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Conjugated linoleic acid in ewe milk fat

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Ewe milk fat from five different herds was studied to determine the content of conjugated linoleic acid (CLA) isomers. Research was carried out by combining gas chromatography-mass spectrometry (GC-MS) of fatty acid methyl esters (FAME) and 4,4-dimethyloxazolyne derivatives (DMOX) with silver ion-high performance liquid chromatography (Ag^+-HPLC). Reconstructed mass spectral profiles of CLA characteristic ions from DMOX were used to identify positional isomers and Ag^+-HPLC to quantify them. Total CLA content varied from 0·57 to 0·97 g/100 g of total fatty acids. FAME and DMOX were separated into a complex mixture of minor isomers and major rumenic acid (9-*cis* 11-*trans* C18:2) by GC-MS using a 100-m polar capillary column. Rumenic acid would represent more than 75% of total CLA. 11-*trans* 13-*trans*, 11–13 *cis/trans* plus *trans/cis* and 7–9 *cis/trans* plus *trans/cis* were the main CLA isomers after rumenic acid. Minor amounts of 8–10 and 10–12 C18:2 isomers were also found. Although most of the isomers were present in each herd's milk, differences in content were observed for some CLA species.

Keywords: Ewe, milk, CLA, fatty acid, GC-MS, DMOX, Ag^+-HPLC.

The generic name conjugated linoleic acid (CLA) is a collective term embracing all octadecadienoic acids (C18:2) with a conjugated double bond system in the 7–9, 8–10, 9–11, 10–12, 11–13 and 12–14 positions and a *cis/cis*, *cis/trans*, *trans/cis* and *trans/trans* geometrical configuration. Data from animal models have been used to suggest that the 9-*cis* 11-*trans* C18:2 isomer or rumenic acid (RA) is responsible for CLA anticarcinogenic properties, as well as growth promoting and antiatherogenic effects, whereas the *10-trans 12-cis* isomer has lean body mass-enhancing properties (Belury, 2002; Pariza, 2004; Khanal, 2004).

Milk and dairy products are the richest common source of CLA in foodstuffs. Cow milk fat contains ~0·6% CLA, with RA representing ~80% of this percentage. The remaining 20% consists of several minor isomers of which 7-*trans*, 9-*cis* (Yurawecz et al. 1998) and 11-*trans* 13-*cis* C18:2 (Sehat et al. 1998) are the most prominent. CLA concentration among the bulk ewe milk samples collected was found to be around 1% of total fatty acids (Jahreis et al. 1999; Prandini et al. 2001; Barbosa et al. 2003). Production of ewe milk is of economic importance in a number of countries, especially in the Mediterranean region and Middle East, where it is used to produce a variety of cheeses and yoghurts.

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Elucidation of individual CLA chemical structures in ewe milk fat has never been carried out. Most studies of ovine dairy products simply quantify the most prominent peak assigned to CLA as fatty acid methyl esters (FAME) by gas chromatography (GC). This main peak could include more than one isomer, and minor CLA isomers masked by the RA peak have not been documented. The RA content could therefore be overestimated. This work would make it possible to measure the amounts of the most relevant CLA isomers and RA more accurately.

The isomer mixtures in milk are complex and no single technique can resolve all isomers. In recent years, different procedures have been used to elucidate CLA isomers in different foodstuffs. The majority of them have been quantified by silver-ion HPLC (Ag^+-HPLC) (Sehat et al. 1998, 1999; Rickert et al. 1999; Adlof, 2003) but gas chromatography mass spectrometry (GC-MS) has been shown to be the most powerful tool for identifying them (Kramer et al. 2001; Roach et al. 2002; Dobson, 2003). A more complete qualitative and quantitative analysis involves applying both techniques, especially when GC is coupled to MS to provide structural information. A detailed positional analysis of the double bonds of fatty acids can be performed subsequently using one of several fatty acid derivatives other than FAME, such as 4,4 dimethyloxazoline (DMOX); this has already been applied to different food materials (Yurawecz et al. 1998; Roach, 1999, 2001). High-resolution MS with a selected ion-recording technique makes it possible to distinguish CLA

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from interfering fatty acids and discriminate among the different isomers. The aim of this work was to determine the CLA isomers present in ewe milk fat using GC-MS of CLA derivatives and to quantify the main ones by Ag^+-HPLC.

Materials and Methods

Samples and Standards

Milk samples were collected at monthly intervals during the milking period from five ovine herds. They were taken from the storage tanks containing milk from the whole herd. The freshly drawn milk was shipped to the laboratory in isothermal containers at 4 °C and kept at this temperature until fat extraction was carried out. Each herd consisted of a different breed: Churra breed (130 head), Awasi breed (130 head), Assaf and Castellana cross-breed (170 head), Manchega breed (2200 head) and Assaf breed (480 head). The herds were located in different places in Spain to ensure a wide variety of milk composition. A total of 45 samples were collected over the year. All the ewe milk fat samples from each herd were combined in equal volumes to obtain five sample mixtures.

A mixture and pure CLA isomer methyl esters (9-*cis* 11-*trans* and 10-*trans* 12-*cis* C18:2) were purchased from Nu-Chek Prep. Inc. (Elysian, MN, USA). In order to form other CLA isomers, Nu-Chek mixtures were isomerised using I_2 (Eulitz et al. 1999). For FAME determination, an anhydrous milk fat (reference material CRM-164) obtained from the EU Commission (Brussels, Belgium) was also employed.

Lipid extraction and fatty acid derivatization

Milk fat extraction was carried out according to ISO (2001). The fat residue extracted was stored frozen at −20 °C until GC analysis. FAME were prepared by base-catalysed methanolysis of the glycerides (KOH in methanol) according to ISO (2002).

The preparation of DMOX derivatives from FAME was based on the Fay & Richli (1991) procedure. 2-Amino-2-methyl-propanol was added to the FAME solution. The vessel was flushed with nitrogen, stoppered and placed in a heating block at 190 °C overnight. After cooling, the reaction mixture was dissolved in 5 ml dichloromethane and washed twice with 3 ml distilled water. The mixture was shaken thoroughly so that the layers settled. The solvent layer was passed through a short column of anhydrous sodium sulphate (prepared in a Pasteur pipette, plugged with a small piece of cotton wool and pre-washed with hexane prior to use) to dry in a fresh tube. The sample was washed from the tube with hexane, dried in a gentle stream of nitrogen on a heating block at 30 °C and dissolved in hexane for GC-MS analyses.

GC-MS analyses

Fatty acid derivatives were analysed on an Agilent chromatograph (model 6890N, Palo Alto, CA, USA) with a mass spectrometry 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 silanized glass wool.

FAME were separated using a CP-Sil 88 fused-silica capillary column (100 m × 0·25 mm i.d. × 0·2 μm film thickness, Chrompack, Middelburg, Netherlands). The column was held at 100 °C for 1 min after injection, temperature-programmed at 7 deg C/min to 170 °C, held there for 55 min, then temperature-programmed at 10 deg C/min to 230 °C and held there for 23 min. Helium was the carrier gas with a column inlet pressure set at 214 KPa and a split ratio of 1 : 20. The injection volume was 0·2 μl.

One microlitre of solution of DMOX derivatives was separated in the same column in the following conditions: the oven temperature was 75 °C for 2 min after injection, then temperature-programmed 5 deg C/min to 180 °C, held there for 30 min, then temperature-programmed 5 deg C/min to 220 °C and held there for 30 min. The column inlet pressure was set at 197 KPa in a splitless system.

Silver-ion HPLC

Ag^+-HPLC separation of CLA methyl esters was carried out using an HPLC (Agilent Technologies, Serie 1100, Palo Alto, CA, USA) equipped with a photodiode array detector operated at 234 and 205 nm. Three ChromSpher 5 Lipids analytical silver-impregnated columns (each 4·6 mm i.d. × 250 mm stainless steel; 5 μm particle size; Varian) 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. The flow was initiated 0·5 h prior to the sample injection and the injection volume was 10 μl.

Results and Discussion

Total CLA content

Total CLA content determined by GC was an average 0·74% of total FAME. Variability among herds was high and two groups of samples were distinguished. One group consisting of three herds had the lowest CLA percentages (Manchega 0·62, Assaf 0·57 and Castellana–Assaf cross 0·62%) and the other group consisting of two herds had the highest CLA percentages (Churra and Awassi with 0·96 and 0·97%, respectively). All of them were within the interval of variation (0·5–2·0) reported in the literature (Jahreis et al. 1999; Prandini et al. 2001; Barbosa et al. 2003) for this ruminant. This variability among herds suggests that diet and/or breed could have a major influence. Previous studies in cow (Kelsey et al. 2003) and ewe (Barbosa et al. 2003) milk fat only observed minor variation in CLA content among breeds fed the same diet.

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Dietary factors, however, are known to greatly affect CLA in milk fat. Such factors include, foods that alter the rumen environment and thereby affect the bacteria responsible for biohydrogenation, and the lipid substrates consumed.

Profile of CLA FAME by GC-MS

Partial GC-MS chromatograms of the CLA region from total ewe milk fat FAME are shown in Fig. 1. All the CLA isomers eluted in a region between 66 and 71 minutes. In total ion chromatogram (TIC), different peaks can be distinguished in this region (Fig. 1A). In order to discriminate CLA from unknown or interfering fatty acids, high-resolution selected-ion recording (SIR) MS of the molecular ion (*m/z*=294) was used (Fig. 1B). Qualitatively, the mass spectra of CLA FAME isomers are indistinguishable from one another and from methylene-interrupted octadecadienoic acid methyl esters such as linoleate. However, because the 294 ion is selective for C18:2 methyl esters, it could be useful for discriminating CLA species from other fatty acids that elute in the same region of the chromatogram.

The CLA region was shown to be rather free of interfering FAME except for one peak corresponding to C21:0 (heneicosanoic acid). This acid has been reported as co-eluting in GC analysis with 8-*cis* 10-*cis* (Kramer et al. 1998) or 10-*trans* 12-*cis* C18:2 (Roach et al. 2000) in the liver lipid of pigs and milk fat of cows, respectively. In this study C21:0 was detected after an isomer (potentially 11-*trans* 13-*cis* C18:2) and probably interfering with other minor CLA molecules. Regarding the elution pattern of the isomerised standard mixture (Fig. 1C), the isomers affected could include a variety of *cis/cis* CLA.

SIR-MS for the molecular ions of other FAME that may possibly interfere in the CLA was also tested. Particular interest was focused on detecting signs of the ion *m/z* 322 corresponding to C20:2 isomers. Other works (Roach et al. 2000) have observed in their studies that C20:2 could interfere with the determination of *trans/trans* CLA isomers. In the present study, however, this fatty acid eluted two minutes after the last CLA isomer.

When we applied TIC containing *m/z* 294 ion selective for C18:2, it was possible to observe a detailed chromatogram where up to 8–9 peaks could be labelled as CLA isomers (Fig. 1B). Theoretically, a tentative identification based on the isomers' retention times and comparison with an isomerised commercial CLA-FAME standard from Nu-Chek separated in the same conditions (Fig. 1C) could provide evidence for determining the correct identity of each isomer (Eulitz et al. 1999). In addition, the elution order of the CLA isomers using the 100 m CP Sil88 column is well established (Kramer et al. 2001, 2002) and could also be another very useful tool for detecting CLA isomers: elution order is *cis/trans, trans/cis, cis/cis* and finally, the *trans/trans* region. The isomerised mixture containing comparable amounts of eight CLA isomers is partially resolved by GC, but this separation is not apparent when one isomer predominate as in milk fat.

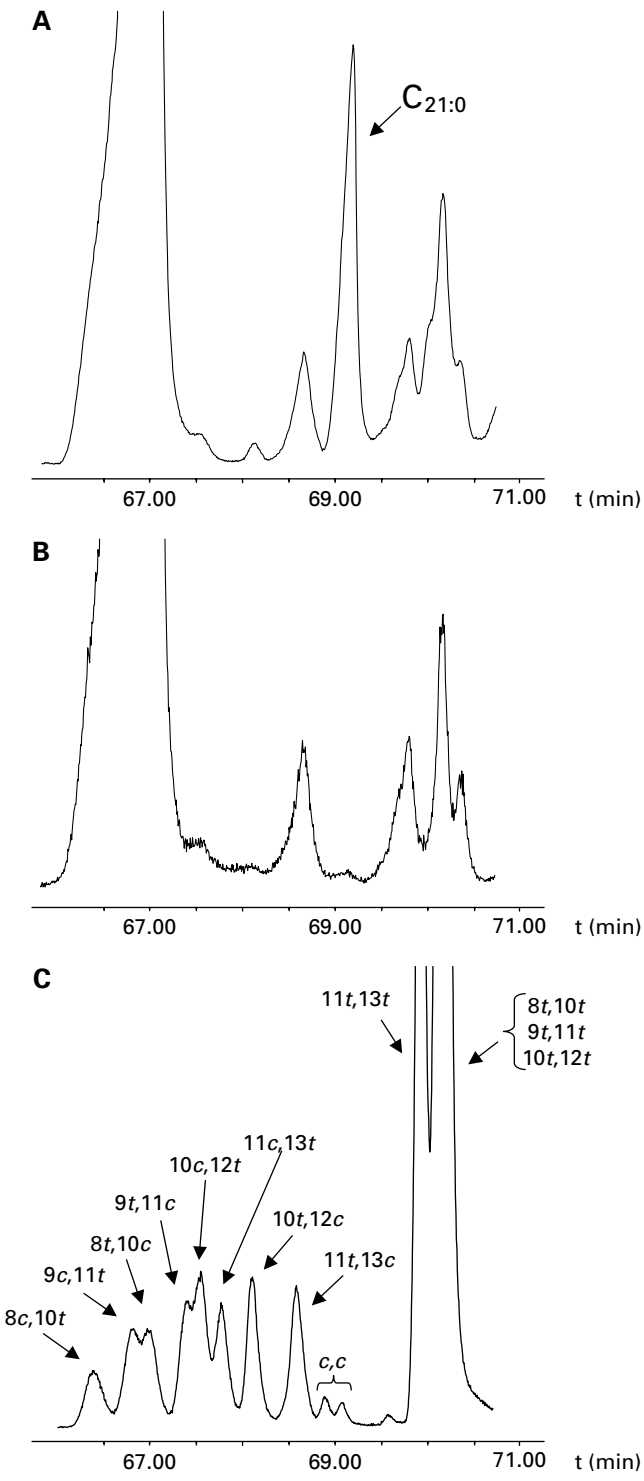


Fig. 1. FAME profile by GC-MS of ewe milk fat (CLA region) of (A) total ion, (B) high resolution selected-ion recording at *m/z* 294 and (C) Total ion of an isomerised standard CLA methyl ester mixture (*Nu-Chek*-Prep Inc.).

The biggest peak in the CLA region in ewe milk fat (Fig. 1A & B) for instance would correspond to 9-*cis* 11-*trans* C18:2, but other minor isomers such as 7–9 and 8–10

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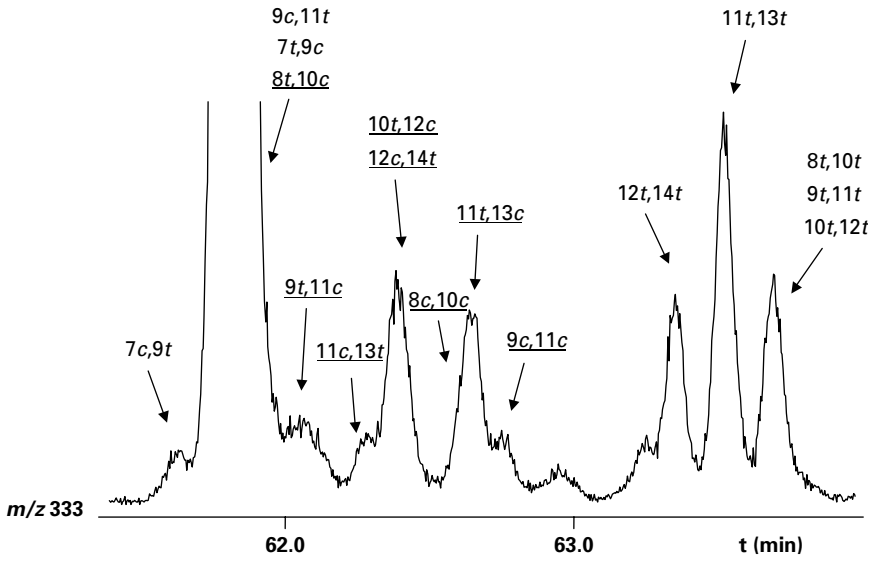


Fig. 2. FAME profile by GC-MS of the dimethyloxazoline (DMOX) derivatives of ewe milk fat obtained by high resolution selected-ion recording at *m/z* 333. Isomers underlined are only tentatively identified.

C18:2 could be masked. On the other hand, other CLAs do not occur as pure peaks and overlap, as do *trans/trans* isomers (last peak in Fig. 1C).

Analysis of DMOX by GC-MS

GC-MS of DMOX derivatives is now being widely used for analysing CLA mixtures because this technique gives chromatographic resolution comparable to that of FAME but retains the conjugated double bond configurations in the products. The mass spectra of CLA DMOX derivatives contain intense ions at *m/z* 113 and 126, and a characteristic molecular ion (*m/z* 333). The *m/z* 333 ion profile for the DMOX derivatives of ewe milk CLA is shown in Fig. 2. The most prominent peak, including RA, is followed by other CLA isomers. The presence of a small peak merging just ahead of that of the major RA isomer was also observed. In order to elucidate the possible structural configuration of every peak two approaches were employed: first, the elution order of DMOX derivatives, similar to the one described above for FAME; second, the characteristic ion spectra. Every positional CLA isomer exhibits a different ion profile.

In Fig. 2 twelve peaks could be distinguished. Comparing their mass spectra (Fig. 3) with the literature (Roach, 1999) three peaks could be assigned: 12-*trans* 14-*trans*, 11-*trans* 13-*trans* and a 7–9 isomer eluting just before the biggest peak (Fig. 2). Although the mass spectra of CLA DMOX derivatives do not elucidate the geometrical isomer configuration (*cis* or *trans*), this configuration would be deduced from the isomer chromatographic retention times. Thus 12–14 and 11–13 were assigned as *trans/trans* isomers because they both eluted at the end of the CLA region in the *trans/trans* area. The mass spectrum of the first peak in Fig. 2 exhibited an ion profile (Fig. 3) similar to the 7–9 configuration reported by Yurawecz et al. (1998) and Roach (1999): an abundant ion at *m/z* 234 preceded by a homologous series of ions separated by gaps of 14 mass units with intervals of 12 mass units found between *m/z* 168 (C6) and 180 (C7), and between *m/z* 194 (C8) and 206 (C9), indicated the presence of two double bonds at 7 and 9 carbons.

Since CLA isomers in milk fat invariably overlap by GC, reconstructed ion chromatograms of selected ions from the DMOX derivative data obtained by GC-MS were used. Recording data for only a limited set of ions (diagnostic ions for CLA positional isomers with double bond positions are well documented) including the characteristic ions for the CLA isomers of interest could be one tentative way of identifying these isomers. However, it should be borne in mind that the *m/z* values for these ions are not always specific to any one positional isomer and that they are present at lower intensities in other positional isomers; therefore, some degree of chromatographic separation would be necessary.

Figure 4 show the elution profile of the characteristic diagnostic ions of CLA positional isomers depicted beside the common molecular ion. When maximum intensities in all the diagnostic ion profiles for the same isomer coincide at the same elution time, then the peak may be attributed to this specific CLA isomer. For co-eluting CLA isomers, the reconstructed ion chromatograms would show a small difference in retention times. This reconstructed ion profile GC-MS method could help assign some minor CLA isomers in ewe milk fat like 7-*cis* 9-*trans* before RA and 7-*trans* 9-*cis* just inside the most prominent CLA peak (Fig. 4A). Although the characteristic ion of the 7–9 positional isomer (*m/z*=234) is also found in 9–11 and 8–10 CLA

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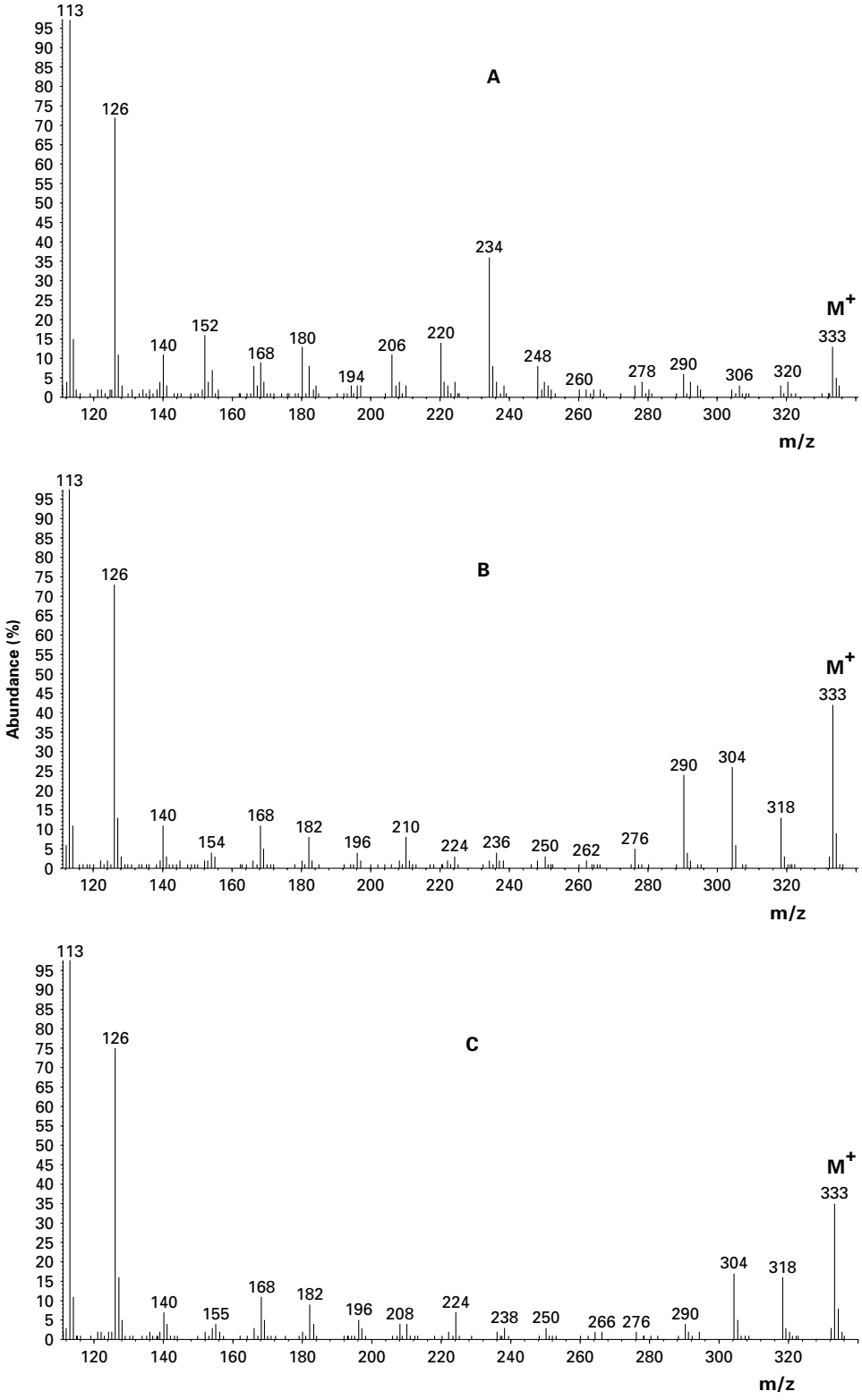


Fig. 3. Mass spectrum of the peak labelled in Figure 2 as 7-*cis* 9-*trans* C18:2 (A), 11-*trans* 13-*trans* C18:2 (B) and 12-*trans* 14-*trans* C18:2 (C) corresponding to dimethyloxazoline derivatives of ewe milk fat.

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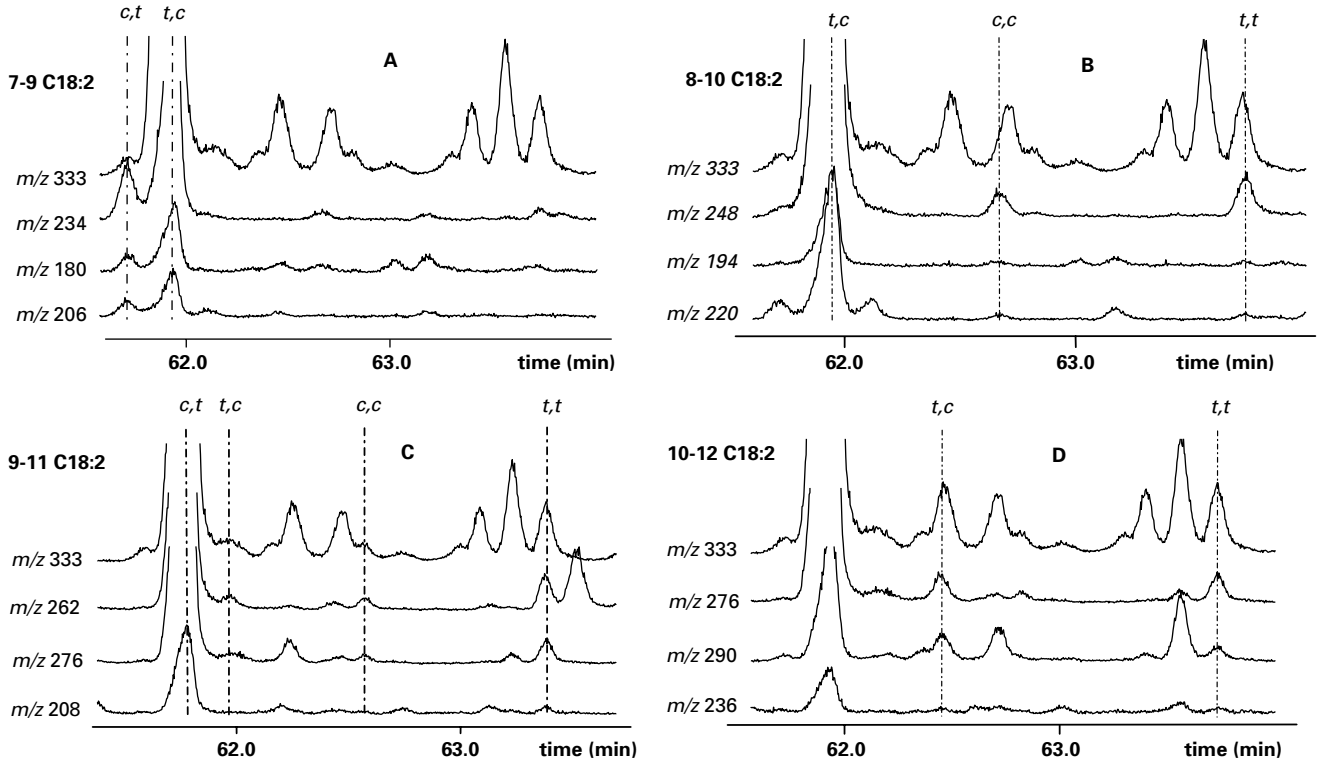


Fig. 4. Reconstructed ion chromatograms for dimethyloxazoline derivatives of CLA isomers from ewe milk fat. Profiles include (A) ion *m/z* 333 and characteristic ions of 7–9 C18:2 positional isomer (*m/z* 234, 180 and 206); (B) ion *m/z* 333 and characteristic ions of 8–10 C18:2 positional isomer (*m/z* 248, 194 and 220); (C) ion *m/z* 333 and characteristic ions of 9–11 C18:2 positional isomer (*m/z* 262, 276 and 208) and (D) ion *m/z* 333 and characteristic ions of 10–12 C18:2 positional isomer (*m/z* 276, 290 and 236).

spectra (Roach, 1999), the *m/z* 180 and 206 ions are exclusive and can only be attributed to 7–9 isomers. Additionally, 7-*cis* 9-*trans* and 7-*trans* 9-*cis* C18:2 elute separately and far from the chromatographic area where the rest of the 7–9 C18:2 positional isomers (*cis/cis* and *trans/trans*) would be found. Therefore, the elution sequence of CLA isomers on a 100 m CP Sil 88 column, together with the CLA DMOX mass spectra (Fig. 3A & 4A), would provide evidence for the existence of trace amounts of *cis*-7 *trans*-9.

The last peak labelled as CLA in the chromatographic area studied includes an overlapping of at least three *trans/trans* isomers: 8–10, 9–11 and 10–12 (Fig. 4B–D). Although the presence of 7-*trans* 9-*trans* C18:2 should not be completely discarded, the absence of *m/z* 180 and 206 ions (Fig. 4A) means that its existence cannot be confirmed.

With the help of this procedure, the detection of 11-*trans* 13-*cis* C18:2 was also possible (spectra not shown). In contrast, with the reconstructed ion chromatograms it was not possible to confirm the presence of other CLA isomers because they did not exhibit exclusive ions (8-*trans* 10-*cis*, 11-*cis* 13-*trans*, 10-*trans* 12-*cis*, and 8-*cis* 10-*cis* C18:2) or signs of these ions were too weak to be detected (9-*trans* 11-*cis*, 12-*cis* 14-*trans*, 9-*cis* 11-*cis* C18:2). These CLA isomers were only tentatively assigned in Fig. 2.

CLA analysis by Ag^+-HPLC

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 234 nm, and non-conjugated FAME respond poorly at this wavelength. Inspection of Fig. 5 indicates that up to 15 different peaks could be attributed to CLA in ewe milk fat. Ag^+-HPLC identification of CLA isomers was based on co-injection with reference material (Fig. 6) because of retention time irreproducibility and in accordance with the elution order previously reported in studies in similar chromatographic conditions (Sehat et al. 1999; Dobson, 2003). The retention volumes of the CLA isomers change with time but with little loss of resolution.

The Ag^+-HPLC profile of ewe milk fat (Fig. 5) was shown to separate the different *trans/trans* compounds (from 12–14 to 7–9 C18:2) followed by a chromatographic zone where *cis/trans* and *trans/cis* isomers eluted. Although some geometrical isomers were not resolved (*cis/trans* from *trans/cis*), species differing in positional double bonds eluted separately. The use of three columns in series was sufficient to begin resolving 7–9, 8–10, 9–11, 10–12, 11–13 and 12–14 C18:2. The most prominent peak corresponds to 9–11 positional isomers. It was preceded by 3 small peaks assigned to 12–14, 11–13 and 10–12

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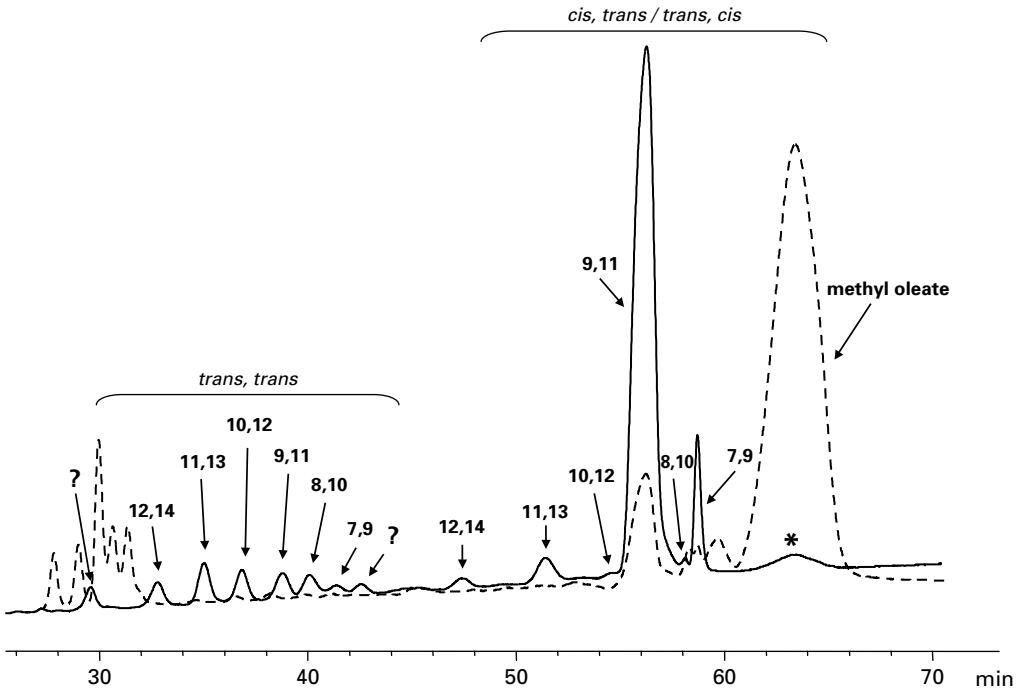


Fig. 5. Ag^+-HPLC profile of ewe milk fat FAME using UV detector at 234 nm (solid line) and 205 nm (broken line) and three columns in series. Asterisk represents methyl oleate.

positional isomers. A tiny shoulder eluting after the 9–11 isomer was attributed to 8–10 C18:2. This was followed by a greater peak corresponding to 7–9 C18:2 (Fig. 5). The difference in retention time between the 8–10 and 7–9 C18:2 was demonstrated chromatographically by co-injecting increasing amounts of a commercial CLA mixture added to a ewe milk fat sample (Fig. 6). With increased amounts of the commercial CLA mixture, the 8–10 C18:2 emerged as the most abundant and before the 7–9 C18:2 peak in the co-injected mixture.

After the *cis/trans* plus *trans/cis* area, *cis/cis* CLA isomers should be located. Nevertheless these compounds were not detected by Ag^+-HPLC. A wide peak (labelled with an asterisk in Fig. 5) could mask some of these isomers. This peak is due to the absorption of methyl oleate. The use of a dual UV wavelength detector meant that it was possible to detect CLA simultaneously and selectively at 234 nm and total fatty acids likewise at 205 nm, thus any interfering peaks due to methyl oleate (Fig. 1) could be observed. At 234 nm, unsaturated fatty acids are generally not detected, unless they are present at a very high concentration in a sample. It should be taken into account that methyl oleate is the most abundant FAME in milk fat. Sehat et al. (1999) also found this pattern when they researched CLA profiles in similar conditions in different dairy foods.

Another two small peaks eluting at the limits of the *trans/trans* region were not identified (Fig. 5). These two peaks could be assigned to 6–8 and 13–15 *trans/trans* C18:2 isomers if the relative elution time and results of previous studies are considered (Rickert et al. 1999; Fritsche et al. 2001). However, it was not possible to confirm them.

Quantitative considerations

From all the data above, it can be seen that most of the CLA in ewe milk corresponds to RA. Given that the 9-*trans* 11-*cis* C18:2 isomer peak seems to be negligible in comparison with the 9-*cis* 11-*trans* C18:2 (Fig. 2 & 4C), the contents of the positional 9–11 C18:2 obtained from Ag^+-HPLC analysis (76·5–82·4% in Table 1) should be attributed almost entirely to RA. Thus, as a minimum percentage, 75% of CLA in ewe milk would be assigned to RA (Table 1). This percentage does not differ substantially from proportions reported for RA in total CLA in cow milk fat (Sehat et al. 1998; Yurawecz et al. 1998).

The percentage of 7–9 C18:2 was the second greatest in three of the five herds studied. Concentrations of 7–9 CLA reported in the literature typically highlight 7-*trans* 9-*cis* C18:2 (Sehat et al. 1998; Yurawecz et al. 1998), whereas there is hardly any mention of the *cis*-7 *trans*-9 isomer. Cow milk fat contents in the 7-*trans* 9-*cis* isomer were generally in the order of 5–10% of total CLA, most of them derived by endogenous synthesis via Δ9-desaturase from 7-*trans* C18:1 produced in the rumen (Bauman et al. 2003). In the present research, final percentages of 7-*trans* 9-*cis* C18:2 in the total CLA would probably be similar or slightly lower, because a small fraction of 7–9 *cis/trans* plus *trans/cis* peak (Fig. 5) could correspond to 7-*cis* 9-*trans*. GC with Fourier Transform Infrared Spectrometry (FTIR) detection, a

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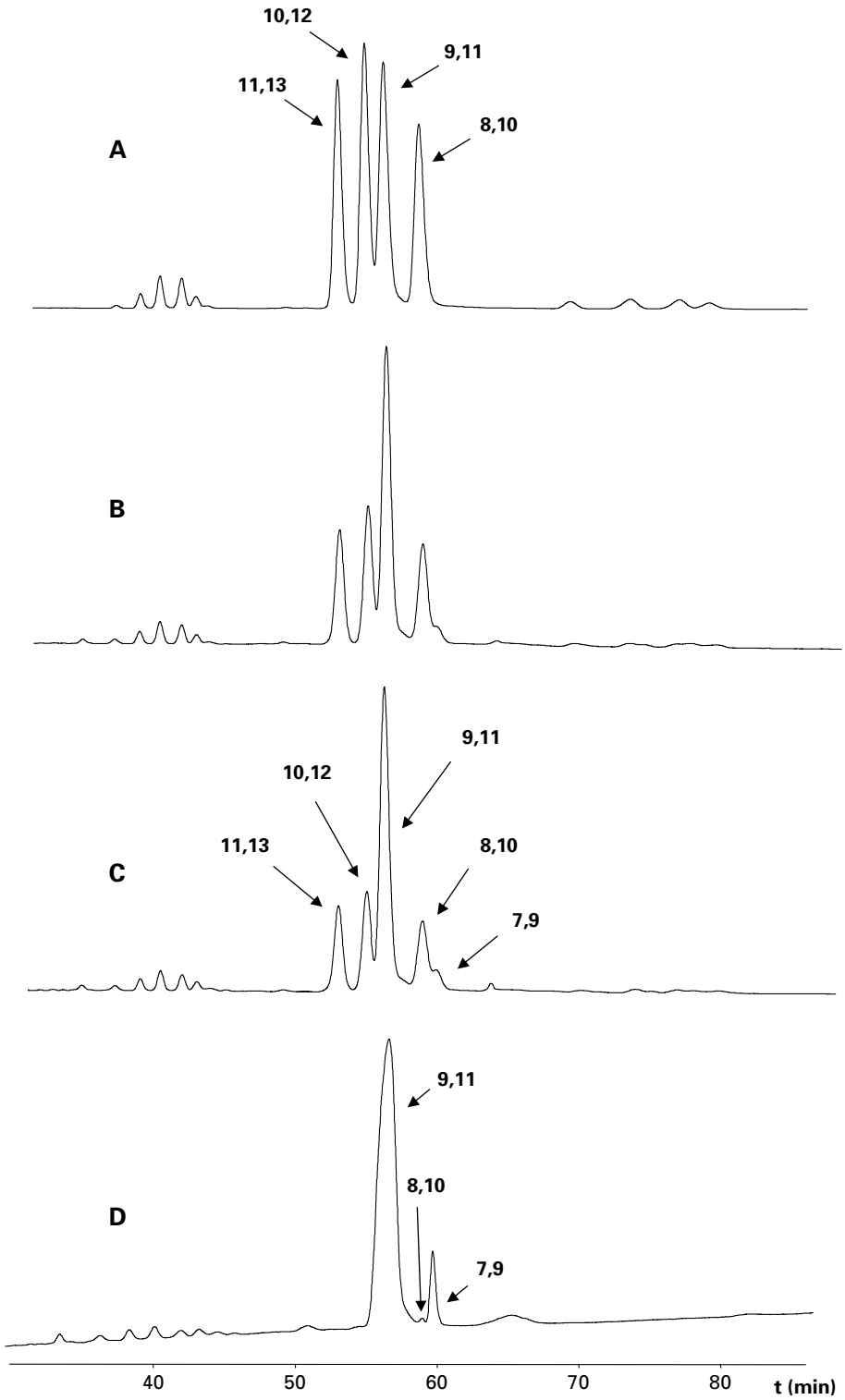


Fig. 6. Ag^{+}-HPLC profiles of FAME from (A) standard *Nu-Chek* mixture, co-injected mixtures of standard *Nu-Chek* plus ewe milk fat FAME (B) 1 : 5, (C) 1 : 10 and (D) ewe milk fat using a UV detector at 234 nm and three columns in series.

powerful technique for determining the geometrical configuration of double bonds in fatty acids, could be applied to confirm the presence of this isomer. Nevertheless, in the context of CLA, GC-FTIR enables the identification of *cis/trans, cis/cis* and *trans/trans* isomers but it does not distinguish between *cis/trans* and *trans/cis* isomers.

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Table 1. Relative composition determined by silver-ion high performance liquid chromatography of conjugated linoleic acid (CLA) isomers in ewe milk fat

Data correspond to the mean values of two methylations and two injections of each methylation

	Herd (individual CLA as % of total CLA)				
CLA Isomers	Assaf	Churra	Castellana X Assaf	Manchega	Awassi
12-*trans* 14-*trans*	1·33 ± 0·23	3·47 ± 0·19	1·31 ± 0·10	1·26 ± 0·20	3·40 ± 0·41
11-*trans* 13-*trans*	1·59 ± 0·11	5·01 ± 0·34	2·23 ± 0·40	1·21 ± 0·27	5·08 ± 0·37
10-*trans* 12-*trans*	1·97 ± 0·15	1·17 ± 0·25	1·61 ± 0·16	1·77 ± 0·19	1·37 ± 0·15
9-*trans* 11-*trans*	1·15 ± 0·14	1·97 ± 0·13	1·35 ± 0·09	1·13 ± 0·18	1·99 ± 0·20
8-*trans* 10-*trans*	1·09 ± 0·26	1·12 ± 0·22	1·12 ± 0·16	1·37 ± 0·10	1·05 ± 0·33
7-*trans* 9-*trans*	0·64 ± 0·21	0·48 ± 0·17	0·49 ± 0·16	0·61 ± 0·21	0·58 ± 0·24
12–14 (*c,t/t,c*)	0·63 ± 0·16	1·83 ± 0·36	0·69 ± 0·18	0·52 ± 0·10	1·45 ± 0·13
11–13 (*c,t/t,c*)	1·14 ± 0·07	4·23 ± 0·16	1·83 ± 0·24	0·76 ± 0·29	3·86 ± 0·37
10–12 (*c,t/t,c*)	0·34 ± 0·01	0·39 ± 0·04	0·36 ± 0·02	0·41 ± 0·08	0·28 ± 0·11
9–11 (*c,t/t,c*)	82·40 ± 0·83	76·91 ± 1·80	82·26 ± 1·18	81·06 ± 1·13	76·50 ± 0·93
8–10 (*c,t/t,c*)	0·71 ± 0·14	0·11 ± 0·01	0·59 ± 0·28	0·22 ± 0·03	—
7–9 (*c,t/t,c*)	7·01 ± 0·42	3·31 ± 0·49	6·15 ± 0·19	9·69 ± 0·48	4·44 ± 0·10

c,t/t,c corresponds to the sum of *cis/trans* plus *trans/cis* C18:2 isomers

Assignments of these isomers (*cis/trans* or *trans/cis*) could be made with reasonable certainty when GC retention data are taken into account. Confirmation of the identification of 7-*cis* 9-*trans* C18:2 had to be carried out using Ag^+-HPLC collection of the 7–9 C18:2 peak and this fraction was further analysed by GC-MS (Sehat et al. 1998).

In contrast to RA and 7-*trans* 9-*cis* C18:2, other CLA isomers found in milk appear to originate exclusively from rumen output (Bauman et al. 2003). The 8–10 and 10–12 *cis/trans* plus *trans/cis* isomer content was very low in all the milks studied (below 1% of total CLA; Table 1). This fact, from a physiological point of view, is remarkable since 10-*trans* 12-*cis* C18:2 is responsible for the observed weight loss/muscle-mass enhancement CLA effects (Belury, 2002; Pariza, 2004). Although the 10–12 C18:2 detected by Ag^+-HPLC should be attributed to the geometrical isomer mentioned above (Fig. 4D where no sign of the m/z=236 –10–12 C18:2 characteristic ion- at a retention time of 10-*cis* 12-*trans* C18:2 was detected), the milk fats here would be a poor source of this isomer.

Ewe milk could be a rich source of 11–13 (*cis/trans* plus *trans/cis* C18:2); milks from Churra and Awassi herds showed the most noticeable percentages in these isomers (up to 4·2% of total CLA; Table 1). From Fig. 2, it appears that 11-*trans* 13-*cis* would be the major geometrical isomer. *Trans/trans* total content was within a range of 7–14% of total CLA, and the Churra and Awassi herds had the highest percentages. Individual t*rans/trans* isomers were easily quantified by Ag^+-HPLC, and 11–13 and 12–14 were the most abundant CLA isomers after RA in the Churra and Awassi herds.

In conclusion: Ag^+-HPLC with three columns in series and GC-MS of FAME and DMOX with very long polar capillary columns have provided the necessary tools for studying the CLA profile in ewe milk fat. The combination of these methods meant that it was possible to detect the most important CLA isomers in ewe milk. On the basis of the results observed in the present study, RA was confirmed as the predominant CLA. Proportions of this isomer (>75% of total CLA) were similar to those generally reported in cow milk fat (Sehat et al. 1998; Yurawecz et al. 1998). Although we have not been able to assess all the individual contents, we have tentatively identified 16 CLA isomers present in these milks. We confirmed that 11-*trans* 13-*trans*, 11–13 *cis/trans* plus *trans/cis* and 7–9 *cis/trans* plus *trans/cis* C18:2 were the main CLA isomers after RA. Differences in the isomer profiles in the herds lead us to believe that diet would affect the CLA species content. There is thus a definite need for feed and fatty acid composition studies in these ruminants to clarify such issues.

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