. Adachi (1965) used the reaction of methylamine with lactulose for its estimation, which involves a preliminary step to eliminate lactose by oxidation with periodate to aldonic acid, followed by its removal by ion-exchangers. Adachi (1965) further improved the procedure using a cysteine–HCl–carbazole–H_2SO_4 reagent, after separation of lactulose by thin layer chromatography (TLC), resulting in a four-fold sensitivity over the procedure of Vachek (1974). Zagrodzki *et al.* (1968) used a keto-specific method based on an anthrone–acetic acid–phosphoric acid–ethanol reagent. In the present communication, further modifications to the procedure of Zagrodzki *et al.* (1968) have been made to improve the analysis of lactulose.

Food Chemistry 0308-8146/92/$05.00

EXPERIMENTAL

Materials

Anthrone, GR; glacial acetic acid, LR; orthophosphoric acid, GR; distilled ethanol; α-lactose (Sigma); lactulose (Sigma); silica gel G, LR; boric acid, AR; acetonitrile, LR.

Standard sugar solutions

Lactulose (1 mg ml^{-1}) and lactose (10 mg ml^{-1}) prepared fresh in distilled water for colorimetry. Lactulose and lactose (50 mg ml^{-1}) prepared fresh in distilled water for TLC.

Lactulose–lactose mixtures

A lactose solution (15%) was subjected to heat-treatment at 70°C for 20 min in a weak alkaline medium (0·04 M $Ca(OH)_2$). Aliquots of the reaction mixture

were taken out at intervals of 5 min up to a period of 25 min to obtain solutions containing varying quantities of lactulose and lactose. For colorimetric estimation, the mixtures were diluted 1 : 50 in water and 0·2 ml aliquots were taken for analysis.

Anthrone reagent

This reagent was prepared fresh according to the procedure of Zagrodzki *et al.* (1968) as follows: anthrone (200 mg) was dissolved in hot glacial acetic acid (6 ml), followed by the addition of absolute ethanol (12 ml) and orthophosphoric acid (2·8 ml) successively. The reagent was kept tightly capped to prevent crystallisation of anthrone due to absorption of moisture.

Thin layer chromatography

The procedure of Martinez-Castro and Olano (1981) was followed. Silica gel G suspended in 0·03 M boric acid (1 : 2) was spread over plates to a thickness of 250 μ and activated at 100°C for 1 h before use. Fifty microlitres of the sugar solutions were spotted over TLC plates which were then developed in acetonitrile/water (50 : 20, v/v) and the separated sugars were identified by spraying diphenylamine–acetone–phosphoric acid reagent. The individual sugars were extracted into 50% ethanol (2 ml) and 0·2 ml was taken for colorimetric analysis.

Zagrodski procedure for the estimation of lactulose

In this procedure an aliquot 92% ethanolic sample solution (0·2 ml) containing 0–500 μg of lactulose was mixed with the anthrone reagent (0·5 ml) and the mixture kept in a boiling water bath for 30 min. After cooling, glacial acetic acid (10 ml) was added. The optical density of the yellow colour formed was read at 427 nm.

The modifications made to improve the procedure of Zagrodski *et al.* (1968) are described below.

Effect of ethanol concentration on colour formation
The quantity of ethanol in the reaction mixture was increased to 1–6 ml instead of 0·2 ml of 92% ethanol in the original procedure. The reaction mixture thus consisted of standard or sample (0·2 ml) to which absolute ethanol (1–6 ml) was added, along with the anthrone reagent (0·5 ml). After the reaction was over, glacial acetic acid (0·5 ml) was added. The effect of varying the concentration of alcohol on colour intensity is shown in Fig. 1.

Effect of acetic acid concentration on colour intensity
The effect of reducing the concentration of glacial acetic acid gradually from 10 ml to 5 ml on the optical density was studied. The results are shown in Fig. 2.

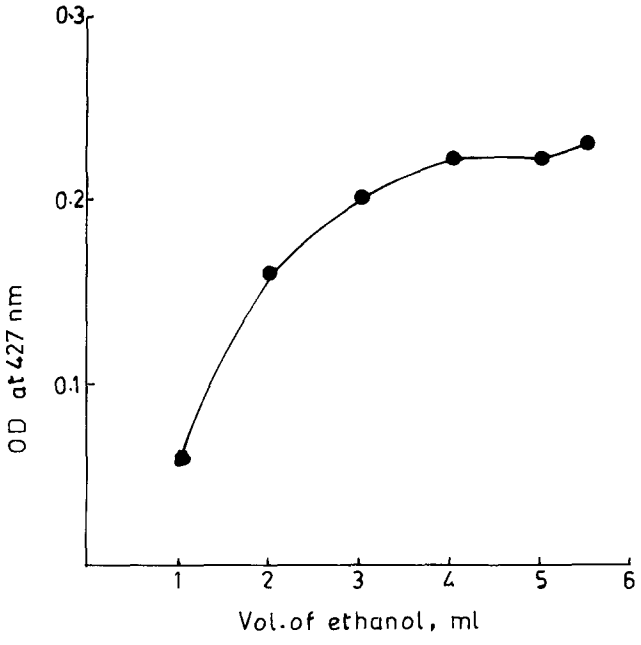

Fig. 1. Effect of volume of ethanol added on the intensity of colour.

Based on the above experiments, the procedure for estimation of lactulose was modified as follows:

Procedure for the estimation of lactulose

Lactulose (10–160 μg) in water (0·2 ml) was taken. Absolute alcohol (4 ml) was added followed by the anthrone reagent (0·5 ml). The mixture was kept in a boiling water bath for 30 min. After cooling, glacial acetic acid (6 ml) was added to dissolve the reaction products. Optical density of the yellow colour was measured at 427 nm. The standard curve for lactulose is shown in Fig. 3.

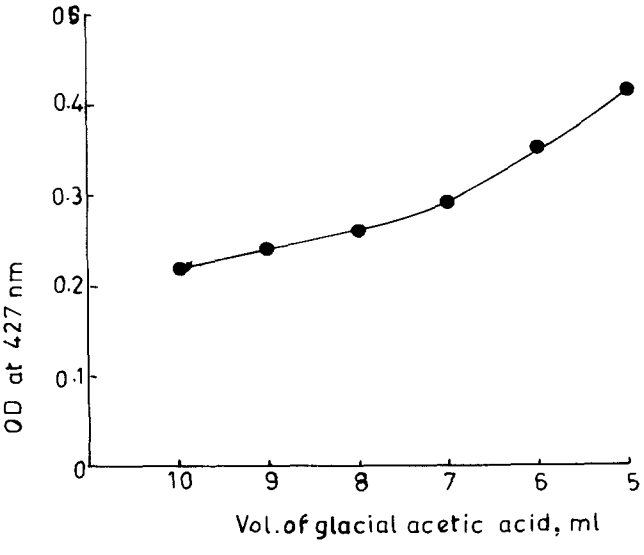

Fig. 2. Effect of volume of glacial acetic acid added on the intensity of colour.

Table 1. Analysis of lactulose in known mixtures with lactose by direct colorimetry and TLC

Sugar mixture	Lactulose (μg)	10	60	120	0	0	0	10	60	120
	Lactose (μg)	0	0	0	290	240	180	290	240	180
Estimated as Lactulose (μg)										
By direct colorimetry		10	60	120	3	3	3	12	67·5	125
By TLC separation		6	58	118	3	3	3	13	66	115

Interference of lactose in the colorimetric method

Since lactose at high concentrations gives the same colour with anthrone reagent as lactulose, the colour produced by different concentrations of lactose alone ranging from 100–300 μg was studied. The results are shown in Fig. 4.

Lactose interference in the assay was investigated by incorporating in the reaction mixture different concentrations of lactose along with lactulose. Three levels of lactulose ranging from 10 to 120 μg and three levels of lactose from 100 to 300 μg were used. The results are shown in Fig. 4.

Lactulose analysis in mixtures by colorimetric and TLC methods

Mixtures containing pure lactose and lactulose, with varying quantities of these sugars were prepared. These were analysed for lactulose by direct colorimetry and after separation by TLC. The data are shown in Table 1. Lactulose was also determined by both methods in reaction mixtures of lactose–lactulose subjected to heat treatment for different time intervals, as mentioned earlier. The results are shown in Table 2.

RESULTS AND DISCUSSION

The results presented in Figs 1–4 and Tables 1 and 2 are discussed below. At a fixed concentration of 100 μg of lactulose in 0·2 ml, varying absolute alcohol content from 1·0 to 6·0 ml increased the optical density of the sample yielding a maximum optical density with 4 ml of alcohol (Fig. 1). Hence for maximum colour yield, a minimum of 4 ml of alcohol was required in the reaction mixture.

In the method of Zagrodzki *et al.* (1968), 10 ml of acetic acid was added after completion of the reaction. Reduction in the quantity of acetic acid to 6 ml resulted in a marked increase in the intensity of colour as shown in Fig. 2. This may be mainly due to a decrease in the total volume of reaction mixture. A uniform quantity of 6 ml of acetic acid was therefore used in all the assays.

The modified procedure of lactulose estimation gave a linear response for lactulose concentrations ranging from 10 μg to 160 μg (Fig. 3). The standard curve of Zagrodzki for lactulose is also shown in the figure. It is

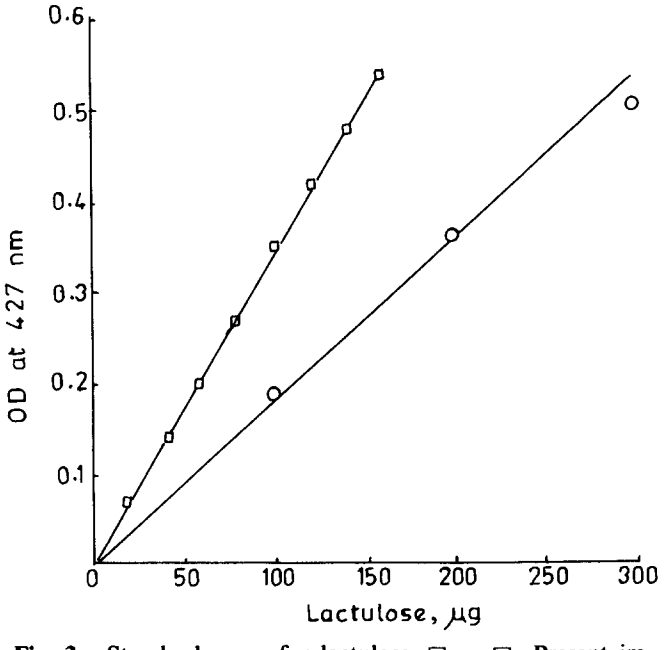

Fig. 3. Standard curve for lactulose. □—□, Present improved method, ○—○, According to Zagdrodzki *et al.* 1968.

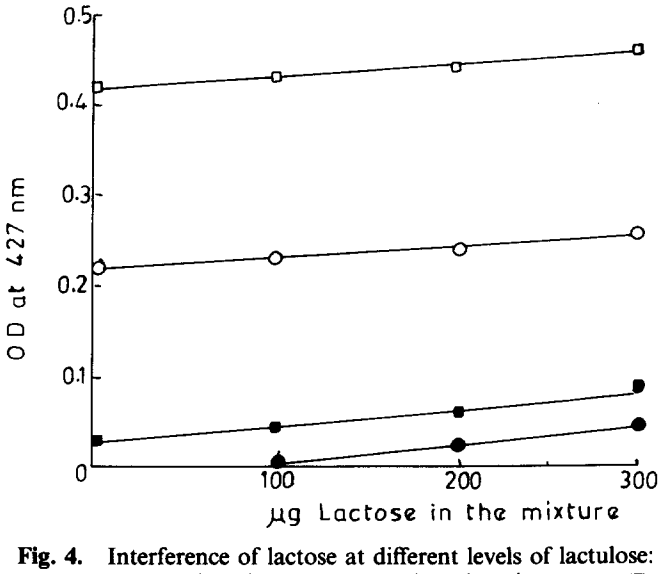

Fig. 4. Interference of lactose at different levels of lactulose: □—□, 120 μg lactulose; ○—○, 60 μg lactulose; ■—■, 10 μg lactulose; ●—●, no lactulose.

Table 2. Comparative TLC and direct colorimetric analysis of lactulose in mixtures during alkaline isomerisation of lactose

Sample No.	Time after reaction mixture drawn (min)	Lactulose (μg)	
		Direct colorimetry	By TLC separation
1	5	21·5	17
2	10	27	23
3	15	32	28·6
4	20	39	36
5	25	47	44

evident the modifications resulted in an improved curve for lactulose. The colour was stable at room temperature over several hours. Figure 4 shows that lactose did not interfere in the estimation of lactulose up to a concentration of 300 μg, beyond which a gradual increase in the colour was observed. In order to determine whether the direct colorimetric procedure can be used instead of the TLC procedure involving a prior separation of lactose, lactulose when present along with lactose in known mixtures, was estimated by both the methods. Table 1 shows that there is good agreement between the two methods when mixtures of sugars were analysed for lactulose.

As a result of the above modifications, the sensitivity of the method increased two-fold over that of Zagrodzki *et al.* (1968). An optical density of 0·35 for 100 μg of lactulose was obtained with the modified procedure, as compared with a value of 0·17 with the earlier method. The increase in sensitivity was due to varying the alcohol and acetic acid contents in the reaction mixture. The modifications also enable taking a greater sample volume than that in the original method. Whereas in Zagrodzki's method (1968) 16 μl of aqueous sample is taken, in the present procedure the sample volume was increased up to 200 μl. Consequently, solutions with low lactulose content can be used directly without concentration. While applying the procedure for estimation of lactulose in lactose–lactulose mixtures, it is necessary to dilute the sample so that the lactose content does not exceed 300 μg. In the presence of lactose, the modified colorimetric method compared well with the TLC procedure.

During the estimation of lactulose in isomerised mixtures of 15% lactose, a maximum difference of 4 μg of lactulose was observed between the two methods (Table 2). Compared to the direct colorimetric procedure, lactulose estimated by TLC procedure was slightly less due to losses during scraping and extraction. The present colorimetric method therefore serves as a convenient procedure for estimation of lactulose in place of the time-consuming TLC method at lactose contents below 300 μg in the mixtures.

ACKNOWLEDGEMENTS

One of the authors (R.N.) acknowledges with thanks Hindustan Ciba-Geigy Ltd, for the Research Scholarship provided for him to carry out the work. The authors also thank the Director, Central Food Technological Research Institute, Mysore, for his interest in the work.

REFERENCES

Adachi, S. (1965*a*). Spectrophotometric determination of lactulose with methylamine, *Analytical Chem.*, **37**, 896–8.

Adachi, S. (1965*b*). A rapid method for the assay of lactulose, *Analytical Biochem.*, **12**, 137–42.

Martinez-Castro, I. & Olano, A. (1981). Ready detection of small amounts of lactulose in dairy products by thin layer chromatography, *Chromatographia*, **14**, 621–2.

Petuely, F. (1957). Bifidusflora bei Flaschenkindern durch bifidogen substanzen (Bifidus Faktor), *Zeitschriff fur Chemie.*, **79**, 174.

Vachek, J. (1971). Spectrophotometric determination of lactulose, *Cslka Farm.*, **20**, 96–8.

Vachek, J. (1974). Determination of lactulose in lactulose syrups, *Idem*, **23**, 61–4.

Zagrodzki, S., Kulagowska, A. & Kroi, B. W. (1968). Bestimmung der Lactulose in Milchproducten, *Qual. Plant. Mater. Veg.*, **16**, 102–12.