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Note

Azorean bovine milk conjugated linoleic acid. Effect of green pasture diet, storage and processing temperature

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Abstract – Conjugated linoleic acids (CLA) that are isomeric forms of the *cis*-9,*cis*-12 linoleic acid have sparked considerable interest in the scientific community due to their reported beneficial health properties. The objective of this study was the quantification of the CLA in commercial Azorean bovine milk and comparison with CLA in Portuguese mainland milk, and evaluation of the effect of storage and the processing temperature on the CLA content in milk. The relative average of CLA (*cis*-9,*trans*-11 isomer) content in Azorean (S. Miguel Island-Portugal) and Portuguese mainland milks represents 1.33 ± 0.09% and 0.79 ± 0.07% of total fatty acid methyl esters (tFAME), respectively. The *cis*-9,*trans*-11-CLA isomer variation in Azorean ultrapasteurized (UHT) milk, throughout the year, ranged from 1.16 ± 0.09% to 1.46 ± 0.09% and reflects the bovine pasture-fed diet (fresh grass) during all seasons and the mild winter in the Azores Islands. The CLA content of Azorean milk is a little higher than that published in the literature for New Zealand (1.20% of tFAME). The effect of the processing temperature on the CLA level shows a decrease in the *cis*-9,*trans*-11-CLA isomer content from 1.56 ± 0.05 (raw milk) to 1.40 ± 0.04 and 1.26 ± 0.04 (% to tFAME) in different pasteurized and UHT-treated milks, respectively. The effect of storage time on the CLA content of commercial UHT milk, kept refrigerated at 6–7 ºC for two months, shows a loss of 1.2%, which is negligible compared with the processing temperature effect. Azorean milk is naturally rich in CLA, and according to recently published literature, has a wide array of health benefits and may be useful in the prevention of some degenerative diseases.

milk / fatty acid / conjugated linoleic acid / gas chromatography

摘要 – 青草饲料、贮藏和加工温度对亚速尔群岛牛奶中共轭亚油酸的影响。 共轭亚油酸 (conjugated linoleic acid, CLA) 是指一类亚油酸的位置和空间共轭二烯异构体，其中 *cis-9, cis-12* 两种异构体被认为具有生理活性。本文定量地研究了亚速尔群岛牛奶中 CLA 的含量，并与葡萄牙本土牛奶中 CLA 含量进行了对比，同时评价了贮藏和加工温度对牛奶中 CLA 含量的影响。亚速尔群岛 (S. Miguel 岛 – 葡萄牙) 牛奶和葡萄牙本土牛奶中 CLA (*cis*-9,*trans*-11)的含量分别占总脂肪酸甲酯中的1.33 ± 0.09% 和0.79 ± 0.07%。由于亚速尔群岛的奶牛全年喂饲青草饲料和及冬季温和的气候，因而 UHT 杀菌的亚速尔群岛牛奶贮藏一年后 *cis*-9,*trans*-11-CLA 含量从 1.16 ± 0.09% 到 1.46 ± 0.09%。亚速尔群岛牛奶中 CLA 含量略高于文献报道的新西兰牛奶中 CLA 含量 (1.20%, 占总脂肪酸甲酯的百分含量) 。巴氏杀菌和 UHT 杀菌分别使 *cis*-9,*trans*-11-CLA 含量从 1.56 ± 0.05%(原料奶) 减少到 1.40 ± 0.04% 和

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1.26 ± 0.04%。市售的UHT奶在6–7 °C保存2个月，CLA含量减少了1.2%，与加工温度对CLA的影响相比，贮藏过程所带来的CLA损失可以忽略不计。目前关于CLA特殊保健功能的文献报道非常多，由于亚速尔群岛牛奶中富含CLA，因此，这种牛奶可能有助于预防一些退行性疾病。

牛奶 / 脂肪酸 / 共轭亚油酸 / 气相色谱

Résumé – Acide linoléique conjugué dans le lait de vache des Açores. Effet de l'alimentation à l'herbe et de la température de traitement et de stockage. Les acides linoléiques conjugués (CLA), qui sont des formes isomères de l'acide linoléique *cis*-9,*cis*-12, ont reçu beaucoup d'intérêt de la part de la communauté scientifique en raison des propriétés bénéfiques pour la santé qui leur sont attribuées. L'objectif de cette étude était de quantifier les CLA dans le lait de vache du commerce des Açores, de les comparer à ceux du lait du continent (Portugal) et d'évaluer l'influence de la température de traitement et de stockage sur la teneur en CLA du lait. La moyenne relative de la teneur en CLA (isomère *cis*-9,*trans*-11) du lait des Açores (Ile St-Miguel) et du lait du continent représente respectivement 1,33 ± 0,09 % et 0,79 ± 0,07 % des esters méthylés des acides gras totaux (tFAME). La variation en isomères CLA *cis*9-*trans*11du lait UHT des Açores au cours de l'année allait de 1,16 ± 0,09 % à 1,46 ± 0,09 % du fait de l'alimentation des vaches toute l'année à l'herbe de pâture en raison de l'hiver doux dans ces îles. La température de traitement avait pour effet de diminuer la teneur en isomère CLA *cis*9-*trans*11 (en % des tFAME) de 1,56 ± 0,05 (lait cru) à 1,40 ± 0,04 et 1,26 ± 0,04 pour les laits pasteurisés et UHT respectivement. L'effet du temps de stockage sur la teneur en CLA du lait UHT du commerce, conservé réfrigéré à 6–7 °C pendant 2 mois, montrait une perte de 1,2 %, ce qui est négligeable comparé à l'effet de la température de traitement. Le lait des Açores est naturellement riche en CLA, ce qui, d'après des publications récentes de la littérature, en fait un produit bénéfique pour la santé, utile dans la prévention de maladies dégénératives.

lait / acide gras / acide linoléique conjugué / chromatographie gazeuse

1. INTRODUCTION

Conjugated linoleic acid (CLA) is a term that refers to a mixture of geometric and positional isomers of *cis*-9,*cis*-12-octadecadienoic acid ($C_{18:2}$, ω-6) for which the two double bonds have a conjugated arrangement instead of methylene interruption. Dietary sources of CLA include milk fat, meat products and vegetable oils, particularly rapeseed, linseed, sunflower and soybean oils [1, 20]. Animal sources are richer in CLA than vegetable sources, and dairy products are thus one of the major dietary sources of which *cis*-9,*trans*-11-CLA is the major isomer (also called rumenic acid) [5, 6, 15]. Foods of ruminant origin generally contain more CLA than foods of non-ruminant origin [17]. CLA is formed in the bovine rumen by the anaerobic bacteria *Butyrivibrio fibrisolvens* as an intermediate step in biohydrogenation of unsaturated fatty acids (via linoleic acid isomerase) [1, 7, 13]. Chin et al. [5] have reported that the intestinal flora of rats is also capable of converting free linoleic acid into CLA. However, the major source is endogenous synthesis in body tissues (adipose and mammary tissue), catalyzed by Δ^9-desaturase, with the precursor being *trans*-11-$C_{18:1}$ (*trans* vaccenic acid) [12]. Figure 1 shows the production of the *cis*-9,*trans*-11-CLA isomer in ruminant fat by rumen biohydrogenation and tissue Δ^9-desaturase [2, 18]. The different conjugated isomers in ruminant milk and tissue are thought to be due to a combination of double-bond migration, and the action of specific *cis, trans* isomerases in the rumen [10]. The biological properties of dietary CLA are currently attracting the attention of the scientific community, because of its wide range of positive health effects, particularly anti-carcinogenic [3, 4, 24, 28], anti-atherogenic [19], anti-obesity [8], anti-diabetic [14], and enhancement of immunity properties [25], demonstrated in studies using different animal models. These effects appear to be mediated by 2 isomers of CLA, and the 2 biologically more active isomers

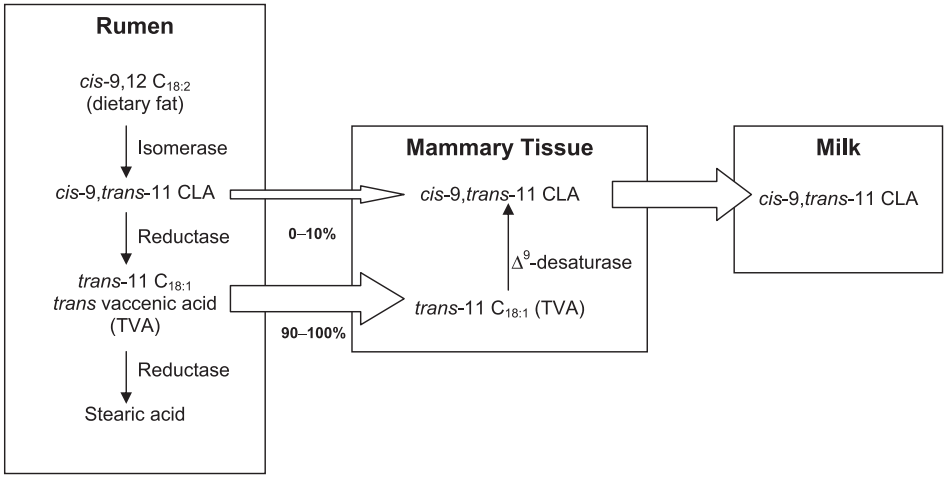

Figure 1. Production of *cis*-9,*trans*-11-$C_{18:2}$ in ruminant fat by biohydrogenation and tissue Δ^9-desaturase (a multienzyme complex that introduces a *cis* double bond between carbons 9 and 10 of fatty acids [2,18]).

are *cis*-9,*trans*-11 and *trans*-10,*cis*-12. In some cases an effect is produced by one of these isomers acting alone. Lately, there has been great deal of interest in the quantification of CLA in various foodstuffs. For example, Park et al. [23] have shown that the *trans*-10,*cis*-12-CLA isomer is responsible for inducing the physiological effect for the reduction of body fat, whereas the *cis*-9,*trans*-11-CLA isomer enhances growth in young animal models [22]. In other cases, the two isomers act together to produce a particular effect. Research has indicated that the *cis*-9,*trans*-11-CLA is found in milk and beef to a greater extent than the *trans*-10,*cis*-12-CLA [21]. The *cis*-9,*trans*-11 is the predominant isomer because there appears to be 2 routes of formation, representing ca. 80% of total CLA in bovine milk with minor amounts of a wide number of other isomers present [4, 27]. CLA is also present in human milk and in maternal placental blood, but not in infant formula, and it has been suggested that this factor explains the lower levels of food allergies, immune deficiencies and diabetes in children that are breast-fed [10].

The Azores (38º 30'N and 28º 00'W) is an archipelago of volcanic green islands in an isolated spot of the North Atlantic 1500 Km West of the Portuguese mainland and 3900 Km from the Eastern coast of the United States of America. The climate of the Azores is heavily influenced by the Gulf Stream, with no frost below elevations of 500 m and average temperatures of 15 ºC in winter and 22 ºC in summer. The soil is fertile and a large percentage, as much as 50%, has been converted into pasture for dairy cows.

Since UNILEITE half-skimmed commercial milk is the most consumed in Azores-S. Miguel Island (70% of the market), it was our goal to characterize the fatty acid (FA) profile, to study the variation in CLA content in bovine milk throughout the year in comparison with different diets from other geographical regions, particularly from the Portuguese mainland, to investigate the impact of cows grazing pasture (fresh grass) on the CLA content in milk, and to evaluate the effect of storage and the processing temperature on the CLA level of Azorean milk fat.

2. MATERIALS AND METHODS

2.1. Milk samples and chemicals

High-quality raw, pasteurized and ultra-pasteurized (UHT) bovine milk samples,

from Azorean lactating Holstein cows, were kindly donated by UNILEITE, U.C.R.L. (Arrifes, S. Miguel, Azores), and also obtained from local stores on the Portuguese mainland. The raw milk was collected in sterilized flasks, refrigerated at 4 ºC (< 8 h) and, after fat adjustment (1.5%), was stored at –20 ºC (storage time of up to 2 weeks) before extraction of FA and analysis. The commercial pasteurized and UHT bovine milk samples are homogenized mixtures of milk taken from cows of different ages, in several lactation phases, and obtained from different farms at different times of the day (morning and evening). All solvents and reagents, analytical grade, were purchased from Fluka Chemika (Steinheim, Switzerland), Seelze-Aktiengesellschaft (Riedel-de-Haën, Germany) and E. Merck (Darmstadt, Germany). The deionized water used for sample preparation was obtained with a Millipore water purification system (Millipore, Bedford, MA, USA). Derivatization reagents (sodium methoxide in methanol and 14% boron trifluoride in methanol) were obtained from Alltech Associates (Deerfield, IL, USA). Fatty acid methyl esters (FAME) and methylated conjugated linoleic acid (CLAME) standards and 2-amino-2-methylpropanol were purchased from Sigma Chemicals (St Louis, MO, USA).

2.2. Sample preparation

The milk samples were taken in triplicate, from raw, pasteurized and commercial lots of UHT half-skimmed milk. FA and, particularly, CLA were determined using a modified methodology described by Jiang et al. [17]. The alterations were introduced to minimize oxidation and the formation of artifacts during extraction and hydrolysis, and to achieve more complete hydrolysis of milk fat phospholipids and triacylglycerols.

2.3. Fat extraction

As CLA is prone to isomerization, care was taken in the extraction methodology to avoid this drawback [11]. A 15-mL milk sample was transferred into a separatory funnel, and 25 mL of isopropanol was added. After vigorous shaking, 20 mL of hexane was added, and the mixture was shaken again for another 3 min using a vortex. The mixture was then centrifuged at 2500 rpm for 10 min at 5 ºC, and the upper layer (hexane plus fat) was quantitatively transferred to a second separatory funnel. The lower layer was extracted twice, in the same conditions, with 10 mL of hexane, and the supernatants were pooled with the previous hexane layer. After addition of 10 mL of 0.5 $mol \cdot L^{-1}$ aqueous Na_2SO_4, the hexane layer (upper layer) was collected into a centrifuge tube and kept at –20 ºC for 20 min. After reaching room temperature, the material was centrifuged at 2500 rpm for 5 min at 5 ºC, quantitatively transferred to a flask and evaporated with a rotary evaporator at 37 ºC. The residue, after being dissolved in dichloromethane (5 mL), was dried with anhydrous sodium sulfate (passing through a Pasteur pipette with ca. 300 mg) in order to remove traces of water. Finally, the sample was dried under a stream of dry nitrogen or with a rotary evaporator to constant weight. The net fat was capped under nitrogen and stored, if necessary, at –20 ºC until further analysis. The gravimetric determination of the fat was performed using a microbalance (Mettler-Toledo, AG Grifensee, Switzerland).

2.4. Hydrolysis and derivatization procedure

Prior to gas chromatography (GC) analysis, the FA must be converted into derivatives, mostly methyl esters. There are standard methodologies used to derivatize unconjugated FA, but some of the methods, particularly those using an acidic catalyst under vigorous experimental conditions, are unsuitable for use with conjugated free FA. According to Park et al. [23], no single method will methylate CLA without any problems, either changing isomer distribution or generating artifacts or both. Thus, the choice of the methylating reagent was critical for the analysis of CLA. For this procedure, we compared the results of three methods: sodium methoxide/methanol, sodium methoxide/methanol using boron trifluoride as a catalyst and a slightly modified method of Jiang et al. [17].

Duplicate samples of 6.3 mg each of milk fat were transferred into a screw-capped (PTFE-faced septa) Pyrex tube, dried under nitrogen and hydrolyzed into a free FA by addition of 0.5 mL of 2 mol·L^{-1} KOH in ethanol. The test tubes were then sealed under N_2 and heated in a heating block with continuous shaking (every 2 min) for 15 min at 80 ºC. After cooling, 1.5 mL of 14% boron trifluoride in methanol was added and the mixture was again heated (heating block) at 80 ºC for 15 min. The reactions were always carried out using freshly prepared reagents to avoid the effects of interisomerization and intraisomerization of CLA isomers. These effects were insignificant, as already described by Werner et al. [29], but at temperatures above 80 °C and for more than 20 min there was a slight change in CLA isomer distribution and artifact formation in a time- and/or temperature-dependent manner. After cooling to room temperature, 1 mL of distilled water (saturated with NaCl) plus 1 mL of hexane were added. The mixture was vortexed vigorously for 1 min, and the upper layer (hexane plus fat) was quantitatively transferred to a 5-mL vial. The lower layer was extracted twice, in the same conditions, with 1 mL of hexane, and the supernatants were pooled with the previous hexane layer. The combined hexane extracts were dried with anhydrous sodium sulfate in order to remove traces of water, and collected in another 5-mL vial. The solution was dried under a stream of dry nitrogen. The residue was reconstituted with 300 µL of hexane, quantitatively transferred into a micro-vial and injected into GC. The whole derivatization procedure was carried out in subdued light to reduce the light-induced isomerization. Figure 2 shows schematically the protocol adopted during this study, that under rigorous conditions (fresh reagents and incubation temperature not higher than 80 ºC) generated less artifacts (allylic methoxides) and a superior recovery of 4.5 ± 1.5% as compared with the other two derivatization methods referred to.

2.5. Analytical gas chromatography

Total fatty acid methyl esters (tFAME) of the extracted fat were analyzed on a Hewlett-Packard model 5890 Series II capillary gas chromatograph fitted with a flame ionization detector, using a HP autoinjector model 7973, linked to a HP 3365 Chemstation. The instrument was fitted with a split/splitless injector (a split ratio of 45:1 was used) and a capillary column of fused silica coated with CP-Sil 88 (0.25 mm i.d. × 100 m in length, 0.20-µm thickness) (Chrompack, Middelburg, The Netherlands). The temperature was held at 60 ºC for 2 min, programed at a rate of 20 ºC·min^{-1} to 175 ºC and then held at this temperature for 50 min. The temperature was again increased to 220 ºC at a rate of 4 ºC·min^{-1} and held for an additional 30 min. Helium was the carrier gas at a flow rate of 1.66 mL·min^{-1} and pressure programing (inlet pressure) was used in constant flow mode at 180 KPa. The injector and detector temperatures were held constant at 250 ºC and 300 ºC, respectively.

The FAME and CLAME standards used were injected in the same analytical conditions. The percentage of each FA, after correction with the detector response factor (determined from a FAME standard sample) for each individual FAME, was calculated by dividing the area under the FA peak by the sum of the areas under the total reported FA peaks. The GC/MS was carried out with a VG Analytical ZAB-SE mass spectrometer (Fisons, PA, USA) interfaced with a Hewlett-Packard model 5890A gas chromatograph using the same analytical conditions described above, after the methyl esters were converted into dimethyloxazoline (DMOX) derivatives according to the Fay and Richli [9] procedure. The mass spectrometer conditions were: electron impact ionization at 70 eV, source temperature 200 ºC, 100 µA trap current, and the sweep time was 1.5 s·decades^{-1} scan, with a mass range of 20 to 500 a.m.u. Diagnostic ions in the gas chromatography-electron ionization (GC-EIMS) of CLA isomers were successfully used for the double-bond location in the CLA mixtures. We also used the selected ion monitoring to confirm the presence of different CLA isomers.

2.6. Statistical evaluation

Results are expressed as mean values (% of tFAME), and the relative standard

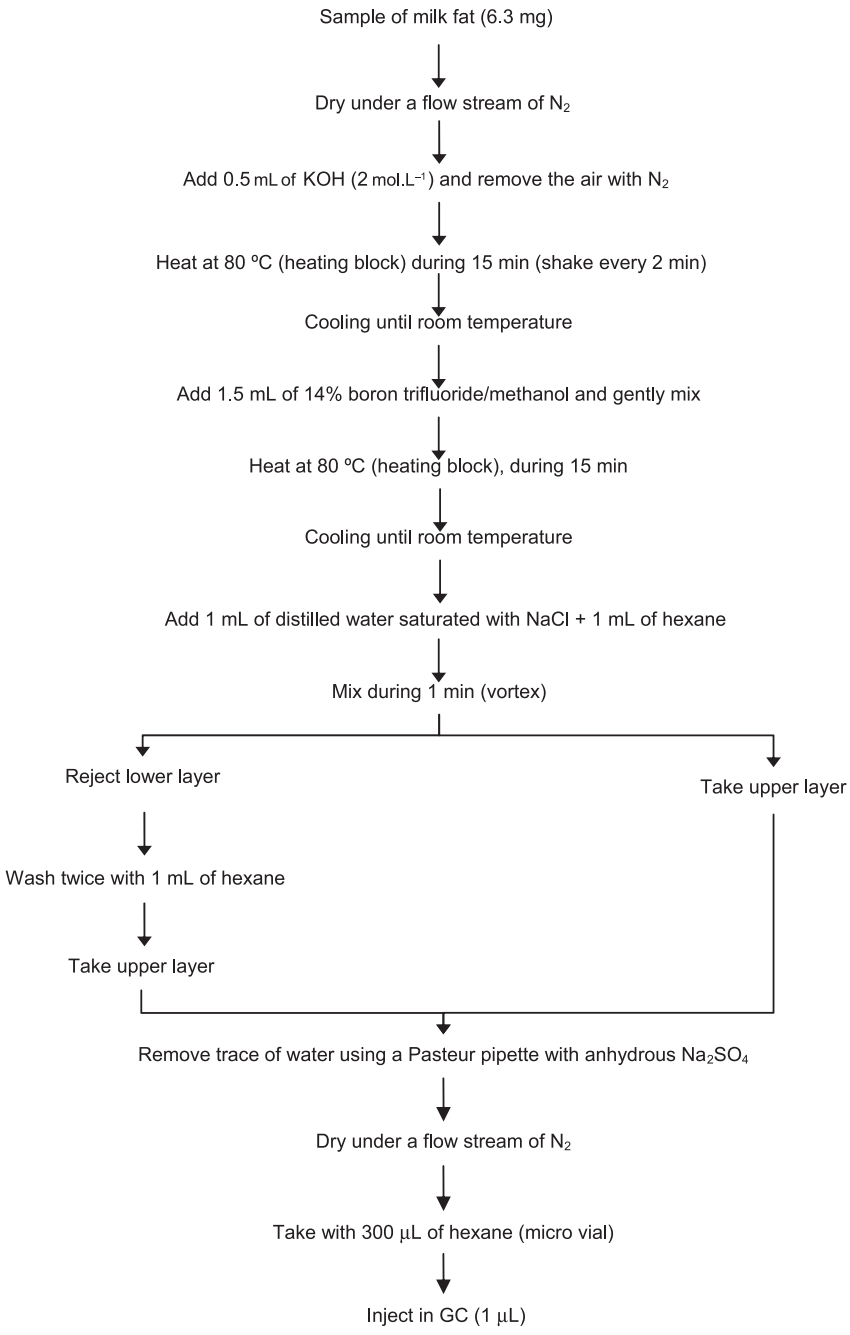

Figure 2. Protocol of the milk fat methylation procedure.

deviation (RSD) is reported. For the recovery determination, known amounts of standard CLA were added to one sample, which was subjected to chromatography analysis, and then the entire analytical scheme was performed, including lipid extraction, hydrolysis, methylation and GC analysis. The sample was spiked at three different concentrations of CLA and the recovery was calculated based on the difference between the total amount determined in the spiked samples and the amount observed in the non-spiked samples. All analyses were carried out in triplicate. The ability to yield repeatable results in GC analysis was evaluated in intraday and interday measurements of the retention time by repeated injections ($n = 5$) of a prepared UHT milk sample of a known level of *cis*-9,*trans*-11-CLA isomer. The results are expressed in relative percentage of each FA, calculated by internal normalization of the chromatographic peak area. FA and, particularly, CLA identification was carried out by comparison of the retention times with the FAME and CLAME pure standards (time window 0.5%) or coelution with the referred standards, and confirmed by GC/MS determinations.

2.7. Effect of storage and processing temperature on the CLA content

Heat, aging and protein as initiators of a free radical-type oxidation of $C_{18:2}$ may be the cause of the CLA level variation during the milk processing. The effect of processing temperature on the Azorean milk CLA content was investigated in raw, pasteurized (temperature of 60 ºC for 20 min) and UHT (temperature of 138 ºC for 3 sec) milk samples ($n = 3$) using the same analytical conditions and samples (from the same raw milk batch) with the same content of lipids. The effect of storage was also investigated between a fresh UHT milk sample and one kept refrigerated at 6–7 ºC for 2 months.

3. RESULTS AND DISCUSSION

From the public health perspective, animal products contribute to the nutrients in our food supply [16]. They also contain micro-components that have some beneficial effects on human health and disease prevention, and CLA represents one of these micro-components that has attracted the attention of the scientific community, because of its wide potential health benefits. There have been no reports on the CLA content in Azorean bovine milk.

The methylation of the FA, and particularly CLA, using the protocol described in Figure 2 were completed, with the results being confirmed by TLC (silica gel-60). A slight, insignificant increase in the *cis*-9, *trans*-11-CLA isomer was observed during the 2-wk period of refrigerated storage, but this effect was negligible up to 2 wk at a storage temperature of –20 ºC.

The position of the double bonds in CLA isomers was determined from the mass spectra of the DMOX derivatives. The diagnostic features of the GC-EIMS were the molecular ion at *m/z* 333, showing the presence of the $C_{18:2}$ DMOX derivative, and the presence of *m/z* 182, 196, 208, 222, 234, 262, 276, the gaps of 12 a.m.u. between *m/z* 196 and 208 and between *m/z* 222 and 234 to locate the double-bond positions, and the intense allylic cleavage ions at *m/z* 182 and 262 (and an intense ion at *m/z* 276) show the presence of the *cis*-9,*trans*-11-CLA isomer. Selected-ion recording (SIR) was a useful tool to verify GC peak assignments based on FID data. For the same positional isomer, the *cis,trans* consistently eluted before the *trans,cis* CLA isomers. The elution temperatures of the DMOX derivatives were about 10 ºC higher than those required by the corresponding FAMEs.

A typical chromatogram of Azorean milk FAME is displayed in Figure 3, with an expansion between the 60–80 min retention times where the CLA isomers were eluted. Figure 4A shows the *cis*-9,*trans*-11-CLA isomer variation throughout the year (2005), ranging from 1.16 ± 0.09% to 1.46 ± 0.09% of tFAME. This small variability reflects the bovine pasture-fed diet (fresh grass) during all seasons and the mild winter in the Azores Islands. The CLA (*cis*-9,*trans*-11 isomer) content in milk is a little higher than that published by Jensen [16] for New Zealand (1.20% of tFAME) (see Tab. I). Figure 4B illustrates the average of

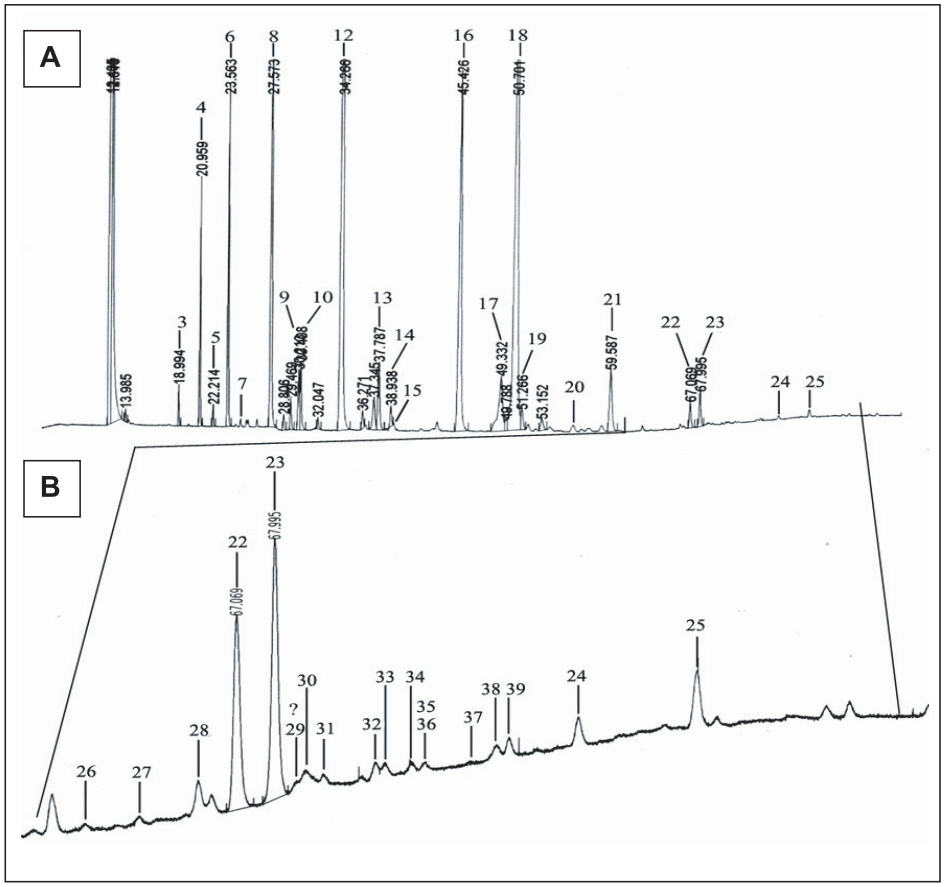

Figure 3. Azorean UHT milk FAME profile. Analytical conditions: Column CP-Sil 88, 100 m × 0.25 mm i.d., 0,2-μm film. Column temperature 60 ºC (2 min hold), 175 ºC at 20 ºC·min^{-1} (50 min hold), 220 ºC at 4 ºC·min^{-1} (30 min hold). Injector 250 ºC and detector (FID) 300 ºC. Flow 1.66 mL·min^{-1} (helium), inlet pressure 180 kPa, split ratio 45:1. Legend: (**A**) Total profile of FAME: 3 – C8:0, 4 – C10:0, 5 – C11:0, 6 – C12:0, 7 – C12:1, 8 – C14:0, 9 – C14:1, 10 – C15:0, 11 – C15:0 (iso), 12 – C16:0, 13 – C16:1, 14 – C17:0, 15 – C17:1, 16 – C18:0, 17 – C18:1(*trans* isomers), 18 – C18:1 (c9), 19 – C18:1 (c7), 20 – ?, 21 – C18:2 (c9,12), 22 – C18:3 (c9,12,15), 23 – C18:2 (c9,t11)-CLA, 24 – C21:0, 25 – ?. (**B**) Chromatogram expansion between 60 and 80 min: 26 – C20:0, 27 – C20:1 (c11), 28 – C18:3 (c6,9,12), 29 – C18:2 (t8,c10)-CLA, 30 – ?, 31 – C18:2 (t10, c12)-CLA, 32 – C18:2 (c8,10)-CLA, 33 – C18:2 (c9,11)-CLA, 34 – C18:2 (c10,12)-CLA, 35 – C18:2 (c11,13)-CLA, 36 – C20:2 (c11,14), 37 – C18:2 (t11,13)-CLA, 38 – C18:2 (t8,10 + t9,11 + t10,12)-CLA, 39 – C20:3 (c8,11,14).

the milk *cis*-9,*trans*-11-CLA isomer content of milk in the two geographical regions, the Azores (S. Miguel-Portugal) and the Portuguese mainland, with 1.33 ± 0.09% and 0.79 ± 0.09% of tFAME, respectively. The lower value for the Portuguese mainland reflects the different diet, particularly during the cold seasons with a mixed ration feeding. Lawless et al. [20] have also observed substantial variation in CLA content of milk fat among individual cows fed the same diet. This situation was not

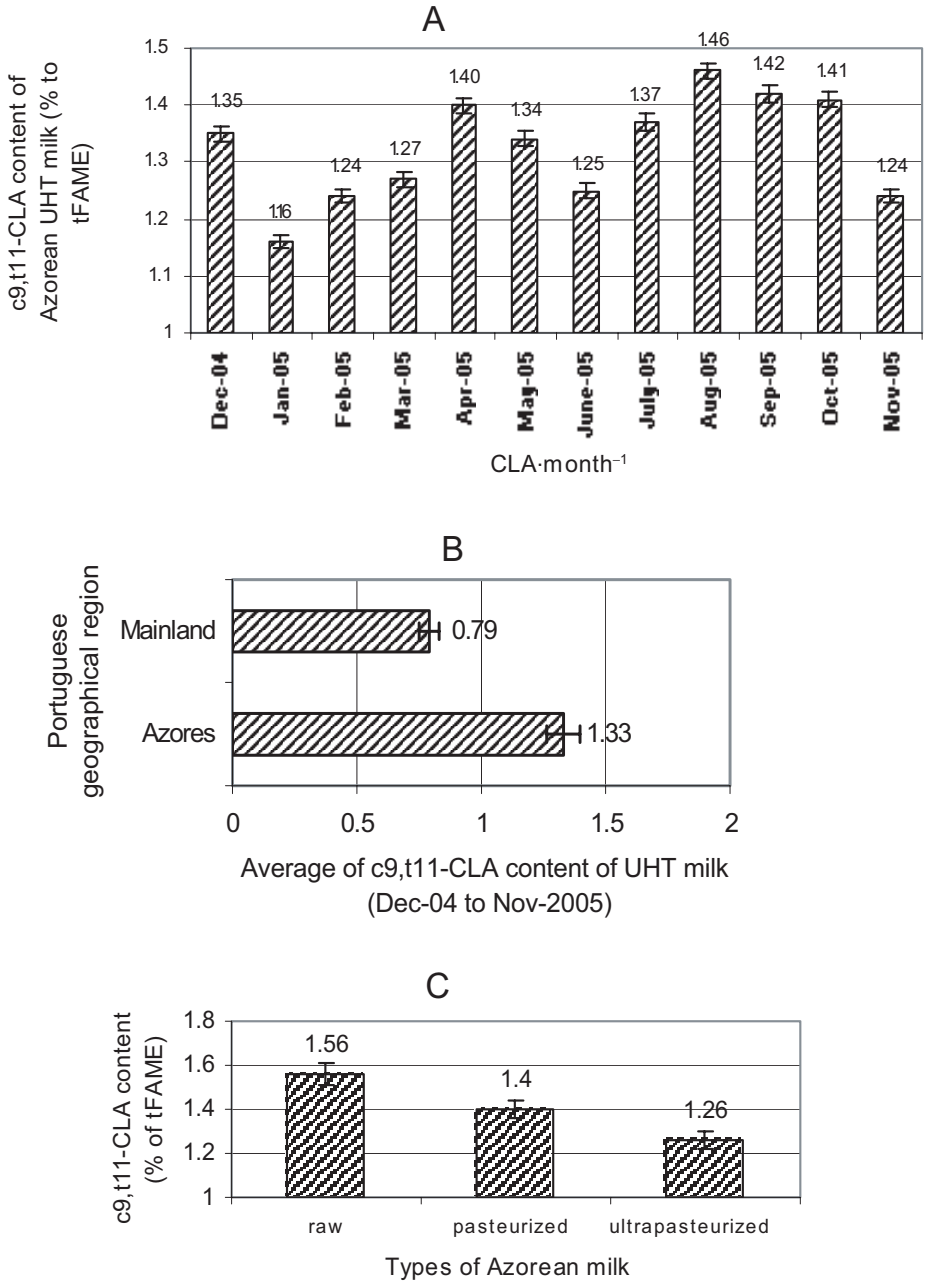

Figure 4. A. Variability in the *cis*-9,*trans*-11-CLA isomer content of Azorean UHT milk throughout the year (2005). **B.** Comparison of the *cis*-9,*trans*-11-CLA isomer content of Azorean UHT milk versus Portuguese mainland UHT milk. **C.** Effect of processing temperatures (pasteurization and ultrapasteurization) on the *cis*-9,*trans*-11-CLA content of Azorean UHT milk.

Table I. Mean contents of the *cis*-9,*trans*-11- CLA isomer of some European countries, the United States and New Zealand in comparison with Azorean and Portuguese mainland milks' content.

Country[1]	Mean	Standard deviation	Minimum	Maximum	*n*
Portugal (Mainland)[a]	0.79[f]	0.09	0.68	0.92	[h]
Portugal (Azores)[b]	1.33[f]	0.09	1.16	1.46	[h]
Germany[c]	0.75[g]	0.38	0.10	1.89	1738
Germany[d]	0.45[g]	0.13	0.10	1.05	909
Germany[e]	0.76[g]	0.15	0.19	1.19	236
Germany[b]	1.20[g]	0.20	0.49	1.89	593
Austria	0.92[g]	0.26	0.52	1.44	13
Belgium	0.76[g]	–	–	–	1
Denmark	0.87[g]	0.19	0.59	0.99	4
Spain	0.95[g]	0.09	0.82	1.11	10
France	0.74[g]	0.27	0.21	1.56	198
United Kingdom	1.03[g]	0.28	0.60	1.40	21
Great Britain	0.87[g]	0.02	0.85	0.89	4
Italy	0.94[g]	0.31	0.63	1.67	12
Ireland	1.41[g]	0.31	0.56	1.82	23
Luxembourg	0.67[g]	–	–	–	1
Netherlands	0.73[g]	0.28	0.36	1.33	63
Sweden	0.56[g]	–	–	–	1
United States	0.45[g]	–	–	–	–
New Zealand	1.20[g]	–	–	–	1

[1] Adapted from Precht and Molkentin [26]. [a] = mixed ration feeding; [b] = pasture feeding; [c] = all feeding periods; [d] = barn feeding; [e] = transition period; [f] = this study; [g] = literature; [h] = from ca. 20 000 L of milk.

investigated because our aim was the quantification of CLA in commercial milk. The effects of pasteurization and other processing temperatures on milk are academic, since these treatments are required and must be used to produce safe commercial milk. The effect of the processing temperature on the CLA level was investigated by comparison of CLA content in three milk samples (raw, pasteurized and UHT from the same raw milk batch). The results are illustrated in Figure 4C and show the decrease in the *cis*-9,*trans*-11-CLA isomer content of Azorean milk from 1.56 ± 0.05 (raw milk) to 1.40 ± 0.04 and 1.26 ± 0.04 (% to tFAME) in different pasteurized and UHT-treated milks, respectively. Heat as initiator of a free radical-type oxidation of $C_{18:2}$ may be the cause of CLA content variation during the temperature milk treatments. The effect of storage time on the CLA content of commercial UHT milk was also investigated between a fresh UHT sample and one kept open and refrigerated at 6–7 ºC, for two months. The results show a loss of 1.2%, which is negligible compared with the processing temperature effect. These results are similar to those published by Jensen [16]. All the quantitative analyses of the *cis*-9,*trans*-11-CLA isomer were achieved using the external standard method, and the isomer is expressed in percentage relative to tFAME.

The efficiency of the FA recovery from milk samples was measured in five replicate extractions, hydrolysis and GC analysis.

Table II. Recovery of the *cis*-9,*trans*-11-CLA isomer from an Azorean UHT milk sample, using the methodology described in Materials and Methods.

Sample[1] (ng)	Added (ng)	Found (ng)	Recovery (%)	RSD[2] (%)
279	100.00	336.93	88.9	6.50
279	150.00	396.01	92.3	4.34
279	200.00	434.02	90.6	5.52

[1] CLA *cis*-9,*trans*-11 isomer content in 1 μL of GC injection volume.
[2] Relative standard deviation.

All lipid classes (triglycerides and phospholipids) were completely hydrolyzed, except the sphingomyelins. However, this type of lipid only contributed ca. 0.15% of the bovine milk fat and mainly contained long-chain FA ($C_{22:0}$ to $C_{24:0}$). The accuracy of the CLA *cis*-9,*trans*-11 isomer determination methodology was evaluated by determining the recovery of CLA in a sample of UHT milk of a known level (previously determined) of the *cis*-9,*trans*-11-CLA isomer. Three different amounts of standard were added to the sample (injection volume of 1 μL containing 279 ng of *cis*-9,*trans*-11-CLA isomer), which was subjected to GC analysis. The recovery was calculated based on the difference between the total concentration determined in the spiked samples and the concentration in the non-spiked samples. The results with the RSD are shown in Table II. The RSD was better than 6.5% and the mean recovery ranged from 88.9% to 92.3%, indicating the high degree of the method's accuracy for determination of the *cis*-9,*trans*-11-CLA isomer under the analytical conditions used. Similar recoveries have been reported by others [16]. The limit of *cis*-9,*trans*-11-CLA isomer detection, defined as the amount of injected sample which gave a signal to noise ratio of 3, was determined to be 4.75 ng. The repeatability of the results increased by keeping the experimental conditions strictly the same during all analyses. The repeatability of the GC analysis evaluated in intraday and interday measurements of the retention time by repeated injections (n = 5) of a known level of *cis*-9,*trans*-11 isomer solution of CLAME (0.279 $\mu g \cdot \mu L^{-1}$) shows that the RSD intraday repeatability was 1.21%, whereas the interday precision (data acquired over a period of 5 days) was better than 3.51%.

Table III. Composition (wt% of tFAME) of Azorean UHT milk long-chain fatty acids (average of one-year period – mean + standard deviation, n = 3).

Fatty acids	Mean ± SD	Minimum	Maximum
C10:0	2.57 ± 0.31	2.14	3.11
C11:0	0.31 ± 0.03	0.27	0.37
C12:0	4.17 ± 0.31	3.81	4.86
C14:0	13.18 ± 0.59	12.37	14.54
C14:1	1.09 ± 0.07	1.01	1.19
C15:0	1.60 ± 0.11	1.44	1.79
C16:0	34.87 ± 1.06	33.01	36.44
C16:1	1.82 ± 0.10	1.64	1.98
C17:0	0.94 ± 0.26	0.62	1.24
C18:0	11.24 ± 0.54	10.36	12.13
C18:1t	2.50 ± 0.25	2.15	2.98
C18:1c	21.99 ± 1.16	20.04	23.73
C18:2	1.99 ± 0.14	1.80	2.17
C20:1	0.60 ± 0.08	0.48	0.77
C18:2conj[a]	1.11 ± 0.08	0.97	1.22
CLA (total)	1.33 ± 0.09	1.16	1.46

[a] *cis*-9,*trans*-11-CLA isomer.

Table III shows the average (one-year period) composition of the long-chain FA of the Azorean UHT milk fat.

4. CONCLUSION

Dairy products are one of the major dietary sources of CLA, which is almost entirely the *cis*-9,*trans*-11-$C_{18:2}$ isomer. The CLA in ruminant milk results from incomplete biohydrogenation of dietary linoleic acid in the rumen and endogenous synthesis in body tissues, catalyzed by Δ^9-desaturase with the precursor being *trans*-11-$C_{18:1}$ (*trans* vaccenic acid). Several

dietary factors can affect the concentration of CLA in milk fat [21]. These include feed consumption, seasonal variation, stage of maturity and preservation of forages. In the present study, substantial variation in CLA content in milk between bovines of two different geographical regions was observed. The CLA content in Azorean milk has little variation throughout the year, and reflects the mild climate all year long. Higher concentrations of CLA are found when cows are fed mainly on fresh grass rather than mixed ration feeding. The temperatures of pasteurization and ultrapasteurization decreased the CLA content in the bovine milk samples. The present findings indicate that storage of milk-based products with CLA content should be kept to a practical minimum time and preferably at a temperature as low as possible.

The published scientific literature about CLA, using animal studies and clinical trials, is growing at a phenomenal rate and indicates the evidence that CLA has a wide array of health benefits and may be useful for preventing some diseases, and consequently improving human health [10]. Since, in the Azores Islands there are ca. 110 000 milk cows for a population of 240 000 people, milk-based products are very common in the Azorean diet. This fact could be an issue for a future epidemiological study, comparing some of the reported physiological effects of CLA (anti-carcinogenic, reduction of body fat gain, reduction of atherosclerosis, enhances the immune function, reduction of symptoms of diabetes and reduction of hypertension) [10] in the Azorean population with those in populations of other geographical regions, less dependent on milk-based products.

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