ELSEVIER

PII: S0308-8146(96)00195-1

Food Chemistry, Vol. 57, No. 4, pp. 575–580, 1996

0308-8146/96 $15.00+0.00

Analytical, Nutritional and Clinical Methods Section

High performance liquid chromatographic analysis of lactose-hydrolysed milk

H. E. Indyk,[a] M. J. Edwards[a] & D. C. Woollard[b]

[a]*Anchor Products, P.O. Box 7, Waitoa, New Zealand*
[b]*Lynfield Dairy and Food Services Centre, Ministry of Agriculture, P.O. Box 41, Auckland, New Zealand*

(Received 30 January 1996; accepted 12 April 1996)

The application of high performance liquid chromatography to the determination of lactose in milk has been investigated. Both ion-exchange and partition modes were employed, and for unmodified milk, returned equivalent values. However, for milk subjected to the action of β-galactosidases, it has been confirmed that at least one significant transferase disaccharide intermediate is chromatographically unresolved during the former resin-based mode, resulting in an erroneously high lactose estimation. Since such transferase oligosaccharides are adequately separated during partition chromatography on amine-bonded silica, it is recommended that resin-based cation-exchange columns are inappropriate for the estimation of lactose in such lactose-reduced milks. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

The simultaneous analysis of carbohydrates in foods and biological tissues is generally achieved through the use of either gas–liquid (GLC) or high performance liquid chromatography (HPLC) (Robards & Whitelaw, 1986). While GLC techniques may be the preferred choice for highly complex mixtures at low levels, the multiple procedures involved during extraction and preparation of volatile derivatives may lead to incomplete derivatisation, multiple anomer formation and manipulative losses. Increasingly however, HPLC techniques have been applied and currently dominate in mono- and disaccharide analysis, in view of several inherent methodological advantages (Chang *et al.*, 1995). These include the capability to detect carbohydrates directly without derivatisation and the absence of anomer separation, as well as the simpler and more robust nature of the technique.

Several chemistries of separation have been developed for both native and derivatised analytes including those utilising silica, amino-bonded silica, *in situ* amino-modified silica, reversed-phase, polyol- and cyclodextrin-bonded silica, polymer based anion- and cation-exchange and size exclusion columns and have been extensively reviewed along with evidence for the multiplicity of mechanisms involved (Honda, 1984; Vidal-Valverde *et al.*, 1985; Hounsell, 1987; Verzele *et al.*, 1987; Ball, 1990). More recently, alternative HPLC methods incorporating post-column derivatisation, light-scattering and anion-exchange-PAD techniques have been described (Escott & Taylor, 1985; Engelhardt & Ohs, 1987; Lee, 1990; Del Nozal *et al.*, 1992; Coquet *et al.*, 1992; Yamauchi *et al.*, 1993; Kim *et al.*, 1995) and have demonstrated advantages for low level clinical applications. However, for food applications, the relative insensitivity of refractive index (RI) detection is not usually a limiting factor and most reports have utilised either the amino-bonded silica or resin-based cation-exchange modes for the separation of mono- and oligosaccharides with RI detection. The differing separation chemistries and hence elution order may be usefully exploited, although sensitivity with fixed-ion resin columns is higher as a result of the absence of acetonitrile in the eluent.

The dominant carbohydrate in mammalian milk is the disaccharide lactose [4-(β-D-galactopyranosyl)-D-glucopyranose] and for many applications, this may be the only carbohydrate required for measurement (West & Llorente, 1981; Brons & Olieman, 1983; Kwak & Jeon, 1988; Harvey, 1988). Conventional heat treatments and spoilage have both been demonstrated to generate monosaccharides at trace levels, requiring GLC for accurate measurement (Troyano *et al.*, 1991). Processed milk products may contain significant amounts of either added (e.g. maltodextrin) or generated (e.g. lactulose) saccharides which will complicate chromatographic analysis. Both amino-bonded silica and cation-exchange HPLC schemes have been advocated and the relative merits compared (Richmond *et al.*, 1982; Pirisino, 1983; Woollard, 1984; Betschart & Prenosil, 1984; Jeon *et al.*, 1984; Jeon & Mantha, 1985; Scott & Hatina, 1988; Bugner & Feinberg, 1990).

Lactose reduction in milk has become a useful strategy in facilitating the consumption of dairy products by the lactose intolerant (Pirisino, 1983; Smart, 1993). Further, whey may be similarly processed to provide a hydrolysate of use as a corn syrup substitute in many food applications. Hydrolysis is achieved through modification with β-galactosidase, an enzyme which may be isolated from several sources. In addition to primarily yielding the cleavage monosaccharides glucose and galactose, the β-galactosidases are able to catalyse a series of transgalactosylation reactions forming several possible galactooligosaccharides and internally rearranged lactose isomers at appreciable levels. Their recent identification as potential bifidus factors has also resulted in commercial exploitation of the transferase activity of β-galactosidase. The accepted mechanism suggests that lactase is more accurately classified as a transferase rather than an hydrolase, with the extent of transferase activity greatly dependent on initial lactose concentration, incubation conditions and enzyme source (Mozaffar *et al.*, 1985; Toba *et al.*, 1985; Prenosil *et al.*, 1987; Smart, 1993).

We evaluated previously reported HPLC methods for possible application to the QC measurement of lactose in commercially produced lactose-reduced milk. Most have employed cation-exchange in view of its enhanced RI sensitivity and capability to resolve glucose and galactose (Pirisino, 1983; Betschart & Prenosil, 1984; Woollard, 1984; Jeon *et al.*, 1984). However, this separation mode may potentially be compromised by chromatographically unresolved disaccharides formed during transgalactosylation.

MATERIALS AND METHODS

Apparatus

The HPLC system comprised a Model 6000A pump, CHM column heater and a Model 410 differential refractive index detector operated at 35 °C internal temperature (Waters, Milford, MA, USA), C6W manual 6-port injector fitted with a 5 or 25 μl loop (Valco, Houston, TX, USA) and an SE 120 chart recorder (Brown Boveri Corp., Nurnberg, Austria). Two analytical column configurations were used for carbohydrate separation:

a. A 25 cm×4.6 mm Alphasil $5NH_2$ (HPLC Technology, Macclesfield, Cheshire, UK) preceded by a C_{18} Guard-Pak insert (Waters), maintained at room temperature. Mobile phase was acetonitrile:water (75:25 v/v, filtered and degassed) at 1.0 ml min^{-1}.
b. A 30 cm×6.5 mm Sugar Pak I (Ca^{2+}) (Waters), maintained at 90 °C was preceded by a Sugar-Pak II precolumn insert. Mobile phase was water (filtered and degassed) held at 60 °C at 0.5 ml min^{-1}.

Reagents

Carbohydrate standards (α-lactose monohydrate, α-D(+)-glucose, D(+)-galactose, β-D(−)-fructose, sucrose and β-D-galactopyranosyl-[1–6]-D-galactopyranose) were obtained from Sigma (St Louis, MO, USA).

Carrez reagents 1 and 2 were prepared by separately dissolving potassium hexacyanoferrate (II) (3.6 g) and zinc acetate dihydrate (7.2 g) (Sigma) in water (100 ml). All water was prepared by a reverse osmosis and ion exchange system to >18 mΩ resistivity (Millipore, Bedford, MA, USA). Acetonitrile was HPLC grade (BDH, Poole, UK).

Grade 540 filter paper (Whatman, Maidstone, UK) and 0.45 μm Minisart cellulose acetate membrane units (Sartorious, GmbH, Gottingen, FRG) were employed for extract clarification.

Enzymatic hydrolysis was achieved with Maxilact L2000, a commercially available purified β-galactosidase derived from *Kluyveromyces (Saccharomyces) lactis* (EC 3.2.1.23, Gist-Brocades, MA Delft, Holland). This is a liquid preparation in glycerol (1 g equivalent to 2000 neutral lactase units) which was diluted 1:1.5 with water prior to use.

Standards

Lactose (anhydrous), glucose and galactose standards were prepared in water at 10, 5 and 5 mg ml^{-1} respectively for studies involving the Alphasil 5 NH_2 column, and diluted 1:10 for the Sugar-Pak system.

Milk hydrolysis

Lactose-reduced milk powder was commercially processed in dairy manufacturing plant incorporating a 1.5 tonne h^{-1} tall-form drier. Standardised whole milk (12.5% total solids) was held at 38–40 °C for 3 h after addition of enzyme preparation (1:800 dilute Maxilact:whole milk, v/v) prior to spray drying. Samples for analysis were withdrawn either before or after conversion to dried product.

Analysis

Milk samples (0.75 g powder or 2.5 g milk) were weighed accurately into graduated tubes (25.0 ml) and dissolved in ca. 15 ml warm water. A control milk powder sample was included in each sample set in order to monitor method performance. Carrez reagents 1 and 2 (0.25 ml each) were added sequentially with mixing and contents allowed to stand for 20 min. Extracts were made to volume with water, filtered (discarding first filtrate) and an aliquot passed through a 0.45 μm membrane. The clear extract was either injected directly (25 μl, system a) or following a 1:10 dilution (5 μl, system b).

For QC application, peak identity was based on comparison against authentic standards, and quantification of lactose and other carbohydrates based on the external standard technique.

Partial characterisation of the dominant unknown oligosaccharides was achieved following their fractionation and collection during chromatography on amine-

modified silica, evaporation to dryness, acid hydrolysis of isolated carbohydrates (1–2.5 N HCl, 90 °C) and injection under cation-exchange conditions in order to resolve and identify monosaccharide composition. The intact isolated oligosaccharides were also evaluated under both LC configurations in order to confirm elution characteristics relative to known carbohydrates.

For comparative purposes, several samples were also analysed using a GLC procedure (Li *et al.*, 1983). Briefly, deproteinised milk sample extracts were dried under nitrogen, converted to the oxime-trimethylsilyl derivatives and injected into a Perkin-Elmer GC equipped with an RSL-300 capillary column (30 m×0.53 mm). Quantification was by the internal standard technique against phenyl-β-D-glucopyranoside.

RESULTS

Figure 1 illustrates the chromatography achieved under both configurations for authentic standards, whole milk and lactose-hydrolysed whole milk extracts.

Both chromatographic techniques revealed galactose at levels consistently lower than glucose in hydrolysed milks, concordant with trans-galactolytic activity during incubation, while intact whole milks contained essentially negligible monosaccharide content.

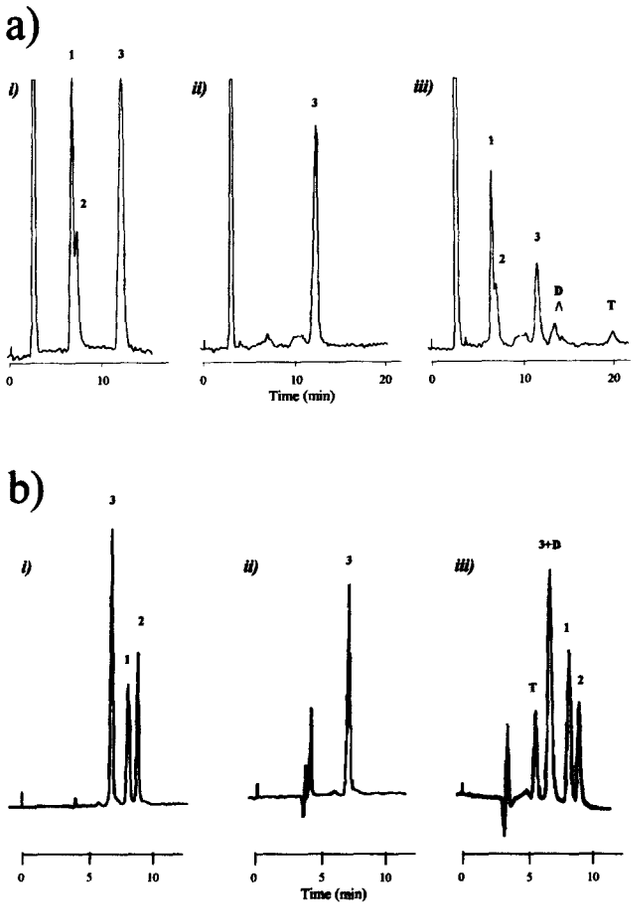

Fig. 1. Analytical chromatography of carbohydrate extracts under a) amine-bonded (Alphasil $5NH_2$) and b) ion-exchange (Sugar Pak I) modes (conditions described in text). (i) authentic standards, (ii) whole milk powder and (iii) lactose-hydrolysed whole milk powder. Peaks: (1) glucose, (2) galactose, (3) lactose, (D) disaccharides, (T) trisaccharides.

Recovery of lactose spiked whole milk has been estimated to be 97.8 and 97.2% (50 and 100% level) irrespective of chromatographic mode. An RSD_R of 2.6% (mean = 37.0 g 100 g^{-1}; $n = 16$) was achieved for the control milk powder lactose content on amine-silica and 3.5% (mean = 35.7 g 100 g^{-1}; $n = 7$) for the resin based system.

Glucose and galactose are poorly resolved on amine-modified silica, which therefore does not facilitate accurate individual monosaccharide quantification. This is a recognised limitation of such polar-bonded phases. However, this chromatographic mode does reveal the presence of two oligosaccharides other than lactose at significant levels in hydrolysed milk (each at ca. 2–5%, estimated against lactose), as well as at least two additional carbohydrates at lower levels (< 1%). Their retention characteristics indicated di- and trisaccharide structures (a conclusion supported by coelution of fractionated unknowns with standards under ion-exchange chromatography), although the dominant unidentified disaccharide did not coelute exactly with authentic β-D-Gal [1-6] D-Gal.

While several oligosaccharides' peaks were separated with the amine-bonded column, fewer were resolved on the cation-exchange column. Thus, while monosaccharide separation was complete and trisaccharide elution was unambiguous under ion-exchange conditions, this mode did not reveal the apparent presence of disaccharides other than lactose in hydrolysed milk. Further, both authentic β-D-Gal [1–6] D-Gal and fractionated putative disaccharide were demonstrated to coelute precisely with lactose on this chromatographic system.

Table 1 lists measured lactose levels for several typical milk samples by both chromatographic modes, with comparison against data obtained by the reference GLC technique.

While there is excellent agreement between all three chromatographic techniques for intact whole milk, a

Table 1. Comparison of lactose in milk powders determined by HPLC and GLC (g/100 g)

Sample	HPLC		GLC
	Amine-Si	Sugar Pak II	
1	11.0	15.9	11.4
2	16.6	21.1	16.9
3	10.0	16.3	10.1
4	9.4	15.7	9.3
5	8.6	14.4	9.0
6	14.2	18.6	14.3
7	40.3	40.0	40.6
8	35.8	35.5	35.2

Samples 1–6 are enzymatically lactose-reduced whole milk powders.
Samples 7 and 8 are unmodified whole milk powders (sample 8 is the control).
Data are the mean of duplicate analyses.

consistently positive bias existed for the resin based LC system for all samples of lactose hydrolysed milk, consistent with possible coelution of lactose and other putative disaccharides originating from transferase activity.

Acid hydrolysis of the two dominant unknown peaks fractionated during amine-silica chromatography, indicated that both carbohydrates contained glucose and galactose. Although partial acid-catalysed isomerisation of glucose to fructose occurred simultaneously during cleavage (an observation also confirmed with authentic lactose under the same conditions), the results indicated a 1:1 galactose:glucose ratio for the disaccharide, with a less certain stoichiometry indicated for the trisaccharide.

DISCUSSION

The availability of lactose hydrolysed milk is recognised to be of special importance to the high proportion of human populations with an intolerance to lactose. Since most populations can tolerate low levels of intact lactose, it is generally accepted that a 50–80% lactose reduced milk will satisfy the physiological requirements of the majority of intolerant groups, although extreme intolerance may require an exclusively lactose-free milk.

The extent of lactose hydrolysis is clearly an important control parameter and several monitoring techniques have been used including enzymatic, colourimetric, polarimetric, cryoscopic and chromatographic, the latter offering the advantages of specificity and concurrent multianalyte capability. Isocratic HPLC-RI is generally preferred to GLC-FID in the QC environment in view of its relative operational and methodological simplicity.

There are several strategies for the preparation of a protein and fat-free sample extract suitable for chromatographic analysis. These include the use of various organic solvents, trichloroacetic acid, perchloric acid, lead acetate, phosphotungstic acid, Carrez reagent and ultrafiltration (Ball, 1990). The Carrez reagents have previously been recommended for the analysis of milk constituents (Kleyn, 1985) and have proven effective in the present application. Apart from a final 0.45 μm membrane filtration step, no further clean-up was found to be necessary, despite the evaluation of additional fractionation with C_{18} solid-phase extraction cartridges. The recommended in-line chromatographic guard columns were found to be effective in eliminating or minimising the influence of potentially interfering contaminants (e.g. lipids, proteins, carotenoids, chloride and other anions).

Resin based cation-exchange columns have predominantly been advocated during studies of enzymatic hydrolysis of pure lactose (Betschart & Prenosil, 1984; Jeon & Mantha, 1985), whey (Woollard, 1984; Jeon *et al.*, 1984) and milk (Pirisino, 1983). Only one study, involving the action of different microbial β-galactosidases on pure lactose substrate, has been reported where the application of amino-silica has been compared directly against cation-exchange, concluding that cation-exchange may be compromised by unresolved disaccharides (Jeon & Mantha, 1985), although Pirisino (1983) commented on the slightly asymmetric lactose peak obtained with a resin column for milk hydrolysate.

The chromatography obtained with the resin-based Sugar-Pak column during the present study of milk hydrolysis by *K. lactis*, conclusively revealed the presence of a trisaccharide as a significant transferase reaction product. However, the limitations of this chromatographic mode were demonstrated through its failure to reveal transferase formation of disaccharide, as a consequence of coelution with intact substrate. Amine-silica chromatographic analysis of hydrolysed milk, however, conclusively showed the unambiguous presence of several transient transferase oligosaccharides, including disaccharide, at levels similar to previous studies of model lactose solutions (Jeon & Mantha, 1985; Smart, 1993).

Conclusive evidence for the presence of a disaccharide coeluting with lactose under cation-exchange chromatographic conditions has been gained through the consistently positive bias relative to amino-silica for lactose content in hydrolysed milks, the quantitative equivalence for unhydrolysed milks with both modes, and the partial characterisation, through acid-catalysed cleavage, of the isolated alleged disaccharide.

Several attempts to identify the dominant transferase reaction products formed with model lactose solutions and a range of microbial β-galactosidases have been reported in the literature (Wierzbicki & Kosikowski, 1972; Shukla, 1975; Huber *et al.*, 1976; Toba & Adachi, 1978; Toba *et al.*, 1981, 1985; Mozaffar *et al.*, 1985; Prenosil *et al.*, 1987; Smart, 1993). All of these studies utilised TLC and GLC techniques either alone or in combination. Most reports indicate a prevalence of di- and trisaccharide intermediates, although some tetra-, penta- and hexasaccharide formation has also been confirmed. The majority of reported studies have demonstrated the specificity of β-galactosidase for transfer of the β-(1–4) linkage to a β-(1–6) configuration, although β-(1–2) and β-(1–3) saccharides have also been indicated at lower levels. The dominant disaccharide formed in the present study is therefore likely to be β-D-galactopyranosyl-(1–6)-D-glucose (allolactose), a conclusion supported in this work through the confirmation of both glucose and galactose incorporation in the isolated carbohydrate. A second, minor disaccharide, with slightly higher retention on amine-silica, is plausibly β-D-galactopyranosyl-(1–6)-D-galactose in view of its coelution with authentic material. The apparent release of glucose, combined with literature evidence, suggests that the major trisaccharide formed in this experimental system is β-D-galactopyranosyl-(1–6)-β-D-galactopyranosyl-(1–6)-D-glucose, although either linkage may well incorporate a 1–2, 1–3 or 1–4 configuration.

CONCLUSION

Previous authors have advocated the use of resin-based separations for the analysis of enzymatically hydrolysed lactose, in view of enhanced monosaccharide separation and sensitivity compared to polar bonded-phase partition systems. The present study of lactose-hydrolysed milk has, however, confirmed the inappropriateness of the predominantly size-exclusion mode, due to its inability to resolve a transferase disaccharide formed in significant amounts. In contrast, and despite lower sensitivity, amine-bonded silica allows for the unambiguous measurement of both lactose and the several oligosaccharides generated during incubation with β-galactosidase. This mode is therefore recommended during liquid chromatographic QC compliance monitoring of lactose in lactose-reduced milk.

ACKNOWLEDGEMENTS

The authors thank Anchor Products for their support during this work. Acknowledgements are also due to the NZ Dairy Research Institute and MAF for their participation with GLC analyses.

REFERENCES

Ball, G. F. M. (1990). The application of HPLC to the determination of low molecular weight sugars and polyhydric alcohols in foods: a review. *Food Chem.*, **35**, 117–152.

Betschart, H. F. & Prenosil, J. E. (1984). High-performance liquid chromatographic analysis of the products of enzymatic lactose hydrolysis. *J. Chromatogr.*, **299**, 498–502.

Brons, C. & Olieman, C. (1983). Study of the high-performance liquid chromatographic separation of reducing sugars, applied to the determination of lactose in milk. *J. Chromatogr.*, **259**, 79–86.

Bugner, E. & Feinberg, M. (1990). Methodology of validation by collaborative study of a method of determining simple sugars. *Analusis*, **18**, 600–607.

Chang, S. K. C., Holm, E., Schwarz, J. & Rayas-Duarte, P. (1995). Food. *Anal. Chem.*, **67**(12), 127R–153R.

Coquet, A., Veuthey, J.-L. & Haerdi, W. (1992). Comparison of post-column fluorescence derivatisation and evaporative light-scattering detection to analyse saccharides selectively by LC. *Chromatographia*, **34**(11/12), 651–654.

Del Nozal, M. J., Bernal, J. L., Gomez, F. J., Antolin, A. & Toribio, L. (1992). Post-column derivatisation of carbohydrates with ethanolamine-boric acid prior to their detection by high performance liquid chromatography. *J. Chromatogr.*, **607**, 191–198.

Engelhardt, H. & Ohs, P. (1987). Trace analysis of sugars by HPLC and post-column derivatisation. *Chromatographia*, **23**(9), 657–662.

Escott, R. E. A. & Taylor, A. F. (1985). The determination of sugars by high performance liquid chromatography using a novel bonded phase column and selective post column colourimetric detection. *J. High Res. Chrom. Chromatogr. Comms.*, **8**, 290–292.

Harvey, J. (1988). A high performance liquid chromatography method for lactose determination in milk. *Austr. J. Dairy Tech.*, **43**, 19–20.

Honda, S. (1984). High-performance liquid chromatography of mono- and oligosaccharides. *Anal. Biochem.*, **140**, 1–47.

Hounsell, E. F. (1987). Carbohydrates. In *HPLC of Small Molecules*, ed. C. K. Lim. IRL Press, Washington, DC, pp. 49–68.

Huber, R. E., Kurz, G. & Wallenfels, K. (1976). A quantitation of the factors which affect the hydrolase and transgalactosylase activities of β-galactosidase (*E. coli*) on lactose. *Biochemistry*, **15**(9), 1994–2001.

Jeon, I. J. & Mantha, V. R. (1985). High performance liquid chromatography analysis of oligosaccharides formed during β-galactosidase action on lactose. *J. Dairy Sci.*, **68**, 581–588.

Jeon, I. J., Galitzer, S. J. & Hennessy, K. J. (1984). Rapid determination of lactose and its hydrolysates in whey and whey permeate by high performance liquid chromatography. *J. Dairy Sci.*, **67**, 884–887.

Kim, Y. S., Liu, J., Han, X. J., Pervin, A. & Linhardt, R. J. (1995). Analysis of fluorescently labeled sugars by reversed-phase ion-pairing high-performance liquid chromatography. *J. Chromatogr. Sci.*, **33**, 162–167.

Kleyn, D. H. (1985). Determination of lactose by an enzymatic method. *J. Dairy Sci.*, **68**, 2791–2798.

Kwak, H. S. & Jeon, I. J. (1988). Comparison of high performance liquid chromatography and enzymatic method for the measurement of lactose in milk. *J. Food Sci.*, **53**(3), 975–976.

Lee, Y. C. (1990). High-performance anion-exchange chromatography for carbohydrate analysis. *Anal. Biochem.*, **189**, 151–162.

Li, B. W., Schuhmann, P. J. & Holden, J. M. (1983). Determination of sugars in yoghurt by gas–liquid chromatography. *J. Agric. Food Chem.*, **31**, 985–989.

Mozaffar, Z., Nakanishi, K. & Matsuno, R. (1985). Formation of oligosaccharides during hydrolysis of lactose in milk using β-galactosidase from *Bacillus circulans*. *J. Food Sci.*, **50**, 1602–1606.

Pirisino, J. F. (1983). High performance liquid chromatographic determination of lactose, glucose, and galactose in lactose-reduced milk. *J. Food Sci.*, **48**, 742–744, 754.

Prenosil, J. E., Stuker, E. & Bourne, J. R. (1987). Formation of oligosaccharides during enzymatic lactose hydrolysis: parts I and II. *Biotech. Bioeng.*, **30**, 1019–1031.

Richmond, M. L., Barfuss, D. L., Harte, B. R., Gray, J. I. & Stine, C. M. (1982). Separation of carbohydrates in dairy products by high performance liquid chromatography. *J. Dairy Sci.*, **65**, 1394–1400.

Robards, K. & Whitelaw, M. (1986). Chromatography of monosaccharides and disaccharides. *J. Chromatogr.*, **373**(1), 81–110.

Scott, F. W. & Hatina, G. (1988). HPLC determination of sugars, starch and oligosaccharides in infant formulas using resin-based, fixed-ion columns. *J. Food Sci.*, **53**(1), 264–269.

Shukla, T. P. (1975). β-Galactosidase technology: a solution to the lactose problem. *CRC Crit. Rev. Food Tech.*, **5**, 325–356.

Smart, J. B. (1993). Transferase reactions of β-galactosidases. New product opportunities. In *Bulletin of the IDF*, 289, Ch. 4, pp. 16–22.

Toba, T. & Adachi, S. (1978). Hydrolysis of lactose by microbial β-galactosidases. Formation of oligosaccharides with special reference to 2-*O*-β-D-galactopyranosyl-D-glucose. *J. Dairy Sci.*, **61**, 33–38.

Toba, T., Tomita, Y., Itoh, T. & Adachi, S. (1981). β-Galactosidases of lactic acid bacteria: Characterization by oligosaccharides formed during hydrolysis of lactose. *J. Dairy Sci.*, **64**, 185–192.

Toba, T., Yokota, A. & Adachi, S. (1985). Oligosaccharide structures formed during the hydrolysis of lactose by *Aspergillus oryzae* β-galactosidase. *Food Chem.*, **16**, 147–162.

Troyano, E., Olano, A., Fernandez-Diaz, M., Sanz, J. & Martinez-Castro, I. (1991). Gas chromatographic analysis of free monosaccharides in milk. *Chromatographia*, **32**(7/8), 379–382.

Verzele, M., Simoens, G. & Van Damme, F. (1987). A critical review of some liquid chromatography systems for the separation of sugars. *Chromatographia*, **23**(4), 292–300.

Vidal-Valverde, C., Martin-Villa, C., Olmedilla, B. & Blanco, I. (1985). High performance liquid chromatographic systems to separate and quantify a mixture of nine sugars and four polyols. *J. Liq. Chromatogr.*, **8**(1), 75–94.

West, L. G. & Llorente, M. A. (1981). High performance liquid chromatographic determination of lactose in milk. *J. Assoc. Off. Anal. Chem.*, **64**(4), 805–807.

Wierzbicki, L. E. & Kosikowski, F. V. (1972). Formation of oligosaccharides during β-galactosidase action on lactose. *J. Dairy Sci.*, **56**(11), 1400–1404.

Woollard, D. C. (1984). The estimation of glucose, galactose and residual lactose in hydrolysed whey products using cation-exchange high performance liquid chromatography. *NZ J. Dairy Sci. Tech.*, **18**, 209–224.

Yamauchi, S., Nakai, C., Nimura, N., Kinoshita, T. & Hanai, T. (1993). Development of a highly sensitive fluorescence reaction detection system for liquid chromatographic analysis of reducing carbohydrates. *Analyst*, **118**, 773–776.