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Conjugated linoleic acid content and isomer distribution during ripening in three varieties of cheeses protected with designation of origin

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Abstract

Cheeses have been identified as important sources of conjugated linoleic acid (CLA), a mixture of positional and geometric isomers with potential anticarcinogenic activity and other beneficial properties. The objectives of this study were to examine the effects of ripening on the overall CLA content as well as on the isomers profile using GC and Ag^{+}-HPLC. Three Spanish cheeses Protected with Designation of Origin (Mahón, Manchego and Cabrales) were manufactured in different cheesemaking plants and monitored at different times during the ripening period. Total CLA content varied from 3 to 9 mg/g of total fatty acids and rumenic acid (9-*cis*, 11-*trans* C18:2, RA) represented more than 75% of total CLA. After RA, 7–9 (*cis*/*trans* plus *trans*/*cis*), 11-*trans*, 13-*trans* and 11-*trans*, 13-*cis* C18:2 were the main CLA isomers. The results achieved confirm that the effect of ripening on the total CLA concentration and isomer distribution was negligible.

Keywords: CLA; Cheese; Milk fat; Ripening

1. Introduction

Conjugated linoleic acid (CLA) is a collective term that refers to geometrical and positional isomers of octadecadienoic acid (C18:2) containing conjugated unsaturation. The major CLA isomer in natural products is 9-*cis*, 11-*trans*, also known as rumenic acid (RA), produced predominantly by ruminants as a first intermediate of biohydrogenation of linoleic acid by rumen bacteria and by endogenous conversion of vaccenic acid (11-*trans* C18:1), an intermediate of polyunsaturated fatty acid biohydrogenation in the rumen, by Δ9-desaturase (Bauman, Corl, & Peterson, 2003). Data from animals models have been used to suggest that RA was responsible for CLA anticarcinogenic, growth-promoting, as well as antiatherogenic properties (Khanal, 2004; Lee, Lee, Cho, & Kim, 2005), whereas 10-*trans*, 12-*cis* C18:2, a minor isomer, has lean body mass-enhancing properties (Pariza, 2004). If these advantages were transferred to humans, increased RA consumption could have a positive effect on the nutritional value of the food containing it.

The principal dietary sources of CLA are milk and dairy products. Concentrations of CLA in milk have been reported to vary markedly between European countries from 0.1 to 1.9 g/100 g (Precht & Molkentin, 2000). The CLA content of milk can be increased through manipulation of the diet, such as grazing on pasture or using supplements of dietary polyunsaturated fatty acids, in the form of oilseeds or as free oils (Stanton, Murphy, McGrath, & Devery, 2003). The effect on CLA content of post-milking related factors, such as processing conditions, storage, ageing, etc., or converting one product into another, such as milk into cheese is still, however, disputed (García-López, Echeverría, Tsui, & Balch, 1994; Ha, Grimm, & Pariza, 1989; Luna et al., 2004; Luna, De la Fuente, & Juárez, 2005; Shanta, Decker, & Ustunol, 1992; Shanta, Ram,

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O'Leary, Hicks, & Decker, 1995; Van Nieuwenhove, González, Pérez-Chaia, & Ruiz-Holgado, 2004). As regards cheeses, the application of heat, the use of different fermentation cultures, or ripening periods could potentially modulate the CLA level in the final foodstuff (Chin, Liu, Storkson, Ha, & Pariza, 1992; Ha et al., 1989; Lin, Boylston, Chang, Luedecke, & Shultz, 1995; Lin, Boylston, Luedecke, & Shultz, 1999; Shanta et al., 1992). Although, from these studies, it can be deduced that the overall CLA content would not be excessively affected by ageing, the results are not conclusive.

Although different works have reported a detailed analysis of CLA minority isomers in different varieties of cheeses (Lavillonière, Martin, Bougnoux, & Sébédio, 1998; Rickert et al., 1999; Sehat et al., 1998), information about their evolution during manufacturing is very limited. Werner, Luedecke, and Shultz (1992) suggested that different starter cultures, processing conditions and ageing periods did influence the CLA isomer distribution in Cheddar, Coulgar and Viking cheese. Gnädig et al. (2004) however, did not detect changes in CLA isomer profile during Emmental cheese manufacturing process. Therefore more research would be necessary to clarify how the proportion of CLA isomers develops during processing and ageing of cheese.

The objectives of this study were to examine the effects of ripening on the overall CLA content and the isomers profile in three Spanish Protected with Designation of Origin (PDO) natural cheeses. PDO cheeses coming under a designation of origin scheme have to be made with milk from selected breeds of livestock adapted to the environment, fed and handled in due accordance with regulations to obtain high quality products. Maintenance or enhancement of the CLA content of PDO cheeses through alterations in manufacturing parameters would contribute to increased nutritional value and consumer acceptability of these dairy products.

2. Materials and methods

2.1. Samples

Three representative PDO cheese varieties on the Spanish market were studied. Mahón is a semi-hard non-cooked pressed cheese, salted in brine, and produced on the island of Menorca from cows' milk. Three Mahón cheese samples (samples A–C) were made in three different plants, one artisanal cheesemaker (sample C) who used raw milk and two industrial manufacturers (samples A and B) that used pasteurised milk. All cheeses were made according to the same cheesemaking procedures. The milk was coagulated for a minimum time of 30–40 min at 32 °C. The curd was cut to obtain chickpea-sized lumps and was then left to settle for 10 min before draining off the whey. After pressing and salting, the cheeses were dried in ventilated rooms and then taken to the maturing rooms where they stayed. To carry out the study of CLA composition during ripening, samples of each of the raw milks and cheeses at 2, 60, 120 and 180 days were taken.

Manchego is a semi-hard pressed cheese made in the La Mancha region from locally produced ewes' milk. Three Manchego cheese samples (samples D–F) were made at three different plants, one artisanal cheesemaker (sample F), who used raw milk, and two industrial manufacturers (samples D and E) who used pasteurised milk. In all cases, cheesemaking was conducted under identical conditions. The milk was coagulated for a minimum time of 45 min at 30 °C. The resultant curd was progressively cut until obtaining lumps of 5–10 mm. The mass was then stirred and reheated to 40 °C and moulded. After moulding, the cheeses were pressed, salted in brine and finally ripened for six months. Samples of each of the different raw milks and cheeses at 2, 60, 120, and 180 days of ripening were taken.

In order to evaluate the influence of moulds during cheese ripening, the most representative of the mould-ripened cheese varieties manufactured in Spain was also studied. Three Cabrales cheese samples (samples G–I) were made in three different plants of the province of Asturias from pasteurised cows' milk, according to the cheesemaking procedures described by Alonso, Juárez, Ramos, and Martín-Alvarez (1987). A commercial starter (*Flora Danica* MSP, Chr. Hansen, Denmark) was added, and moulds of *Penicillium* were grown naturally and ripened in natural mountain caves. Samples of all of the cheeses at 2, 30, 60 and 90 days of ripening were taken.

The main characteristics of all these cheeses are shown in Table 1. Bulk milk was used in all cases for cheesemaking.

2.2. Analytical methods

Milk and cheese fat extraction was carried out according to ISO-IDF (2001). The fat extract was stored at −20 °C before fatty acid derivatisation. Fatty acid methyl esters (FAME) were prepared by base-catalysed methanolysis of the glycerides (KOH in methanol) according to ISO-IDF (2002). The preparation of dimethyloxazoline (DMOX) derivatives from FAME was based on the Fay and Richli (1991) procedure.

FAME were separated by gas chromatography (GC) using a CP-Sil 88 fused silica capillary column (100 m × 0.25 mm i.d. × 0.2 μm film thickness, Chrompack, Middelburg, The Netherlands) on a Perkin–Elmer chromatograph (model 8420, Beaconsfield, UK) equipped with a flame ionisation detector. The column was held at 100 °C for 1 min after injection, temperature programmed at 7 °C/min to 170 °C, held there for 55 min, then temperature programmed at 10 °C/min to 230 °C and held there for 23 min. Helium was used as a carrier gas at 214 kPa. The sample (0.2 μl) was injected into a splitless injector. The areas of the CLA peaks were calculated as mg/g of fat using nonadecanoic acid (C19:0) methyl ester as internal standard. To obtain response factors, an anhydrous

Table 1
Characteristics of the Mahón, Manchego and Cabrales cheeses studied

Sample	Name of variety	Milk	Milk heat treatment	Microbiota	Period of ripening (months)
A	Mahón	Cow	Pasteurisation	Lactic acid bacteria	6
B	Mahón	Cow	Pasteurisation	Lactic acid bacteria	6
C	Mahón	Cow	No	Without starter	6
D	Manchego	Ewe	Pasteurisation	Lactic acid bacteria	6
E	Manchego	Ewe	Pasteurisation	Lactic acid bacteria	6
F	Manchego	Ewe	No	Without starter	6
G	Cabrales	Cow	Pasteurisation	*Flora Danica* + *Penicillium* mould	3
H	Cabrales	Cow	Pasteurisation	*Flora Danica* + *Penicillium* mould	3
I	Cabrales	Cow	Pasteurisation	*Flora Danica* + *Penicillium* mould	3

milk fat (reference material CRM-164) consisting of known amounts of fatty acids obtained from the European Commission (Brussels, Belgium) was also used. All fatty acid analyses were carried out in triplicate.

DMOX derivatives were analysed on an Agilent chromatograph (model 6890N, Palo Alto, CA, USA) equipped with a mass spectrometric detector. The filament trap current was 400 μA at 70 eV. Injections were under data system control with an auto-injector and a glass split injector insert packed with silanised glass wool. One microlitre of solution of DMOX derivatives was separated on the same column CP-Sil 88 in the following conditions: the oven temperature was 75 °C for 2 min after injection, then temperature programmed at 5 °C/min to 180 °C, held there for 30 min, then temperature programmed 5 °C to 220 °C, and held there for 30 min. Helium was used as a carrier gas at 197 kPa. The sample (1.0 μl) was injected in a splitless mode.

Silver-ion HPLC (Ag^+-HPLC) separation of CLA methyl esters was carried out using a chromatograph (Agilent Technologies, Series 1100, Palo Alto, CA, USA) equipped with a photodiode array detector at 234 nm. Three ChromSpher 5 Lipids analytical silver-impregnated columns (250 mm × 4.6 mm i.d. stainless steel, 5 μm particle size; Varian Ltd., Oxford, UK) were used in series. The mobile phase was 0.1% acetonitrile in hexane and operated isocratically at a flow rate of 1.0 ml/min as described by Sehat et al. (1998). The flow was initiated 0.5 h prior to sample injection and injection volume was 10 μl. All fatty acid analyses were carried out in triplicate. In order to identify the different compounds, a mixture (9-*cis*, 11-*trans*; 8-*trans*, 10-*cis*; 11-*cis*, 13-*trans*; 10-*trans*, 12-*cis* C18:2 and small amounts of a variety of all *cis* and all *trans* C18:2 isomers) and pure CLA methyl ester isomers (9-*cis*, 11-*trans* and 10-*trans*, 12-*cis* C18:2) were purchased from Nu-Chek Prep. Inc. (Elysian, MN, USA).

Analysis of variance was performed using the Statgraphic Statistical System (Rockville, MD).

3. Results and discussion

3.1. Total CLA content

Tables 2–4 show the CLA content determined by GC-FID in different types of cheese and its evolution during ripening. CLA levels varied widely from one cheese to another. The highest concentrations of CLA were found in Mahón cheese, where amounts near to 10 mg/g of fat were achieved. Manchego cheeses, some of the most consumed on the Spanish market, exhibited CLA levels around 6 mg per 100 mg of fat, whereas the smallest amounts were detected in Cabrales (<4 mg/g). These data are very similar to those obtained in previous studies with other blue cheeses. CLA concentrations reported in cheeses ripened with mould were around 5 mg/g of fat (Fritsche & Steinhart, 1998; Chin et al., 1992) although other authors (Lin et al., 1995) detected slightly higher contents.

The results of earlier studies on CLA content in different varieties of cheeses provide data with very wide variation. CLA values compiled by Parodi (2003) in cheeses manufactured in different countries ranged from 2.7 to 14.4 mg/g of fat, the interval in which this study's results are included. Chin et al. (1992) and Fritsche and Steinhart (1998) for example, found CLA ranges of 3–7 and 4–17 mg/g of fat in different varieties of cheese produced in the USA and Germany, respectively.

3.2. Effect of milk pasteurisation

As can be seen in Tables 2 and 3, those cheeses manufactured with pasteurised milk (samples A, B, D and E) after two days of manufacturing had CLA levels that were barely different from those in milk. This fact was common to cheeses manufactured with raw milk (samples C and F) that had not therefore been subject to any heating, which would indicate the negligible effect of this treatment on CLA content.

The influence of temperature on CLA formation during the manufacture of dairy products has been a topic that has aroused some controversy. In the first studies (Aneja & Murthi, 1991; Ha et al., 1989; Shanta et al., 1992) it would seem that moderate heating (<100 °C) could favour the formation of CLA during the manufacturing stages of different dairy products. However, data from most recent studies seem to show the opposite. The application of different thermal treatments during the manufacture of yoghurts (Luna et al., 2004) and processed cheeses (Luna et al., 2005) did not alter the CLA content. The use of different temperatures for cooking and moulding during the manufacture of Emmental cheese did not produce any

Table 2
Total conjugated linoleic acid (CLA) and rumenic acid (RA) content (mg/g fat) determined by gas chromatography of Mahón cheeses collected at different stages of manufacturing and ripening

	Mahón A		Mahón B		Mahón C	
	RA	Total CLA	RA	Total CLA	RA	Total CLA
Milk	7.5 ± 0.6^{a}	8.6 ± 0.7^{a}	7.8 ± 0.1^{a}	9.1 ± 0.1^{a}	5.3 ± 0.3^{a}	6.3 ± 0.3^{a}
2 days	7.1 ± 0.6^{a}	$8.1 \pm 0.8^{a,b}$	7.8 ± 0.1^{a}	$9.0 \pm 0.1^{a,b}$	5.7 ± 0.4^{a}	6.6 ± 0.4^{a}
2 months	6.4 ± 0.3^{a}	7.5 ± 0.1^{b}	8.0 ± 0.1^{a}	9.5 ± 0.1^{c}	5.7 ± 0.2^{a}	6.4 ± 0.5^{a}
4 months	7.5 ± 0.5^{a}	8.5 ± 0.3^{a}	7.8 ± 0.1^{a}	9.1 ± 0.1^{a}	5.4 ± 0.1^{a}	6.4 ± 0.1^{a}
6 months	6.5 ± 0.2^{a}	7.4 ± 0.2^{b}	7.4 ± 0.1^{b}	8.5 ± 0.2^{b}	5.2 ± 0.2^{a}	6.0 ± 0.4^{a}

[a,b,c] Values in the same column without a common letter are significantly different: $p \leqslant 0.05$.

Table 3
Total conjugated linoleic acid (CLA) and rumenic acid (RA) content (mg/g fat) determined by gas chromatography of Manchego cheeses collected at different stages of manufacturing and ripening

	Manchego D		Manchego E		Manchego F	
	RA	Total CLA	RA	Total CLA	RA	Total CLA
Milk	5.7 ± 0.4^{a}	6.5 ± 0.5^{a}	4.9 ± 0.3^{a}	5.7 ± 0.3^{a}	5.6 ± 0.3^{a}	6.5 ± 0.4^{a}
2 days	5.4 ± 0.2^{a}	6.2 ± 0.2^{a}	4.8 ± 0.2^{a}	5.7 ± 0.3^{a}	5.7 ± 0.3^{a}	6.4 ± 0.5^{a}
2 months	5.6 ± 0.7^{a}	6.1 ± 0.1^{a}	4.6 ± 0.1^{a}	5.4 ± 0.1^{a}	5.6 ± 0.4^{a}	6.5 ± 0.3^{a}
4 months	5.1 ± 0.2^{a}	6.0 ± 0.1^{a}	4.3 ± 0.2^{a}	5.2 ± 0.2^{a}	5.2 ± 0.4^{a}	6.1 ± 0.4^{a}
6 months	5.0 ± 0.2^{a}	5.6 ± 0.3^{a}	4.4 ± 0.2^{a}	5.2 ± 0.2^{a}	5.3 ± 0.2^{a}	6.2 ± 0.2^{a}

[a] Values in the same column without a common letter are significantly different: $p \leqslant 0.05$.

Table 4
Total conjugated linoleic acid (CLA) and rumenic acid (RA) content (mg/g fat) determined by gas chromatography of Cabrales cheeses collected at different stages of ripening

	Cabrales G		Cabrales H		Cabrales I	
	RA	Total CLA	RA	Total CLA	RA	Total CLA
2 days	3.4 ± 0.5^{a}	3.8 ± 0.5^{a}	2.7 ± 0.5^{a}	3.2 ± 0.4^{a}	3.2 ± 0.2^{a}	3.5 ± 0.3^{a}
1 month	3.1 ± 0.6^{a}	3.4 ± 0.7^{a}	2.7 ± 0.4^{a}	3.1 ± 0.4^{a}	3.1 ± 0.6^{a}	3.3 ± 0.5^{a}
2 months	3.2 ± 0.1^{a}	3.5 ± 0.2^{a}	2.7 ± 0.4^{a}	3.0 ± 0.4^{a}	3.2 ± 0.1^{a}	3.6 ± 0.2^{a}
3 months	3.0 ± 0.1^{a}	3.4 ± 0.2^{a}	2.6 ± 0.3^{a}	3.0 ± 0.3^{a}	2.8 ± 0.3^{a}	3.4 ± 0.1^{a}

[a] Values in the same column without a common letter are significantly different: $p \leqslant 0.05$.

changes either in CLA levels in this product (Gnädig et al., 2004). Only more severe heating conditions, including very high temperatures (200 °C), different from those traditionally applied during the manufacture of most types of cheeses significantly affected CLA levels (Precht, Molkentin, & Vahlendieck, 1999). RA is also prone to positional isomerization *via* a thermally-induced concerted reaction at 200 °C to render 8-*trans*, 10-*cis* C18:2 as has been recently described (Destaillats, Japiot, Chouinard, Arul, & Angers, 2005).

3.3. Influence of ripening in CLA levels

The period of ageing hardly altered the CLA content in most of the cheeses examined (Tables 2–4). The comparison of these results with earlier research is difficult given the different types of cheese reported in the literature and their apparent dissimilarity. Chin et al. (1992) after examining a large number of varieties observed that cheeses with a more prolonged ripening period were those with lower CLA levels. Conversely, in other works, higher amounts of CLA were detected in cheeses with more months of ripening (Ha et al., 1989; Lin et al., 1995; Parodi, 2003; Zlatanos, Laskaridis, Feist, & Sagredos, 2002). Most of these studies, however, just report the overall CLA content of individual samples of different types of cheeses acquired on the market with no detailed monitoring of CLA levels at different points throughout the ripening period in the same variety and sample. This fact means that the comparisons established are of limited use from a scientific point of view. Monitoring during different ripening stages in Cheddar (Lin et al., 1999), in different varieties of Swedish hard cheeses (Jiang, Björck, & Fondén, 1997), in Edam (Ryhänen et al., 2005) and in Emmental (Gnädig et al., 2004) provided the same data as observed in this research and reinforces the idea that ripening does not substantially modify the CLA content in milk fat.

It is not easy to attribute the slight decrease in the total CLA content in samples A and B (Table 2). Lin et al. (1999) also observed a small decrease in CLA levels in Cheddar cheeses with six months of ripening compared to an identical sample at three months. These authors sta-

ted that this decrease could be due to proteolytic activity in the microbial flora enzymes present in cheese. Peptides and other low molecular weight protean compounds from this activity could act as hydrogen donors in the reactions that convert CLA into monoenoic or saturated acids. Although the existence of proteolytic activity is well known during the ripening of Mahón (Marcos & Esteban, 1993) and it could be that CLA is converted into other fatty acids by the mechanism suggested by Lin et al. (1999), further studies would be necessary to confirm this hypothesis.

In the light of these results (Table 4), the characteristic presence of the *Penicillium roqueforti* mould in Cabrales cheese would not result either in significant changes in CLA content. Neither the use of starter cultures with different types of lactic bacteria (Jiang et al., 1997) nor the addition of selected strains of *Propionibacterium freudenreichii* with different lipolytic activity (Gnädig et al., 2004), for example, affected CLA content in the cheese fat. Nonetheless, in this field, the progress made in selection studies of lactic bacteria with reinforced capacity for generating CLA from other PUFA gives hope as to its future application in the manufacture of cheeses.

3.4. Evolution of the CLA isomer profile in cheeses

Most of the CLA detected in the cheeses studied correspond to the 9-*cis*, 11-*trans* isomer. The percentages of RA in the total CLA content determined by GC-FID ranged between 83% and 93% and coincided with the data reported in works with other cheese varieties (Chin et al., 1992; Lavillonière et al., 1998; Shanta et al., 1995) carried out with an identical instrumental technique. However, the use of CG-FID with capillary columns is not able to resolve a large part of the CLA minority isomers and does not allow a reliable quantification of some of them. Moreover, the RA peak could mask other different CLA isomers, which would affect the measurement of the real RA content.

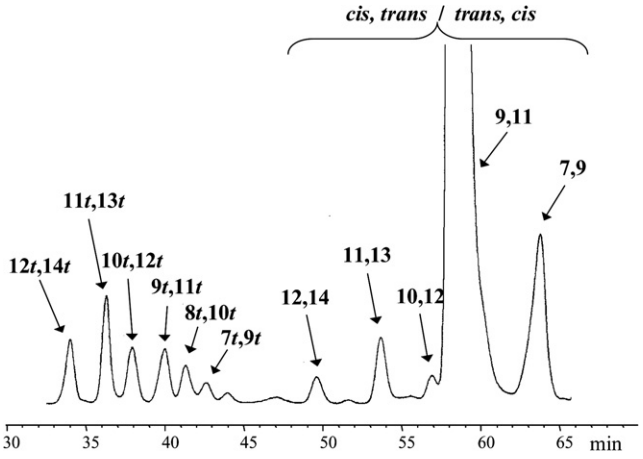

Fig. 1. Ag^+-HPLC profile of Manchego cheese fat FAME using UV detection at 233 nm and three columns in series.

The complementary use of Ag^+-HPLC is currently the most effective way of separating and quantifying CLA isomers. CLA FAME are selectively detected by their characteristic UV absorbance at 233 nm and non-conjugated FAME respond poorly at this wavelength. Ag^+-HPLC profile of CLA-FAME from cheese fat was seen to separate the different *trans*/*trans* (from 12–14 to 7–9 C18:2) compounds, followed by a chromatographic zone where *cis*/*trans* and *trans*/*cis* isomers eluted (Fig. 1). Although geometrical isomers (*cis*/*trans* from *trans*/*cis*) are not resolved by this technique, species differing in positional double bonds eluted separately.

Table 5 shows the variation intervals of the CLA isomers in the three cheese varieties examined at the end of ripening period. Given that the 9-*trans*, 11-*cis* C18:2 isomer peak seems to be negligible in comparison with the 9-*cis*,

Table 5
Intervals of variation of conjugated linoleic acid (CLA) isomers determined by silver-ion HPLC in Mahón, Manchego and Cabrales cheeses at the end of the ripening period (6, 6 and 3 months, respectively)

Isomer	Cheese variety (% of total CLA)		
	Mahón	Manchego	Cabrales
trans/*trans*			
12,14	1.33–1.69	1.59–2.06	0.75–1.41
11,13	2.67–4.13	2.13–2.63	1.36–2.21
10,12	1.05–1.19	1.10–1.35	1.19–2.14
9,11	1.14–1.55	1.30–1.52	0.71–1.20
8,10	0.30–0.44	0.53–0.68	0.75–1.10
7,9	0.41–0.79	0.29–0.38	0.41–1.17
cis/*trans* + *trans*/*cis*			
12,14	0.48–0.59	0.79–1.15	0.19–0.30
11,13	2.10–3.71	1.31–2.13	1.59–1.91
10,12	0.18–0.38	0.37–0.56	0.51–0.64
9,11	81.7–84.1	79.5–81.7	83.3–84.3
7,9	3.71–6.05	6.34–7.11	5.00–5.11
$\sum$*trans*/*trans*	6.90–9.79	6.94–8.62	5.17–9.23
$\sum$*cis*/*trans* + *trans*/*cis*	88.2–94.8	88.3–92.6	90.6–92.3

11-*trans* C18:2 (Fig. 2, peaks 1 and 2), the positional 9–11 C18:2 content obtained from Ag^+-HPLC analysis should be almost entirely attributed to RA. Thus, a percentage higher than 75% of CLA in cheeses would be assigned to RA. The percentage of 7–9 C18:2 isomers (*cis*/*trans* plus *trans*/*cis*) was the second greatest (3.7–7.1%), while a noticeable content of 11–13 C18:2 isomers was also measured. Concerning the rest of the isomers, concentrations were hardly higher than 2%.

7–9 (*cis*/*trans* plus *trans*/*cis*) CLA isomers reported in the literature for cheeses were generally in the order of 3–5% of total CLA (Gnädig et al., 2004; Rickert et al., 1999). Most of them would correspond to 7-*trans* 9-*cis* C18:2 derived from milk by endogenous synthesis via Δ9-desaturase from 7-*trans* C18:1 produced in the rumen (Bauman et al., 2003). Cheeses were also rich in 11–13 (*cis*/*trans* plus *trans*/*cis* C18:2); Mahón showed the most noticeable percentages in these isomers (Table 5). 11-*trans* 13-*cis*, could be the major geometrical isomer, as deduced by the retention time of DMOX derivative by GC–MS peak 4 in Fig. 2. 10–12 *cis*/*trans* plus *trans*/*cis* CLA isomers content was low in all the cheese varieties studied (below 1% of total CLA), coinciding with previous research in cheese fat (Gnädig et al., 2004; Zlatanos et al., 2002). This fact, from a physiological point of view, is remarkable since 10-*trans*, 12-*cis* C18:2 is responsible for the observed CLA weight loss/muscle-mass enhancement effects (Pariza, 2004). Although most 10–12 C18:2 detected by Ag^+-HPLC could be attributed to the geometrical configuration *trans*/*cis*, these dairy products are a poor source of this isomer.

All *trans* total content, distributed among six isomers, ranged between 5% and 9% of total CLA (Table 5). Mahón had the highest percentages, and 11–13 all *trans* C18:2 was the third most abundant CLA isomer after RA and 7–9 C18:2. The rest of the cheese varieties exhibited profiles with similar amounts of *trans*/*trans* isomers. Other studies, however, measured very high levels of 11–13 all *trans* CLA isomers in different varieties of cheeses (Ha et al., 1989) and also detected noticeable amounts of all *trans* CLA isomers in different Cheddar cheeses (Werner et al., 1992). Differences in the CLA isomer profile with this research should be attributed to the methylation procedure used by these authors. Acid methylation using BF_3 as a catalyst induces CLA isomerization. It has been demonstrated (Park, Albright, Cai, & Pariza, 2001) that incubation with this catalyst can decrease the 9-*cis*, 11-*trans* C18:2, but all *trans* isomers and artifacts increased. More recent research carried out with base catalysts (Lavillonière et al., 1998; Rickert et al., 1999; Sehat et al., 1998) detected lower percentages of *trans*/*trans* isomers and levels of geometrical 9–11 isomers similar to those measured in the present work.

The detection and quantification of CLA all *cis* isomers is more complex. Analyses by GC-FID of FAME did not allow these isomers to be separated from the chromatographic peak corresponding to the C21:0 methyl ester. The application of Ag^+-HPLC did not allow them to be quantified since they elute in the same zone as oleic acid.

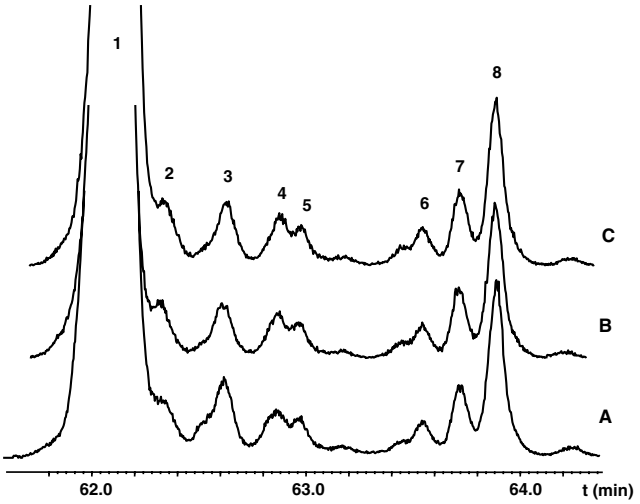

Fig. 2. Profiles by GC–MS of the DMOX derivatives of milk fat (A) and Manchego cheese at two days (B) and six months (C) of ripening obtained by high resolution selected-ion recording at *m*/*z* 333. Peaks were tentatively assigned to **1**: 9-*cis*, 11-*trans* + 7-*trans*, 9-*cis* + 8-*trans*, 10-*cis;* **2**: 9-*trans*, 11-*cis*; **3**: 11-*cis*, 13-*trans* + 10-*trans*, 12-*cis*; **4**: 11-*trans*, 13-*cis*; **5**: 9-*cis*, 11-*cis*; **6**: 12-*trans*, 14-*trans*; **7**: 11-*trans*, 13-*trans* **8**: 8-*trans*, 10-*trans* + 9-*trans*, 11-*trans* + 10-*trans*, 12-*trans*.

This acid, in high concentrations, as occurs in dairy products, absorbs at 233 nm, thus masking the presence of CLA all *cis* isomers. The use of GC–MS of DMOX derivatives (Fig. 2) made it possible to distinguish a peak whose spectrum corresponds to the 9–11 C18:2 positional isomer (Roach, 1999). The assignation of the all *cis* geometrical configuration would be done in accordance with its elution time peak 4 in Fig. 2. Although this procedure does not allow the isomer content to be quantified reliably, it is indeed useful for detecting its existence. The physiological importance of 9-*cis*, 11-*cis* C18:2 is still virtually unknown. However, there is some evidence that it could have more powerful effects on tumours than RA (Tanmahasamut, Liu, Hendry, & Sidell, 2004). The existence of different all CLA *cis* isomers in cheeses has been detected in different varieties (Lavillonière et al., 1998; Ryhänen et al., 2005; Sehat et al., 1998) although their content was very small and probably not more than 1% of total CLA (Rickert et al., 1999).

Table 6 shows the evolution of the different CLA isomers from the raw milk and Manchego cheese throughout the ripening. As can be observed, changes occurred in the isomer profile during the period of time evaluated (six months) are limited. This same trend was also observed in the different Cabrales and Mahón samples (data not shown). No changes in the CLA isomer profile during the manufacture process were observed either by Gnädig et al. (2004) in Emmental cheese, although the period of monitoring in this study was limited to 70 days. Even though Werner et al. (1992) reported that ageing may have affected CLA isomer distribution, since the concentration of 9-*cis*, 11-*cis* C18:2 and other 9–11 CLA isomers was greater in the aged than unaged cheese, these authors did not monitor a uniform

Table 6
Conjugated linoleic acid (CLA) isomers determined by silver-ion HPLC of pasteurised ewes' milk fat and Manchego cheeses (sample H) after 2 days and 2, 4 and 6 months of ripening

Isomer	Milk	Cheese (% of total CLA)			
		2 days	2 months	4 months	6 months
trans/trans					
12,14	2.10 ± 0.07^{a}	2.13 ± 0.02^{a}	2.05 ± 0.05^{a}	2.06 ± 0.06^{a}	2.06 ± 0.05^{a}
11,13	2.55 ± 0.05^{a}	2.66 ± 0.01^{b}	$2.63 \pm 0.02^{a,b}$	$2.62 \pm 0.04^{a,b}$	$2.63 \pm 0.07^{a,b}$
10,12	1.05 ± 0.04^{a}	1.11 ± 0.01^{a}	1.12 ± 0.01^{a}	1.09 ± 0.02^{a}	1.10 ± 0.02^{a}
9,11	1.32 ± 0.01^{a}	1.56 ± 0.02^{b}	1.55 ± 0.05^{b}	1.57 ± 0.01^{b}	1.52 ± 0.03^{b}
8,10	$0.58 \pm 0.04^{a,b}$	0.61 ± 0.01^{a}	0.56 ± 0.01^{b}	$0.59 \pm 0.02^{a,b}$	$0.58 \pm 0.01^{a,b}$
7,9	0.26 ± 0.04^{a}	0.29 ± 0.01^{a}	0.30 ± 0.03^{a}	0.26 ± 0.02^{a}	0.29 ± 0.01^{a}
cis/trans + trans/cis					
12,14	1.12 ± 0.02^{a}	1.13 ± 0.03^{a}	1.12 ± 0.01^{a}	1.13 ± 0.02^{a}	1.15 ± 0.01^{a}
11,13	2.09 ± 0.07^{a}	2.17 ± 0.03^{a}	2.16 ± 0.02^{a}	2.12 ± 0.01^{a}	2.13 ± 0.06^{a}
10,12	0.40 ± 0.05^{a}	0.31 ± 0.04^{a}	0.35 ± 0.01^{a}	0.33 ± 0.02^{a}	0.37 ± 0.08^{a}
9,11	79.15 ± 0.49^{a}	79.42 ± 0.50^{a}	79.59 ± 0.02^{a}	79.57 ± 0.07^{a}	79.50 ± 0.08^{a}
7,9	7.12 ± 0.10^{a}	7.21 ± 0.04^{a}	7.15 ± 0.02^{a}	7.18 ± 0.10^{a}	7.22 ± 0.14^{a}
$\sum$*trans/trans*	7.86	8.36	8.21	8.19	8.18
$\sum$*cis/trans + trans/cis*	89.88	90.24	90.37	90.33	90.37
Unidentified peaks	2.26	1.40	1.42	1.48	1.45

[a,b] Values in the same row without a common letter are significantly different: $p \leqslant 0.05$.

source of raw material. Furthermore, the acid methylation procedure used (Werner et al., 1992) has been shown to be very active in transforming RA into all *trans*, all *cis* and *trans/cis* 9–11 CLA isomers.

The total CLA content in the different cheeses studied varied greatly. Changes in the CLA levels that occur during manufacture are comparatively small. The alterations in the CLA concentration throughout ripening are lower than the differences that occur in the raw material from which the cheeses are manufactured. These results stress the importance of the initial CLA content in raw milk with regard to its final concentration in products for consumption. As regards the isomer profile, alterations during ripening are also very limited, which highlights the negligible effect that the ripening period has. These data indicate that the most suitable strategies for improving CLA content in these dairy products must enhance the content of these acids in raw milk.

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