Simultaneous HPLC–DAD quantification of vitamins A and E content in raw, pasteurized, and UHT cow's milk and their changes during storage

Citation	Chotyakul, N., Pateiro-Moure, M., Saraiva, J. A., Torres, J. A., & Pérez-Lamela, C. (2014). Simultaneous HPLC–DAD quantification of vitamins A and E content in raw, pasteurized, and UHT cow's milk and their changes during storage. European Food Research and Technology, 238(4), 535-547. doi:10.1007/s00217-013-2130-7
DOI	0.1007/s00217-013-2130-7
Publisher	Springer
Version	Accepted Manuscript
Terms of Use	http://cdss.library.oregonstate.edu/sa-termsofuse

Simultaneous HPLC-DAD quantification of vitamins A and E content in raw, pasteurized, and UHT cow's milk and their changes during storage

Nattaporn Chotyakul[ab], Miriam Pateiro-Moure[b], Jorge Alexandre Saraiva[c], J. Antonio Torres[a*], Concepción Pérez-Lamela[b]

[a] *Food Process Engineering Group, Department of Food Science & Technology, Oregon State University,100 Wiegand Hall, Corvallis, OR 97331-6602, USA*

[b] *Nutrition and Bromatology Group, Analytical and Food Chemistry Department, Faculty of Food Science and Technology, University of Vigo, Ourense Campus, 32004 Ourense, Spain*

[c] *QOPNA, Food Chemistry and Biochemistry Group, Chemistry Department, University of Aveiro, 3810-193, Aveiro, Portugal*

**Corresponding author E-mail address: j_antonio.torres@oregonstate.edu*

Abstract

An improved extraction and HPLC method for the simultaneous extraction and quantitation of retinol, α-tocopherol, α-tocotrienol and β-carotene was developed to analyze commercial whole/semi-skim/skim samples of raw/pasteurized/UHT milk in transparent plastic/glass bottles and Tetra Brik^{TM} containers. The sample preparation method required prior saponification at 40ºC for 15 min followed by n-hexane extraction. An isocratic acetonitrile:methanol (65:35 v/v) mobile phase and UV detection were chosen for HPLC quantification. The liposoluble vitamin content in raw, pasteurized conventional/organic, and UHT milk ranged 0.055-5.540 (retinol), 0.135-1.410 (α-tocopherol), and 0.040-0.850 mg/L (β-carotene). No significant differences ($p>0.05$) were observed on losses of retinol, α-tocopherol, and β-carotene content in UHT whole milk after 5 days at 4ºC in the dark. After 14 days at 4ºC in the dark the contents of retinol, α-tocopherol, and β-carotene remained higher in milk with higher fat content and were higher in unopened containers. In UHT whole milk samples containing 0.02% NaN_3, retinol (33%) and α-tocopherol (11%) but not β-carotene (2%) decreased significantly ($p<0.05$).

Keywords: Cow’s milk; Food analysis; Fat soluble vitamins; HPLC; vitamin A; vitamin E

1. Introduction

Milk contains the liposoluble vitamins A, D, E and K and it is often enriched with vitamins A, C, and E [1]. Assuming the recommended daily intake (RDI) for dairy products in Spain, consuming three milk servings/day provides about 16% and 3% of the RDI for vitamins A (1 mg/d) and E (15 mg/d), respectively [2]. Vitamin A is a generic term referring to a variety of chemical substances showing vitamin A activity including retinol, retinal, retinoic acid, retinyl esters, and provitamin A carotenoids such as β-carotene. In dairy and animal food products it is most often present as the retinyl palmitate ester which is hydrolyzed in the small intestine cells to the alcohol retinol [3]. Retinol and its derivatives are found in animal tissues and dairy products, while β-carotene is present in foods of plant and animal origin. In the case of milk, the conversion of β-carotene in the feed to retinol may occur in the mammary gland of lactating dairy cows [4].

Vitamin E inhibits the oxidation of polyunsaturated fatty acids [5] and is found in the form of eight isomers differing in biological activity, namely four tocopherols (α-, β-, γ- and δ-) and the four corresponding tocotrienols [6]. In milk, the vitamin E content is highly variable and present mostly as α-tocopherol [7]. Compared to the other isomers, α-tocopherol is less stable during processing and storage, but it has the highest biological activity with beneficial antioxidant effects on the prevention of chronic diseases [8,9]. Environmental factors such as oxygen, light, temperature, and also the intrinsic food factors water activity, lipid content and pH influence their stability [5].

Liposoluble vitamins content vary with processing conditions, season, cow species, and cow feed composition. Krukovsky et al. [10] determined the carotenoid and α-tocopherol content in milk from Holstein, Guernsey, Brown Swiss and Jersey cows during both pasture and barn feeding. Vitamin E is bound to milk lipids and thus it is expected to decrease when milk fat content is reduced [11]. The composition and milk fat content depend also on feed, season, quality/quantity of feed, and lactation stage. The presence of α-tocopherol in milk may be attributed to the cow feed α-tocotrienol content, particularly that coming from grasses [11]. Guernsey and Holstein milk showed the highest and lowest α-tocopherol level, i.e., 3 and 2 mg per 100g milk fat, respectively. The season effect was studied by Agabriel et al. [12] who analyzed bulk milk in French farm tanks. The difference in α-tocopherol content was attributed to the proportion of grazed grass or grass silage in the feed. Kalac [13] reported also that the

α-tocopherol content in milk fat depends on cow feeding showing higher values for cows fed grass (0.38 mg per 100 g milk fat) than maize silage (0.21 mg per 100 g milk fat). Higher content of α-tocopherol in summer than in winter milk has been reported and associated to cow feed [14,15].

Extremely sensitive detection methods are required to quantify tocopherols and tocotrienols in dairy products [16]. Liposoluble vitamins are analyzed by direct solvent extraction or after saponification [9]. Efficiency of these extraction methods varies and thus must be optimized for milk. HPLC is most often used to quantify vitamins including liposoluble vitamins in milk [17], typically using UV-visible and diode-array detection (DAD) methods [18-21].

In this study, an improved method was developed by studying the simultaneous extraction of vitamins A and E using *n*-hexane directly, or by using *n*-hexane or dichloromethane after saponification following three methods. Also developed was a rapid and sensitive HPLC method for the simultaneous quantification of retinol, retinyl acetate, β-carotene (all with vitamin A activity), α-tocotrienol, and α-tocopherol (all with vitamin E activity). The extraction and HPLC method developed was used to quantify these compounds in samples of raw, pasteurized, and UHT milk with different fat content (whole, semi-skim, and skim) obtained from retail stores and processing plants. Since vitamin content differences have been reported for organic and conventional raw, whole, semi-skim and skim milk distributed in different types of packaging [22-24], organic milk and different container types were included in this study. Finally, the losses of vitamin A and E during consumer milk handling were also studied. This included the refrigerated storage of containers kept close and opened (to simulate contact with the environment) with and without the addition of sodium azide.

Materials and Methods

Milk samples

Organic/conventional, whole/semi-skim/skim, raw/pasteurized/UHT milk in various packaging types was collected in Spain from commercial sources. Raw whole milk was obtained from two processing plants (brands A and B), while pasteurized and UHT milk was obtained from the same two processing plants and also from retail outlets (brands C, D and E). Samples of raw and pasteurized whole, semi-skim, skim milk were

transported in plastic and glass bottles for brands A and B. UHT whole, semi-skim and skim milk were obtained in Tetra Pak™ containers. All transparent containers were covered with aluminum foil to minimize light exposure before analysis which was conducted usually within a few hours after samples were collected. When this was not possible, samples were kept at -20 ºC for a few days and thawed at 4 ºC before analysis. Composition of the milk samples reported by the milk suppliers is shown in Table 1.

Chemicals and reagents

The calibration standards retinol, β-carotene and α-tocopherol and the internal standards retinyl acetate and α-tocotrienol were obtained from Sigma-Aldrich (Madrid, Spain) (Table 2). Tert-butylhydroquinone (TBHQ, 97%), n-hexane (Chromasolv®) and acetonitrile (Chromasolv®) were obtained from the same supplier. Dichloromethane, phenolphthalein, absolute HPLC-grade ethanol, anhydrous sodium sulfate, and potassium hydroxide (85%) were supplied by Panreac (Vigo, Spain). Stock standard solutions of 100 mg/L α-tocotrienol and 100 mg/L of retinol, retinyl acetate, β-carotene, α-tocopherol, and β-carotene were prepared in acetonitrile, except for β-carotene which was prepared in *n*-hexane. To minimize vitamins loss by oxidation, all solutions were bubbled with nitrogen for 1 min and stored in amber vials under refrigeration (4ºC).

Chromatography and detection procedures

The chromatographic system used was a Liquid Chromatography HPLC system from Thermo Fisher Scientific (Waltham, MA, USA) consisting of a Spectra System SCM1000 vacuum degasser, a Spectra System P4000 pump, a Spectra System AS3000 autosampler, and a Surveyor PDA plus detector connected to a PC computer running ChromQuest™ 5.0. The most common mobile phases used in the determination of vitamins A and E are methanol (MeOH), acetonitrile (ACN), and mixtures of these solvents with water [25]. Methanol:acetonitrile and methanol:water mixtures were tested as mobile phase (Table 3) on a 150 mm×4.6 mm 5 µm particle Luna C_{18} analytical column (Phenomenex, Madrid, Spain) with a 4 mm×2 mm guard column with the same packing material. Isocratic conditions of the mobile phase at a flow rate of 1 mL/min for 30 min and DAD detection were used to optimize all tested quantification methods. HPLC column temperature was held constant at 35 ºC. Sample injection

volume initially set at 20 µL was increased to 100 µL to detect low vitamin concentrations. The wavelengths used were 296 nm for α-tocopherol and α-tocotrienol, 326 nm for retinol and retinyl acetate, and 450 nm for β-carotene (Table 2).

Milk saponification and solvent extraction procedures

Four extraction methods were tested for the extraction of vitamins from milk with antioxidants added to prevent the oxidation of fat soluble vitamins during extraction (Table 4). Butylated hydroxytoluene (BHT) and ascorbic acid (AA) are antioxidants commonly used for this purpose. Lee et al. (2003) found that BHT was more suitable than AA and caused fewer background interferences in the chromatographic separation of tocopherols by yielding a single 292 nm peak when using UV detection. However, BHA and BHT are only fairly heat stable, whereas TBHQ is more heat-stable (Reische et al. 2002). Therefore, TBHQ was used instead of BHT to minimize vitamin losses during the saponification heating steps. In all cases, saponification was conducted in the dark under a nitrogen atmosphere.

Method I. Saponification was not used in this extraction method based on the work by Rodas-Mendoza et al. (2003). As recommended by Eitenmiller et al. [26], 1 mL milk samples without saponification were extracted with n-hexane with and without 1 g TBHQ added as antioxidant (Method I). Milk (1 mL) was mixed with 4 mL absolute ethanol, stirred for 2 min, and then for an additional 4 min after adding 400 µL n-hexane. After centrifugation at 2,500 rpm for 5 min at room temperature, the clear organic top layer was concentrated to dryness under nitrogen gas at 40 °C using a TurboVap LV concentration workstation (Caliper Life Sciences, Barcelona, Spain), reconstituted in 2 mL acetonitrile-methanol (65:35), and passed through 0.45 µm Chromafil®Xtrafilter before HPLC injection.

Method II. This extraction procedure is based on the work by Bognar (1986) and Albalá-Hurtado et al. (1997). AA (0.5g) was added to a 25 mL milk sample and then mixed with 50 mL absolute ethanol and 10 mL 60% potassium hydroxide solution. The mixture was then kept overnight at room temperature with mild stirring. After saponification, the mixture was extracted 5 times with n-hexane (3x50 mL plus 2x25

mL). The pooled extracts were then washed with 50 mL water until the aqueous layer appeared colorless when adding 2-3 phenolphthalein drops. Then, 1 g TBHQ and 2 g anhydrous sodium sulfate (Na_2SO_4) were added to the organic fraction as antioxidant and to remove water traces, respectively. The mixture was filtered through a 0.45 µm MS®Nylon membrane filter and concentrated to dryness under nitrogen gas at 40 °C using a TurboVap LV concentration workstation. The extract was reconstituted in 10 mL acetonitrile-methanol (65:35) and passed through 0.45 µm Chromafil®Xtrafilter before HPLC injection.

Method III. In this method published by FAO [27], 0.25 g AA, 50 mL methanol, and 5 mL 50% KOH were added to 5 mL milk samples, held under bubbling nitrogen for 1 min, and then stirred in a water bath set at 80°C for 45 and 15 min, and at 40°C for 15 min. Saponified samples were then extracted with 4x50 mL of n-hexane. As in method II, the mixture was washed with 50 mL of water and 2-3 phenolphthalein drops were added. After washing the organic phase with water, the remaining procedures were conducted as in method II.

Method IV. In Method IV suggested by Rodas-Mendoza et al. [28], 150 mL dichloromethane, 75 mL methanol and 2 g TBHQ were added to 25 mL milk samples and shaken at room temperature for 30 min. The saponified sample was extracted with 2x10 mL dichloromethane and 2x50 mL water and then the remaining procedures including adding 2-3 drops phenolphthalein were followed as in methods II and III.

Vitamins A and E determination by HPLC-DAD

Vitamin content determinations in milk were conducted in duplicates. Individual vitamins and their mixture were spiked into milk samples at concentrations of 1 mg/L for the retinol and β-carotene standards and the internal standard retinyl acetate, while 10 mg/L was used for the α-tocopherol standard and the α-tocotrienol internal standard. Milk sample peaks were identified by comparisons of retention time and wavelength at maximum absorbance for the standards and the internal standards. Six concentrations in duplicates were used to prepare calibration curves in the 0.025-1 mg/L range for vitamin

A (retinol, retinyl acetate and β-carotene), and 0.25-10 mg/L for vitamin E (α-tocopherol and α-tocotrienol) yielding the linearity parameters shown in Table 3.

Based on 6 replications of blank sample injections, limits of detection (LOD) and quantification (LOQ) were estimated as 3 and 10 times, respectively, the value of the standard deviation of the peak height at the retention time for retinol, α-tocopherol, α-tocotrienol and β-carotene [29]. Reproducibility was determined by analyzing six replications (n=6) of each of the four standards (retinol, α-tocotrienol, α-tocopherol, β-carotene). The precision was estimated as the RSD (%) value of the injection of multiple extractions of the same milk sample (UHT whole milk, n=6). Finally, recovery studies were performed by spiking milk samples and solvents used to dilute standards with known amounts of each vitamin. The recovery response was calculated as the vitamin content difference between spiked and non-spiked samples (Table 4).

Degradation of retinol, α-tocopherol, and β-carotene during consumer storage

The effect of consumer milk storage at 4ºC on the retention of retinol, α-tocopherol, and β-carotene was studied for brand A (raw conventional whole milk) and brand B (raw whole, pasteurized whole, and pasteurized semi-skim organic milk). The test conditions considered in this study were based on the assumption that consumers store open milk bottles under refrigeration for up to 2 weeks. In the first study, a bottle of each milk type was opened and samples taken from the same container after 0, 2, 5, 8, and 14 days were kept protected from light until analyzed. In the second study, the losses of retinol, α-tocopherol, and β-carotene in UHT whole milk (brand A) was studied i) by sampling at 0, 3, 10, 20, 30 days always from the same bottle, as above, but with 0.02% sodium azide (NaN_3) when opening it to control microbial growth; and, ii) by sampling from a new milk bottle at and of 0, 4, 11, 21, 31 days to simulate refrigerated storage of milk in closed containers.

Statistical analysis

Statistical analysis was performed with Minitab Statistical Software (Minitab Inc., State College PA). A one-way ANOVA was performed using Tukey post hoc honestly significant difference (HSD) for differences between mean liposoluble vitamin contents (α = 0.05) considering the same thermal treatment (raw, pasteurized and UHT milk) using fat content, packaging, and brand as factors.

Results and discussion

Development of the HPLC analysis protocol

The optimization of the chromatographic conditions for the determination of vitamins A (retinol, β-carotene) and E (α-tocopherol) was performed using the analytical column Luna C_{18}. The first mobile phase tested was methanol:water (94:6, v/v) using 20 and 100µL volume loops with the latter improving the resolution of the β-carotene peak. The chromatographic separation was generally good but resolution of the α-tocotrienol peak was particularly incomplete and the α-tocopherol retention time was excessively short. Using the larger loop size, methanol was tested to improve the chromatographic separation to quantify β-carotene. Sensitivity improved but the retention times were excessively long suggesting that methanol may be too polar for the elution of β-carotene [30]. Although a methanol mobile phase has been used by other authors to quantify retinol and α-tocopherol [31], it did not improve the separation of the vitamers in this work. Similar results were observed for a 90:10 (v/v) MeOH:ACN mobile phase. A ternary ACN:MeOH:H_2O (91:8:1, v/v/v) mobile phase as reported by Gruszka and Kruk [32] produced variable retention times and incomplete peaks resolution for retinol, retinyl acetate and β-carotene. It was modified to a binary ACN:MeOH mobile phase and tested at small methanol increments but this did not improve the peak shape and chromatographic separation. Peak separation for all the vitamers in this study was achieved (Figure 1) when using a higher methanol concentration (i.e., ACN:MeOH at 65:35, v/v) and a higher column temperature (35 ºC) as in the work by Lee et al. [33] but using in this study only one C_{18} column instead of two, and by increasing the injection volume to 100 µL to compensate for the lower sensitivity of UV detectors such as the one used in this study. The retention times obtained at a flow rate of 1 mL/min were 3.5, 9.6 and 26 min for retinol, α-tocopherol and β-carotene, respectively, while the internal standards retinyl acetate and α-tocotrienol eluted at 4.3 min and 5.4 min, respectively. In milk samples, retinol and β-carotene appeared to co-elute with retinyl acetate even though this was not observed when injecting solutions of these compounds as suggested by the high value for β-carotene in method II (Table 4). Consequently, spiking of milk with retinyl acetate was not used when analyzing commercial milk samples even though other authors have used it as an internal standard [34-36].

Development of the extraction protocol

Samples without (method I), or with saponification to facilitate vitamin separation from milk fat (methods II-IV), were extracted with *n*-hexane, a most commonly used solvent [37,38], or dichloromethane (Table 4). The latter, used in a novel, fast and simple milk analysis procedure developed by Stefanov et al. [39] was evaluated in this study because its stronger polarity may dissolve milk compounds better than the frequently-used diethyl ether reported to yield higher recoveries (89.9±1.4%) than n-hexane (14.1±0.7%) when quantifying *α*-tocopherol [40,25]. However, *n*–hexane yielded higher recoveries (83-103%, method III) than dichloromethane (44-61%, method IV). Three saponification methods were tested in this study. In method II, the saponification time was excessive and caused large vitamin losses whereas in method IV precipitation interfered with the extraction of the saponified solution. However, method III adapted from FAO (1997) and tested with samples stirred for 15 min in a 40°C water bath, yielded high recoveries. During the saponification and evaporation steps, AA and TBHQ were used as antioxidants. According to Lee and others (2003), good antioxidants allow separation of individual compounds as single peaks without interferences. TBHQ used in method III (Table 4) allowed separating α-tocopherol and α-tocotrienol in standards and milk extracts. In summary, method III was chosen to determine vitamins A and E in milk in this work because it resulted in high recoveries with low standard deviations while methods I, II and IV showed lower efficiency and highly variable standard deviations (Table 4).

Performance of the extraction and quantification method selected

The reproducibility of the protocol method III yielding high recoveries of 86, 103, 83, and 99% for retinol, β-carotene, α-tocotrienol, and α-tocopherol, respectively (Table 4), was determined by analyzing six replicates (n=6) of three standard solutions (0.05, 0.25, 1 mg/L for retinol and β-carotene and 0.5, 2.5, and 10 mg/L for α-tocotrienol and α-tocopherol). The relative percentage standard deviation (% RSD) showed variability under 2% for retinol, α-tocotrienol and α-tocopherol, and about 2% for β-carotene. The precision was estimated as the RSD value of the injection of six extractions of the same milk sample (n=6) yielding values of 3, 2, and 5% for retinol, α-tocopherol and β-carotene, respectively, in UHT whole milk. However, α-tocotrienol could not be

quantified because the LOQ in this study was higher (0.1 mg/L) than the reported concentration in raw whole milk [0.0176 mg/L, 11].

The quantification linearity (R^2) for the HPLC method here reported was about 0.999, with a lowest value of 0.975 for the simultaneous quantification of vitamins A and E. LOD values were 6, 53, 27 and 4 µg/L for retinol, α-tocotrienol, α-tocopherol and β-carotene, respectively, while the corresponding LOQ values were 10, 100, 100 and 10 µg/L. These values are better than those in previous reports. For example, Karpinska et al. [41] reported for retinol and α-tocopherol LOD values of 3.83 and 1.81 mg/L, respectively, and corresponding LOQ values of 12.7 and 6.37 mg/L. Albalá-Hurtado et al. [38] reported for infant milk formulae LOD/LOQ limits also higher than those found in this study, i.e., 0.01/0.02 mg/L for vitamin A (retinol) and 0.30/0.40 mg/L for vitamin E (α-tocopherol). Higher values were also found by Lee et al. [33] for retinol, α-tocopherol and β-carotene.

Analysis of commercial samples

Fat content of the samples included in this study ranged 3.5-3.92%, 1.5-1.6% and 0.24-0.30% for the whole, semi-skim and skim commercial milk samples (Table 1), respectively. Alfa-tocotrienol was not detected in all milk samples because of the lower LOD values of this study as compared to previous reports [41]. Samples of brand A (lots I, II, and III), B, C, D, and E were compared (α=0.05) as the difference between mean values for each vitamer in raw, pasteurized and UHT milk using fat content as factor from the same processed milk in different brands.

The vitamin A and E content in raw and pasteurized conventional (brand A) and organic milk (brand B), and UHT samples of conventional milk (brands A, C, D, and E) samples measured in this study (Table 5) were compared with published values (Table 6). These comparisons required conversion of published values from w/w to w/v units using reported density values for milk [42].

Retinol: The retinol levels in conventional raw whole milk brand A lots I to III, ranging from 0.730 to 0.895 mg/L, were higher than the minimum of the corresponding range 0.269-0.362 mg/L reported by Paul and Southgate [43] for whole milk (3.8% fat) and also higher than the reported average vitamin A content in milk of 0.413 mg/L (range

0.103-1.033 mg/L) reported by Renner et al. [44]. For raw milk with similar fat content (3.92%), the retinol level in raw whole milk brand A lot II (0.73 mg/L) was higher than the value reported by Ollilainen and other [45]. Whereas organic raw whole milk brand B lots I and II showed retinol content of 1.020±0.27 and 1.075±0 mg/L, respectively were lower than the corresponding concentrations reported by Ellis et al. [24] in raw organic milk (14.575±2.60 mg/L) and even in raw conventional milk (16.785±3.74 mg/L). No significant differences ($p>0.05$) were observed for raw whole milk brands A and B from different lots. The retinol content of raw organic versus conventional whole milk was reported by Bergamo et al. [46]. Retinol was 6% lower in conventional milk, whereas in this study it was almost 30% lower. Pasteurized milk brand A showed 1.065±0.01, 5.540±0.27 and 4.140±0.23 mg/L for whole, semi-skim, and skim milk, respectively, i.e., higher than the corresponding values of 0.537 mg/L, 0.258 mg/L, and traces, reported by Early [p. 31, 47]. Pasteurized semi-skim and skim milk of brand A yielded significantly higher ($p<0.05$) retinol content than whole milk reflecting the vitamin A and D fortification declared on the container label. Organic pasteurized whole milk brand B lots I and II showed retinol contents of 0.930±0.11 and 0.880±0 mg/L, with non-significant statistic differences ($p>0.05$) among lots, but with statistic significant difference ($p<0.05$) when milk fat was removed for thermal milk treatment for semi-skim brand B lot II which contained only 0.450 mg/L retinol. Results for UHT commercial milk samples brands A (lot III), C, D and E showed that milk fat content affect the retinol content with statistically significant differences ($p<0.05$) for each brand (Table 5). The retinol content range in whole, semi-skim, skim UHT milk brands C, D, and E was 0.745-0.820, 0.230-0.290, and 0.055-0.085 mg/L. Ollilainen et al. [45] reported an average content of all-trans retinols of 0.168, 0.337, 0.539 mg/L in 1.9%, 3.9% fat cow's milk and human milk, respectively (Table 6).

α-tocopherol: The α-tocopherol level in conventional raw whole milk brand A lots I-III varied from 0.590 to 0.730 mg/L, while organic raw whole milk brand B yielded values of 1.105±0.01 (lot I) and 1.125±0 mg/L (lot II) lower than the corresponding concentrations reported by Ellis et al. [24] in raw conventional milk (44.665±9.85 mg/L) and raw organic milk (42.372±9.85 mg/L). No significant differences ($p<0.05$) in α-tocopherol were detected for raw whole milk samples of the same brand except for brand A lot I. An about 47% higher α-tocopherol content was also found in organic as

compared to conventional milk whereas Ellis et al. [24] reported that α-tocopherol was 5% higher in conventional than in organic milk. Pasteurized milk brand A yielded values of 0.700±0.03, 0.815±0.01 and 0.400±0.01 mg/L for whole, semi-skim, and skim milk, respectively and the statistic showed significant effect ($p<0.05$) of fat content. Organic pasteurized semi-skimmed and whole milk brand B showed that *α*-tocopherol content varied from 0.445 to 0.930 mg/L, with statistically significant differences ($p<0.05$) among lots and milk with different fat level. The α-tocopherol content range in whole, semi-skim, skim UHT milk brands C, D, and E was 0.135-0.620 mg/L. Kaushik and others [11] reported a 0.453±2.2 mg/L α-tocopherol concentration in raw whole milk (3% fat) that was higher than in reduced (2% fat, 0.273±3.9 mg/L), low (1% fat, 0.147±1.7 mg/L) and non-fat milk (0.5% fat, 0.047±0.5 mg/L). The level of α-tocopherol in UHT commercial milk samples brand A (lot III), C, D and E showed a significant effect ($p<0.05$) of fat content for all brands except for brand D skim and semi-skim UHT milk ($p>0.05$) (Table 5).

β-carotene: Significant differences ($p<0.05$) were found among lots of raw whole milk samples with values ranging from 0.160 to 0.180 mg/L for brand A lots I-III which were higher than the minimum of the corresponding range 0.134-0.238 mg/L reported by Paul and Southgate [43] for whole milk (3.8% fat) and also higher than the reported average β-carotene 0.207 mg/L (range 0.031-0.516 mg/L) by Renner et al. [44]. Organic raw whole milk brand B lots I and II yielded levels of 0.330±0.002 and 0.290±0.001 mg/L, respectively lower than the corresponding concentrations reported by Ellis et al. [24] in raw organic milk (5.526±1.35 mg/L) and raw conventional milk (5.154±2.10 mg/L). A significant effect ($p<0.05$) of the milk fat content was observed for brands A and B pasteurized milk samples. Organic samples contained about 45% more β-carotene than conventional samples which was higher than reported by Ellis et al. [24] (about 7%). The β-carotene of pasteurized milk brand A samples was 0.165±0.002, 0.100±0.200 and 0.040±0.0003 mg/L for whole, semi-skim, and skim milk, respectively. The β-carotene content for organic pasteurized milk brand B ranged from 0.125 to 0.270 mg/L lower than the concentration (5.681 mg/L) reported by Hulshof et al. [48]. UHT milk samples brand A (lot III), C, D and E showed a significant effect ($p<0.05$) of the milk fat content (Table 5). The liposoluble vitamin content range in whole, semi-skim, skim UHT milk brands C, D, and E was 0.125-0.140, 0.070-0.85,

and 0.04-0.06 mg/L for β-carotene. Ollilainen et al. [45] reported an average content of β-carotene for 0.099, 0.173, 0.031 mg/L in 1.9%, 3.9% fat cow's milk and in human milk, respectively (Table 6).

Degradation of retinol, α-tocopherol, β-carotene

The analysis of retinol, α-tocopherol, and β-carotene in milk samples differing fat content showed losses generally increasing during the 14 days storage time (Table 7). In most cases, short storage periods (up to 5 days) had no statistically significant effect ($p>0.05$) on the retinol, α-tocopherol, and β-carotene content. In most cases, storage for 5-14 days led to significant losses ($p<0.05$) for retinol and β-carotene, and similarly also for α-tocopherol after 8-14 days ($p<0.05$). Retinol, α-tocopherol, and β-carotene concentration at the end of storage time (14 days) remained higher in milk with higher fat content. After 14 days, loss of retinol was 18% (raw, brand A and B), 15% (pasteurized whole milk brand B) and 20% (pasteurized semi-skimmed milk brand B) whereas α-tocopherol loss was 27% (raw milk brand A), 23% (raw milk brand B), 15% (pasteurized whole milk brand B), and 9% (pasteurized semi-skimmed milk brand B). Finally, β-carotene showed losses of 11% (raw milk brand A), 19% (raw milk brand B), 20% (pasteurized whole milk brand B), and 10% (pasteurized semi-skimmed milk brand B).

The effect of storage on the loss of retinol, α-tocopherol, and β-carotene concentration in UHT whole milk is shown in Table 8. Retinol concentration decreased significantly ($p<0.05$) to 33% after 30 days in milk samples containing 0.02% NaN_3 (condition i), whereas when opening new containers (condition ii) at every sampling time, smaller (0-6%) but significant ($p<0.05$) losses were found after 11 days and only 8% reduction after 31 days. In general, the α-tocopherol content determined for each storage time under conditions (i) and (ii) were statistically different ($p<0.05$) after 10 and 11 days, respectively, and became constant for longer storage period (20-31 days). Losses were about 11% and 13% for condition (i) and (ii), respectively. No changes in β-carotene were found under conditions (i) and (ii) during storage time of this study except for the latter showing a loss of only 9% after 31 days.

4. Conclusions

Four extraction methods to simultaneously determine by HPLC the concentrations of retinol, α-tocotrienol, α-tocopherol, and β-carotene in cow's milk were compared. Method III chosen in this work using small sample and solvent volumes yielded minimum losses of all target vitamins (86±2%, 103±0%, 83±1%, and 99±4% recovery values for retinol, α-tocotrienol, α-tocopherol, and β-carotene, respectively). When combined with HPLC/DAD it allowed the simultaneous determination of vitamers in several brands of raw, pasteurized conventional and organic milk and commercial UHT milk with different fat content ranging 0.055-5.540 (retinol), 0.135-1.410 (α-tocopherol), 0.040-0.850 (β-carotene) mg/L. Short storage periods at 4ºC in the dark (up to 5 days) had no statistically significant effect ($p>0.05$) on the retinol, α-tocopherol, and β-carotene content. After 14 days, the concentration of retinol, α-tocopherol, and β-carotene range was higher in milk with high fat content. The effect of storage on the loss of retinol, α-tocopherol, and β-carotene concentration in UHT whole milk, retinol concentration decreased significantly ($p<0.05$) in milk samples containing 0.02% NaN_3 whereas unopened containers (condition ii) every sampling time, significant ($p<0.05$) losses were found after 11 days. The content of α-tocopherol determined for each time of storage for both condition i) and ii) became constant for longer storage period (20-31 days). No significant changes ($p>0.05$) in β-carotene were found in both cases for condition i) and ii) over the time period excepted for condition ii) at 31 days.

5. Acknowledgements

The authors acknowledge funding from the INCITE program of the Galician Council of Innovation and Industry (Ref.09TAL019383PR), general support to the research groups of Universities of Vigo and Aveiro provided by the European Regional Development Fund (ERDF/FEDER), Fundação para a Ciência e a Tecnologia (FCT, Portugal), European Union, QREN, FEDER and COMPETE for funding the QOPNA research unit (project PEst-C/QUI/UI0062/2013), and Formula Grants no. 2011-31200-06041 and 2012-31200-06041 from the USDA National Institute of Food and Agriculture. The authors of this work gratefully acknowledge CAPSA and Santa Mariña de Loureiro for their cooperation on this research.

REFERENCES

1. MacGibbon AKH, Taylor MW (2003) Composition and structure of bovine milk lipids. In: Fox PF, McSweeny PLH (eds) Advanced dairy chemistry, vol 2: Lipids. Springer Science + Business Media, Inc, New York, NY, pp 1-35

2. Herrero C, Granado F, Blanco I, Olmedilla B (2002) Vitamin A and E content in dairy products: their contribution to the recommended dietary allowances (RDA) for elderly people. Journal of Nutrition Health and Aging 6 (1):57-59

3. Sommer A, West KP (1996) Vitamin A deficiency: health, survival, and vision. Oxford University Press, Oxford, UK

4. Baldi A, Pinotti L (2008) Lipophilic microconstituents of milk. In: Bösze Z (ed) Bioactive Components of Milk. Springer Inc, pp 109-128

5. Miquel E, Alegria A, Barbera R, Farre R, Clemente G (2004) Stability of tocopherols in adapted milk-based infant formulas during storage. International Dairy Journal 14:1003-1011

6. Kamal-Eldin A, Gorgen S, Pettersson J, Lampi AM (2000) Normal-phase high-performance liquid chromatography of tocopherols and tocotrienols - comparison of different chromatographic columns. Journal of Chromatography A 881:217-227

7. McDowell LR (2000) Vitamins in animal and human nutrition. Vitamin E, 2nd edn. Iowa State University Academic Press, Ames, IA

8. Brigelius-Flohe R, Traber MG (1999) Vitamin E: function and metabolism. The FASEB Journal 13:1145-1155

9. Chavez-Servin JL, Castellote AI, Lopez-Sabater MC (2006) Simultaneous analysis of Vitamins A and E in infant milk-based formulae by normal-phase high-performance liquid chromatography-diode array detection using a short narrow-bore column. Journal of Chromatography 1122 138-143

10. Krukovsky V, Whiting F, Loosli JK (1950) Tocopherol, carotenoid and vitamin A content of the milk fat and the resistance of milk to the development of oxidized flavors as influenced by breed and season. Journal of Dairy Science 33 (11):791-796

11. Kaushik S, Wander R, Leonard S, German B, Traber MG (2001) Removal of fat from cow's milk decreases the vitamin E contents of the resulting dairy products. Lipids 36:73-78

12. Agabriel C, Cornu A, Journal C, Sibra C, Grolier P, Martin B (2007) Tanker milk variability according to farm feeding practices: vitamins A and E, carotenoids, color, and terpenoids. Journal of Dairy Science 90:4884-4896

13. Kalac P (2011) The effects of silage feeding on some sensory and health attributes of cow's milk: A review. Food Chemistry 125:307-317

14. Ball GFM (1998) Bioactivity and analysis of vitamins in foods. Chapman & Hall Publishing Co., London, UK

15. Lindmark-Månsson H, Fondéna R, Pettersson HE (2003) Composition of Swedish dairy milk. International Dairy Journal 13:409-425

16. Podda M, Weber C, Traber MG, Packer L (1996) Simultaneous determination of tissue tocopherols, tocotrienols, ubiquinols, and ubiquinones. Journal of Lipid Research 37:893-901

17. Bernal J, Mendiola JA, Ibanez E, Cifuentes A (2011) Advanced analysis of nutraceuticals. Journal of Pharmaceutical and Biomedical Analysis 55:758-774

18. Liyanage C, Hettiarachchi M, Mangalajeewa P, Malawipathirana S (2008) Adequacy of vitamin A and fat in the breast milk of lactating women in south Sri Lanka. Public Health Nutrition 11 (7):747-750

19. Plozza T, Trenerry VC, Caridi D (2012) The simultaneous determination of vitamins A, E and β-carotene in bovine milk by high performance liquid chromatography-ion trap mass spectrometry (HPLC-MS). Food Chemistry 134 (1):559-563. doi:10.1016/j.foodchem.2012.02.121

20. Szlagatys-Sidorkiewicz A, Zagierski M, Jankowska A, Łuczak G, Macur K, Bączek T, Korzon M, Krzykowski G, Martysiak-Żurowska D, Kamińska B (2012) Longitudinal study of vitamins A, E and lipid oxidative damage in human milk throughout lactation. Early Human Development 88 (6):421-424

21. Turner T, Burri BJ (2012) Rapid Isocratic HPLC method and sample extraction procedures for measuring carotenoid, retinoid, and tocopherol concentrations in human blood and breast milk for intervention studies. Chromatographia 75 (5-6):241-252. doi:10.1007/s10337-012-2193-9

22. Moyssiadi T, Badeka A, Kondyli E, Vakirtzi T, Savvaidis I, Kontominas MG (2004) Effect of light transmittance and oxygen permeability of various packaging materials on keeping

quality of low fat pasteurized milk: chemical and sensorial aspects. International Dairy Journal 14:429-436

23. Zygoura P, Moyssiadi T, Badeka A, Kondyli E, Savvaidis I, Kontominas MG (2004) Shelf life of whole pasteurized milk in Greece: effect of packaging material. Food Chemistry 87:1-9

24. Ellis KA, Monteiro A, Innocent GT, Grove-White D, Cripps P, McLean WG (2007) Investigation of the vitamins A and E and b-carotene content in milk from UK organic and conventional dairy farms. Journal of Dairy Research 74:484-491

25. Escrivá A, Esteve MJ, Farré R, Frígola A (2002) Determination of liposoluble vitamins in cooked meals, milk and milk products by liquid chromatography. Journal of Chromatography A 947:313-318

26. Eitenmiller RR, Ye L, Landen WO (2008) Vitamin analysis for the health and food sciences. 2 edn. CRC Press, FL:Boca Raton

27. Anonymous (1997) Producción y manejo de datos de composición química de alimentos en nutrición. Oficina Regional de la FAO para América Latina y el Caribe, Santiago de Chile, Chile

28. Rodas-Mendoza B, Morera-Pons S, Castellote-Bargalló AI, López-Sabater MC (2003) Rapid determination by reversed-phase high-performance liquid chromatography of vitamins A and E in infant formulas. Journal of Chromatography A 1018:197-202

29. Vial J, Jardy A (1999) Experimental comparison of the different approaches to estimate LOD and LOQ of an HPLC method. Analytical Chemistry 71:2672-2677

30. Nelis HJCF, De Leenheer AP (1983) Isocratic nonaqueous reversed-phase liquid chromatography of carotenoids. Analytical Chemistry 55 (2):270-275

31. Arnaud J, Fortis I, Blachier S, Kia D, Favier A (1991) Simultaneous determination of retinol, α-tocopherol and β-carotene in serum by isocratic high-performance liquid chromatography. Journal of Chromatography B: Biomedical Sciences and Applications 572 (1-2):103-116

32. Gruszka J, Kruk J (2007) RP-LC for determination of plastochromanol, tocotrienols and tocopherols in plant oils. Chromatographia 66:909-913

33. Lee BL, New AL, Ong CN (2003) Simultaneous determination of tocotrienols, tocopherols, retinol, and major carotenoids in human plasma. Clinical Chemistry 49 (12):2056-2066

34. Escriva A, Esteve MJ, Farre R, Frigola A (2002) Determination of liposoluble vitamins in cooked meals, milk and milk products by liquid chromatography. Journal of Chromatography A 947 (2):313-318

35. Gentili A, Caretti F, Bellante S, Ventura S, Canepari S, Curini R (2013) Comprehensive profiling of carotenoids and fat-soluble vitamins in milk from different animal species by LC-DAD-MS/MS hyphenation. Journal of Agricultural and Food Chemistry 61:1628-1639

36. Chauveau-Duriot B, Doreau M, Nozière P, Graulet B (2010) Simultaneous quantification of carotenoids, retinol, and tocopherols in forages, bovine plasma, and milk: validation of a novel UPLC method. Analytical and Bioanalytical Chemistry 397:777-790

37. Bognar A (1986) Determination of vitamin A in food using high-pressure liquid chromatography.Results of a collaborative study of the vitamin analysis working group following the LMBG paragraph 35. Zeitschrift für Lebensmittel-Untersuchung und -Forschung A (Berlin) 182 (6):492-497

38. Albalá-Hurtado S, Novella-Rodríguez S, Veciana-Nogués MT, Mariné-Font A (1997) Determination of vitamins A and E in infant milk formulae by high-performance liquid chromatography. Journal of Chromatography A 778:243-246

39. Stefanov I, Vlaeminck B, Fievez V (2010) A novel procedure for routine milk fat extraction based on dichloromethane. Journal of Food Composition and Analysis 23:852-855

40. Stancher B, Zonta F (1983) High-performance liquid chromatography of fat-soluble vitamins: Simultaneous quantitative analysis of vitamins D2, D3, and E. Study of percentage recoveries of vitamins from cod liver oil. Journal of Chromatography A 256:93-100

41. Karpińska J, Mikołuć B, Motkowski R, Piotrowska-Jastrzębska J (2006) HPLC method for simultaneous determination of retinol, α-tocopherol and coenzyme Q10 in human plasma. Journal of Pharmaceutical and Biomedical Analysis 42:232-236

42. Chandan RC (2007) Milk composition, physical and processing characteristics. In: Hui YH (ed) Handbook of Food Products Manufacturing: Health, Meat, Milk, Poultry, Seafood, and Vegetables, vol 2. Wiley-Interscience, Hoboken, NJ, pp 347-378

43. Paul AA, Southgate DAT (1985) McCance and Widdowson's the composition of foods. North-Holland Biomedical Press, Elsevier, London, UK

44. Renner E, Schaafsma G, Scott KJ (1989) Micronutrients in milk. In: Renner E (ed) Micronutrients in milk and milk-based food products. Elsevier Science Publishers Ltd., London, UK, pp 1-70

45. Ollilainen V, Heinonen M, Linkola E, Varo P, Koivistoinen P (1989) Carotenoids and retinoids in finnish foods: dairy products and eggs. Journal of Dairy Science 72:2257-2265

46. Bergamo P, Fedele E, Iannibelli L, Marzillo G (2003) Fat-soluble vitamin contents and fatty acid composition in organic and conventional Italian dairy products. Food Chemistry 82 (4):625–631

47. Early R (1998) The technology of dairy products. 2 edn. Thomson Science Publishing, London, UK

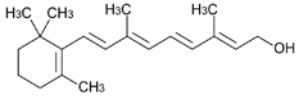
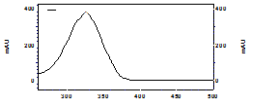
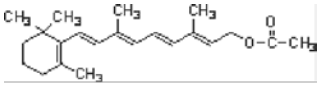
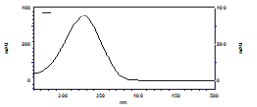
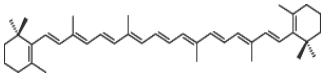
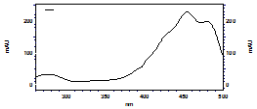
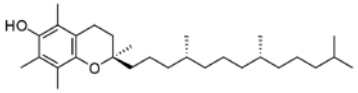
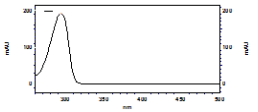
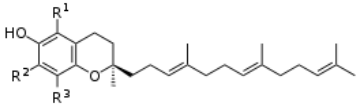
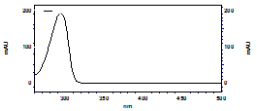
48. Hulshof PJM, Roekel-Jansen T, Bovenkampy P, West CE (2006) Variation in retinol and carotenoid content of milk and milk products in The Netherlands. Journal of Food Composition and Analysis 19:67-75

Table 1.Characteristics of commercial milk samples

Treatment	Brand	Type	Fat (g/100 mL)	Protein (g/100 mL)	Carbohydrate (g/100 mL)	pH
Raw	A	Raw (I)	3.77	3.20	4.65	6.74
	A	Raw (II)	3.92	3.22	4.65	6.73
	A	Raw (III)	3.62	3.24	4.72	n.a.
	B*	Raw (I)	3.2-3.6	n.a.	n.a.	n.a.
	B*	Raw (II)	3.2-3.6	n.a.	n.a.	n.a.
Pasteurized	A	Whole	3.51	3.22	4.74	n.a.
	A	Semi-skim	1.50	3.25	4.81	6.73
	A	Skim	0.24	3.31	4.82	6.74
	B*	Whole	3.2-3.6	n.a.	n.a.	n.a.
	B*	Semi-skim	1.2	n.a.	n.a.	n.a.
UHT	A	Whole	3.50	3.21	4.74	n.a.
	C	Whole	3.60	4.50	3.00	n.a.
	C	Semi-skim	1.50	3.00	4.60	n.a.
	C	Skim	0.30	3.10	4.70	n.a.
	D	Whole	3.60	3.00	4.60	n.a.
	D	Semi-skim	1.55	3.10	4.60	n.a.
	D	Skim	0.30	3.15	4.60	n.a.
	E	Whole	3.60	3.00	4.80	n.a.
	E	Semi-skim	1.60	3.00	4.80	n.a.
	E	Skim	0.30	3.00	4.80	n.a.

* denotes organic milk sample; n.a. = information not available

Table 2. Physical and chemical characteristics of the studied vitamins.

	Compound	Chemical structure	Formula	Absorbance [d] λ_{max} (nm)	Spectrum
Vitamin A	**Retinol**		$C_{20}H_{30}O$	326	
	Retinyl acetate		$C_{22}H_{32}O_2$	326	
	β-carotene		$C_{40}H_{56}$	450	
Vitamin E	**α-tocopherol**		$C_{29}H_{50}O_2$	296	
	α-tocotrienol		$C_{29}H_{44}O_2$	296	

[a]Molecular weight
[b]Melting point (ºC)
[c]Density at 20ºC (g mL^{-1})
[d]Maximum absorbance in ACN: MeOH (65:35)

Table 3. Effect of the mobile phase on the calibration curve based on the HPLC peak area for liposoluble vitamins A and E

Mobile phase	Compounds	Concentration (mg/L)	Calibration line	
			Slope[a]	r^2
MeOH:Water (94:6 v/v)	Retinol	0.025-2.5	9.3×10^5	0.998
	Retinyl acetate	0.10-5.0	6.4×10^5	0.998
	β-carotene	0.50-10.0	1.4 x10	0.975
	α-tocopherol	1.0-20.0	8.3×10^3	0.999
MeOH (100%)	β-carotene	0.050-5.0	3.2×10^6	1.000
ACN:MeOH:Water (91:8:1 v/v)	α-tocopherol	0.25-15.0	4.0×10^4	0.999
	α-tocotrienol	0.25-15.0	2.3×10^4	0.999
ACN:MeOH (65:35 v/v)	Retinol	0.025-1.0	4.0×10^6	0.999
	Retinyl acetate	0.025-1.0	4.0×10^6	0.999
	β-carotene	0.025-1.0	1.0×10^7	0.999
	α-tocopherol	0.25-10.0	4.3×10^5	0.999
	α-tocotrienol	0.25-10.0	2.3×10^5	0.999

Table 4. Effect of the extraction method on the %recovery for vitamins A and E

Vitamin	Compound	Solvent extraction[a]		Saponification and solvent extraction[a]		
		Method I[b]	Method I[c]	Method II[d]	Method III	Method IV
A	Retinol	68 (23)	93 (12)	47 (13)	86 (2)	61 (35)
	β-carotene	12 (2)	10 (46)	168 (6)	103 (0)	25 (9)
E	α-tocotrienol	64 (3)	61 (4)	74 (2)	83 (1)	39 (11)
	α-tocopherol	62 (4)	70 (4)	80 (3)	99 (4)	44 (10)

[a]Values in brackets are CV (%)

[b]extracted without antioxidant

[c]extracted with antioxidant (TBHQ)

[d]spiked with retinyl acetate intended to be used as internal standard

Table 5. Liposoluble vitamin content (average ± standard deviation, mg/L) in commercial raw, pasteurized and UHT milk samples of the same brand and container type but differing in fat content

Vitamin	Brand	Raw whole[1,2]	Pasteurized[1,2]			UHT[3]		
			Whole	Semi-skim	Skim	Whole	Semi-skim	Skim
Retinol	A (I)	$0.895 \pm 0.07^{1,a}$	n.a.	$5.540 \pm 0.27^{1,d}$	n.a.	n.a.	n.a.	n.a.
	A (II)	$0.730 \pm 0^{1,a}$	n.a.	n.a.	$4.140 \pm 0.23^{1,c}$	n.a.	n.a.	n.a.
	A (III)	$0.790 \pm 0^{1,a}$	$1.065 \pm 0.01^{1,a}$	n.a.	n.a.	1.260 ± 0^{d}	n.a.	n.a.
	B* (I)	$1.020 \pm 0.27^{2,a}$	$0.930 \pm 0.11^{2,a}$	n.a.	n.a.	n.a.	n.a.	n.a.
	B* (II)	$1.075 \pm 0^{2,a}$	$0.880 \pm 0^{2,a}$	$0.450 \pm 0.03^{2,b}$	n.a.	n.a.	n.a.	n.a.
	C	n.a.	n.a.	n.a.	n.a.	0.775 ± 0.12^{c}	0.230 ± 0.06^{b}	0.085 ± 0.01^{a}
	D	n.a.	n.a.	n.a.	n.a.	0.820 ± 0.05^{c}	0.260 ± 0.01^{b}	0.055 ± 0^{a}
	E	n.a.	n.a.	n.a.	n.a.	0.745 ± 0.01^{c}	0.290 ± 0.02^{b}	0.055 ± 0.01^{a}
α-tocopherol	A (I)	$0.730 \pm 0.03^{1,b}$	n.a.	$0.815 \pm 0.01^{1,c}$	n.a.	n.a.	n.a.	n.a.
	A (II)	$0.635 \pm 0.01^{1,a}$	n.a.	n.a.	$0.400 \pm 0.01^{1,a}$	n.a.	n.a.	n.a.
	A (III)	$0.590 \pm 0.02^{1,a}$	$0.700 \pm 0.03^{1,b}$	n.a.	n.a.	0.685 ± 0.02^{e}	n.a.	n.a.
	B* (I)	$1.105 \pm 0.01^{2,c}$	$0.930 \pm 0.03^{2,d}$	n.a.	n.a.	n.a.	n.a.	n.a.
	B* (II)	$1.125 \pm 0^{2,c}$	$0.825 \pm 0.01^{2,c}$	$0.445 \pm 0.01^{2,a}$	n.a.	n.a.	n.a.	n.a.
	C	n.a.	n.a.	n.a.	n.a.	1.410 ± 0.03^{g}	0.305 ± 0^{b}	0.620 ± 0^{d}
	D	n.a.	n.a.	n.a.	n.a.	0.600 ± 0^{d}	0.295 ± 0.01^{b}	0.300 ± 0.01^{b}
	E	n.a.	n.a.	n.a.	n.a.	0.825 ± 0.03^{f}	0.425 ± 0^{c}	0.135 ± 0.01^{a}
β-carotene	A (I)	$0.160 \pm 0^{1,a}$	n.a.	$0.100 \pm 0.20^{1,b}$	n.a.	n.a.	n.a.	n.a.
	A (II)	$0.170 \pm 0^{1,ab}$	n.a.	n.a.	$0.040 \pm 0^{1,a}$	n.a.	n.a.	n.a.
	A (III)	$0.180 \pm 0.01^{1,b}$	$0.165 \pm 0^{1,d}$	n.a.	n.a.	0.160 ± 0^{e}	n.a.	n.a.
	B* (I)	$0.330 \pm 0^{2,d}$	$0.270 \pm 0^{2,f}$	n.a.	n.a.	n.a.	n.a.	n.a.
	B* (II)	$0.290 \pm 0^{2,c}$	$0.225 \pm 0^{2,e}$	$0.125 \pm 0^{2,c}$	n.a.	n.a.	n.a.	n.a.
	C	n.a.	n.a.	n.a.	n.a.	0.125 ± 0^{d}	0.070 ± 0^{b}	0.040 ± 0^{a}
	D	n.a.	n.a.	n.a.	n.a.	0.140 ± 0.01^{d}	0.075 ± 0^{bc}	0.050 ± 0^{ab}
	E	n.a.	n.a.	n.a.	n.a.	0.135 ± 0.01^{d}	0.850 ± 0^{c}	0.060 ± 0.01^{b}

1 Transparent plastic bottle covered with aluminum foil
2 Transparent glass bottle covered with aluminum foil
3 Tetra Pak™ container
Different subscript letters indicate statistical differences among samples collected from the same processing plant, same brand. Finally, *denotes organic milk and n.a. = not available; n.d. = not detectable. I, II, and III indicate different lots of the same milk brand and type.

Table 6. Vitamins A and E concentrations in cow's milk reported in several works in the literature

Milk sample	Retinol	α-tocopherol	β-carotene	References
Raw conventional milk (brand A), mg/L	0.73-0.89	0.59-0.73	0.16-0.18	*
Raw organic milk (brand B), mg/L	1.02-1.08	1.11-1.12	0.29-1.02	*
Conventional milk, mg/L	16.785±3.74	44.665±9.85	5.154±2.10	Ellis et al. (2007)
Organic milk, mg/L	14.575±2.60	42.372±9.85	5.526±1.35	Ellis et al. (2007)
Bovine milk, mg/L	0.45	1.5	0.12	Plozza et al. (2012)
3.8% milk fat, mg/L	0.269-0.362	n.a	0.134-0.238	Paul and Southgate (1985)
3.9% milk fat, mg/L	0.337	n.a	0.173	Ollilainen et al. (1989)
1.9% milk fat, mg/L	0.168	n.a	0.099	Ollilainen et al. (1989)
Whole milk (3% fat), mg/L	n.a	0.453±2.2	n.a	Kaushik et al. (2001)
Reduced fat (2% fat), mg/L	n.a	0.273±3.9	n.a	Kaushik et al. (2001)
Low-fat (1% fat), mg/L	n.a	0.147±1.7	n.a	Kaushik et al. (2001)
Non-fat (0.5% fat), mg/L	n.a	0.047±0.5	n.a	Kaushik et al. (2001)
Pasteurized conventional whole milk (brand A), mg/L	1.07	0.07	0.16	*
Pasteurized conventional semi-skim milk (brand A), mg/L	5.54	0.82	0.1	*
Pasteurized conventional skim milk (brand A), mg/L	4.14	0.4	0.04	*
Pasteurized organic whole milk (brand B), mg/L	0.88-0.93	0.82-0.93	0.22-0.27	*
Pasteurized organic semi-skim milk (brand B), mg/L	0.45	0.44	0.12	*
Pasteurized whole milk, mg/L	0.537	n.a	n.a	Early (1998)
Pasteurized semi-skim milk, mg/L	0.258	n.a	n.a	Early (1998)
Pasteurized skim milk, mg/L	Trace	n.a	n.a	Early (1998)
UHT whole milk (brand C – E), mg/L	0.74-0.82	0.6-1.41	0.06-0.08	*
UHT semi-skim milk (brand C – E), mg/L	0.23-0.29	0.3-0.43	0.03-0.62	*
UHT skim milk (brand C – E), mg/L	0.06-0.08	0.07-0.09	0.04-0.06	*

* Values reported in this work. α-tocotrienol was not detected and was not reported in the references here listed

Table 7: Effect of storage at 4°C in the dark on the retinol, α-tocopherol, β-carotene content (n = 3, average ± standard deviation, mg/L) in milk with different fat content

		Time, days					
		0	2	5	8	14	Final % Loss
Retinol	*Brand A*[1]						
	Raw whole milk	0.835 ± 0.005^{c}	0.825 ± 0.005^{c}	0.809 ± 0.005^{c}	0.767 ± 0.004^{b}	0.685 ± 0.003^{a}	18%
	Brand B[*,2]						
	Raw whole milk	1.075 ± 0.001^{b}	1.071 ± 0.022^{b}	0.929 ± 0.005^{a}	0.903 ± 0.009^{a}	0.886 ± 0.006^{a}	18%
	Pasteurized whole milk	0.878 ± 0.001^{e}	0.851 ± 0.003^{d}	0.833 ± 0.001^{c}	0.814 ± 0.002^{b}	0.744 ± 0^{a}	15%
	Pasteurized semi-skim milk	0.452 ± 0.025^{b}	0.426 ± 0^{b}	0.362 ± 0.001^{a}	0.366 ± 0.018^{a}	0.363 ± 0.008^{a}	20%
α-tocopherol	*Brand A*[1]						
	Raw whole milk	0.715 ± 0.003^{c}	0.671 ± 0.006^{b}	0.653 ± 0.003^{b}	0.650 ± 0.008^{b}	0.521 ± 0.014^{a}	27%
	Brand B[*,2]						
	Raw whole milk	1.122 ± 0.005^{b}	1.124 ± 0.034^{b}	0.944 ± 0.006^{a}	0.888 ± 0.084^{a}	0.866 ± 0.002^{a}	23%
	Pasteurized whole milk	0.824 ± 0.010^{b}	0.786 ± 0.049^{b}	0.787 ± 0.016^{b}	0.787 ± 0.016^{b}	0.697 ± 0.004^{a}	15%
	Pasteurized semi-skim milk	0.445 ± 0.010^{b}	0.448 ± 0.007^{b}	0.448 ± 0.015^{b}	0.443 ± 0.006^{b}	0.404 ± 0.004^{a}	9%
β-carotene	*Brand A*[1]						
	Raw whole milk	0.179 ± 0.001^{b}	0.174 ± 0.001^{b}	0.174 ± 0.001^{b}	0.173 ± 0.003^{b}	0.159 ± 0.001^{a}	11%
	Brand B[*,2]						
	Raw whole milk	0.293 ± 0.001^{d}	0.279 ± 0.001^{c}	0.247 ± 0.003^{ab}	0.252 ± 0.005^{b}	0.239 ± 0.005^{a}	19%
	Pasteurized whole milk	0.225 ± 0.003^{b}	0.225 ± 0.020^{b}	0.203 ± 0.001^{ab}	0.198 ± 0.002^{ab}	0.179 ± 0.003^{a}	20%
	Pasteurized semi-skim milk	0.125 ± 0.002^{b}	0.124 ± 0.004^{b}	0.121 ± 0.001^{ab}	0.119 ± 0.003^{ab}	0.113 ± 0^{a}	10%

† Container type: [1] transparent plastic bottle covered with aluminum foil before analysis, [2] transparent glass bottle covered with aluminum foil before analysis. Average values with same superscript in a row are not significantly different ($p > 0.05$). Finally, * denotes organic milk

Table 8: Effect of storage at 4°C in the dark on the retinol, α-tocopherol, β-carotene content (n = 3, average ± standard deviation, mg/L) in UHT whole milk in Tetra Pak™ containers†

	Time, days					
	0	3	10	20	30	Final % Loss
Same container with 0.02% sodium azide [1]						
Retinol	0.80 ± 0.001^{e}	0.766 ± 0.008^{d}	0.671 ± 0.009^{c}	0.586 ± 0.002^{b}	0.534 ± 0.004^{a}	33%
α-tocopherol	0.481 ± 0.001^{c}	0.459 ± 0.008^{b}	0.451 ± 0.002^{b}	0.434 ± 0.004^{a}	0.428 ± 0.006^{a}	11%
β-carotene	0.124 ± 0.005^{a}	0.129 ± 0^{a}	0.128 ± 0.001^{a}	0.127 ± 0.001^{a}	0.122 ± 0.05^{a}	2%
	0	4	11	21	31	Final % Loss
- New bottle [2]						
Retinol	0.80 ± 0.001^{d}	0.767 ± 0.014^{c}	0.755 ± 0.002^{bc}	0.747 ± 0.001^{ab}	0.737 ± 0.001^{a}	8%
α-tocopherol	0.482 ± 0.001^{b}	0.463 ± 0.001^{b}	0.46 ± 0.011^{b}	0.423 ± 0.011^{a}	0.417 ± 0.010^{a}	13%
β-carotene	0.124 ± 0.005^{b}	0.130 ± 0^{b}	0.127 ± 0.002^{b}	0.130 ± 0.003^{b}	0.113 ± 0.001^{a}	9%

† Testing conditions included 1: 0.02% sodium azide (NaN_3) added once with milk analyzed from the same container, 2: milk analyzed from a new container for every sampling time. Average values with the same superscript in a row are not significantly different ($p > 0.05$).

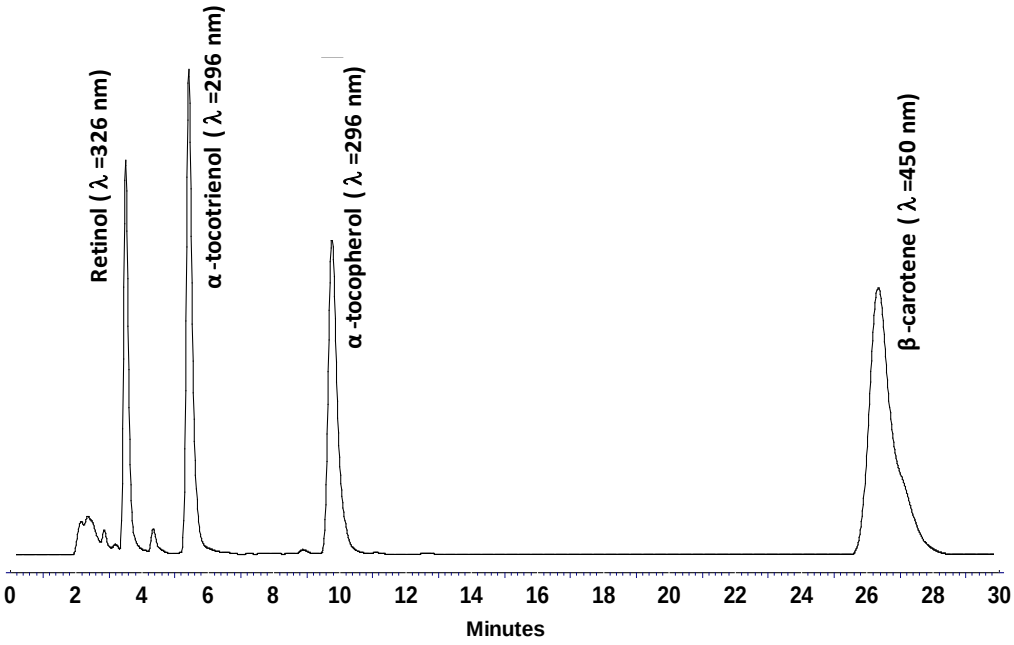


Figure 1. Chromatogram for a 1 mg L^{-1} and 10 mg L^{-1} standard solutions of vitamin A (retinol and β-carotene) and vitamin E (α-tocotrienol and α-tocopherol), respectively

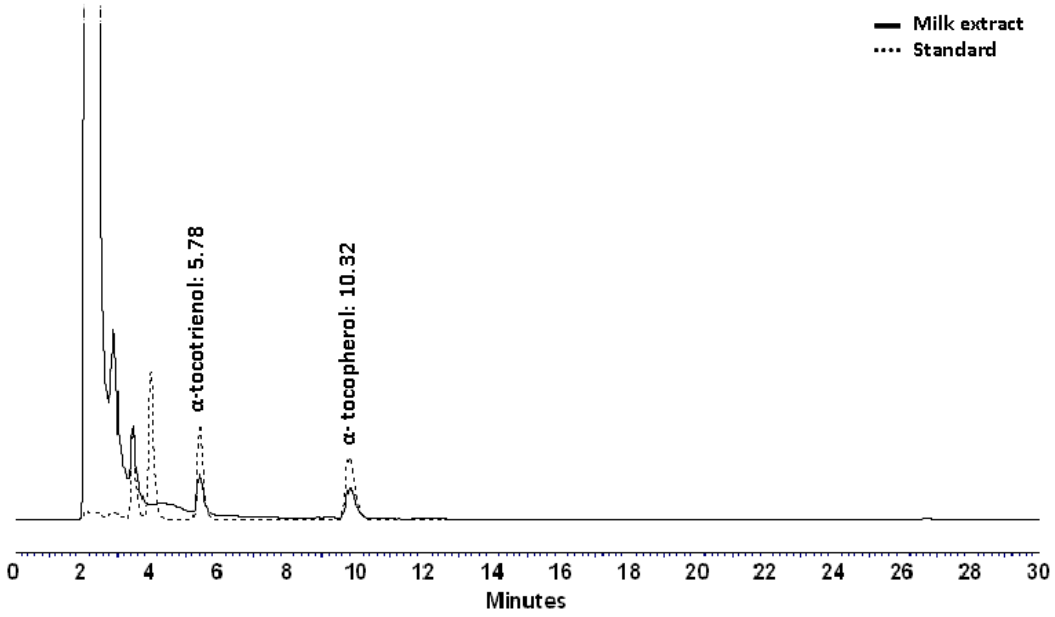


Figure 2. Chromatograms of 10 mg/ L standard solutions of vitamin E (α-tocopherol and α-tocotrienol) and milk samples with antioxidant TBHQ (2 mg/L) and spiked with 10 mg/L vitamin E