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Heat-treated milk differentiation by a sensitive lactulose assay

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Abstract

A sensitive enzymatic assay for determining lactulose (detection limit = 2.5 mg/l; RSD <4%) was applied to 90 samples of various milk categories, such as, pasteurized milk (fresh and high-temperature), UHT milk (indirect, direct, by infusion and by injection), and in-container sterilized milks. Results showed that it was possible to distinguish, by lactulose content, not only in-container sterilized milk (744 mg/l), indirect UHT (341 mg/l) and direct UHT (165 mg/l), but also UHT milk produced by mild technologies such as milk treated by infusion (107 mg/l), high-temperature pasteurized milk (58 mg/l), and low temperature pasteurized milk (3.5 mg/l).
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1. Introduction

Continuous developments in the heat treatment processes of milk and in alternative procedures, such as microfiltration (Larsen, 1996), have given rise to a number of commercial milks, such as pasteurized (low and high-temperature), microfiltered and pasteurized/UHT, UHT (direct and indirect systems), and in-container sterilized milk. The establishment of thermal parameters, defined under specific temperature/time conditions, contributes to the classification of heat-treated milks. These thermal parameters are mainly employed to identify and optimize processes, assess heat-loads and identify the degree of thermal damage (Glaeser, 1996; Morales, Romero, & Jimenez-Perez, 2000; Pellegrino, Resmini, & Luf, 1994).

Lactulose was proposed by the International Dairy Federation and the European Union as a parameter capable of differentiating between UHT milk and in-container sterilized milk (EU Commission, 1992; IDF, 1992, 1993). Both international bodies suggested 600 mg/l of lactulose as a marker for distinguishing between the two milk types, so as to guarantee the quality of UHT milk.

With regard to pasteurized, microfiltered and pasteurized, high temperature pasteurized milk and reconstituted powdered milk, upper limits (threshold) of lactulose concentrations cannot be proposed since the methods available for lactulose determination are neither sufficiently sensitive (level of detection 50 mg/l) (Andrews, 1986; De Block, Merchieres, Van Renterghem, & Moermans, 1996; Olano, Calvo, & Corzo, 1994), nor routinely applicable (Andrews, 1986; Boehringer Mannheim, 1995; Mayer, Genrich, Kunnecke, & Bilitewski, 1996; Moscone, Bernardo, Marconi, Amine, & Palleschi, 1999).

In this study, a robust enzymatic–spectrophotometric method, that we had previously developed (Amine, Moscone, Bernardo, Marconi, & Palleschi, 2000), was adapted to improve its sensitivity and validated at low and very low lactulose concentrations. In particular, this

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method was applied to milks of different heat-processes, such as fresh pasteurized milk, high-temperature pasteurized milk, direct UHT milk treated by both injection and infusion systems, indirect UHT-treated milk, and in-container sterilized milk, in order to distinguish the heat treatments used.

2. Materials and methods

2.1. Samples

The samples included fresh pasteurised (PAST) milk ($n = 10$), high-temperature pasteurized (HT PAST) milk ($n = 10$), direct UHT-treated milk using an injection system (INJ UHT) ($n = 20$), direct UHT-treated milk using an infusion system (INF UHT) ($n = 20$), indirect UHT-treated milk using a plate or a tubular heat-exchange system (IND UHT) ($n = 20$), and in-container sterilized (STER) milk ($n = 10$).

The sterilized milk samples (UHT and STER) were produced at the production plant and at the pilot plant of the Dairy Research and Development Centre of Parmalat, Sala Baganza, Parma, Italy. Pasteurized milk samples (PAST and HT PAST) were purchased at supermarkets in Rome and Parma (Italy).

2.2. Lactulose analysis

2.2.1. General

The lactulose analysis was performed by adapting the enzymatic–spectrophotometric assay standardized by Amine et al. (2000) to improve its sensitivity. The method is based on the assumption that free fructose is absent in milk but derives from lactulose hydrolysis. Lactulose is hydrolysed to D-galactose and D-fructose with β-galactosidase. The liberated D-fructose is equivalent to the amount of lactulose. In the presence of the fructose dehydrogenase (FDH) and the electron carrier phenazine methosulfate (PMS), fructose and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) produce a coloured compound (MTT Formazan), measured by its light absorbance at 570 nm.

2.2.2. Sample treatment

A milk sample of 10 ml was pipetted into a 50 ml conical flask and 1.75 ml of both Carrez I and II were gradually added successively; the resulting solution was stirred for 2–3 min, then 6.5 ml of citric/phosphate buffer was added. The solution was then thoroughly mixed for 2–3 min, left to rest for 30 min and filtered through a filter paper eliminating the first 2–3 ml of filtrate. 1 ml of milk filtrate, 0.2 ml of β-galactosidase and 0.3 ml of citric/phosphate buffer were added in succession to each glass-stoppered test tube (7.5×1.0 cm). The test tubes were placed in a water bath at 65±0.1 °C for 12 min then cooled in tap water.

2.2.3. Absorbance measurement

One-hundred microlitres of MTT, 50 μl of PMS, milk hydrolysate (200 μl for STER/UHT milk and 500 μl for HT PAST/PAST milk) and citric/phosphate buffer were mixed in a 1 cm light path cuvette. The reaction started with 2 units (16 μl) of FDH for STER/UHT/HT PAST milk and 6 units (48 μl) of FDH for PAST milk. The volume of the buffer was modulated to maintain the final volume at 1.0 ml. The increase in absorbance at 570 nm was measured after 12 min at room temperature.

A blank was prepared for each milk sample, using the same procedure as the above, without adding the FDH solution.

2.3. Reagents

These were, fructose dehydrogenase (FDH) (EC 1.1.99.11, 112 units mg/solid) (Sigma Chemical Co., St. Louis, MO, 63178, USA) and β-D-galactosidase from *Aspergillus orizae* (EC 3.2.1.23, 9 units mg/solid) (Sigma).

All other chemicals, of high purity, were purchased from Sigma (St. Louis, MO, USA).

2.4. Apparatus

Absorbance measurements were made with a Varian DMS UV–visible spectrophotometer (Varian Inc., Walnut Creek, CA, 94598, USA) using a 1 cm cuvette light path.

3. Results and discussion

The analytical performances of the enzymatic assay were tested at low and very low concentrations of lactulose. Two calibration curves were plotted by adding varying amounts of lactulose standard to PAST milk (from 2.5 to 20 mg/l using 6 FDH units and 500 μl of hydrolysate volume, and from 10 to 100 mg/l using 2 FDH units and 500 μl of hydrolysate volume). The standard curves were linear and described by the equations $y = 0.0061x$, $R^2 = 0.988$, $P \leqslant 0.001$ and $y = 0.0118x + 0.0135$, $R^2 = 0.999$, $P \leqslant 0.001$, for 6 and 2 FDH units, respectively.

Excellent precision ($n = 6$) was confirmed at low lactulose concentration, since the absorbance RSDs were 3.0 and 3.7% for PAST and HT PAST milk, respectively.

The accuracy of this system was checked by running a recovery test after the addition of increasing amounts of lactulose (from 2.5 to 20 mg/l) to PAST milk (Table 1). The results of the recovery test were satisfactory, with values ranging from 101 to 105%.

Table 1
Recovery test of different amounts of lactulose added to fresh pasteurized milk

Lactulose added (mg/l)	Lactulose recovered (mg/l)	Recovery (%)	RSD ($n=3$)
2.5	2.56	102	2.9
5.0	5.11	103	3.5
7.5	7.55	101	2.7
10.0	10.27	103	3.2
15.0	15.41	103	2.1
20.0	21.03	105	3.1

The detection limit of lactulose in milk samples was significantly improved up to 2.5 mg/l using 6 FDH units and 500 μl of hydrolysate. This was significantly lower than those of any of the available methods varying from 10 mg/l of the enzymatic-spectrophotometric method to 200 mg/l of the HPLC reference method (Andrews, 1986; Boehringer Mannheim, 1995; Cataldi, Angelotti, & Bufo, 1999; De Block et al., 1996; De Rafael, Calvo, & Olano, 1996; IDF, 1998; Martinez-Castro & Olano, 1981; Moscone et al., 1999; Olano et al., 1994). The above detection limit is sufficiently low to allow the quantification of lactulose in all types of heat-treated milks, from low pasteurized to in-container sterilized milks.

The present method for the quantification of lactulose in milk samples, besides being significantly more sensitive, is also more rapid than those routinary used (Boehringer Mannheim, 1995; IDF, 1998; Olano et al., 1994). The complete time of analysis (deproteinization + - analysis) is 60 min for one sample and 100 min for 20 samples.

In order to verify the use of lactulose as a process indicator for pasteurized and direct UHT milks, 90 milk samples of different heat-classes were analysed. The lactulose values of the pasteurised (PAST and HT PAST), direct UHT (INF UHT and INJ UHT), indirect UHT (IND UHT) and in-container sterilized (STER) milks are given in Fig. 1.

The lactulose content of STER milk varied from 493 to 1147 mg/l, with an average value of 744 mg/l, which was significantly higher than any of the UHT milks (max lactulose content 421 mg/l in IND UHT sample). The limit of 600 mg/l proposed by the European Union could be lowered to 400 mg/l to protect the quality of UHT milk from excessive heat-load, as adopted by Germany (Pellegrino, De Noni, & Resmini, 1995). Since the method is highly sensitive, lactulose formation can be monitored in pasteurized milk and in UHT milk produced by gentle technologies, such as the infusion system.

The lactulose content of HT PAST milk, varies from 32 to 79 mg/l, with an average of 58 mg/l. This enables differentiation from both PAST milk (range 2–6 mg/l; average 3.5 mg/l) and UHT milks such as INJ UHT (range 126–233 mg/l; average 165 mg/l) or IND UHT (range 275–421 mg/l; average 348 mg/l). The lactulose content of INF UHT milk (range 89–120 mg/l; average 107 mg/l) is between those of INJ UHT and HT PAST milk, which confirms that this sterilization process reduces thermal damage to raw milk, thus protecting its physicochemical, nutritional and organoleptic properties (de Jong, Waalewijn, & Van der Linden, 1996).

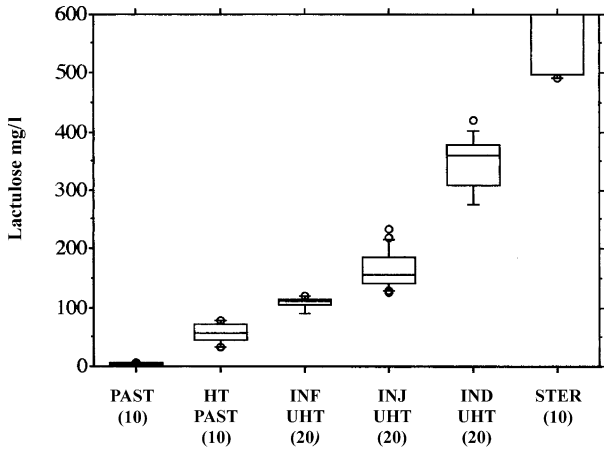

Fig. 1. Box and whisker plots of lactulose contents in milks of different heat-processes: PAST = fresh pasteurized milk; HT PAST = high-temperature pasteurized milk; IND UHT = indirect UHT-treated milk; INJ UHT = direct UHT-treated milk using an injection system; INF UHT = direct UHT-treated milk using an infusion system; STER = In-container sterilized milk. () Number of samples per class. The central box covers the middle 50% of the data values, between the lower and upper percentiles. The whiskers extend out from the 10th and 90th percentiles. The central line is at the median. When unusual values occur for away from the bulk of the data, they are plotted as separate points.

Having a sensitive analytical method available means that a maximum limit (threshold) for lactulose can be defined, not only to differentiate between UHT and sterilized milks, but also between and within different types of UHT and pasteurized milks.

Lactulose concentrations may be used in combination with other indices (furosine, β-lactoglobulin, soluble whey protein, BSA, α-lactoalbumin, HMF, peroxidase and alkaline phosphatase) to differentiate, with greater certainty, between various types of pasteurized and sterilized milks and to assess the heat damage in reconstituted powdered milk, and in sterilized creams [Corzo, Delgado, Troyano, & Olano, 1994; De Rafael et al., 1996; EU Commission, 1992; Glaeser, 1996; IDF, 1992; Morales et al., 2000; Pellegrino et al., 1994 (Chapter. 20), 1995; Van Renterghem & De Block, 1996]. The method proposed in this study is simple, rapid, sensitive and meets the needs of both the quality control and milk processing laboratories, thus contributing to product quality assurance. Moreover, this method can be

readily adapted to an enzymatic test kit, making it suitable for running a large number of analyses.

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