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Analysis of galactosylisomaltol in milk systems using HPLC

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Abstract

While performing the HPLC separation of hydroxymethyfurfural (HMF) from in-bottle sterilised milk samples, two unknown compounds, named by us as **X** and **Y**, were observed. Preliminary data indicated that the amount of compound **X** in the samples was dependent on the intensity of the heat treatment applied to milk. Compound **X** was isolated from heated solutions of lactose/caseinate and milk, and purified by semi-preparative rp-HPLC. Applying HPLC-DAD, UV, IR, GC–MS and NMR analysis it was possible to identify it as galactosylisomaltol. The analysis of HMF and galactosylisomaltol enables to follow simultaneously, the Amadori product degradation through 1,2- or 2,3-enolisation in milk systems. Higher levels of galactosylisomaltol were found in in-bottle sterilised samples as compared with UHT-treated and pasteurised milks. Galactosylisomaltol was not found either in raw or pasteurised milk.

Keywords: Milk; Galactosylisomaltol; Maillard reaction; HPLC

1. Introduction

During thermal processing and storage of milk and milk products, non-enzymatic browning (Maillard reaction) occurs between the carbonyl group of lactose and the amino group of available lysine, forming lactulosyllysine as a stable compound. The protein bound Amadori compound is not available as a source of amino acids which results in a decreased nutritional value of processed milk, that is particularly important in infant formulas (Finot, Deutsch & Bujard, 1981). Although the Maillard reaction has been extensively studied in the last decades, it is not completely understood yet, in part due to the large number of intermediate compounds formed and in part due to the many variables which can affect it, such as temperature and heating time (Labuza, 1979), water activity (a_w) (Eichner & Karel, 1972), pH (O'Beirne, 1986), moisture content (Franzen, Singh & Okos, 1990; Yeo & Shibamoto, 1991) and chemical composition of the food system (Bell, 1997; Buera, Chirife, Resnik & Wetzler, 1987).

5-Hydroxymethyl-2-furancarboxaldehyde (hydroxymethylfurfural, HMF) is a well-known decomposition product of 3-deoxyglucosones and has been used as an indicator of intensity of heat treatments applied to milk since 1959 (Keeney & Bassette, 1959). Traditionally, total HMF determination in milk is carried out by colourimetric measurement at 443 nm of the yellow complex formed with 2-thiobarbituric acid (TBA). This method suffers a lack of specificity, because of the generalised reactivity of TBA towards aldehydic groups (Morales, Romero, & Jimenez-Perez, 1996), although rp-HPLC techniques are now available for accurate measurement of HMF in milk (e.g. Morales, Romero & Jimenez-Perez, 1992). While applying this method to UHT-treated milk samples, Morales et al. (1992) described the formation of two unknown compounds, designed as **X** and **Y**, that were eluted very close to HMF. Compound **X** was eluted about 1.2 min before HMF and showed a stronger temperature dependence than HMF. Recently, the chromatographic conditions for HMF separation were improved in order to avoid interference of these unknown compounds at any range of thermal processed milk (Morales et al., 1995). In the literature, it is known the formation of pyrralinone reductones, furfural and isomaltol derivatives during the heating of lactose with amino acids in aqueous solution (Kramhöller, Ledl, Lerche & Severin, 1992; Ledl, 1984; Goodwin, 1983). These groups of compounds could give us some light concerning the structure of the compound **X**.

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The aim of this work was the isolation and identification of compound **X** from an enriched milk or milk-like solution.

2. Materials and methods

2.1. Chemicals

All chemicals used were the purest available. HMF and 2-furancarboxaldehyde were purchased from Sigma (St. Louis, MO), acetic acid and sodium acetate from Analar (Essex, UK), lactose 1-hydrate from Merck (Darmstadt, Germany), and methanol HPLC grade from Scharlau (Barcelona, Spain). Sodium caseinate (spray-dried) was obtained from DMV-International (Veghel, The Netherlands).

2.2. Preparation of an enriched galatosylisomaltol solution

Skimmed pasteurised milk was heated at 90°C for 3 h in an air-forced oven. Milk (100 ml) was deproteinised with 20 ml of 24 g/100 ml trichloroacetic acid (TCA). After filtration through a filter paper (Whatman No. 42) a yellow-brown solution was obtained. Supernatant was partially concentrated by air-vacuum rotary evaporator at 60°C. Different solvents were tested on solid phase extraction (Plus C_{18}, Waters, Milford, MA) for clarification of the solution. Finally, the fraction eluted with 20% (v/v) methanol was collected, and the solvent was evaporated under reduced pressure at 35°C.

2.3. Heating model systems

Five model systems dissolved in 50 mM Na-phosphate buffer (pH 6) were prepared with 50 g/l lactose (system A), 50 g/l lactose + 30 g/l sodium caseinate (system B), skimmed milk containing lactose (49 g/l) and protein (32 g/l) (system C), 25 g/l glucose (system D) and 25 g/l glucose + 30 g/l sodium caseinate (system E). Systems were heated in stoppered tubes at 90°C for 3 and 6 h. After cooling in an ice water bath, 2 ml of solution was mixed with 1 ml of 0.3 N oxalic acid and 1 ml of 40 g/l trichloroacetic acid. After standing for 0.5 h at room temperature, the solution was centrifuged and the supernatant analysed by HPLC according to Morales et al. (1995).

2.4. HPLC analysis

A Kontron Instruments (Milan, Italy) chromatographic system was used for semi-preparative analysis, with a pump (MD-420), UV–VIS detector (MD-432) and a DT-450/MT-2 v.3.90 computing integrator connected to a PC. For UV scanning analysis (from 220 to 320 nm) a Spectra Physics (San Jose, CA) was used. The HPLC system consisted of a P200 pump, an A53000 sampler, and a Focus forward optical scanning detector. The sample (5 ml) was diluted with 2.5 ml of 0.3 N oxalic acid solution and deproteinised with 2.5 ml of TCA solution (40 g/l). The solution was kept for 10 min at room temperature and then centrifuged. A filtered solution was used for reversed-phase HPLC analysis. A degassed mobile phase was prepared with sodium acetate buffer (0.08 M), and the pH was adjusted to 3.6 with acetic acid. An Extrasyl ODS-2 S5 analytical column (25×0.40 cm, 5 μm, Tecknokroma, Barcelona, Spain) was used for HMF, furancarboxaldehyde and galactosylisomaltol separation at room temperature. A semi-preparative Spherisorb ODS2 column (25×1 cm, 5 μm, Tecknokroma, Barcelona, Spain) was used for peak collection.

2.5. Spectroscopy

UV spectra were recorded with a Shimadzu UV-1601 (Duisburg, Germany) recording spectrophotometer. IR spectra were recorded in a potassium bromide disk with a Perkin–Elmer spectrophotometer model 1720X.

2.6. Gaschromatography–mass spectrometry

Pure compound **X** was dissolved in CH_2Cl_2 (Ultra-Resi-Analysed, Baker, Deventer, The Netherlands) and analysed by GC–mass spectrometry (GC–MS) on a Shimadzu QP-5000 and identified by comparison with the NIST62 spectra library. The chromatograms are recorded using the software Chemstation CLASS5K. A capillary column SPB-1701 (30 m×0.2 mm, film 1 μm, Supelco, Bellefonte, USA) was used. The temperature program was: 37°C×10 min, 4°C/min to 200°C, then isothermal. The carrier gas was helium with a flow rate of 1 ml/min (at 70°C) and linear velocity of 27.3 cm/s (at 70°C), split injection with a split ratio of 10; injector temperature 220°C; detector temperature 250°C. Mass conditions: ions were generated by EI at 70 eV, detector gain 1500 V.

2.7. NMR analysis

^{1}H NMR and ^{13}C NMR were determined in D_2O using tetramethylsilane as internal standard on a Bruker AMX-300 (300 MHz). Chemical shifts were expressed in ppm (δ). Data of galactosylisomaltol: ^{1}H NMR (δ) 2.39, s, 3H, CH_3CO; 3.67, m, 3H; 3.78, m, 2H; 3.9, d, 1H; 5.52, d, J 7 Hz, 1H; 6.68, 1H, d, CH-4; 7.57, d, 1H, CH-5). ^{13}C NMR (δ) 28.75, 63.30, 70.96, 72.86, 75.09, 78.48, 104.55,107.02, 140.09, 151.29, 156.78, 191.92.

2.8. Commercial milk samples

Processed commercial samples of whole, half-skimmed and skimmed milks with well-defined heating treatments were obtained from several Spanish dairies

throughout the country. Different industrial treatments, such as pasteurisation (Alfa-Laval equipment at 78°C/15 s), UHT-direct processed (Rossi–Catelli equipment at 145°C/4 s and APV equipment at 148°C/3.2 s) and in-bottle sterilisation (Stork equipment at 120°C/14 min) were selected. The sample was collected the same day of production, stored at 4°C and analysed within 3 days.

3. Results and discussion

Fig. 1a shows the classical chromatographic separation of total (21.38 ± 1.25 μmol/l) and free (2.50 ± 0.13 μmol/l) HMF in an in-bottle sterilised skimmed milk. Besides HMF (retention time = 7.8 min) and furancarboxaldehyde (retention time = 15.7 min), two unknown peaks named by us as **X** (retention time = 6.2 min) and **Y** (retention time = 8.8 min) are also separated in these samples. Identification of HMF and furancarboxaldehyde was achieved by comparison of the retention time and UV spectra with standard compounds.

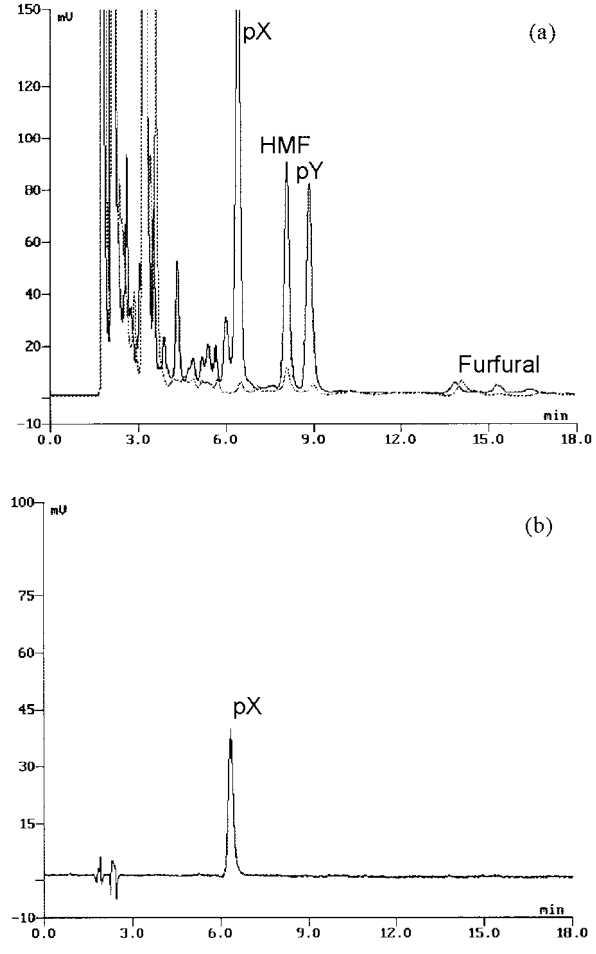

Fig. 1. Classical separation of total and free (dotted line) HMF of (a) a skimmed in-bottle sterilised milk (Stork, 120°C/14 min), and (b) isolated fraction of p**X**.

The formation of **X** increased linearly during milk heating and a buffered lactose/caseinate solution kept on a boiling water bath for 6 h (data not shown). In this sense, the formation of compound **X** could be related to the thermal treatment applied to milk. Several milk model systems containing lactose/caseinate (50:30 g/l) and milk were tested in order to achieve the most favourable conditions for formation of **X**. Finally, an enriched solution of compound **X** was prepared from pasteurised skimmed milk as described in Materials and Methods, since, keeping the same heating conditions, and ratio between reactants, more **X** was formed from milk than from lactose/caseinate systems. A yellow–brown solution was obtained and clarified by solid phase extraction. The clarification step is necessary in order to avoid the injection of highly non-polar compounds which could be largely retained in the column. Then, 2 ml of solution was passed through a pre-packed C_{18} cartridge and the fraction eluted with a 20% methanolic solution was analysed by HPLC. Under these conditions, the recovery of **X** was approximately 53.2%. To obtain a satisfactory purification, the pale yellow solution was injected (500 μl) onto a semi-preparative column and eluted isocratically with methanol:water (15:85, v/v) at a flow rate of 3 ml/min while recording at 280 nm. Fig. 2a shows the DAD spectra of **Y**, **X**, and HMF which show maxima at 274, 278, and 282 nm, respectively.

Fractions of approximately 1.4 ml were manually collected from each injection (Fig. 1b) and their purity was tested analytically by HPLC (Fig. 1c). Additionally, several combinations of methanol:water were tried as an eluent to verify whether the isolated **X** was a unique peak. Chromatographic peak **X** was recorded at 280 and 254 nm and the ratio of response in the purified fraction was compared with the enriched fraction. Only highly pure fractions were gathered, nitrogen was bubbled, and the solution was freeze-dried: approximately 5.2 mg of a colourless compound **X** were obtained. Fig. 2b shows the spectra profile of a rediluted sample of freeze-dried **X** which agrees with the spectrum obtained from a fresh enriched fraction using a DAD detector. The spectrum of compound **X** was recorded in neutral (50 mM Na–phosphate buffer, pH 6.7) and acid (10 mM Na–acetate buffer, pH 3.6) solution but no shift of the maximum wavelength was observed.

Analysed by GC/MS without derivatisation compound **X** had retention time 29.058 min: its mass spectrum was characterised by the presence of an intense ion at 126 m/z (80%, possibly the molecular ion) and the base peak at 111 m/z corresponding to a loss of 15 from 126 m/z. Less important peaks were detected at 97 m/z (1%, M-29), 83 m/z (8%, M-43), 69 m/z (6%, M-57), while the fragments at 55 m/z (40%) and 43 m/z (90%) were more intense. The mass-spectra library NIST suggested that it could be 2-acetyl-3-hydroxyfuran (isomaltol). The

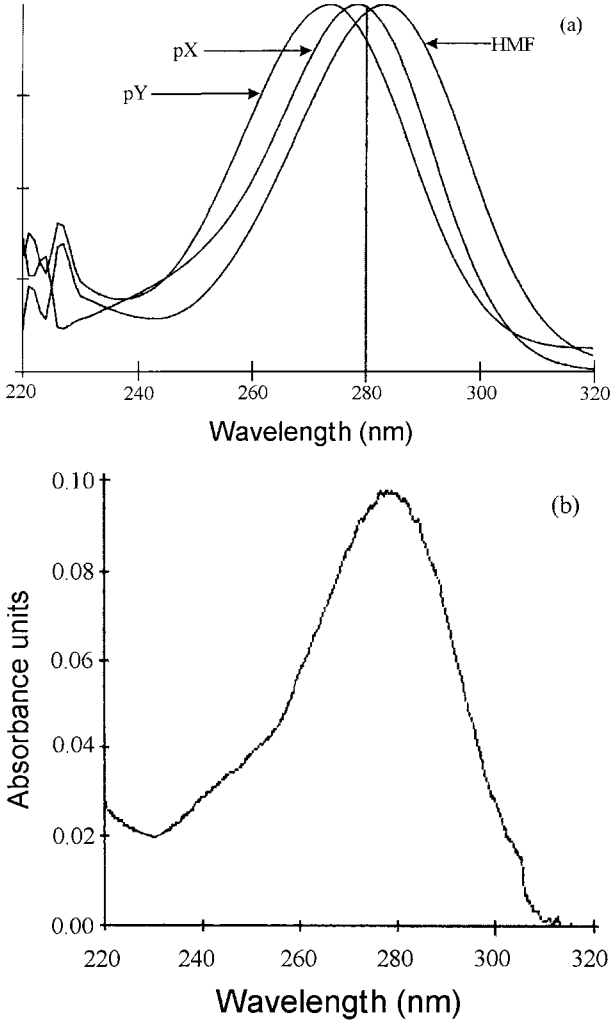

Fig. 2. (a) DAD spectrum of chromatographic peaks corresponding to p**X**, HMF and p**Y**; (b) spectrum of a freeze-dried fraction of p**X**.

loss of a methyl group (−15) is typical of acetyl substituent on an aromatic or heteroaromatic ring. These data agree with those reported by Ito (1977) for isomaltol formation through the thermal degradation of sucrose.

The IR spectrum of the isolated compound showed bands at 3127 (-OH), 2856 (-OH....O), 1603 (-C=C), 1575 (-C=C-O), 1474 (-CH in a furan ring), 1403 (-CH_3-C=O), 1103 (-HC=CH-), 1067 cm^{-1} (-C-O-C- in a furan ring). Additionally, bands at 1272 and 1108 cm^{-1} were recorded as reported by Ito (1977) and a characteristic band at 1023 cm^{-1} were shared with a standard of tetrahydrofuran. These results agree with those reported by Hodge and Nelson (1961); Ito 1997 and Bartulin, et al. (1992).

The sample was submitted also to ^1H NMR and ^{13}C NMR analysis. Doing this it was immediately clear that besides the expected peaks of isomaltol (2.39 δ, s, 3H, CH_3CO; 6.68 δ, 1H, d, CH-4; 7.57 δ, d, 1H, CH-5), there was a sugar structure for the presence of three complex peaks between 3.6 and 4.0 δ corresponding to 6H and a douplet at 5.03 δ for the anomeric CH. The $J=7$ Hz of the last signal suggested the presence of the β-anomer. It was therefore possible to assign to compound **X** the structure of galactosylisomaltol which was compatible also with the ^{13}C-NMR. These results suggest that this compound submitted to GC–MS without derivatisation is decomposed in the GC injector to give isomaltol. Finally, the identification was confirmed by comparing compound **X** with a standard of galactosylisomaltol kindly provided by Dr. Pischtsrieder (University of Munich, Germany).

Kramhöller et al. (1992) described the formation of β-pyranones, cyclopentenones and isomaltol derivatives when lactose solutions were heated in the presence of primary or secondary amines through the formation of 1-deoxyhexosuloses (a, Fig. 3). Degradation of the Amadori product in nearly neutral solution leads to the formation of 1-deoxyhexuloses as main products (Ames, 1992). Galactosylisomaltol (e, Fig. 3) is formed from the thermal degradation of 4-*O*-β-D-galactopyranosyl-2-hydroxy-2-methyl-2H-pyran-3-(6H)one (b, Fig. 3) and 4,5-dihydroxy-2-(β-D-galactopyranosyloxy)-5-methyl-2-cyclopenten-1-one (c, Fig. 3) in milk like systems (Kramhöller et al., 1992). Galactosylisomaltol and the β-pyranone derivatives have been already detected in heated milk (Kramhöller et al., 1992). When Amadori compounds are heated for a short time (70°C/15 min), β-pyranone is formed as main product together with minor amounts of cyclopentenone (Kramhöller, et al., 1993). Ledl, et al. (1986) stated that the usefulness of 4-*O*-β-D-galactopyranosyl-2-hydroxy-2-methyl-2H-pyran-3-(6H)one as an indicator of heat treatment intensity is limited, because it is rather rapidly converted to isomaltol derivatives, such as galactosylisomaltol (d, Fig. 3). Moreover, the pyranone derivative, measured by polarography, has been detected in heated milk and levels are increasing linearly during heating of milk in a boiling water bath for up to 8 h (Ledl et al. 1986). It could be reasonable to expect the formation of isomaltol from dehydration of galactosylisomaltol as described by Ledl et al. 1986) at pH values between 1 and 3 or at high temperatures but this conditions are far from usually applied in milk products.

This behaviour under slightly acid conditions was tested in a pure solution of **X** (0.75 μg/ml) heated in the presence of an oxalic acid solution (0.3 or 0.6 N) up to 60 min in a boiling water bath. Fig. 4 confirms that compound **X** degrades in oxalic acid solution while it is stable in water (isomaltol was not determined since it is not eluted in the chromatographic conditions described).

Additional experiments were performed in order to support with evidence the formation of galactosylisomaltol from model systems containing lactose/glucose and/or proteins. Klostermeyer (1986) stated that galac-

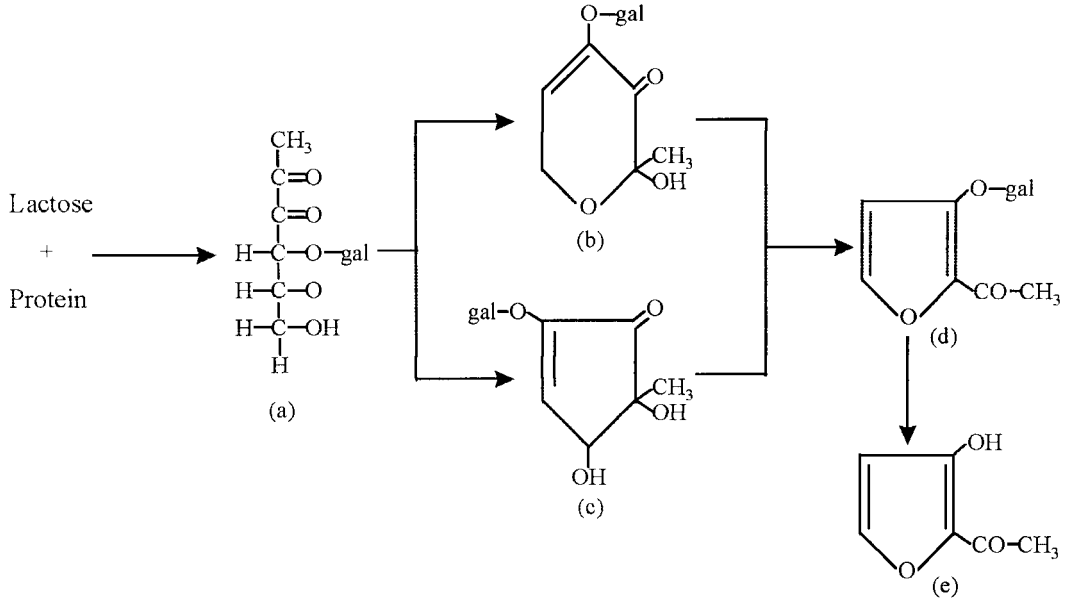

Fig. 3. Chemical pathway for formation of isomaltol from milk derived from studies of Kramhöller et al. (1992).

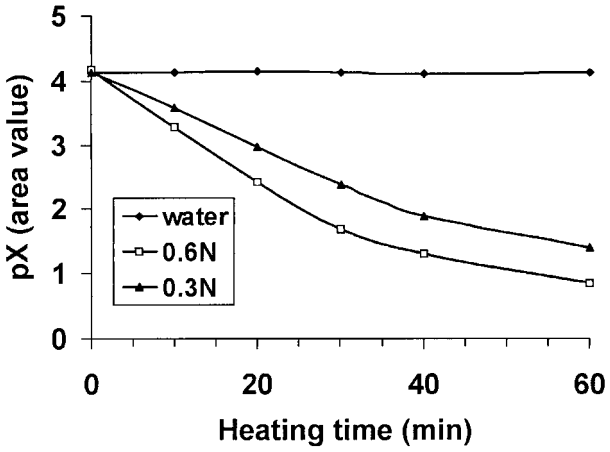

Fig. 4. Degradation of a p**X** solution (0.75 μg/ml) dissolved in water (◆) and oxalic acid solution 0.3 N (▲) and 0.6 N (□) up to 60 min in a boiling water bath.

tosylisomaltol and isomaltol are only derived from reaction of disaccharides with amines. In our experiments, **X** (galactosylisomaltol) was just found in the systems B and C which agrees with the reaction scheme postulated by Ledl et al. (1986) and Kramholler et al. (1992). Galactosylisomaltol (Fig. 5b) was formed from a lactose/caseinate model systems, but not when a monosaccharide or disaccharide was heated without any amino compound (Fig. 5a). 1-Deoxyosones derived from lactose degrade in a somewhat different manner in respect to those derived from glucose and different chromatographic profiles are obtained (Fig. 5b). It is reasonable to expect the formation of 1-deoxyosones during milk treatment, because they derive from 2,3-enolisation of the Amadori intermediate, that is favoured at a higher pH value over 1,2-enolisation. The amounts of galactosylisomaltol, considered as an advanced Maillard reaction product, progressively increases more drastically than HMF or furancarboxaldehyde do as the heating time or temperature increase. Galactosylisomaltol was never detected either in unheated model systems or in raw milk samples.

The thermal behaviour of galactosylisomaltol was studied in a milk-resembling model systems consisting of sodium caseinate (30 g/l) plus lactose (49 g/l) dissolved in a simulated milk salt solution adjusted at pH 6.65. The system was heated at 110 and 120°C in an oil bath for up to 30 min. The formation of galactosylisomaltol in lactose sodium-caseinate systems appeared to be strongly temperature dependent. Fig. 6 show that an induction period is needed before galactosylisomaltol is largely formed since several reactions take place and each reaction has its own rate. This observation agrees with thermal behaviour of other advanced Maillard products, such as lysylpyrraline (Morales & van Boekel, 1996), and the development of overall fluorescence or colour (i.e. Morales & van Boekel, 1997). Higher levels of galactosylisomaltol were obtained in samples heated at 120°C for 30 min (38.3 ± 1.9 μmol/l, $n = 3$). The limit of detection was settled at 0.03 μmol/l and the quantitative analysis was performed by the external standard method.

Table 1 summarised the amounts of galactosylisomaltol, as compared with HMF, obtained in several industrial processed samples. Higher values of galactosylisomaltol were obtained in the more drastic processes such as in-bottle sterilisation. The absence of galactosylisomaltol in pasteurised milk is probably due to the induction period necessary for the development of the reaction.

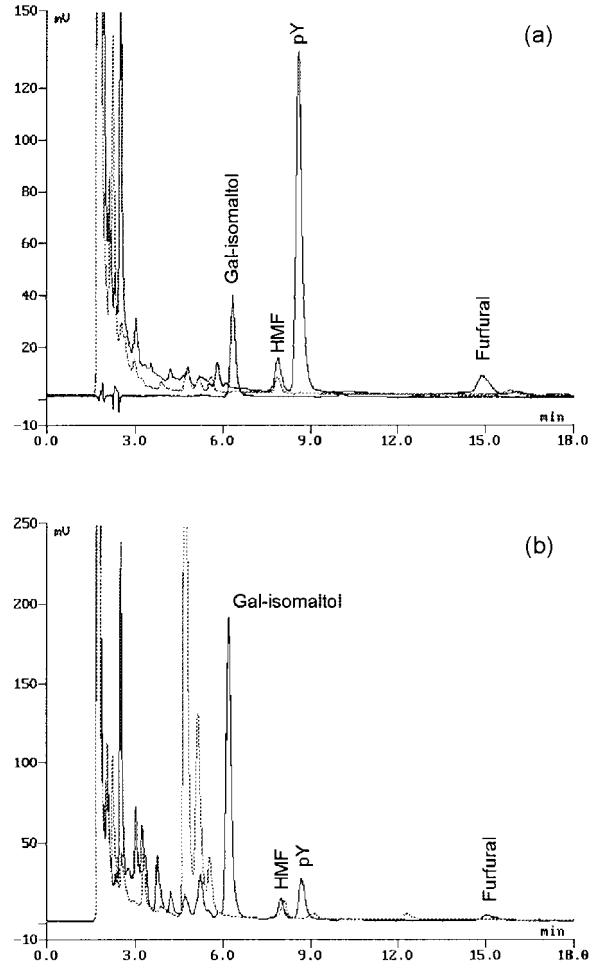

Fig. 5. Chromatographic profiles of (a) a lactose and glucose solution (dotted line) heated for 6 h and isolated galactosylisomaltol, and (b) a lactose or glucose/caseinate (dotted line) solution heated for 6 h.

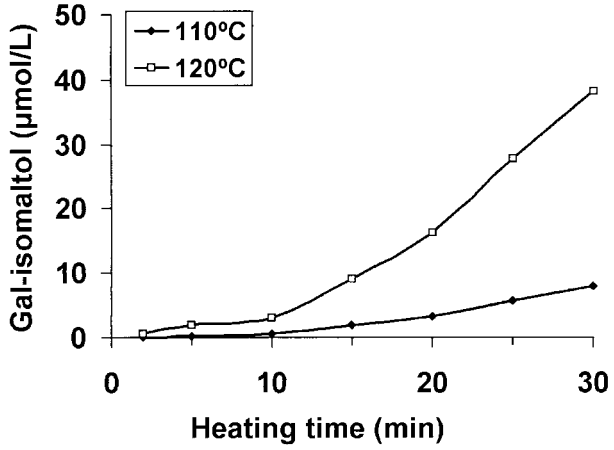

Fig. 6. Formation of galactosylisomaltol (µmol/l) in milk-resembling model systems heated at 110°C (◆) and 120°C (□) for up to 30 min.

Several questions, such as its stability, and its dependence on the thermal treatment remain to be solved in future studies, in order to assess the usefulness of galactosylisomaltol as a heat-induced index.

Table 1
Galactosylisomaltol and HMF content (mean valuies and standard deviation, $n=3$) in several industrial scale processed milk

Process	Equipment	Fat content (g/kg)	GaI-isomaltol (µmol/l)	HMF (µmol/l)
Pasteurization	Alfa–Laval	36	n.d.[a]	n.d.
		3	n.d.	n.d.
UHT-direct	Rossi–Catelli	35	0.51 ± 0.03	0.29 ± 0.04
		3	0.75 ± 0.05	0.60 ± 0.07
	APV	36	0.53 ± 0.02	
		2	0.67 ± 0.05	
Sterilization	Stork	35	2.52 ± 0.11	1.34 ± 0.11
		3	5.58 ± 0.22	2.50 ± 0.13

[a] n.d., not detected.

4. Conclusions

Galactosylisomaltol was described as one of the unknown peaks separated during the chromatographic analysis of HMF in milk products and its chromatographic separation is reliable and not time-consuming. These results open the possibility of monitoring in the same chromatographic analysis the two main reaction pathways during the Maillard reaction in milk (1-deoxyosone and 3-deoxyosone routes). Additional experiments should be conduced in the future to assess the effectiveness of a galactosylisomaltol index for the classification of technological processes during milk-sterilisation or for the detection of the addition of overheated ingredients to milk products.

Acknowledgements

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