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# Development of an improved extraction and HPLC method for the measurement of ascorbic acid in cows' milk from processing plants and retail outlets

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## Abstract

An improved extraction (2.5% $HPO_3$, 5 mM dithiothreitol) and HPLC quantification using a C-18 column at 35ºC and 0.1M acetic acid (98%) and acetonitrile (2%) mobile phase was developed to quantify total ascorbic acid (AA) in commercial whole/semi-skim/skim raw/pasteurized/UHT milk packaged in opaque bags, transparent plastic, cardboard, and Tetra Brik™. AA content ranged 0.21-10 and 3.4-16 mg/L in milk from retail outlets and processing plants, respectively, and was higher in organic milk. For same processor/lot samples, pasteurized milk showed higher AA content than UHT milk. This was not true for retail outlets samples. AA content was similar for whole/semi-skim and semi-skim/skim milk but not for whole/skim comparisons. Among UHT samples, the AA content trend was whole<semi-skim<skim and lower for UHT milk in opaque plastic and Tetra Brik™ container. After 14d at 4ºC in the dark, AA losses ranged 35-83% depending on milk type and preservation method with a higher AA retention in unopened containers.

**Keywords:** Cow milk; vitamin analysis; ascorbic acid; HPLC-DAD

**Running Title:** Vitamin C in retail/processing plant cow milk

## Introduction

Vitamins are essential in physiological processes including vitamin C which cannot be synthesized by humans and thus must be present in the diet. Some foods are supplemented with vitamins such as specialized dairy products for infants, children, and teenagers. Significant vitamin losses occur during food processing and storage reflecting food processing temperature and time, pH, redox potential, packaging material, and storage conditions such as light, temperature, oxygen, and others (Oamen et al. 1989, Ochiai and Nakagawa 1992, Albala-Hurtado et al. 2000). Vitamin C, a water-soluble vitamin present naturally in foods mostly as ascorbic acid (AA), is an excellent antioxidant with multiple metabolic functions in both plant and animal cells (Lindmark-Mansson and Akesson 2000). AA is used as a technological marker of the severity of processing and storage effects on food quality (Pizzoferrato 1992, Romeu Nadal et al. 2008a, Romeu Nadal et al. 2008b). AA is also important when assessing novel alternatives to stabilize foods including pressure processing. AA losses during pressure-assisted thermal processing have been reported (Ramirez et al. 2009).

The main AA degradation pathway is oxidation to dehydroascorbic acid (DHAA) yielding diketo-L-gulonic acid (DKGA) (Murchie et al. 2005). This reaction is the basis for the use of AA as an antioxidant. Oxidation of AA to DHAA can be reversed by reducing agents which are used in analytical procedures to quantify the total amount of AA. The first reducing agent investigated for this purpose was dithiothreitol (DTT) used at room temperature at a concentration of 10 mM (Scott and Bishop 1986). DTT has been effectively used in determinations of total AA in several foods including milk (Weiss 2001) but its efficiency is pH-dependent (Ryley and Kajda 1994). Takayanagi *et al*. (2009) showed that 5 mM DTT was sufficient to protect AA against degradation during sample handling. Although other reductants such as mercaptoethanol, homocysteine, and cysteine can prevent AA oxidation during analytical determinations, DTT is the most common alternative (Ryley and Kajda 1994, Mesías-García et al. 2010).

The AA content in cow milk is low varying with the season and the feed used. Walstra *et al.* (2001) reported that the total AA content in raw cow milk ranged between 1.7 and 2.8 mg/100 g, while Bilic (1991) found values for AA and DHAA of 0.59 mg/100 g and 0.15 mg/100 g, respectively. In pasteurized cow milk, Marconi and Panfili (1998) reported a value of 0.70 mg/100 g while Scott *et al.* (1984) found AA values of 1.45 mg/100 mL. Pizzoferrato (1992)

reported amounts between 0.25-0.51 mg/100 mL in UHT cow´s milk. The main problem associated with AA determinations in milk is its low concentration. Avoiding potential interferences from other food components is also a concern. High-performance liquid chromatography (HPLC) is frequently used to quantify AA in foods including milk (Okamura 1980, Gliguem and Birlouez Aragon 2005, Eitenmiller et al. 2008). The aims of this study were to develop and apply an improved sample extraction and HPLC quantification method to determine the total AA in commercial cow milk samples; to evaluate the influence of various conditions (heat treatment, packaging material, production system, and milk brand) on their total AA content; and, to quantify losses of total AA in milk samples stored at 4ºC for up to 14 days in the dark.

## Materials and Methods

### Chemicals and reagents

Reagents were all of analytical grade including the AA standard (Grupo Productos Aditivos, Madrid, Spain), DL-dithiothreitol (DTT) and phosphotungstic acid hydrate (Fluka Analytical, Madrid, Spain), zinc acetate (Merck, Darmstadt, Germany), glacial acetic acid and *meta*-phosphoric acid (Panreac, Barcelona, Spain), and HPLC grade water (Sigma-Aldrich, Madrid, Spain). The AA standard stock solution, and dilutions in 2.5% *meta*-phosphoric acid ($HPO_3$) to yield a pH value similar to the solution used to extract AA from milk samples, were prepared quickly and stored immediately in amber bottles. DTT (5mM) was added to minimize AA oxidation. Seven AA standard solution concentrations in the 0.25-10 mg/L range were used to build an HPLC calibration curve. AA standards were prepared daily and kept at 4 °C until used. Standard solutions were injected only once to minimize possible effects of AA degradation.

### Chromatographic quantification method

The HPLC system (Thermo Fisher Scientific Inc., Waltham, MA) used consisted of a Spectra system SCM 1000 vacuum degasser, a Spectra system P4000 pump, a Spectra system AS3000 autosampler, and a Surveyor $PDA^+$ detector connected to a computer running Chrom Quest 5.0. Different columns and mobile phases with a 0.5 mL/min flow rate were tested during the

quantification method optimization (Table 1). Milk sample AA peak identification for the optimized method was carried out by comparison of retention time, wavelength and spectrum with those obtained for AA standards. AA quantification in milk was performed in triplicate samples.

**Milk samples**

Organic/conventional, whole/semi-skim/skim, raw/pasteurized/UHT milk in various packaging types and from seven commercial trademarks was investigated (Fig. 1). Raw whole milk was obtained from two processing plants (brands A and D), while pasteurized and UHT milk were obtained from the same two processing plants, and also from local retail outlets (brands B, C, E, F, and G). Raw milk samples were collected in 100 mL plastic containers filled with no headspace and kept covered with aluminum foil until analyzed. Pasteurized whole, semi-skim and skim milk were obtained in three packaging types (opaque plastic bags, transparent plastic bottles, cardboard boxes). UHT whole, semi-skim, and skim milk were obtained in opaque plastic bottles and Tetra Brik™ containers. Transparent packages were covered with aluminum foil before analysis completed usually within a few hours after sample collection. When this was not possible, samples were kept at -20 ºC for a few days and thawed at 4 ºC before analysis. Composition of milk samples is shown in Table 2.

**Sample extraction**

*Method 1*. This method was based on the work reported by Zafra-Gómez *et al.*(2006) modified to improve AA extraction. Milk samples (4 mL) were mixed with 1 mL of an extracting solution prepared by dissolving 9.1 g zinc acetate, 5.5 g phosphotungstic polyhydrated acid in 5.8 mL glacial acetic acid, and adding then HPLC grade water to reach a 100 mL final volume. The milk and extraction solution mix was vortexed for 10 s and centrifuged at 4000 rpm and 10 °C for 10 min. The clear layer was filtered through 0.45 µm Chromafil® Xtra into an amber vial and injected directly into the HPLC system. The analysis was completed in less than 1 h from extraction to chromatographic separation.

*Method 2*. Sample preparation was carried out as in Method 1 but using 2.5% *meta*-phosphoric acid ($HPO_3$) as extraction solution to improve AA extraction from milk as suggested by Romeu-Nadal et al. (2006).

**AA Degradation during storage at 4 °C**

The degradation of AA during storage was analyzed assuming first order kinetics (İpek et al. 2005, van Loey et al. 2005):

$$\ln \frac{A_t}{A_o} = -kt \quad (1)$$

where $A_o$ and $A_t$ are the AA concentration (*mg/L*) at time 0 and *t* (*day*), respectively, and *k* is the reaction rate constant ($day^{-1}$). The time period considered in this study was based on the assumption that milk consumers open a container, store it under refrigeration, and then open it once a day for one week. The study was extended to 14 d which was the maximum shelf-life expected.

*Study 1: Consumer milk handling effect*. AA degradation was studied in raw whole, pasteurized semi-skim and skim and UHT whole milk for brand A, and in raw and pasteurized whole milk for brand D. Samples from UHT containers opened at time 0 were taken after 0, 0.25, 0.5, 1, 2, 5, 7, and 14 d at 4ºC. Thus, milk was subjected to microbial and atmospheric oxygen conditions representing consumer use. Before analysis, samples were protected from light and kept at 4$^{o}$C.

*Study 2: Effect of sodium azide addition on the consumer milk handling effect*. AA degradation was studied in UHT whole milk (brand A) by sampling from the same open container, without and with 0.02% sodium azide added to control microbial growth, and following the above periodicity.

*Study 3: Milk storage effect.* AA degradation was studied in UHT whole milk (brand A) by opening new containers at each sampling time following the above periodicity to determine storage effects in closed containers stored at 4$^{o}$C.

**Statistical analysis**

Statistical analysis was performed with SPSS (Windows Version 18.0). Data was tested for normality by the Kolmogorov-Smirnov (K-S) test. One and multi-way analysis of variance (ANOVA) were used to test for differences between mean AA content ($\alpha = 0.05$) in raw, pasteurized and UHT milk, using fat content, packaging, and brand as factors. A paired two-sample test was used to compare conventional and organic milk, pasteurized and UHT milk, and type of container for UHT milk from the same brand.

## Results and Discussion

### Extraction procedure

The extracting solution used in Method 1 was immiscible with acetonitrile and DTT causing several problems including column clogging. The Method 2 extracting solution (based on *meta*-phosphoric acid) was miscible with acetonitrile and DTT, and was used in this work as the preparation procedure was quicker and easier. *Meta*-phosphoric acid as extracting solution for AA analysis has been reported in previous works, with indications that it precipitates proteins and stabilizes AA (Kim et al. 1987, Eitenmiller et al. 2008).

### Column and mobile phase selection

Several mobile phases and three columns were tested to establish optimum analysis conditions yielding the retention times and absorption wavelengths reported in Table 1. Using an amino column (150 x 2.1 mm, 3 µ particle size, Hypersil® APS1, Thermo Electron Corporation, UK) with mobile phases containing high acetonitrile content (mobile phases 1-3) caused precipitation of the extracted milk sample in the column and could not be used. Using the same column with mobile phases 4 and 5 resulted in retention times with high standard deviation values, i.e., 2.9±0.6 and 4.9±0.3, respectively, and variable maximum absorption wavelengths (Table 1). Tests with the $C_{18}$ Kinetex column (100 x 4.6 mm, 2.6 µ particle size, Phenomenex®100A, Phenomenex, Torrance, CA) with mobile phases 6 and 7 showed an unacceptably low AA retention time (about 0.8 min). Tests with the $C_{18}$ Luna column (150 x 4.6 mm, 5µ particle size, Phenomenex® 100A) using mobile phase 6 showed the same co-elution problems as the $C_{18}$ Kinetex column while a 5 min AA retention time and good separation were observed when using mobile phase 5. However, 2.0% acetonitrile was necessary to retain column efficiency, resulting

in mobile phase 8, which showed good peak separation and slightly shorter retention times. In conclusion, mobile phase 8, column Luna $C_{18}$, and a guard column containing the same packing material were selected. A 35 ºC column temperature and 0.50 mL/min isocratic elution rate resulted in a 20 min quantification time. The injection volume was 20 µL and the detection was at 245 nm. Fig. 2 shows an analysis for AA in 2.5% *meta*-phosphoric acid and 5 mM DTT using these conditions.

**Ascorbic acid quantification**

After optimization of the extraction, column, mobile phase, and elution conditions, the method was evaluated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), reproducibility, and precision. HPLC run time was set at 20 min ensuring that the column was ready for the next sample. The calibration curve showed $r^2$ values of 0.999. LOD and LOQ values were defined as the concentration in the milk sample extract ($n$ = 3) with signal to noise ratio of 3 and 10, respectively (Anonymous 1980, Vial and Jardy 1999). The LOD (0.050 mg/L) was lower than values reported for fortified foods (around 1 mg/kg, Fontannaz et al. 2006), baby foods (30 mg/kg, Su et al. 1995) and milk (1 mg/kg, Mannino and Pagliarini 1988) but higher than values (0.010 mg/L) reported by Rodríguez-Bernaldo de Quirós *et al.* (2009) for fruit juices and soft drinks. In addition, the LOQ (0.10 mg/L) was lower than all the reported values in the works cited for LOD comparison. Method reproducibility was determined by analyzing six replicates ($n$=6) of three standards (0.5, 1, and 5 mg/L) showing about 5.0% relative standard deviation (RSD). Method precision (2.0%) was estimated as the RSD value for multiple extractions of the same milk sample (UHT whole milk, $n$=6). Finally, the results showed high recovery levels (tested by spiking the samples with 10 mg/L of AA, Table 3) ranging 77-83%, 62-82%, and 53-88% for whole, semi-skim, and skim milk, respectively with RSD values lower than 5.0%. When DTT was used in the analysis, the corresponding values increased up to 90%, 95% and 96% with RSD values lower than 3.0%. These results indicate that the method was suitable for AA analysis in milk.

**Commercial milk samples**

Milk fat content ranged 3.5-3.9%, 1.5-1.6%, and 0.23-0.30% for whole, semi-skim milk and skim milk (Table 2). AA concentration ranged 0.21-10 mg/L in brand B, C, E, F, and G samples

collected from retailers, and 3.4-16 mg/L in brand A and D samples collected from the processing plant (Table 4). Brand D (organic milk) showed the highest values, while brands B, C (except for Tetra Brik™ container milk), and F showed the lowest, with the remaining brands (A, E, and G) presenting intermediate values.

*Milk collected from processing plants.* Three sample lots (I, II, and III) of brand A and one of brand D were analyzed (Table 4). AA content in brand A raw milk ranged from 4.9 ± 0.14 (Lot II) to 9.8 ± 0.41 mg/L (Lot I), and was 16 ± 0.17 mg/L for brand D organic milk. UHT processing of brand A milk reduced AA content by about 20 to 40%. Pasteurization of brand A and D milk caused lower losses ranging 10-20%. Multi-way variance analyses showed statistically significant differences between lots ($p<0.05$). Multi-range ANOVA tests showed AA content similarities between lots I and III for raw and pasteurized milk but not for UHT milk from all three lots (Table 4). Different feeding, sampling date, and thermal treatments are possible causes for this observation. Processing temperature and time are the most important factors affecting AA degradation (Steskova et al. 2006). UHT milk can be processed by direct (~2 s at 150 ºC) and indirect heating (15 s at 142 ºC) (Walstra et al. 2001) while pasteurization requires at least 15 s at 72 ºC, although actual time and temperature used by the processor may be higher than these values (Salgado et al. 2011). Pasteurization conditions of brand A and D were 15-20 s at 66-68 ºC and 14 s at 75 ºC, respectively, while the thermal treatment for the brand A UHT milk was 2 s at 148 ºC. When comparing samples from the same brand A lot, pasteurized milk showed higher AA content than UHT milk for all lots, an observation consistent with previous reports (Anonymous 1980, Ryley and Kajda 1994) and reflecting thermal process severity differences (Okamura 1980, Haddad and Loewenstein 1983). For the same brand A milk processing lot, significant differences ($p<0.05$) were observed between pasteurized and UHT whole and semi-skim milk (Lots III and I, respectively), but not for skim milk (Lot II). Therefore, thermal treatment severity was not the only factor influencing the AA content.

*Milk collected from retail outlets.* Thermal pasteurization conditions for brand B milk were 12 s at 78 ºC but this information was unavailable for brand C. Both brands showed similar low AA content (under 0.36 mg/L and 0.42 mg/L, respectively) with statistical differences only between whole and skim milk from the same brand ($p<0.05$). The UHT thermal treatment for brands E

and G was similar, i.e., 3 and 2.4 s at 148 ºC, respectively, while the brand C conditions were more severe than those used typically (9 s at 150 ºC). Processing conditions for brand F were 2.8 s at 142 ºC**.** One and multi-way variance analyses ($p<0.05$) were conducted to assess milk fat content effects. With regards to pasteurized milk, statistical differences in AA content were found for the fat content for brands B and C, using multi-way analysis. Multi-range tests showed similarities between AA content for whole/semi-skim and semi-skim/skim milk samples but not for whole/skim comparisons (Table 4). For the 5 UHT milk samples, statistical differences in AA content were found also for fat content showing a whole < semi-skim < skim trend.

The packaging effect was studied for UHT milk comparing opaque plastic bottles and Tetra Brik™ containers. Statistical differences ($p<0.05$) were found for UHT milk as affected by packaging type with lower AA content observed in opaque plastic as compared to Tetra Brik™ containers, except for brand B whole milk which showed very low AA content. AA levels ranged from 0.21-10 mg/L and 0.22-3.4 mg/L in Tetra Brik™ and opaque plastic containers, respectively (Table 4). These results are similar to previous reports on the effect of packaging on the AA stability in milk. For example, Gliguem and Birlouez Aragon (2005), reported that after 1 month at room temperature AA degradation in UHT milk reached 99% and 51% in 3-layered and in 6-layered packaging, respectively. AA degradation differences may reflect oxygen permeation differences and a higher milk exposure to light in opaque plastic as compared to Tetra Brik™ containers (Gliguem & Birlouez Aragon, 2005). Although **s**tatistical differences in AA content were found, the retail outlet samples storage time and conditions were factors that could not be controlled, and the AA content for the raw milk corresponding to the pasteurized and UHT milk samples collected was unavailable. However, considering that AA indicates the process and storage severity, these large differences in AA content do suggest quality differences in the commercial retail outlet milk.

*Effect of milk type (conventional/organic).* Organic milk brand D showed the highest AA content (Table 4). Lund (1991) reported also that organic milk showed higher AA concentration than conventional milk and reported AA values of about 15 mg/L. Since organic cows graze on fresh grass and clover, the milk they produced has been reported to have a higher vitamin content (Kalac 2011).

*Consumer storage effects.* AA concentration in milk stored at 4°C decreased gradually as reported by Oamen et al. (1989) following first order kinetics for up to 6-7 days of storage (Fig. 3). Çakmakçi & Turgut (2005) reported that AA in pasteurized milk decreased significantly for the first 24 h of 4ºC storage, then gradually between1-3 d, changing much less afterwards (3-5 d). After 14 d, conventional (brand A, Lots I, II, and III) and organic (brand D) whole milk showed a 35-66% and 83% AA content decrease, respectively, with respect to raw milk. The AA degradation rate constant ($k$, $day^{-1}$) was significantly higher ($p<0.05$) in brand D (0.347) than in A with non-significant differences among lots (0.153, 0.166, and 0.154 for lots I, II and III, respectively, Fig. 3a). At the same temperature (4ºC), the $k$ value for AA loss in orange juice was lower than in milk (Kennedy et al., 1992).

The thermal process effect (brand A, Lot III) on AA losses (Fig. 3b) showed a significantly higher reaction rate constant ($p<0.05$) for pasteurized milk (0.298 $day^{-1}$) than for raw (0.154 $day^{-1}$) and UHT (0.057 $day^{-1}$) milk. In the case of the fat content level effect on AA losses analyzed for pasteurized milk (Fig. 3c), the reaction rate constant changed from 0.397, 0.345 and 0.298 $day^{-1}$ for brand A semi-skim, skim and whole milk, respectively, and were statistically different ($p<0.05$). The AA degradation rate constant ($k$, $day^{-1}$) for pasteurized whole milk was significantly higher for organic brand D milk (0.0353 $day^{-1}$) than in brand A, Lot I (0.298). However, since after 6-7 days of faster AA degradation following first order kinetics, the AA loss rate decreased or approached zero, AA losses after 14 d was about 81%, 62%, and 52% for UHT, pasteurized, and raw milk, respectively. Gliguem & Birlouez Aragon (2005) reported a 51% AA decrease in fortified UHT milk kept one month at room temperature, and up to 75% decrease after four months. Other authors have reported AA losses during milk storage also (Kennedy et al. 1992, Rodríguez-Comesaña et al. 2002, Castro et al. 2004, Burdurlu et al. 2006). A rapid decrease of about 90% in AA levels was reported during the first 48 h for pasteurized milk in plastic or glass containers (Öste et al. 1997), whereas in this work a 48% reduction was observed within the first 48 h in pasteurized whole milk (brand A, Lot III) in transparent glass containers covered with aluminum foil. Steskova *et al.*, (2006) reported no significant of AA losses during 7 days after pasteurization when milk packages were opened, but another work reported that AA was converted to other forms (Schroeder et al. 1985).

*Storage studies*. AA degradation rate constants were 0.006, 0.022 and 0.057 $day^{-1}$, respectively with statistical differences ($p<0.05$), for new container, same container, and same container with sodium azide added after opening it (Fig. 4). After 14 d, AA loss was highest (84%) in milk with 0.02% sodium azide, whereas the lowest reduction (18%) was observed when a new container was opened at each sampling time. When sampling always from the same container, an AA loss of 50% of was observed. These results indicate that adding sodium azide increased AA losses, whereas milk stored in unopened bottles retained more AA. Further research is required to fully interpret the sodium azide effect but it may reflect an interaction with ascorbic acid.

## Conclusions

An extraction and HPLC methodology was developed and optimized to analyze AA content in commercial milk samples obtained from processing plants and retail outlets. AA concentration in samples of raw, pasteurized, and UHT conventional and organic milk with different fat content of several brands, and commercialized in various container types ranged widely, i.e., from 0.21 to 16 mg/L. AA content variation could be attributed to processing conditions, feed composition, and container type. AA levels varied for pasteurized and UHT milk with different fat levels, with a whole<semi-skim<skim AA concentration observed as a general trend. Organic milk showed the highest AA level. In spite of the unknown factors for milk collected from retail outlets, it was concluded that the Tetra Brik™ container is a packaging choice superior to opaque plastic bottles. After 14 days of storage at 4ºC in the dark, the decrease of AA ranged from 83% to 35% depending on milk type and preservation method used. A higher retention of AA was observed when the milk container was kept unopened.

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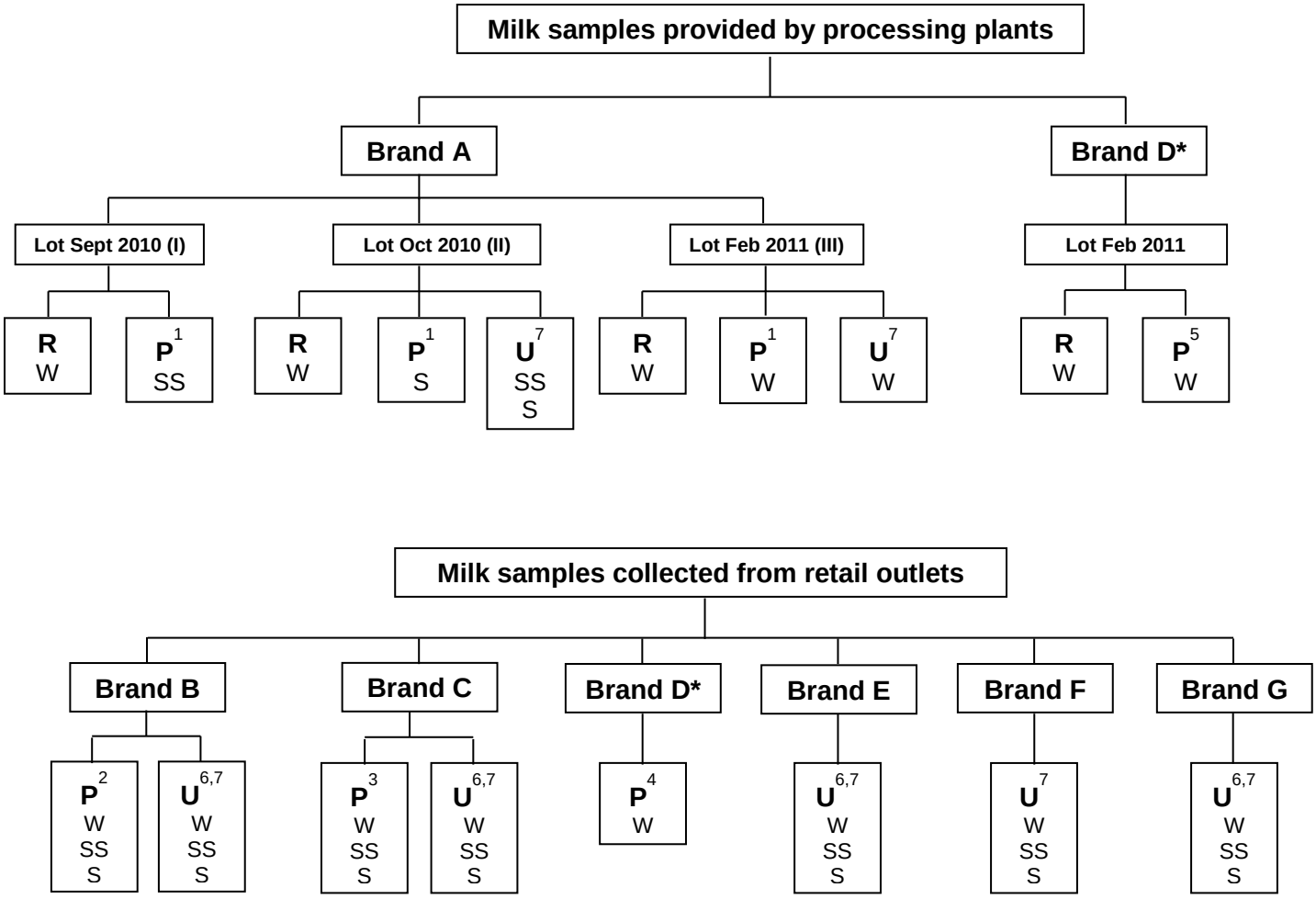


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447 **Figure 1**. Milk samples collected from processing plants and retail outlets indicating brand (A to
448 G), fat content (W: whole, SS: semi-skim, S: skim), container type (1: transparent plastic bottle
449 covered with aluminum foil before analysis, 2: transparent plastic bottle, 3: cardboard boxes, 4:
450 opaque plastic bag, 5: transparent glass bottle covered with aluminum foil before analysis, 6:
451 opaque plastic bottle, 7: Tetra Brik$^{TM}$ container), type of milk (*: organic), and thermal
452 treatment (R: raw, P: pasteurized, U: UHT). Roman numerals in milk samples provided by
453 processing plants indicate samples from the same milk lot

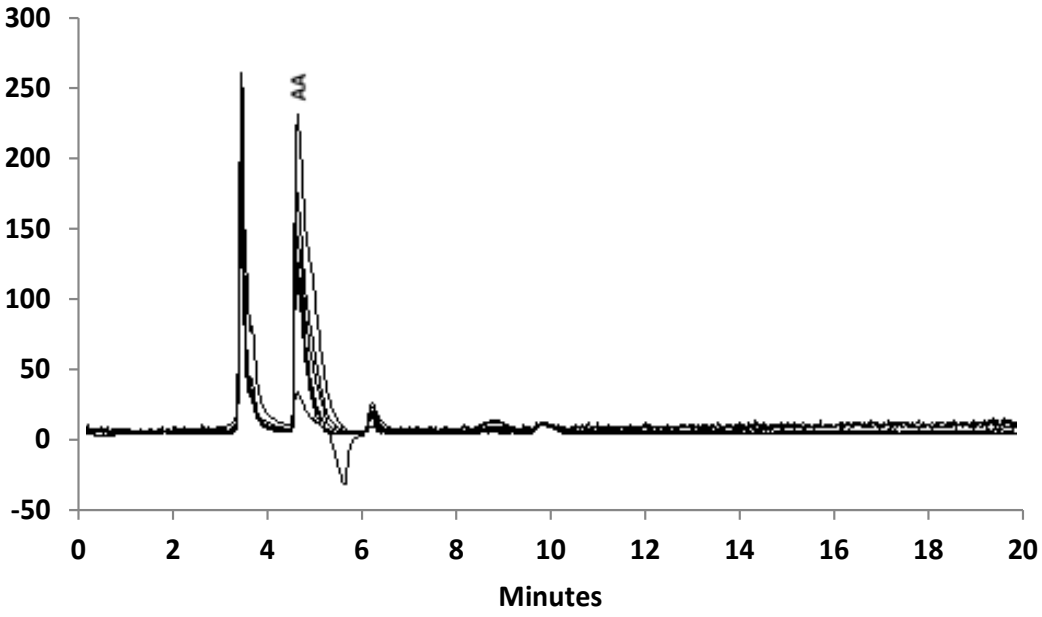


**Figure 2.** Chromatograms for 0.25 - 10 mg/L ascorbic acid (AA) standards in 2.5% *meta*-phosphoric acid ($HPO_3$) and 5 mM dithiothreitol (DTT). HPLC quantification using a $C_{18}$ Luna column (150 x 4.6 mm, 5µ particle size, Phenomenex® 100A) at 35ºC and a 0.1M acetic acid (98%) and acetonitrile (2%) mobile phase.

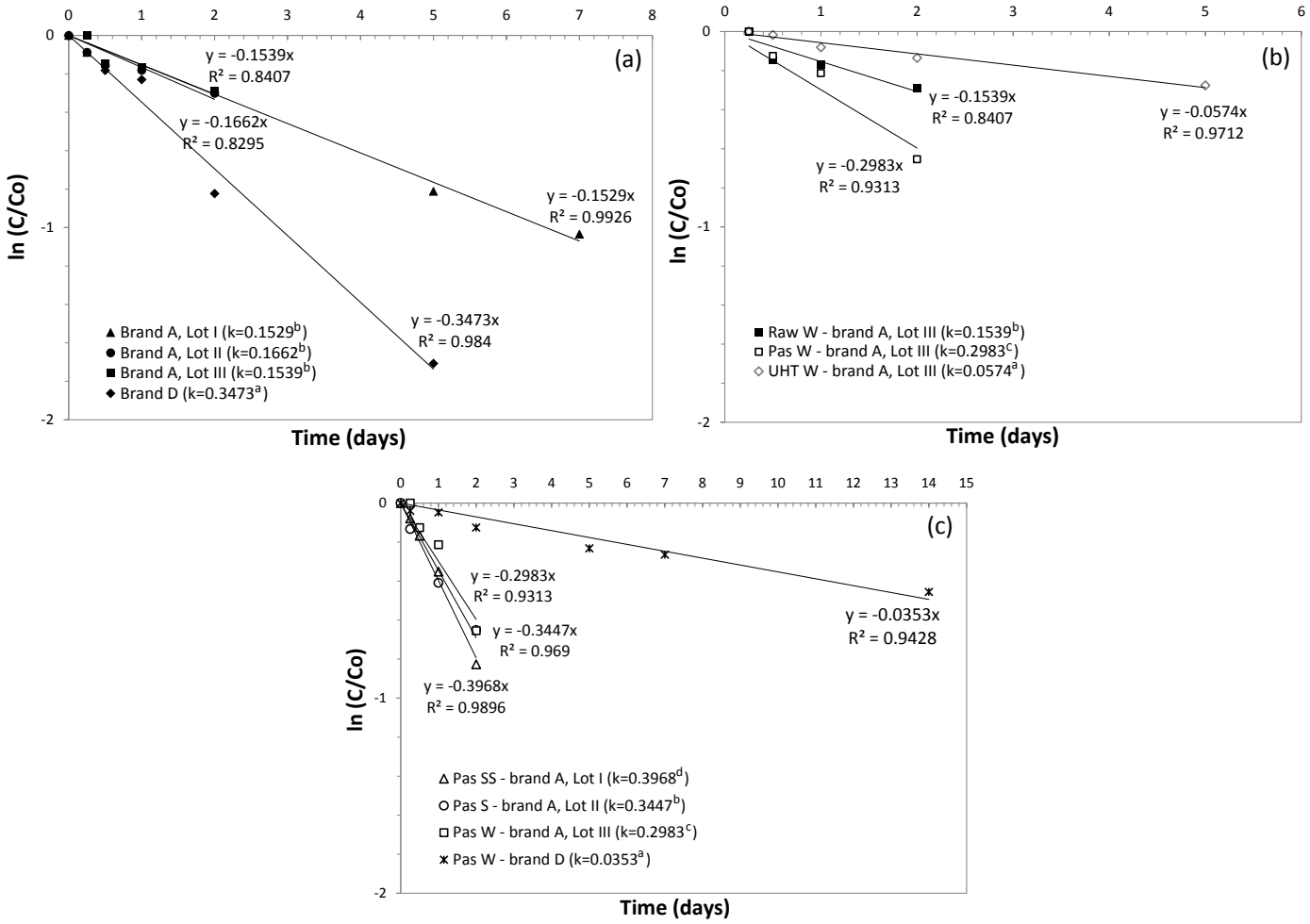


**Figure 3.** Ascorbic acid degradation kinetics in milk packaged in transparent glass bottles covered with aluminum foil before analysis: raw whole milk (a); effect of thermal process on milk brand A, Lot III (b); effect of fat content level on pasteurized whole, semi-skim and skim milk (c). Different low case subscript letters on the first order kinetic constant values (*k*) shown in each graph for the milk studied indicate statistically significant differences ($p<0.05$).

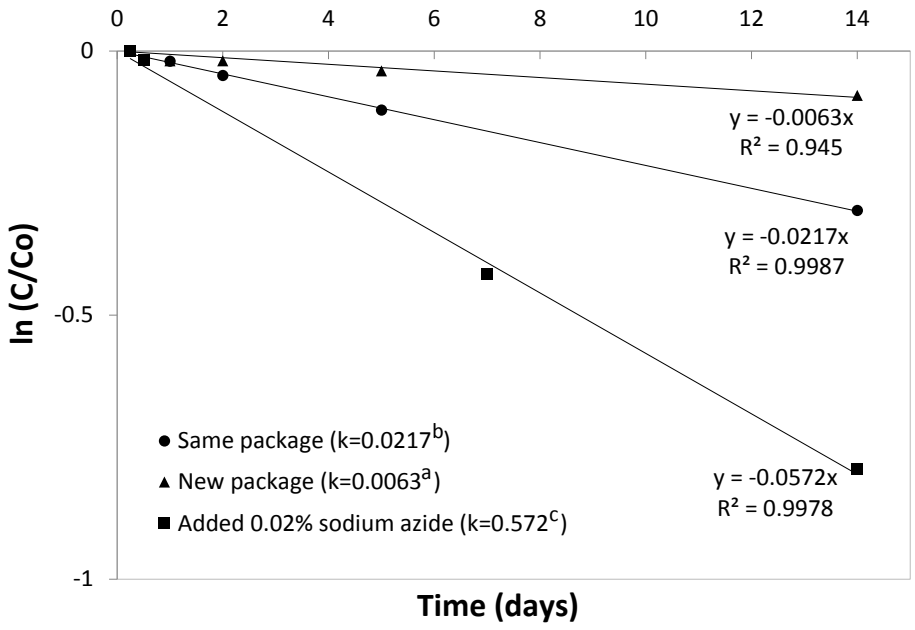


**Figure 4.** Ascorbic acid degradation in UHT whole milk in Tetra Brik$^{TM}$ container: (●) sample from the same container, (▲) sample from new containers, (■) sample from same container after adding 0.02% sodium azide ($NaN_3$). Different low case subscript letters on the first order kinetic constant values (k) indicate statistically significant differences ($p<0.05$).

**Table 1.** Milk ascorbic acid analysis conditions

| Mobile phase and column | | Retention time (min) mean± SD, n = 2 | Absorption (nm) |
|---|---|---|---|
| **No.** | ***Amino column (150 x 2.1 mm)*** | | |
| 1 | 75% ACN + 25% 0.05M $KH_2PO_4$ | 6.6 ± 1.9 | 253, 267 |
| 2 | 50% ACN + 50% 0.05M $KH_2PO_4$ | 2.7 ± 0.5 | 240, 244 |
| 3 | 19% ACN + 81% 0.005M $H_2SO_4$ | 3.2 ± 1.2 | 243 |
| 4 | 95% 0.08M $CH_3COONH_4$ in ACN + 5% ACN | 2.9 ± 0.6 | 250, 256 |
| 5 | 0.1M Acetic acid | 4.9 ± 0.3 | 236, 245 |
| | ***Kinetex C18 column (100 x 4.6 mm)*** | | |
| 6 | 95% 0.01M $CH_3COONH_4$ in ACN + 5% ACN | 0.8 ± 0.00 | 258, 259 |
| 7 | 2% Acetic acid | n.d.(1) | n.d. |
| | ***Luna C18 column (150 x 4.6 mm)*** | | |
| 6 | 95% 0.01M $CH_3COONH_4$ in ACN + 5% ACN | 1.8 ± 0.01 | 265 |
| 5 | 0.1M Acetic acid | 5.1 ±0.01 | 245 |
| 8 | 98% 0.1M Acetic acid + 2% ACN | 4.9 ± 0.00 | 245 |

(1) n.d. = not determined

**Table 2.** Composition of commercial milk samples

| Treatment | Brand | Type | Energy value (kcal) | Protein (g/100mL) | Carbohydrate (g/100mL) | Fat content (g/100mL) |
|---|---|---|---|---|---|---|
| Raw[8] | A | Whole I | n.a | 3.2 | 4.7 | 3.8 |
| | A | Whole II | n.a. | 3.2 | 4.7 | 3.9 |
| | A | Whole III | n.a. | 3.2 | 4.7 | 3.6 |
| | D* | Whole | n.a. | n.a. | n.a. | n.a. |
| Pasteurized | A[1] | Whole | n.a. | 3.2 | 4.7 | 3.5 |
| | A[1] | Semi-skim | n.a. | 3.3 | 4.8 | 1.5 |
| | A[1] | Skim | n.a. | 3.3 | 4.8 | 0.24 |
| | B[2] | Whole | 51 | 3.1 | 4.6 | 3.5 |
| | B[2] | Semi-skim | 45 | 3.1 | 4.6 | 1.5 |
| | B[2] | Skim | 34 | 3.1 | 4.6 | 0.30 |
| | C[3] | Whole | 62 | 3.0 | 4.5 | 3.6 |
| | C[3] | Semi-skim | 43 | 3.0 | 4.6 | 1.5 |
| | C[3] | Skim | 33 | 3.1 | 4.7 | 0.30 |
| | D*[, 5] | Whole | n.a. | n.a. | n.a. | n.a. |
| UHT | A[7] | Whole | n.a. | 3.2 | 4.7 | 3.5 |
| | A[7] | Semi-skim | n.a. | 3.3 | 4.7 | 1.5 |
| | A[7] | Skim | n.a. | 3.3 | 4.9 | 0.23 |
| | B[6] | Whole | 64 | 3.1 | 4.7 | 3.6 |
| | B[6] | Semi-skim | 46 | 3.2 | 4.7 | 1.6 |
| | B[6] | Skim | 34 | 3.2 | 4.7 | 0.30 |
| | C[7] | Whole | 62 | 3.0 | 4.5 | 3.6 |
| | C[7] | Semi-skim | 43 | 3.0 | 4.6 | 1.5 |
| | C[7] | Skim | 33 | 3.1 | 4.7 | 0.30 |
| | E[7] | Whole | 63 | 3.1 | 4.6 | 3.6 |
| | E[7] | Semi-skim | 45 | 3.2 | 4.7 | 1.6 |
| | E[7] | Skim | 34 | 3.2 | 4.7 | 0.25 |
| | F[7] | Whole | 61 | 3.0 | 4.6 | 3.6 |
| | F[7] | Semi-skim | 45 | 3.1 | 4.6 | 1.6 |
| | F[7] | Skim | 32 | 3.2 | 4.6 | 0.30 |
| | G[7] | Whole | 64 | 3.0 | 4.8 | 3.6 |
| | G[7] | Semi-skim | 46 | 3.0 | 4.8 | 1.6 |
| | G[7] | Skim | 35 | 3.0 | 4.8 | 0.30 |

Raw milk samples were collected in 100 mL plastic containers filled with no headspace and covered with aluminum foil before analysis while the container for other milk samples were as follows: (1) Transparent plastic bottle covered with aluminum foil before analysis; (2) Transparent plastic bottle; (3) Cardboard box; (4) Opaque plastic bag; (5) Transparent glass bottle covered with aluminum foil before analysis; (6) Opaque plastic bottle; (7) Tetra Brik™ container.
* = organic milk and n.a. = not available

**Table 3.** HPLC ascorbic acid (AA) determination and recovery from commercial milk samples with and without dithiothreitol (DTT) in the extracting solution (n = 3)

| | **Concentration (mg/L) ± RSD(*)** | | **Recovery (%)** | |
|---|---|---|---|---|
| **Sample** | **w/o DTT** | **w/ DTT** | **w/o DTT** | **w/ DTT** |
| ***Transparent plastic bottle, Brand B*** | | | | |
| Pasteurized whole milk | 0.26±0.014 | 0.37±0.020 | 83±0.80 | 90±1.70 |
| + 10 mg/L AA | 8.6±0.08 | 9.3±0.08 | | |
| Pasteurized semi-skim milk | 0.36±0.024 | 0.44±0.002 | 62±1.7 | 67±0.8 |
| + 10 mg/L AA | 6.6±0.17 | 7.2±0.08 | | |
| Pasteurized skim milk | 0.36±0.031 | 0.40±0.071 | 88±1.4 | 87±1.7 |
| + 10 mg/L AA | 9.1±0.12 | 9.1±0.15 | | |
| ***Cardboard box , Brand C*** | | | | |
| Pasteurized whole milk | 0.35±0.080 | 0.47±0.010 | 77±1.5 | 90±2.3 |
| + 10 mg/L AA | 8.1±0.15 | 9.5±0.23 | | |
| Pasteurized semi-skim milk | 0.33±0.040 | 0.46±0.030 | 75±0.30 | 95±0.60 |
| + 10 mg/L AA | 7.8±0.03 | 10±0.06 | | |
| Pasteurized skim milk | 0.42±0.050 | 0.47±0.043 | 84±0.9 | 96±0.9 |
| + 10 mg/L AA | 8.8±0.092 | 10±0.093 | | |
| ***Tetra Brik™ container, Brand E*** | | | | |
| UHT whole milk | 6.7±0.020 | 7.5±0.23 | 81±2.1 | 78±0.9 |
| + 10 mg/L AA | 15±0.21 | 15±0.090 | | |
| UHT semi-skim milk | 5.4±0.35 | 6.4±0.18 | 82±3.2 | 75±0.5 |
| + 10 mg/L AA | 13±4.85 | 14±4.60 | | |
| UHT skim milk | 8.4±0.32 | 8.5±0.17 | 55±2.5 | 70±0.9 |
| + 10 mg/L AA | 14±4.85 | 16±4.85 | | |

(*) RSD = relative standard deviation

**Table 4. Ascorbic acid content (n = 3, average ± standard deviation, mg/L) in raw, pasteurized and UHT milk samples†**

| Treatment | Container††† | Fat content | Processing plant samples | | Retail outlet samples | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Brand A†††† | Brand D* | Brand B | Brand C | Brand E | Brand F | Brand G |
| Raw†† | | Whole | 9.8 ±0.41[a] (I) | 16 ±0.17[a] | n.a. | n.a. | n.a. | n.a. | n.a. |
| | | | 4.9 ±0.14[b] (II) | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| | | | 9.6 ±0.23[a] (III) | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| Pasteurized | 1-5 | Whole | 9.1 ±0.15[1,a] (III) | 12 ±0.23[4, b]<br>14 ±0.33[5, b] | 0.26±0.010[2,A] | 0.35±0.08[3,A] | n.a. | n.a. | n.a. |
| | | Semi | 10 ±0.23[1,a] (I) | n.a. | 0.36±0.031[2,AB] | 0.33±0.04[3,AB] | n.a. | n.a. | n.a. |
| | | Skim | 3.4 ±0.070[1,c] (II) | n.a. | 0.36±0.030[2,B] | 0.42±0.05[3,B] | n.a. | n.a. | n.a. |
| UHT | 7 | Whole | 7.9 ±0.10[d] (III) | n.a. | 0.21±0.010[A] | 3.3 ±0.050[D] | 6.7 ±0.020[A] | 0.96±0.11[A] | 5.1 ±0.14[A] |
| | | Semi | 6.2 ±0.38[e] (I) | n.a. | 0.35±0.010[B] | 8.9 ±0.34[E] | 5.4 ±0.35[A] | 0.87±0.10[A] | 7.1 ±0.17[B] |
| | | Skim | 4.0 ±0.22[c] (II) | n.a. | 0.35±0.0010[B] | 7.4 ±0.19[E] | 8.4 ±0.32[B] | 0.41±0.11[B] | 10 ±0.17[C] |
| | 6 | Whole | n.a. | n.a. | 0.22±0.030[A] | 0.41±0.00[B] | 2.9 ±0.0040[C] | n.a. | 1.0 ±0.010[D] |
| | | Semi | n.a. | n.a. | 0.28±0.020[A] | 0.70±0.0010[C] | 3.4 ±0.030[C] | n.a. | 1.2 ±0.060[D] |
| | | Skim | n.a. | n.a. | 0.29±0.010[A] | 0.81±0.13[C] | 1.3 ±0.030[D] | n.a. | 1.3 ±0.070[D] |

† Different low case subscript letters indicate statistically significant differences among samples collected from the same plant, brand, processing lot and container type. Different upper case subscript letters indicate statistical differences ($p<0.05$) among samples collected from retail outlets. For samples of brands B, C, E, F and G, the multi-way factor ANOVA analysis considered fat content and packaging type.

†† Raw milk samples collected in 100 mL plastic containers with no headspace and covered with aluminum foil before analysis

††† Commercial containers were: (1) Transparent plastic bottle covered with aluminum foil before analysis; (2) Transparent plastic bottle; (3) Cardboard box; (4) Opaque plastic bag; (5) Transparent glass bottle covered with aluminum foil before analysis; (6) Opaque plastic bottle; (7) Tetra Brik™ container.

†††† Milk samples from the same plant A lots identified with Roman numerals I to III

* = organic milk and n.a. = not available.