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Chemical Indicators of Heat Treatment in Fortified and Special Milks

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Carbohydrate and furosine contents in 12 commercial fortified and special milk samples (pasteurized goat's and ewe's milks; ultrahigh-temperature (UHT) goat's milk, UHT milks fortified with calcium, magnesium, fiber, or royal jelly and honey; and lactose-hydrolyzed milks) were analyzed. Except for lactose-hydrolyzed milks, furosine, lactose, lactulose, galactose, glucose, *N*-acetylgalactosamine, *N*-acetylglucosamine, and *myo*-inositol contents were similar to the previously reported values for UHT or pasteurized milk samples. In lactose-hydrolyzed milks, lactulose was not detectable and lactose was present in low amount; high levels of glucose, galactose, fructose, tagatose, and furosine were also detected in this type of milk. Results found in commercial milks were compared to those obtained in laboratory-prepared UHT milks with lactose hydrolyzed prior to heating. Hydrolysis of lactose before thermal treatments promoted elevated accumulation of reducing sugars (galactose and glucose) that could be partially converted to the corresponding isomers (tagatose and fructose) during heating. In addition, the reducing sugars could also react with the amino groups of proteins, giving rise to the corresponding Amadori compound. According to the obtained results, heating prior to hydrolysis of lactose is suggested to avoid a considerable loss of available lysine.

KEYWORDS: Milk; heat treatment; carbohydrate; furosine; lactose

INTRODUCTION

The dairy industry is continually searching for products with high quality and stable properties due to the increasing interest of consumers not only in nutritious and tasty foods but also in products with specific characteristics that provide health benefits as well. Nowadays, certain consumers can obtain benefits from the existing commercial types of milk such as milks fortified with calcium and magnesium (*1*) or royal jelly and honey (*2*), lactose-hydrolyzed milks (*3*) and goat's (*4*) or ewe's milk (*5*), among others. Depending on each case, these milks can represent an important source of nutrients in people with hypertension, osteoporosis, lactose intolerance, and allergic reactions to bovine milk (*6*−*8*).

High-quality milks require an adequate process control to ensure the heat load applied during processing. Assessment of the extent of the heat damage in milk due to nonenzymatic browning can be achieved by the analysis of lactulose (formed by lactose isomerization) and furosine (generated from the acid hydrolysis of the Amadori compound lactulosyl-lysine, the first stable compound of the Maillard reaction) (*9*). Both indicators are used to distinguish milks submitted to different heat treatments (*10*), and furosine is also a good indicator of the adulteration of milk derivatives with reconstituted dried milk (*11*−*13*). In addition, the concentration of other compounds derived from the carbohydrate fraction of milk such as glucose, galactose, *N*-acetylgalactosamine, and *N*-acetylglucosamine may vary with the action of enzymes on glycoproteins, thermal processing, or inadequate storage conditions, so measuring their levels could provide an indication of milk quality (*14*, *15*).

Despite the number of studies conducted to assess the usefulness of lactulose and furosine as quality indicators in milk, to the best of our knowledge, scarce information is available on the content of these parameters in fortified and special milks. The presence of carbohydrates (in milks with soluble fiber, royal jelly, or honey) and salts (in milks with calcium and magnesium) can affect the progress of the isomerization of lactose and/or Maillard reaction during heat treatment of milk. It has been reported that lactose isomerization can be influenced by different salts (*16*−*19*). Moreover, it is also known that lactose concentration in raw milk plays an important role in the formation of lactulose (*20*). In the present work, we have evaluated the extent of nonenzymatic browning by means of lactulose and furosine in commercial samples of goat's and ewe's milk, milks fortified with calcium, magnesium, fiber, royal jelly, and honey, and lactose-hydrolyzed milks. Other quality indicators derived from the carbohydrate fraction have been also determined. In addition, a laboratory-scale study on the effect of previous hydrolysis of lactose on nonenzymatic browning during ultrahigh-temperature (UHT) treatments of milk is also reported.

MATERIALS AND METHODS

Milk Samples and Heat Treatments. A total of 12 commercial samples were obtained from local stores: 2 samples of goat's milk (1

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Table 1. Values of pH, Protein Content, Galactose, Glucose, *N*-Acetylgalactosamine, *N*-Acetylglucosamine, *myo*-Inositol, Lactose, and Lactulose Contents, and Furosine Content in Fortified and Special Commercial Milks

sample	pH	protein (%)	galactose (mg/100 mL)	glucose (mg/100 mL)	*N*-acetyl-galactosamine (mg/100 mL)	*N*-acetyl-glucosamine (mg/100 mL)	*myo*-inositol (mg/100 mL)	lactose (mg/100 mL)	lactulose (mg/100 mL)	furosine (mg/100 g of protein)
pasteurized goat's milk	6.5	4.0	31.9 ± 0.1	14.9 ± 0.4	0.9 ± 0.0	Nd[a]	6.6 ± 0.0	3689.7 ± 239.6	1.8 ± 0.1	35.8 ± 1.4
UHT goat's milk	6.7	2.8	3.7 ± 0.1	2.3 ± 0.1	1.1 ± 0.0	Nd	8.4 ± 0.2	3757.2 ± 275.8	32.5 ± 0.6	154.3 ± 3.5
pasteurized ewe's milk	6.6	5.3	1.3 ± 0.2	4.3 ± 0.4	1.3 ± 0.0	Nd	9.3 ± 0.1	4400.9 ± 50.7	8.5 ± 0.6	8.6 ± 0.1
Ca-fortified 1	6.7	4.4	10.9 ± 1.6	9.7 ± 0.2	4.4 ± 0.3	11.0 ± 0.3	3.7 ± 0.1	5798.6 ± 103.0	38.1 ± 1.3	127.8 ± 5.9
Ca-fortified 2	6.8	3.8	9.7 ± 0.1	7.5 ± 0.1	4.1 ± 0.1	10.2 ± 0.8	4.0 ± 0.0	4961.8 ± 86.5	36.0 ± 2.2	189.6 ± 6.5
Ca-fortified 3	7.0	3.7	12.4 ± 0.6	7.4 ± 0.2	5.0 ± 0.1	9.0 ± 0.4	3.2 ± 0.1	4195.1 ± 27.2	45.2± 0.2	130.0 ± 3.5
Ca-fortified 4	6.9	3.7	7.5 ± 0.1	4.8 ± 0.0	4.3 ± 0.3	8.2 ± 0.4	3.1 ± 0.2	4410.8 ± 338.1	12.2 ± 0.0	66.4 ± 2.9
Mg-fortified	7.0	3.3	10.4 ± 0.2	6.9 ± 0.2	3.8 ± 0.3	8.7 ± 0.5	2.7 ± 0.2	4508.0 ± 365.3	52.5 ± 2.1	204.1 ± 7.5
fiber-fortified	6.8	3.1	13.1 ± 0.7	6.5 ± 0.3	3.3 ± 0.3	8.4 ± 0.0	2.9 ± 0.0	3886.5 ± 198.7	18.4 ± 0.1	67.5 ± 3.2
jelly/honey-fortified	6.7	3.2	29.4 ± 1.5	115.7 ± 1.9	1.1 ± 0.0	2.5 ± 0.3	1.3 ± 0.1	4247.8 ± 52.9	19.4 ± 0.9	87.1 ± 1.1
lactose-hydrolyzed 1	6.7	3.3	1361.4 ± 0.1	1761.2 ± 0.3	3.1 ± 0.1	7.7 ± 0.3	2.2 ± 0.1	732.6 ± 35.0	Nd	375.8 ± 23.1
lactose hydrolyzed 2	6.5	3.0	1374.7 ± 92.6	1767.4 ± 98.8	3.4 ± 0.2	7.2 ± 0.1	2.2 ± 0.1	593.7 ± 21.0	Nd	432.4 ± 6.6

[a] Not detectable.

pasteurized with the addition of bifidobacteria and 1 UHT), 1 sample of pasteurized ewe's milk, and 9 samples of UHT milk (2 lactose-hydrolyzed, 4 samples fortified with calcium salts or milk proteins and 1 fortified with magnesium salts, 1 fortified with fiber, and 1 fortified with royal jelly and honey). All commercial samples were analyzed before the recommended storage time. Bovine raw milk was obtained from a local farm and kept refrigerated for up to 4 h until it was processed. Prior to thermal treatments, to obtain milk with low levels of lactose, part of the raw milk treated with sodium azide (0.05%) was incubated at 4 °C with β-galactosidase (10 μL/10 mL of milk) from *Saccharomyces fragilis* (Lactozym 3000 L HP-G, Novo Nordisk) for 16 h. The hydrolysis was stopped by heating at 95 °C for 2 min and subsequent cooling in an ice−water bath. Portions (7 mL) of milk were sealed in capillary stainless steel tubes (10 m × 1 mm inside diameter) and heated for 10−40 s in the temperature range of 135−150 °C in a thermostatically controlled constant-temperature bath of silicone. Heating was stopped by rapid cooling of the capillary tubes in a bath of ice−water. All experiments were performed in duplicate. As control, milk without β-galactosidase was heated in a similar manner.

Analytical Determinations. The pH of samples was measured at 20 °C in a MicropH2001 pH-meter with glass electrode (Crison Instruments, Barcelona, Spain). The protein content was determined by means of the Kjeldahl method (*21*).

Furosine Determination. To determine the content of furosine, 2 mL of milk was hydrolyzed with 6 mL of 10.6 N HCl at 110 °C for 23 h and was analyzed by RP-HPLC, according to the method of Resmini et al. (*22*), using a C8 Alltech furosine-dedicated column (250 × 4.6 mm; Alltech, Laarne, Belgium) with a linear, binary gradient. Quantification was performed according to the external standard method using a commercial standard of furosine (Neosystem, Strasbourg, France). Data were expressed as milligrams per 100 g of protein.

Carbohydrate Determination. Lactose and lactulose were determined by GC as their trimethylsilyl derivatives of the free carbohydrate fraction using a 3 m × 1.0 mm internal diameter, stainless steel column (Chrompack, Middelburg, The Netherlands) packed with 2% OV-17 on nonsilanized 120/140 Volaspher A-2 (Merck, Damstadt, Germany), following the method of Olano et al. (*23*) for UHT milks and the method of De Rafael et al. (*24*) for pasteurized milks. Phenyl-β-glucoside (Sigma Chemical Co., St. Louis, MO) was used as internal standard.

Free monosaccharides, *N*-acetylgalactosamine, *N*-acetylglucosamine, and *myo*-inositol were analyzed by GC as their trimethylsilyl derivatives using a fused silica capillary column (25 m × 0.2 mm) coated with methyl silicone, following the method of Troyano et al. (*25*). Methyl-α-D-galactopyranoside (Sigma) was used as internal standard.

Statistical Analysis. Statistical analysis was performed using the statistical software SPSS 9.0.1. for Windows (*26*).

RESULTS AND DISCUSSION

Table 1 shows the mean values of the analytical determinations carried out in the commercial samples studied. Milk samples showed pH values in the range of 6.5−7.0. Similar values of pH have been previously reported by other authors in bovine, caprine, and ovine milks (*27*−*31*). In goat's and ewe's milk, protein content was in the range of 2.8−5.3%. The highest level of protein was found in ewe's milk, whereas the lowest value was detected in UHT goat's milk, destined for people allergic to cow's milk proteins. These contents are in agreement with those found by other authors (*27*−*33*). In the case of bovine milk, the four samples fortified with calcium presented elevated levels of proteins (3.7−4.4%), probably due to the use of products containing proteins as the calcium source. The rest of the samples were found to have values similar to those reported in the literature (*34*, *35*).

In most of the samples, galactose, glucose, *N*-acetylgalactosamine, *N*-acetylglucosamine, and *myo*-inositol concentrations were close to those previously found in commercial heat-treated milks (*9*, *14*, *15*, *36*−*38*), indicating adequate storage conditions and quality of initial milk. Pasteurized goat's milk had high levels of glucose and galactose, probably due to enzymatic activities derived from the presence of bifidobacteria. Milk with royal jelly and honey also presented elevated contents of galactose and glucose. As is known, glucose is present in honey in very high concentration (*39*) and galactose in lower amount (*40*). Moreover, high levels of galactose could be also attributed to the action of heat-resistant enzymes derived from poor microbiological quality of the raw milk (*15*, *36*). As expected, lactose-hydrolyzed milks presented high concentrations of galactose and glucose due to the action of β-galactosidase.

Except in lactose-hydrolyzed milks, lactose contents were close to the values previously reported in the literature (*29*, *34*). The high lactose level (5798.6 mg/100 mL) found in calcium-fortified milk 1 may be due to the addition of whey powder.

The lactulose content in pasteurized goat's milk was within the ranges observed by De Rafael et al. (*24*) (0.7−2.8 mg/100 mL) and Akalin and Gönç (*41*) (1.2−1.9 mg/100 mL) for commercial pasteurized bovine milk and was below the maximum proposed for milks submitted to high pasteurization (5 mg/100 mL) (*42*). Pasteurized ewe's milk was found to have an elevated content of lactulose (8.5 mg/100 mL), suggesting a possible excessive heat treatment.

The rest of the samples, with the exception of lactose-hydrolyzed milks, presented values of lactulose corresponding to UHT milks (12.2−52.5 mg/100 mL) (*9*, *11*, *12*, *41*, *43*−*48*), below the limit of 60 mg/100 mL recommended by the International Dairy Federation (*49*) for UHT milks. The highest contents of lactulose (45.2 and 52.5 mg/100 mL) were found

Table 2. Glucose, Galactose, Fructose, and Tagatose Contents in Commercial Milks with Lactose Hydrolyzed and in Milks Hydrolyzed in the Laboratory and Heated under UHT Conditions

sample		glucose (mg/100 mL)	galactose (mg/100 mL)	fructose (mg/100 mL)	tagatose (mg/100 mL)
lactose-hydrolyzed commercial milks	1	1761.2 ± 0.3	1361.4 ± 0.1	6.3 ± 0.2	13.5 ± 0.9
	2	1767.4 ± 98.8	1374.7 ± 92.6	6.0 ± 0.3	14.1 ± 0.9
lactose-hydrolyzed laboratory prepared milks	135 °C, 40 s	2220.6 ± 6.6	1747.8 ± 13.7	3.8 ± 0.2	8.7 ± 0.2
	140 °C, 40 s	2150.9 ± 159.4	1679.7 ± 143.7	4.4 ± 0.4	10.4 ± 1.1
	150 °C, 30 s	2121.3 ± 54.1	1660.7 ± 34.1	5.2 ± 0.1	12.1 ± 0.7

in milks with pH values of 7. Previous findings of Martínez-Castro and Olano (*50*) demonstrated that increases of pH above 6.7 could give rise to substantial increases in the formation of lactulose during heat treatment of milk. Samples with similar pH values showed noticeable differences in lactulose contents, which indicate different intensities of the heat treatments undergone by the samples.

Except lactose-hydrolyzed milks, all of the analyzed samples showed levels of furosine ranging from 8.6 to 204.1 mg/100 g of protein. Other authors (*11*, *48*, *51*−*56*) found furosine concentrations within the range of 25−300 mg/100 g of protein for UHT commercial milks. Pasteurized ewe's milk presented a value of furosine (8.6 mg/100 g of protein) similar to those reported by Villamiel et al. (*13*) for commercial pasteurized cow's milk. However, pasteurized goat's milk was found to have a very high level of furosine (35.8 mg/100 g of protein) with regard to the content of lactulose (1.8 mg/100 mL). Villamiel et al. (*13*) found one pasteurized milk sample with 31.4 mg/100 g of protein and 1.2 mg/100 mL of lactulose, and they suggested the presence of reconstituted milk powder.

As can be observed in **Table 1**, lactose-hydrolyzed milks presented levels of lactose, lactulose, and furosine different from the other samples studied. As expected, the levels of lactose were very low, corresponding to a hydrolysis degree of ∼90%. Similar lactose values were obtained by Corradini et al. (*57*) in UHT milks also treated with β-galactosidase. Because glucose and galactose are sweeter than lactose, an excessive hydrolysis extent could negatively affect the taste of milk. Therefore, the hydrolysis conditions should be controlled to obtain values between 80 and 90% of hydrolysis (*3*, *58*).

Lactulose was not detected in milks with β-galactosidase (detection limit of the analytical method was 0.7 mg/100 mL); however, the concentrations of furosine were very elevated (375.8 and 432.4 mg/100 mg of protein), higher than those obtained in the rest of the commercial samples analyzed. Evangelisti et al. (*59*) found 134.2 and 206.1 mg of furosine/100 g of protein in lactose-hydrolyzed UHT milks after manufacturing. No significant increase in the level of furosine was detected when samples were then stored during 3 months at 4 °C, whereas after the same period of storage but at 20 °C the amounts of furosine were 401.4 and 554.6 mg/100 g of protein, respectively. Corradini et al. (*57*) analyzed UHT commercial milks with low lactose content and stored at 4 °C for 90 days, and they detected values of furosine in the range from 71.5 to 148.1 mg/100 g of protein. In UHT commercial milks with lactose hydrolyzed, Marconi et al. (*60*) found levels of furosine in the range of 162.1−1070.8 mg/100 g of protein; values of furosine >900 mg/100 g of protein were attributed to a prolonged conservation over the time prescribed.

The presence of high amount of furosine may be due to the progress of Maillard reaction during processing or storage of milk or adulteration with milk powder. To elucidate the formation of furosine in this type of milk, a study on the formation of furosine and lactulose in milks hydrolyzed in the laboratory with β-galactosidase was carried out. Two sets of samples (one control and other with hydrolyzed lactose) were submitted to different UHT heating. Lactose, lactulose, furosine, glucose, galactose, tagatose and fructose levels were evaluated.

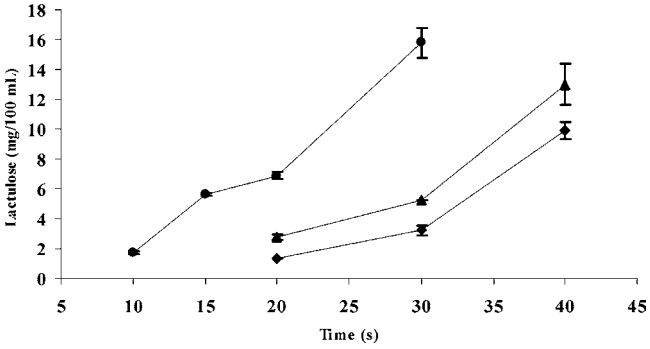

Figure 1. Formation of lactulose in milk without β-galactosidase and heated at 135 (◆), 140 (▲), and 150 °C (●). The graph shows mean values ± SD.

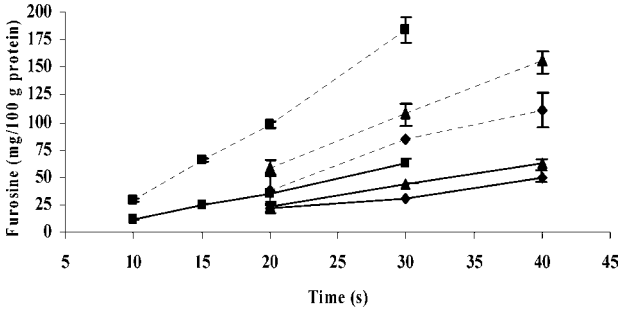

Figure 2. Formation of furosine in milk with (- - -) and without (—) β-galactosidase and heated at 135 (◆) and 140 (▲), and 150 °C (■). The graph shows mean values ± SD.

Lactose contents were 4788.1 and 558.2 mg/100 mL in control and lactose-hydrolyzed milks, respectively. The latter was very close to the values found in commercial lactose-hydrolyzed UHT milk samples (**Table 1**).

Because lactose isomerization depends on the initial concentration of lactose (*20*), lactulose formation was detected only after UHT treatments of control milk samples (**Figure 1**). Lactulose formation increased with heat treatment intensity and reached values in the range of 1.2−15.8 mg/100 mL.

Furosine (**Figure 2**) was found in both types of milk. As in the case of lactulose formation, furosine increased with time and temperature of treatment. The content of furosine in heat-treated control and lactose-hydrolyzed milks ranged from 10.8 to 63.2 mg/100 g of protein and from 17.1 to 183.9 mg/100 g of protein, respectively. Control milks heated for 40 s at 135 and 140 °C and for 30 s at 150 °C presented values of lactulose and furosine similar to those reported by other authors for UHT milks (*11*, *61*), whereas the other heat-treated milk samples were found to have contents of lactulose and furosine similar to those reported for high pasteurization treatments (*13*).

Furosine formation was significantly higher ($P < 0.05$) in lactose-hydrolyzed milks as compared to control milk samples. This result is due to the elevated concentration of reducing monosaccharides after the hydrolysis of lactose, which can react with the amino groups of lysine to give rise to the corresponding Amadori compound.

Glucose, galactose, tagatose, and fructose concentrations were also determined and compared with those obtained in the commercial UHT lactose-hydrolyzed samples. The results are shown in **Table 2**. The most striking feature was the formation of tagatose and fructose. Their contents increased with heating intensity, reaching values, under the most severe conditions (150 °C, 30 s), close to those of commercial UHT lactose-hydrolyzed milks (12.1 and 5.2 mg/100 mL for tagatose and fructose, respectively). Troyano et al. (*38*) observed lower levels of tagatose (0.2−0.6 mg/100 mL) even in commercial sterilized milks.

According to the obtained results we can conclude that, except for lactose-hydrolyzed milks, fortified and special milks analyzed in this study presented similar values of chemical indicators as compared to ordinary commercial milks. In the case of lactose-hydrolyzed milks, the contents of tagatose and fructose (originated by isomerization of galactose and glucose, respectively) found in the commercial milk samples together with the high furosine formation seem to indicate that the hydrolysis of lactose in the commercial samples could have been accomplished prior to the heat treatment. Moreover, although milks were analyzed during the period of shelf life, Maillard reaction development cannot be dismissed during the storage time. The levels of furosine and lactulose in lactose-hydrolyzed commercial milks are not comparable to the contents of these indicators in milks without lactose hydrolyzed submitted to similar heating. To avoid an excessive formation of furosine and, consequently, loss of available lysine and changes in the organoleptic characteristics, it may be advisable to hydrolyze lactose under aseptic conditions after thermal treatment conditions.

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