

## Conjugated Linoleic Acid Contents in Cheeses of Different Compositions During Six Months of Ripening

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### Abstract

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The study deals with the effects of the origin of milk (cow, ewe, goat, at different proportions), seasonality, and ripening time on the contents of conjugated linoleic acid (CLA) in 224 samples of cheese. The sum of the *cis*9, *trans*11 and *trans*10, *cis*12 isomers was determined by GC-FID, after the extraction and methylation of the fatty acids of the samples, observing that the mean amount of CLA was 2.22, 2.72, and 3.54 mg/g of cheese, depending on the proportions of cow's, goat's, or ewe's milks, respectively. The contents in cow's, ewe's, and goat's milk, together with the ripening time and seasonality, were seen to have significant effects ( $P < 0.05$ ) on the concentration of CLA. The Pearson correlation revealed an inverse correlation between the content of CLA and the % of cow's milk ( $r = -0.269$ ,  $P < 0.01$ ) and seasonality ( $r = -0.290$ ,  $P < 0.01$ ), and a direct correlation between CLA content and the % of ewe's milk ( $r = 0.312$ ,  $P < 0.01$ ) and the month of ripening ( $r = 0.188$ ,  $P < 0.01$ ).

**Keywords:** conjugated linoleic acid; cheese ripening; gas chromatography

The term conjugated linoleic acid (CLA) describes a group of polyunsaturated acids that are isomers of linoleic acid with double conjugated bonds, mainly at carbons 9 and 11 or 10 and 12. For each positional isomer, four pairs of geometric isomers are possible. There are 56 geometric and positional isomers of conjugated octadecadienoic acid (LAVILLONNIÈRE *et al.* 1998; RODRÍGUEZ-TADEO *et al.* 2006; VAN NIEUWENHOVE *et al.* 2007; DOMAGALA *et al.* 2010).

The structural complexity of CLA means that it is very difficult to identify the isomers with biologi-

cal activity. Several components may be responsible, although to date investigations have revealed that two do have biological activity: namely, *cis*9, *trans*11 CLA and *trans*10, *cis*12 CLA (LÓPEZ BOTE *et al.* 2004; BONDIA-PONS *et al.* 2007). The *cis*9, *trans*11 isomer stimulates the growth, reduces the severity of diabetes, strengthens bones, decreases cholesterol levels, and has antiatherogenic properties (JUNG *et al.* 2006; BONDIA-PONS *et al.* 2007; SILVA HERNÁNDEZ *et al.* 2007; MELUCHOVÁ *et al.* 2008). In contrast, the *trans*10, *cis*12 isomer elicits a reduction in fat levels (PARK *et al.* 2007). Other

authors have reported a synergic effect between both isomers (LÓPEZ BOTE *et al.* 2004).

In cheeses, the concentrations of CLA vary between 3.4–4.5 mg/g of fat (LIN *et al.* 1995), and this variation is mainly explained by the form of animals feeding. Thus, when fresh pasture is available, the CLA content in the milk fat is much higher than when the animals are given forage mixtures (ZLATANOS *et al.* 2002; NONI & BATTELLI 2008), although the effect of the bacteria strains used in the starter cultures for the elaboration of the cheese must also be taken into account since such bacteria are able to convert free linoleic acid into its conjugated form. Other factors also affect the amount of CLA and among them the lactation period, animal genetics (MA *et al.* 1999), and pH (related to seasonality) are relevant. Thus, the acid environment induced by feeding at the start of spring and in winter may be negative as regards the production of CLA (DHIMAN *et al.* 1999).

The application of heat, the use of different fermentation cultures, or ripening periods could potentially modulate CLA levels in cheese (HA *et al.* 1989; CHIN *et al.* 1992; SHANTA *et al.* 1992; LIN *et al.* 1995; LIN *et al.* 1999). No changes in CLA content were observed during Emmental cheese manufacturing process, and the changes in cooking and moulding temperatures did not influence the CLA content either (GNÄDIG *et al.* 2004). However, as follows from these studies, the effect on CLA contents during the process of milk transformation into cheese is highly debatable. The alterations in the CLA concentration in the course of ripening are lower than the differences that occur in the raw materials from which the cheeses are manufactured (LUNA *et al.* 2007). The comparison between cheeses obtained from milk of the same ruminant species but by different production technologies revealed significant differences in the FA profiles but not in the CLA level. This suggested that the factors involved in the cheese-making process, such as heat treatment of milk and/or curd, the addition of starter cultures, and ripening conditions, do not generally affect the CLA content in milk fat (PRANDINI *et al.* 2011).

The method of lipid extraction in CLA determination may inhibit the actions of lipases and phospholipases (ROACH *et al.* 2002; DOMAGALA *et al.* 2010) and the analytical technique employed can also affect CLA content (in this sense, since GC uses highly polar capillary columns, it is unable to separate the minor isomers of CLA), meaning

that quantification may not be totally reliable (PARK *et al.* 2007).

In view of the importance of conjugated linoleic acid in current diets and the large number of factors affecting its content, we were prompted to determine the influence of different factors, e.g. variable proportions of cow's, ewe's, and goat's milk, seasonality (winter/summer), and the evolution in the course of 6 months ripening – on CLA concentrations in cheeses.

## MATERIAL AND METHODS

To perform the present study, a total of 224 cheeses of known compositions were elaborated and controlled. They were made of milk collected directly from farms in winter (112 cheeses) and summer (112 cheeses) because previous studies had shown that the fatty acid profile varies with the season because of the change in the animal feeding regimen that directly affects the milk fatty acid profile (PEREA *et al.* 2000; FERNÁNDEZ-GARCÍA *et al.* 2006). Cheeses with 16 different compositions were elaborated, prepared with known, varying amounts of milk from cows, ewes, and goats, with percentages ranging between 0% and 100%, as shown in Table 1 (GONZÁLEZ-MARTÍN *et al.* 2007). Cheeses were prepared in the laboratory according to the following procedure: raw milk

Table 1. Composition in % of the reference cheeses elaborated (winter and summer milk)

Cheeses	Cow's milk	Ewe's milk	Goat's milk
1	100	0	0
2	0	100	0
3	75	25	0
4	50	50	0
5	25	75	0
6	0	0	100
7	25	0	75
8	50	0	50
9	75	0	25
10	0	25	75
11	0	50	50
12	0	75	25
13	33	33	33
14	10	45	45
15	45	10	45
16	45	45	10

(40 l), not standardised, was incubated with 15 mg/l direct-vat-set starters made of *Streptococcus lactis*, *S. cremoris* and *S. diacetylactis*, (MA400; Arroyo Laboratories, Santander, Spain) at 30°C. After 10 min at 32°C, 12.5 mg/l of calf rennet (90% chymosin, 10% trypsin, 1:150 000 strength) was added to each vat. Coagulation was allowed to take place over 20–70 minutes. When the curds had developed the desired firmness, evaluated subjectively, they were cut with a cheese harp until pieces similar in size to a grain of rice were obtained. Then, the curd was stirred for 30 min, and heated for 10 to 20 min at 37°C until it had reached the desired consistency to improve its drainage with sieves. The curd was packed in round hoops (1 kg) and pressed for 6 h at 1.47 bar at 2°C. After pressing, the cheeses were salted by soaking them in sodium chloride brine (18%) at 18°C for 6 hours. The cheeses were then transferred to a drying chamber, where temperature (15°C) and relative humidity (70%) were controlled. Seven pieces of these cheeses, identical in their composition, were elaborated, using one of the pieces at each sampling time (6 days, 1, 2, 3, 4, 5, and 6 months), with which the control of the ripening processes could be ensured.

**Determination of conjugated linoleic acid.** This started with the extraction of fat (FIL-IDF 32:1965) and methylation of the lipids according to the procedure proposed by (MURRIETA *et al.* 2003). The conjugated linoleic acid was determined by CG-FID (GC 6890 N; Agilent Technologies, Santa Clara, USA). A fused silica capillary column of 100 mm length, 0.25 mm *i.d.* and 0.2 mm film thickness (Supelco, Inc., Bellefonte, USA) was used following the conditions described elsewhere (REALINI *et al.* 2004), using helium as the carrier gas at a pressure of 38.30 psi and a flow rate of 1 ml per minute. 1 ml of sample was injected into the column in split mode (20:1), with an injector temperature of 250°C. Identification of the conjugated linoleic acid was accomplished by comparing the retention times with pure standards of the *cis9*, *trans11* and *trans10*, *cis12* isomers (Larodan, Malmö, Sweden).

**Statistical analyses.** To study the different factors – month of ripening, seasonality, and the percentages of cow's, ewe's, and goat's milks – the SPSS package (Statistical Package for the Social Sciences) for Windows 15.0 was employed for all the samples analysed. The mean values of the quantitative variables were compared by Analysis of Variance (ANOVA), considering the differences

Table 2. Correlations with the content of conjugated linoleic acid (CLA)

	Level of significance
Cow's milk	99% (–0.269)*
Ewe's milk	99% (0.312)*
Seasonality	99% (–0.290)
Ripening time	99% (0.188)*

\*Pearson correlation SIG

being significant when *P* was lower than 0.05. With a view to studying which means were different, a Tukey multiple comparison test was implemented, which controls the rate of type 1 error. Pearson correlation coefficient tests with a significance test (two-tailed) were performed to study the correlation between the variables and the concentrations of CLA in the cheeses elaborated (Table 2).

## RESULTS AND DISCUSSION

Table 3 shows the minimum, maximum, and mean concentrations together with the standard deviation (SD) of conjugated linoleic acid (sum of *cis9*, *trans11* and *trans10*, *cis12*) contents in the 224 samples of cheese analysed with GC-FID. The variation in the range obtained, 0.14–8.40 mg CLA/g of cheese, for the total number of samples had lower values than those reported for processed cheeses (LIN *et al.* 1995; RODRÍGUEZ-TADEO *et al.* 2006) and melted cheeses (CHIN *et al.* 1992; SEÇKIN *et al.* 2005) from cows, ewes, and goats.

Figure 1 shows the mean CLA values in the cheese samples whose composition included mainly cow's, ewe's, or goat's milks (this situation is considered when the cheese contains amounts equal to or greater than 75% of milk from each of these species). The mean CLA concentrations found were 2.22 ( $\pm$  0.16), 2.72 ( $\pm$  0.22) and 3.54 ( $\pm$  0.19) mg/g of cheese, depending respectively on the contents of cow's, ewe's, or goat's milks. This result is in agreement with the findings of MELUCHOVA *et al.* (2008), who reported that the concentration of CLA was higher in ewe's milk than in that of

Table 3. Chemical reference data

Analyte	<i>n</i>	Mini	Max	Mean	SD
Conjugated linoleic acid (mg/g cheese)	224	0.14	8.40	2.86	1.27

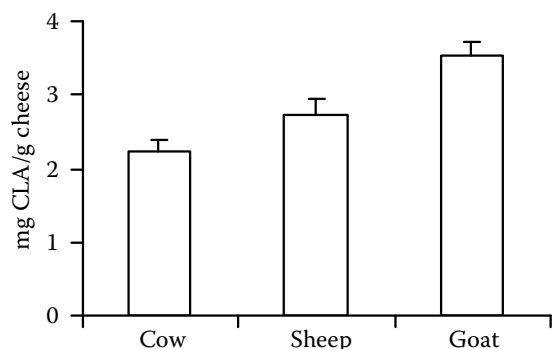


Figure 1. Mean concentration of conjugated linoleic acid (CLA) with respect to the type of milk (cows, ewes, goats) present in the highest amounts in the cheeses

cows: 1% vs. 0.6 of the total amount of fatty acids, although they do not coincide with those reported by PARK *et al.* (2007), who reported higher CLA values for cow's milk.

Among the factors affecting the CLA levels of cheeses, the first factor was the effect of the content in milk from the different species. According to different authors, the CLA values obtained in cow's milk vary considerably. In this sense, values of 0.81–1.65% and 2.23–2.95% (total FAMES basis) have been reported for Bitto and Ossolano cheeses respectively (NONI & BATTELLI 2008); of 6.34 and 3.45 mg CLA/g of fat in Mahón and Cabrales cheeses (LUNA *et al.* 2007), and values of 0.55 and 1.58 mg CLA/g in Mozzarella and Cheddar cheeses (SHANTHA 1992). Accordingly, the values reported here would be within the broad range described in the literature.

The statistical analysis applied indicates that the percentage of cow's milk has a significant effect ( $P < 0.05$ ) on the concentration of CLA in the cheeses studied.

Also, upon performing a comparative study using the % cow's milk factor used in the elaboration of the cheeses, the Tukey model revealed that the means for the samples with between 100% and 75% of cow's milk were different, with a significance of 0.133, from the other proportions of cow's milk used in the study.

The Pearson correlation indicates that the percentage of cow's milk is negatively correlated ( $r = -0.269$ ,  $P < 0.01$ ) with the concentration of CLA.

CLA contents in ewe's cheese tend to be higher than those found in goat's and cow's milks cheeses, mainly due to the fact that in most Mediterranean countries ewe's diets are essentially based on grazing. These results are consistent with the findings

of other authors, in which sheep cheese showed the highest mean values of CLA in comparison with cow and goat cheeses (PRADINI *et al.* 2010).

Thus, values between 3.0 mg/g and 5.4 mg/g have been reported for Pecorino Foggiano (SANTILLO *et al.* 2007), 6.3 mg/g in ewe's cheeses from the Manchego region (LUNA *et al.* 2007), and between 1.2 mg/g and 2.5 mg/g in Feta cheese (uncured) (ZLATANOS *et al.* 2002).

The % of ewe's milk has a significant effect ( $P < 0.05$ ) on the concentration of CLA in the cheeses studied. Finally, on studying the % of ewe's milk used in the elaboration of the cheeses, the Tukey test revealed significant differences between the samples elaborated from 100% ewe's milk with respect to those elaborated with 75% of milk from this species, whereas that of ewe's milk is positively correlated ( $r = 0.312$ ,  $P < 0.01$ ), at a level of significance of 0.05 (2-tails), with the concentration of CLA.

Cheeses made from goat's milk have received little attention, and the values reported are fairly similar to those observed here, with a range between 7–6.9 mg/g of fat in French cheeses (MA *et al.* 1999; PARODI 2003). In all cases studied, the percentage of goat's milk was not statistically significant.

The second factor studied here was the seasonality. The effect of seasonality leads to variations in the concentrations of CLA in the fat from the different species of ruminants due to the variations in nutritional factors. In order to analyse the influence of this factor, two groups of samples were set up (112 from milk collected during the winter and 112 from those collected in the summer). The results showed that the mean CLA concentrations in winter and summer were, respectively, 3.23 ( $\pm 0.29$ ) and 2.49 ( $\pm 0.23$ ) mg/g of cheese, thus the cheeses elaborated in winter, regardless of the species, had higher mean CLA values throughout the ripening process.

The data published show that the CLA contents in diets based on forage vary considerably during different phenological stages (NUDDA *et al.* 2005; SEÇKIN *et al.* 2005; CHAMBA *et al.* 2006; COAKLEY *et al.* 2007; NONI & BATTELLI 2008). The seasonal difference (summer and winter) in milk fat due to the dietary regimen of animals elicits variations in the CLA contents in the cheese, due to the changes in the rumen microflora, which is strongly affected by the animal's diet. This was shown in the work by KIM *et al.* (2009), who found higher values at

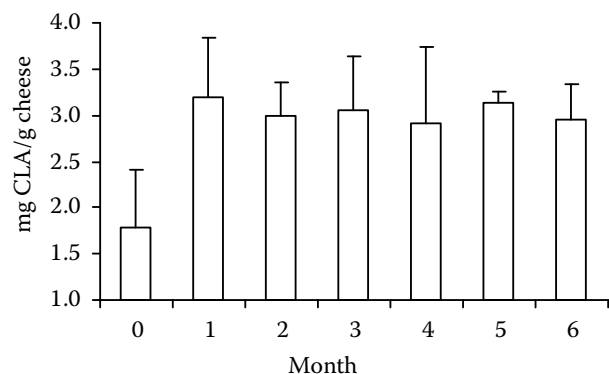


Figure 2. Variation in the mean content of CLA with the ripening time

the beginning of the Korean summer, with 7.1 mg CLA/g of fat, and the lowest ones in January, with 3.9 mg CLA/g of fat.

Various authors have suggested that fresh grass promotes the synthesis of CLA through a greater activity of  $\Delta 9$ -desaturase in the udder (KHANAL & OLSON 2004; NUDDA 2005; MELUCHOVÁ *et al.* 2008; KIM *et al.* 2009). Moreover, the high concentrations of soluble fibre and fermentable sugars in fresh grass can create an environment in the gastrointestinal tract of ruminants, without lowering the pH, that is favourable for the growth of the bacteria responsible for synthesising CLA and the production of vaccenic acid (DHIMAN *et al.* 1999).

In comparison with diets based on pasture, diets containing cereals and maize decrease CLA contents (MA *et al.* 1999; NUDDA 2005), whereas the presence of sunflower in the diet leads to an increase in the content of 9*cis*-11*trans* in cow's milk (COAKLEY 2007).

Seasonality had a significant effect ( $P < 0.05$ ), on the concentration of CLA in the cheeses studied. The Pearson correlation tests reveal an inverse correlation between the CLA content and sea-

sonality ( $r = -0.290$ ,  $P < 0.01$ ), i.e., the samples of cheese elaborated in winter have a higher CLA concentration than those elaborated in summer.

The third factor studied here was cheese ripening. Figure 2 shows the effect of 6 months of ripening on the 224 samples of the cheese analysed. Minimum amount of  $1.79 (\pm 0.62)$  mg CLA/g cheese and maximum of  $3.19 (\pm 0.65)$  mg CLA/g of cheese are seen, the minimum corresponding to 0 and the maximum to 1 month of ripening.

Some authors have reported higher CLA levels in cheeses with the increase in the ripening time (LIN *et al.* 1995; ZLATANOS *et al.* 2002; PARODI 2003). In contrast, others have described that the period of ripening does not alter CLA contents (WERNER 1992; LUNA *et al.* 2007), because, on one hand, a longer time for the bacteria to act involves an increase in the CLA contents, whereas, on the other, oxidising reactions could lead to the destruction of the double bonds, thus decreasing CLA levels (LIN *et al.* 1995; DOMAGALA *et al.* 2010).

Nevertheless, on studying the evolution of CLA levels during the ripening period for the different species of animals (Figure 3), it may be seen that the contents of CLA in the cheeses made of cow's milk undergo a significant increase from the first sampling (6 day) up to 6 months, whereas the ewe's cheeses have the lowest CLA concentration at 0 months and maximum after the second month of ripening, thereafter following a constant evolution over time. This is in contrast to the cheeses elaborated with goat's milk, which do not follow any clear trend, the minimum being attained at 0 months and the maximum appearing in months 1 and 5.

Ripening has a significant effect ( $P < 0.05$ ) on the concentration of CLA in the cheeses studied. The Tukey multiple comparison test indicated that the first sampling point (6 day) is the only one that

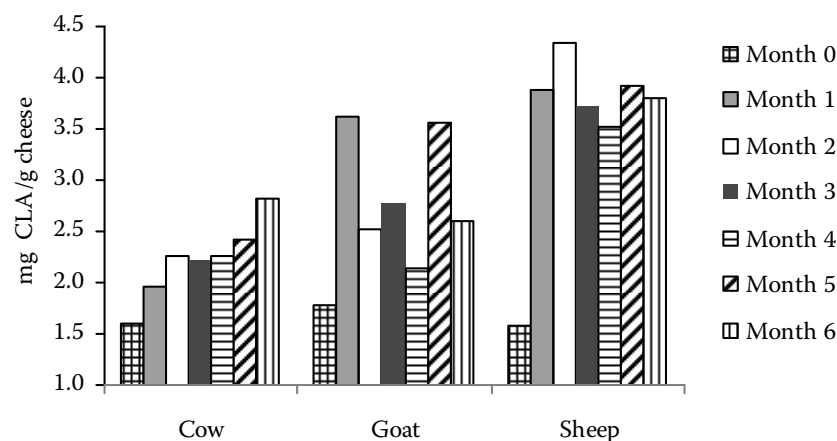


Figure 3. Evolution of the mean concentration of CLA during ripening with respect to the type of milk present at the highest amount in the cheese

shows a different mean; the rest of the sampling points (months 1–6) do not show significant differences in their means at a  $p$  value of 0.97. The Pearson correlation tests reveal a direct correlation between the CLA content and the month of ripening ( $r = 0.188$ ,  $P < 0.01$ ).

From the above results it may be deduced that there may be a significant influence of the factors studied on the CLA content of the cheeses, the effect of the ripening period factor being more difficult to observe because each type of milk evolves in a different way.

## CONCLUSIONS

Significant differences were found in the contents of conjugated linoleic acid due to the type of milk used to elaborate the cheeses studied here, higher concentrations being observed in ewe's cheese than in those made from cow's and goat's milks. Ripening elicits significant effects, especially from month 0 to month 1.

The concentration of conjugated linoleic acid was strongly affected by the season in which the milk was collected for the elaboration of the cheeses, the winter cheeses having significantly higher values.

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