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Note

Automated anion-exchange chromatographic method for the quantitation of lactulose in physiological fluids and processed milks

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Lactulose (4-O-β-D-galactopyranosyl-D-fructofuranose) occurs in lactose containing solutions and is present in the body fluids of human subjects ingesting processed milks and milk products [1, 2]. It is formed by the non-enzymic conversion of the glucose half of the lactose molecule (4-O-β-D-galactopyranosyl-D-glucopyranose) to fructose, in alkaline solution especially if heated. It is not actively transported or hydrolysed by the human intestine and produces osmotic and fermentative diarrhoea when given in a concentrated syrup as a laxative [3]. Pre-packed liquid milks used for infant feeding are sterilised by heating, which promotes lactulose production and its content in these products may reach concentrations which cause or encourage diarrhoea in sensitive patients [4]. Accurate measurement of the small amount of lactulose which diffuses across the gut wall, and is excreted unchanged in the urine after an oral load, has been used to assess mucosal permeability in gastro-intestinal diseases [5].

Lactulose is difficult to separate from other commonly occurring sugars by thin-layer chromatography, but can be resolved by overnight paper chromatography and can be identified by its reaction with keto-specific reagents such as naphtho-resorcinol [1]. However, quantitation requires spectrophotometric scanning of the reacted spots under strict conditions [6], or elution, derivative formation and gas—liquid chromatography [7]. A rapid, automatic, quantitative method for the estimation of lactulose, even in the presence of massive amounts of lactose, has been developed using anion-exchange liquid chromatography of the borate complexes of sugars and a modified copper-bicinchoninate detection reagent [8]. The technique can be performed on an

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amino acid analyser with the appropriate resin and reagents, and minor modification to the reaction manifold of the instrument [9].

EXPERIMENTAL

Reagents

Lactulose and other reference sugars were obtained from Sigma London (Poole, U.K.), disodium bicinchoninate from Pierce and Warriner (Chester, U.K.) and the anion resin Aminex A-29 (8% divinylbenzene, 7 ± 1 μm diameter beads) from Bio-Rad (Watford, U.K.). The mixed resin used for sample preparation (Duolite MB 5113) and AnalaR grade chemicals for buffers and reagents were obtained from BDH (Poole, U.K.).

Sample preparation

The mixed ion-exchange resins were converted to the hydrogen and acetate forms and dried as previously described [1, 6]. Calibration and internal standard solutions of sugars were prepared in 0.25 m*M* thiomersal and stored at 4°C. This preservative was removed by the sample preparation resin and so desalted solutions were stored frozen. They were stable for at least three months if stored at −16°C. Dried milks (1 g) were homogenised with thiomersal solution (10 ml). These solutions and liquid milks (1 ml) were added to de-ionised water (7 ml) in a test tube. Internal standard (1 ml of 10 m*M* xylose) and saturated aqueous picric acid (1 ml) were added and the tube thoroughly mixed. After centrifuging (1500 *g* for 5 min), the extract (2 ml) was transferred to another tube and dry, mixed ion-exchange resin was added to approximately 60% of the final volume to remove excess picric acid and other ions including sugar phosphates, amines and uronic acids [1, 6]. After shaking (5 min) and centrifuging, the clear, colourless solution (0.4 ml) was mixed with the strong borate buffer (1 *M*) used for column elution (0.1 ml, see below) and loaded into the sampler cups of the apparatus. Frozen faeces (2 g) were homogenised with acetone (2 ml) and 5 m*M* palatinose internal standard (2 ml). After centrifuging, the extract (3 ml) was de-ionised with resin. A portion of the supernatant (1 ml) was shaken with an equal volume of chloroform to remove the final traces of lipid. Following centrifugation, part of the upper phase (0.4 ml) was transferred to a small flask, evaporated to dryness (50°C, rotary evaporator) and the residue dissolved in strong borate buffer (1.0 ml) which was loaded into the sampler cup.

Urine (2 ml) was mixed with 2 m*M* palatinose (1 ml) and this solution was de-ionised and borate added as described for milk. Calibration standards were treated as liquid samples and diluted, de-ionised and buffered as described above.

Chromatography

The analysis was performed on a Chromaspek J 180 automatic ion-exchange chromatograph with a high-pressure sample loop loader (Hilger Analytical, Margate, U.K.). The complete cycle time, including regeneration, was 1 h using a heated (69°C) 350 × 3 mm column of anion-exchange resin (A-29) eluted at 0.25 ml/min. The sample (100 μl) was loaded 18.5 min after the start of the

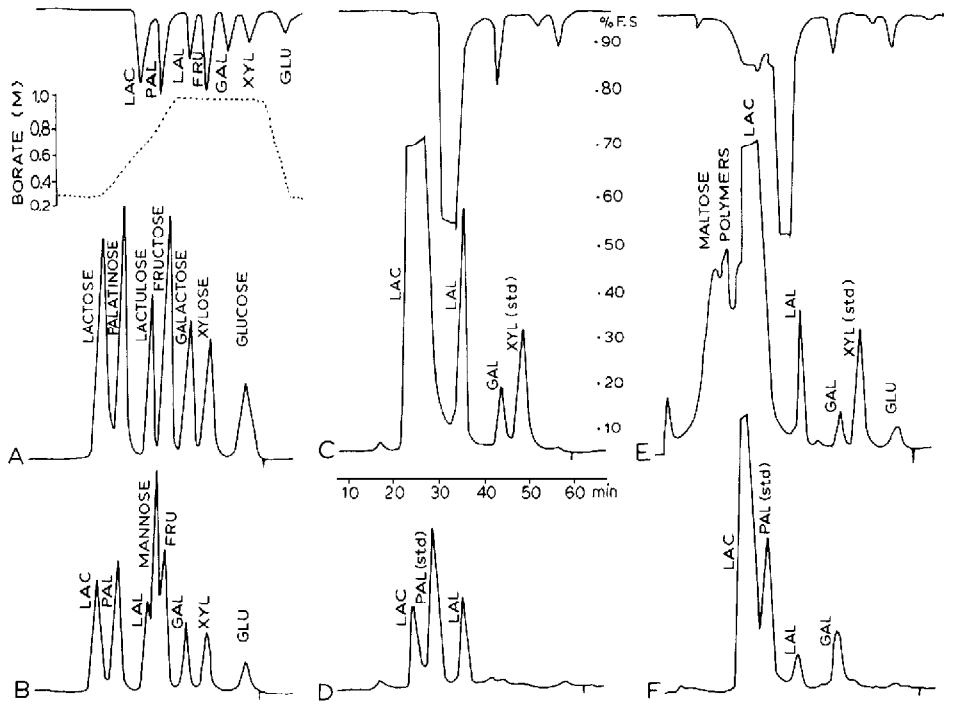

Fig. 1. Chromatograms of lactulose-containing mixtures. Upright traces 570 nm, inverted traces 440 nm. A and B, standard mixtures; C, pre-packed milk; D, faecal sample; E, pre-packed milk with added malto-dextrose; F, urine sample.

regeneration period of the cycle. Sugars were eluted using a gradient of increasing borate molarity and, to a less extent, pH, by mixing a weak (5.6 g borax, 4.4 g boric acid per litre, pH 8.5) and a strong (56 g borax, 19 g boric acid per litre, pH 8.7) buffer as determined by the profile of the rotating drum of the apparatus (Fig. 1).

Detection

The colour reagent of Sinner and Puls [8] was modified to maintain sensitivity in the presence of a higher concentration of borate ions. Potassium carbonate (200 g) was dissolved in approximately 900 ml of de-ionised water. This solution was heated to 70—80°C and 1.2 g of sodium bicinchoninate added. When this had completely dissolved, the solution was cooled and made up to 1 litre. Copper reagent (43 ml, 6.8 g copper sulphate + 26.4 g citric acid per litre) were added, the solution mixed and allowed to stand at least 30 min before use. If stored refrigerated this solution was stable for fourteen days. At room temperature the absorbance of this solution slowly increased after two days.

The column effluent (0.25 ml/min) was introduced into a stream of 2 *M* potassium carbonate (0.15 ml/min) which had been segmented with nitrogen (0.25 ml/min). This stream passed through a mixing coil, colour reagent (0.4 ml/min) was introduced and after a further preliminary mixing coil, it was heated at 90°C for 9 min whilst passing through a coil in an oil bath (Fig. 2). The absorbance of this partially cooled solution was measured at 570 and 440 nm in the flow colorimeter. The heights of the chart recorder peaks relative to

TABLE I

THE LACTULOSE CONTENT OF VARIOUS NATURAL SAMPLES

Source	Lactulose (m*M*)											
	n	Mean	Range	Reference values [2]	Recovery (%)				Between-batch imprecision			
					Conc. range	*n*	Mean	Range	*n* − 1	Mean	± S.D.	C.V. (%)
SMA — Gold Cap (Wyeth)	7*	11.82	10.13—13.54	10.0—11.40					10	12.62	0.24	1.90
SMA — Low birthweight	2*	—	4.72— 5.23	5.2— 5.3								
Premium (Cow & Gate)	7*	14.78	13.83—15.87	12.2—13.70	2.0—20	12	99	94—103				
Powdered Milks (concentration in 10% solution of dried product)	7**	1.17	0.51— 1.82	0.50								
Fresh cow's milk	3*		<0.10	—	2.0—20	5	98	95—102				
Infants (2 weeks to 6 months) receiving pre-packs												
Urine	30	0.41	0.15— 1.60	—	0.2— 5	10	101	95—106	8	0.86	0.02	2.60
Faeces	20	2.15	0.50— 8.02	—	0.2—10	5	102	92—105	5	2.71	0.15	5.60

*Each with a different batch number.

**One sample each of goat's milk, Ostermilk, Localsol, Complan, SMA, Premium and Marvel.

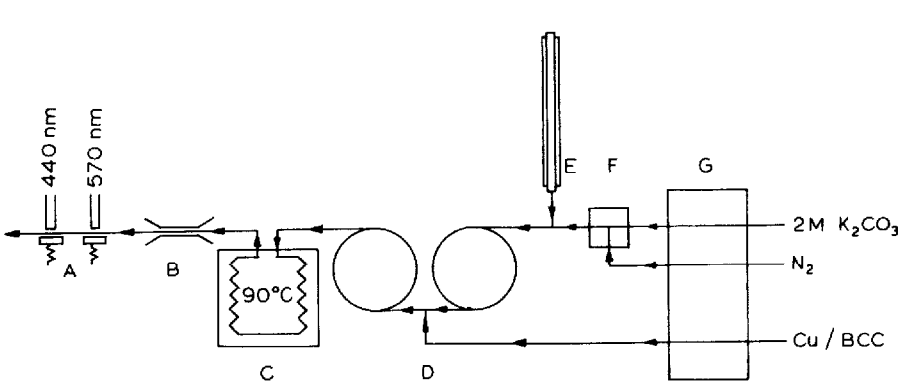

Fig. 2. Flow diagram for post-column detection of sugars using alkaline copper bicinchoninate reagent. A, Photometric detectors; B, vane cooler; C, oil bath; D, mixing coils (2 × 5 turns); E, column; F, gas injection block; G. peristaltic pump.

those obtained from calibration solutions and adjusted for internal standard results were used for quantitation.

RESULTS AND DISCUSSION

Typical chromatograms are illustrated in Fig. 1. The relationship between peak height and amount of lactulose loaded onto the column was linear between 0 and 150 n*M* at 570 nm ($r \geqslant 0.997$) and between 0 and 250 n*M* at 440 nm ($r \geqslant 0.998$). Typical lactulose concentrations in various specimens, its recovery when added to selected specimens, and the extent of between-batch imprecision are listed in Table I. The lactulose content of milk products was similar to that reported by Beach and Menzies [2] (Table I) who used a spectrophotometric scanning technique on paper chromatograms. A similar procedure [1], but using visual comparison with standards (C.V. ± 10%) specimens listed in Table 1. Lactulose concentrations as high as 30 m*M* were found in excessively sterilised or incorrectly stored pre-packed milks and similarly high concentrations were found in ileostomy fluids or faeces from patients with obvious intestinal hurry.

The position of lactulose in the chromatogram varied with the concentration of borate ions in the elution buffers. When the borate ion concentration of the buffer was less than 0.6 *M*, lactulose migrated after mannose but co-migrated with fructose. With buffers of higher molarity, it preceded mannose which partially overlapped both lactulose and fructose (Fig. 1B). The latter was considered preferable as mannose was not present in milks and seldom occurred free in body fluids [10]. A buffer of lower borate molarity was required for complete elution of large amounts of lactose before the emergence of lactulose. In this case, palatinose would appear on the tail of the lactose peak and xylose was a preferable internal standard.

Samples are made alkaline with borate buffer at the final stage of preparation and the possibility that lactose could be transformed to lactulose whilst the samples awaited analysis was investigated. Lactulose was not detected in a standard solution of lactose (10 m*M*) stored in borate for 48 h and the lactulose content of two extracts of prepacked milk did not increase

during the same time period after borate was added. This suggests that the borate complex of lactose is less reactive than the simple sugar.

The highest concentration of borate ions in the column elution buffer (pH 8.8) used by Sinner and Puls [8] was 0.5 *M*. As higher molarities were required to resolve lactulose, extra carbonate had to be added to the reaction mixture to maintain the optimum pH of 10.5. Sodium carbonate was replaced by the more soluble potassium salt but the 2,2′-bicinchoninate precipitated if the final carbonate concentration exceeded 1.5 *M*. Satisfactory conditions were best achieved by the two-stage addition illustrated in Fig. 2. This ensured smooth chart recorder traces (Fig. 1) although solutions of high ionic strength were mixed without the benefit of detergents which caused precipitation in the reaction manifold.

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