Analysis of Conjugated Linoleic Acid Isomers and Content in French Cheeses

F. Lavillonnière[a], J.C. Martin[a,*], P. Bougnoux[b], and J.-L. Sébédio[a]

[a]I.N.R.A., Unité de Nutrition Lipidique, 21034 Dijon Cédex, France, and [b]Hôpital Bretonneau, Laboratoire de Biologie des Tumeurs, 37044 Tours Cédex, France

ABSTRACT: Conjugated linoleic acid (CLA) occurs in food as a result of microbial enzymatic reactions, free radical-type oxidation, and heat treatment. CLA is found in animal products, such as meat and dairy products, especially in cheeses. The CLA composition of 12 different French cheeses was determined by a combination of different analytical methods: reversed-phase high-performance liquid chromatography (RP-HPLC), gas chromatography–mass spectrometry (GC–MS), GC–Fourier transform infrared (GC–FTIR), and silver nitrate thin-layer chromatography ($AgNO_3$-TLC). New isomers (Δ8,10- and Δ11,13-octadecadienoic acids with all possible *cis* and *trans* configurations) that co-eluted with previously identified isomers (Δ9*c*,11*t*-; Δ9*t*,11*c*-; Δ10*c*,12*t*-; Δ10*t*,12*c*-; Δ11*c*,13*c*-; Δ9*c*,11*c*-; Δ10*c*,12*c*-; Δ9*t*,11*t*-; Δ10*t*,12*t*-octadecadienoic acids) were detected. Δ9*c*,11*t*-Octadecadienoic acid was the major CLA isomer in these cheeses. All isomers were present in each product, whatever the production process. However, CLA content in the cheeses varied from 5.3 to 15.80 mg/g of cheese fat, which depended primarily on the origin of the milk (season, geography) and somewhat on the production process.
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KEY WORDS: Cheese fat, conjugated linoleic acid (CLA) isomers, dimethyloxazoline, GC–FTIR, GC–MS.

The term "conjugated linoleic acid" (CLA) refers to a mixture of positional and geometric isomers of linoleic acid. The mechanisms that lead to the conversion of linoleic acid to CLA in foods are not clearly understood, but it is usually proposed that heat treatments (1), free-radical type isomerization of linoleic acid (2), and microbial enzymatic reactions in the rumen (3,4) contribute to the formation of CLA. These isomers of Δ9*cis*,12*cis*-octadecadienoic acid are probably formed from the disruption of the methylene-interrupted sequence by a migration of the double bond along the carbon chain, together with a possible modification of the double-bond geometry. Three positional isomers (Δ9,11-, Δ10,12-, and Δ11,13 octadecadienoic acids) have already been identified where double bonds may be in the *cis* or *trans* configuration. Thus, 12 isomers of CLA may occur in processed cheeses (5).

*To whom correspondence should be addressed at I.N.R.A., Unité de Nutrition Lipidique, 17, rue Sully, BV 1540, 21034 Dijon Cédex, France. E-mail: jcmartin@dijon.inra.fr.

CLA was first isolated and identified as an anticarcinogenic agent by Pariza *et al.* (6). Anticarcinogenicity has been demonstrated in mammary cancer in rats (7), in forestomach cancer in mice (8), and in human cancer cells (9). Moreover, CLA is known to be an anticholesterolemic and an antiatherogenic agent (10,11). Nevertheless, it is not known whether the biological activity of CLA is related to one or several isomers.

CLA is found in many foods, including dairy products, meat, and certain vegetable products. CLA content in animal products is usually higher than in plant materials. Dairy products, especially cheeses, are the major source of CLA in animal foodstuffs, where its content ranges from 3 to 9 mg/g of fat (12). The following study reports the CLA content of French cheeses. The samples are representative of the cheese culture and processes used for French cheese production. We also identified and determined the structure of the CLA isomers present in these cheeses.

EXPERIMENTAL PROCEDURES

Twelve different kinds of cheese were purchased in a local store. They were produced in different regions of France by different cheese-making processes. Three Comté cheeses, produced in three locations of the Jura massif (plains, medium-altitude, and high-plateau), were analyzed before ripening (20 h after manufacture) to evaluate their CLA content. Two other Comté cheeses, matured for 5 mon and 1 yr, respectively, were also analyzed to assess the CLA content every 3 cm throughout the thickness and radius of the cheeses.

Determination of the CLA content in cheeses. Cheese fat was extracted according to Wolff and Castera-Rossignol (13). About 5 g of grated, frozen cheese was mixed with anhydrous Na_2SO_4 (5 g) and homogenized in 20 mL isopropanol (IP) with an Ultra-Turrax (Bioblock Scientific, Strasbourg, France). Hexane (30 mL) was added, and the mixture was homogenized. After 10 min of magnetic stirring, the supernatant was removed and filtered. The cheese fat was extracted twice more according to this procedure. The filtered supernatants were pooled and chromatographed on a column (2 cm i.d.), equipped with a sealed-in coarse fritted disk, protected with glass wool, and containing a lower layer of anhydrous Na_2SO_4 (20 g), separated from an upper layer of Celite 545 (5 g; Prolabo, Gradignan, France), which was capped with glass wool.

The fat extract was eluted with about 100 mL of hexane/isopropanol (HIP) (3:2, vol/vol) and stored in HIP at –20°C prior to analysis after reduction of solvents under vacuum.

Fatty acid isopropyl esters (FAIPE) were prepared from about 10 mg of fat, dissolved in 2 mL HIP in a Teflon-lined screw-cap tube. Isopropanol (1.3 mL) and 0.2 mL of 36 N H_2SO_4 were added. The tubes were tightly capped and vigorously shaken, and the reaction was allowed to proceed at 100°C for 1 h. At the end of the reaction, the tubes were cooled, and the FAIPE were extracted with three 1-mL volumes of hexane after addition of 5 mL distilled water. FAIPE were dried over anhydrous Na_2SO_4 and stored in hexane at –20°C prior to analysis.

Fatty acid methyl esters (FAME) were prepared from 10 mg of cheese fat, dissolved in 2 mL hexane with 0.2 mL CH_3ONa (2 N) in methanol for 30 min at room temperature. FAME were extracted twice with 2 mL hexane after addition of 5 mL distilled water. The pooled hexane phases were dried with anhydrous Na_2SO_4. After evaporation of solvent with nitrogen, FAME were redissolved with acetone and stored at –20°C.

Five to 10 mg of FAME, dissolved in 100 µL acetone, was fractionated isocratically at room temperature on a reversed-phase column (Shandon, Cergy-Pontoise, France; Nucleosil C18, 250-mm length, 10 mm i.d.) with acetonitrile as a mobile phase (4 mL/min, 35 min/run). FAME were detected by refractometry, and the fraction containing CLA was monitored in the ultraviolet (UV) at 234 nm. CLA co-eluted with myristic acid (14:0), palmitoleic acid (16:1Δ9) and linoleic acid (18:2Δ9,12). The solvent was evaporated under vacuum, and the residue was redissolved in hexane.

Gas chromatography (GC) analyses of FAIPE and FAME were carried out on a Hewlett-Packard 5890 chromatograph (Les Ulis, France), fitted with a flame-ionization detector and a split-splitless injector, both set at 250°C. Carrier gas was helium (set at 1.1 mL/min). Elution was performed on a BPX-70 column (SGE, Villeneuve Saint Georges, France; 50 m length, 0.32 mm i.d., 0.25 µm film thickness, cyanopropylsiloxane phase).

For the FAIPE, the column was operated at 60°C for 6 min. The temperature was then increased at a rate of 8°C/min to 170°C and held at this temperature for 35 min. For the FAME collected by high-performance liquid chromatography (HPLC), the column was operated at 60°C for 6 min, and the temperature was then increased at a rate of 8°C/min to 160°C and held for 60 min.

Quantitation of fat in cheeses was estimated with tritridecanoin (tri-13:0) as an internal standard. Tritridecanoin was chosen because its carbon number approximates the mean value for milk fat (14) and because the natural tridecanoic acid (13:0) content is low in this type of fat (<0.2%). Fifty mg of tri-13:0 was added at the beginning of the extraction. The resulting tridecanoic acid isopropyl esters were detected by GC and used to assess the fat content. The fat content was expressed as mg/g cheese.

CLA content was quantitated by GC from the 18:2 Δ9*c*,12*c* content of both the HPLC CLA fraction and FAIPE. CLA isomers in the HPLC fraction were analyzed by GC and identified by comparison of retention times with authentic standards. The quantities of CLA were then expressed as a percentage of total fatty acids and as mg/g cheese.

The reproducibility of the CLA determination was checked by determining the coefficient of variation from five parallel runs on one sample of cheese. The coefficient of variation ranged from 0.26% for CLA content to 16% for fat content.

Determination of double-bond geometry and position in CLA. FAME collected by HPLC were fractionated by silver nitrate thin-layer chromatography ($AgNO_3$-TLC) on Silica gel plates (Merck, Chelles, France; 20 × 20 cm, 0.25 mm thickness) that were previously dipped in a 10% solution of $AgNO_3$ in acetonitrile (15); toluene was the developing solvent. The band containing CLA was identified with authentic standards. The CLA band was scraped off, and the lipids were eluted from silica with three 5-mL vol of hexane after addition of 5 mL of methanol/1% aqueous NaCl (90:10, vol/vol).

Dried FAME were saponified in 2 mL 1 N KOH in 95% ethanol. The reaction was allowed to proceed at room temperature for 12 h. The resulting free fatty acids were partitioned with 2 to 3 drops of pure acetic acid, 5 mL water and three 5-mL vol of hexane/diethyl ether (1:1, vol/vol).

Free fatty acids, dissolved in 95% ethanol, were reduced with 1 mL hydrazine (16). The reaction was allowed to proceed at 40°C for 75 min under a stream of O_2. The free fatty acids were extracted with 40 mL hexane after addition of 120 mL water, and converted into FAME by addition of BF_3/methanol (14%, wt/vol). After extraction, the *cis* and *trans* monoenes were separated by $AgNO_3$-TLC.

GC-Fourier transform infrared (GC–FTIR) analysis of FAME derivatives was carried out on a Carlo Erba 5160 chromatograph (Milano, Italy), fitted with a Bucker IFS 85 FT-IR (Karlsruhe, Germany) and a flame-ionization detector by a Bucker GC–IR interface. Elution was performed on a BPX-70 column (50 m length, 0.32 mm i.d., 0.25 µm film thickness, cyanopropylsiloxane phase). The column was operated at 50°C for 2 min. The temperature was then increased at a rate of 20°C/min to 210°C and held for 100 min. It was further increased at a rate of 15°C/min to 220°C and held for 10 min.

FAME (500 µg) were dried under nitrogen in an ampoule and converted into dimethyloxazoline derivatives (DMOX) with 500 µL 2-amino-2-methylpropanol (17). The reaction was allowed to proceed at 170°C for 8 h for samples that contain free CLA, and 18 h at 180°C for monoene FAME. After heating, DMOX were extracted twice with 2 mL dichloromethane after addition of 2 mL distilled water. The dichloromethane phase was washed with distilled water to neutral pH. GC–mass spectrometry (MS) spectral analysis of DMOX derivatives was carried out on a Hewlett-Packard MSD 5970 B quadrupole with an ion source of 70 eV, fitted with a Hewlett-Packard 5890 II model chromatograph. The DMOX derivatives were eluted on a BPX 70 column. The column was operated for 1 min at 50°C, and the temperature was then increased

to 160°C at a rate of 15°C/min and held for 35 min. Identical proportions of different CLA peaks of the DMOX and FAME, as determined by GC analysis, indicated that no isomerization occurred during DMOX derivatization.

RESULTS

Table 1 shows the different processes of cheese production of the selected samples. At least three types of cheese can be differentiated based on processing: Cheeses A to E (type I) did not undergo physical treatment during processing. Cheeses F to H (type II) underwent mechanical operations. Cheeses I to L (type III) had both a mechanical and a moderate heat treatment. The nature of the microorganisms present in each cheese was unknown. The seasonal origin of milk (winter or summer) for each cheese is also indicated.

Identification of the CLA isomers. Figure 1 shows the HPLC profile of the total FAME. The presence of CLA in this fraction was confirmed by UV detection at 234 nm. The fatty acids of this fraction for each cheese were analyzed by GC. The CLA peak distribution is featured in Table 2. All samples showed a similar isomer profile. Peaks 1 to 7 were identified as CLA isomers by comparison with an authentic standard of CLA. The GC–MS analysis of DMOX derivatives of CLA showed the presence in all cheeses of four positional isomers: the Δ8,10-, Δ9,11-, Δ10,12 and Δ11,13-octadecadienoic acids. The position of the double bonds was given by four prominent ions for each isomer. The presence of two additional abundant ions with high intensities also indicated a conjugated double-bond system (Table 3) (18).

The GC–MS profiles of the DMOX derivatives of the monoenes, formed after hydrazine reduction of the isolated CLA fraction, showed an intense peak at *m/z* 126, characteristic of a DMOX derivative. Moreover, there is an even-mass homologous series at *m/z* 126 + *m/z* 14, derived from the cleavage at each bond. In the region of a double bond, these regular series are interrupted by a *m/z*-12 gap (19). The

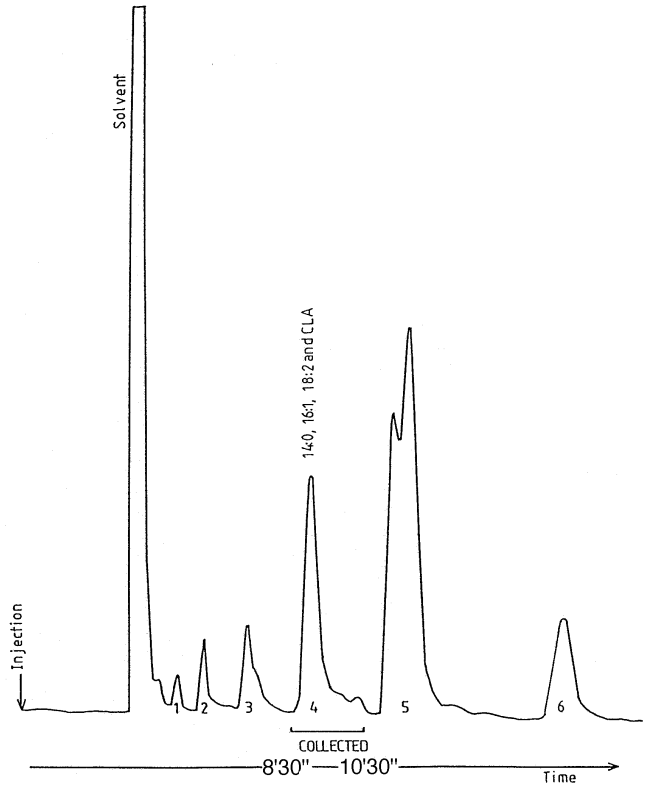

FIG. 1. Fractionation of total fatty acid methyl esters by high-performance liquid chromatography on a C18 reversed-phase column.

DMOX derivatives of both the *cis* and the *trans* monoene fractions allowed us to identify 18:1Δ8, 18:1Δ9, 18:1Δ10, 18:1Δ11, 18:1Δ12, and 18:1Δ13 with *m/z*-12 gap observed at *m/z* 182, *m/z* 196, *m/z* 210, *m/z* 224, *m/z* 238, and *m/z* 252, respectively (Table 3). This confirms the double-bond assignments found with the DMOX derivatives of the parent dienes.

Figure 2 shows the GC–FTIR of the dienes. In Figure 2A, the two peaks at 978 and 949 cm^{-1} are characteristic of a mono-*trans* conjugated fatty acid. The *cis-cis* dienes lack ab-

TABLE 1
Characteristics of French Cheese Samples

Cheeses	French appellation	Type	Milk[a]	Mechanical action	Heating treatment	Ripening (<4 mon)	Ripening (>4 mon)
A	Camembert, pasteurisé	I	PCM-W			•	
B	Camembert lait cru	I	RCM-W			•	
C	Münster	I	PCM-W			•	
D	Chevre	I	PGM-W			•	
E	Bleu	I	PCM-W			•	
F	Reblochon	II	RCM-W	•		•	
G	Tomme	II	PCM-W	•		•	
H	Cantal	II	PCM-S	•			•
I	Comté	III	RCM-S	•	•		•
J	Beaufort	III	RCM-S	•	•		•
K	Emmental	III	PCM-S	•	•		•
L	Toastinette	III	—[b]	•	•		

[a]PCM, pasteurized cow milk; RCM, raw cow milk; PGM, pasteurized goat milk; W, winter milk (November to April); S, summer milk (May to October).
[b]Milk of unknown origin.

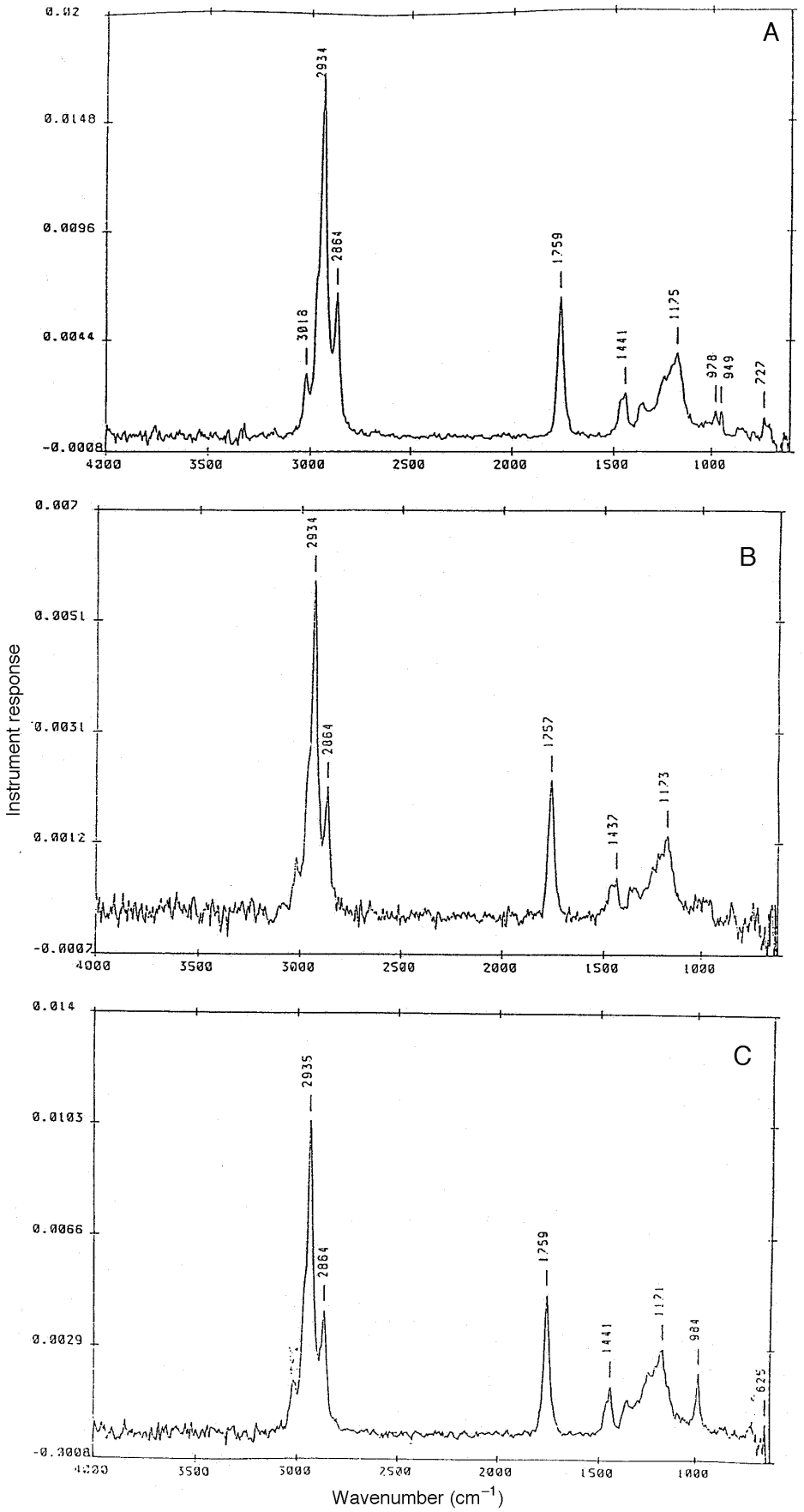

FIG. 2. Fourier transform infrared (FTIR) spectrum. (A) *cis-trans* Conjugated dienes; (B) *cis-cis* conjugated dienes; (C) *trans-trans* conjugated dienes .

TABLE 2
Distribution of CLA Isomers in 12 French Cheeses[a]

	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7
A	88	2.1	1.4	2.5	0.6	1	4.4
B	90	1.3	0.4	2.2	0.5	1.1	4.5
C	89	1.7	0.7	2.8	0.6	0.8	4.3
D	87.1	1.7	1.4	3.8	1.4	0.7	3.9
E	91	2.5	0.6	1.4	0.7	0.4	3.4
F	90.5	1.4	0.5	3.3	0.7	0.5	3.1
G	90	1	0.5	3.8	0.7	0.7	3.3
H	89.1	1.2	0.4	3	0.5	1	4.8
I	87	0.8	0.6	4.9	0.5	1.7	4.5
J	91	1.4	0.6	2.4	0.5	0.6	3.5
K	91	1	0.4	3.4	0.3	0.6	3.3
L	86	1.2	0.6	5	0.7	1.5	0.5

[a]Expressed as weight percentage of total CLA content. CLA, conjugated linoleic acid. See Figure 4 for peak identification.

sorption peaks in that region of the spectrum (Fig. 2B). Finally, the *trans-trans* dienes showed only one peak at 986 cm^{-1} (Fig. 2C).

Figure 3 presents the GC–FTIR profiles of the two monoene fractions, obtained after hydrazine reduction of CLA and fractionation by $AgNO_3$-TLC. In Figure 3A, the peak at 3013 cm^{-1} is unambiguously identified as a *cis* monoene double bond. There is no peak at 966 cm^{-1}. Therefore, there are no *trans* monoenes in this fraction. Conversely, in Figure 3B, the peak at 966 cm^{-1} showed that this fraction contains only *trans* monoenes.

Figure 4 reports the GC elution profile of CLA, whose double-bond positions and geometry have been determined by combination of the GC–MS and GC–FTIR studies as well as by using a 18:2Δ9,11 standard (20). Each peak contained at least two nonresolved critical pairs, except peak 6, which contained only 18:2Δ11*t*,13*t*.

Determination of CLA contents. Figure 5 presents a partial GC chromatogram of FAIPE, obtained from the total lipids of cheese. Up to 26 FAIPE were identified and quantitated, from butyric acid (4:0) to linolenic acid (C18:3Δ9*c*,12*c*,15*c*) (Table 4). The remaining unidentified peaks accounted for only 1.8 to 2.8% of the total fatty acids. In Table 4, we also report individual contents of fatty acids that are possibly associated with biohydrogenation of linoleic acid and linolenic acid, namely, CLA, 18:1Δ11*t*, and 18:0 (21,22). The fatty acid profiles were similar for all samples, regardless of milk type and process. Among all samples, CLA content ranged from 0.53% of total fatty acids (cheese D, type I) to 1.58% (cheese G, type II).

We intended to develop insight on the origin of CLA in cheeses by examining the relationships between CLA contents and contents of the other fatty acids in cheeses. Significant and positive correlations were only found between CLA and the other fatty acids that are also involved in microbial biohydrogenation, e.g., between CLA and 18:0, CLA and 18:1Δ11*t*, 18:2Δ9*c*,12*c* and CLA, as well as between 18:3Δ9*c*,12*c*,15*c*, and CLA (Fig. 6).

Finally, we also studied the influence of the geographic factor upon the CLA content. We chose different kinds of Comté cheeses that were made with milk collected during the same period (September) and originating from the same cow strain (Montbéliard) but grazing either in the plains, at medium altitude or on the high plateau of the Jura mountains. Analyses were made before ripening to prevent any differences due to this factor (Table 5). Cheeses produced from milk of cows grazing in the plains or at medium altitude contained more CLA (20.8 and 20.6 mg/g of fat, respectively) than cheeses from milk of cows grazing on the high plateau (15 mg/g of fat). However, the overall CLA profiles were similar, with peaks 18:2Δ9*c*,11*t* + 18:2Δ8*c*,10*t* accounting for 85 to 89% of the total CLA.

The homogeneity of the CLA content in cheeses was checked by analyzing small samples taken all along the distance (every 3 cm) from the core to the outside edge of Comté cheeses that had matured for 5 mon and 1 yr, respectively. Essentially no differences above the analytical uncertainty were observed between samples when dealing with the CLA content or CLA isomers distribution (Table 5).

TABLE 3
Characteristic Ions of DMOX Derivatives of CLA and of Their Resulting Monoenes[a]

Isomers	Molecular ion (M^+, *m/z*)	Diagnostic ions (*m/z*)
18:2Δ8,10	333	182; 194; 208; 220; 248; 262
18:2Δ9,11	333	196; 208; 222; 234; 262; 276
18:2Δ10,12	333	210; 222; 236; 248; 276; 290
18:2Δ11,13	333	224; 236; 250; 262; 290; 304
18:1Δ8	335	182; 194
18:1Δ9	335	196; 208
18:1Δ10	335	210; 222
18:1Δ11	335	224; 236
18:1Δ12	335	238; 250
18:1Δ13	335	252; 264

[a]Obtained after hydrazine reduction. DMOX, dimethyloxazoline.

TABLE 4
Total Fatty Acid Composition of French Cheeses[a]

	Cheese		
Fatty acids	Type I	Type II	Type III
Short-chain saturated[b]	7.4 ± 0.8	7.0 ± 0.7	7.1 ± 0.3
Medium-chain saturated[c]	10.0 ± 3.4	7.2 ± 0.7	7.1 ± 0.6
Long-chain saturated[d]	53.5 ± 4.0	52.2 ± 3.7	51.8 ± 1.0
Monounsaturated[e]	24.4 ± 1.3	28.1 ± 4.5	27.5 ± 1.9
18:0	8.9 ± 0.9	9.7 ± 1.8	10.2 ± 0.5
18:1Δ11*t*	1.3 ± 0.2	2.6 ± 1.1	2.7 ± 0.5
18:2Δ9*c*,12*c*	1.5 ± 0.3	1.7 ± 0.4	1.8 ± 0.4
18:3Δ9*c*,12*c*,15*c*	0.4 ± 0.0	0.8 ± 0.1	0.8 ± 0.1
CLA	0.6 ± 0.1	1.1 ± 0.5	1.3 ± 0.2
Not identified	2.3 ± 0.4	1.8 ± 0.9	2.8 ± 1.0

[a]Results are expressed as weight percentage (mean ± SEM) of total fatty acids. See Table 1 for cheese-type characteristics. For abbreviation see Table 2.
[b]Sum of C4:0, C6:0, and C8:0.
[c]Sum of C9:0, C10:0, C11:0, C12:0, and C13:0.
[d]Sum of *iso*C14:0, C14:0, *iso*C15:0, C15:0, *iso*C16:0, C16:0, *iso*C17:0, C17:0, and C18:0.
[e]Sum of C10:1, C14:1Δ9*c*, C16:1Δ7*c*, C16:1Δ9*c*, C17:1, C18:1Δ9*c*, C18:1Δ11, and C18:1Δ11*t*.

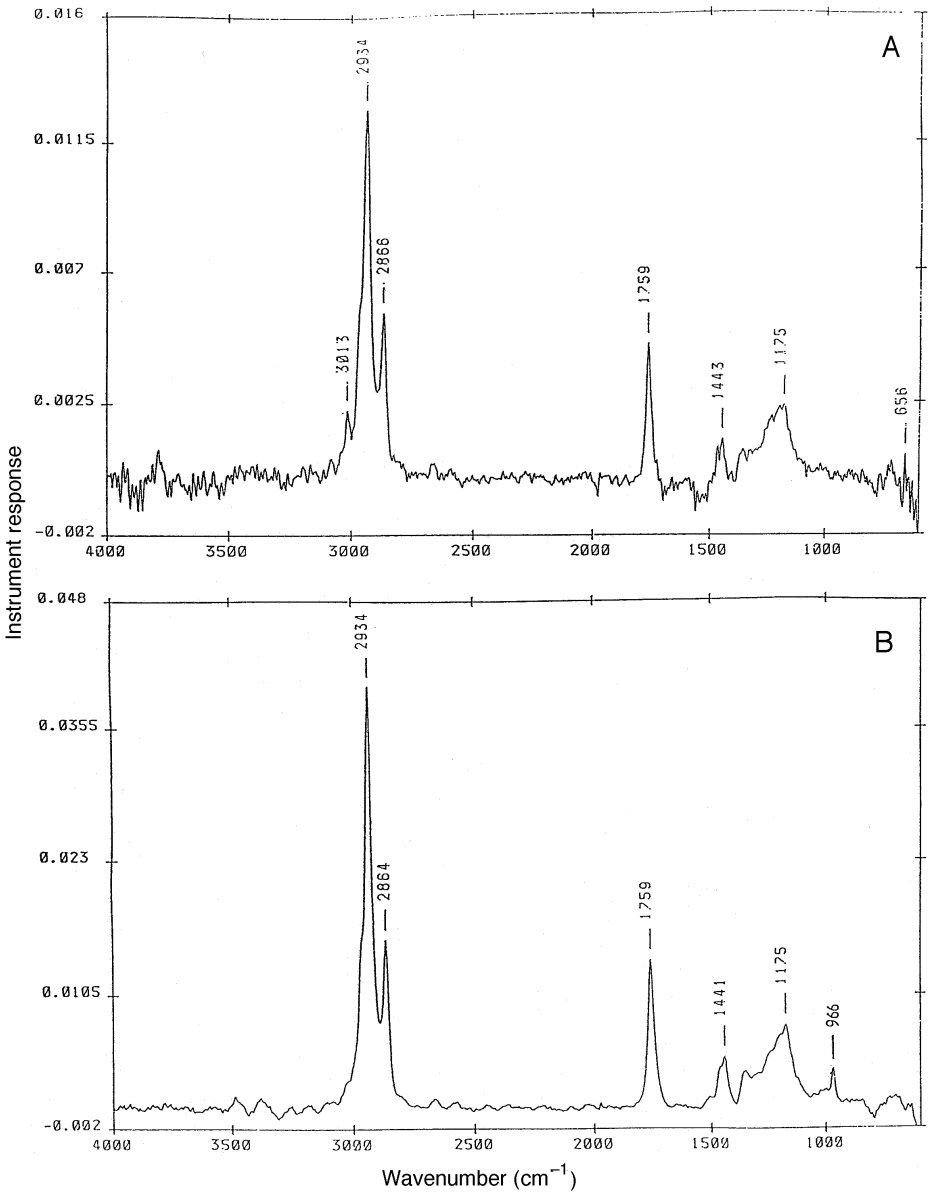

FIG. 3. (A) FTIR absorption spectrum obtained for the *cis* monoene methyl esters fraction. (B) FTIR absorption spectrum obtained for the *trans* monoene methyl esters fraction. For abbreviation see Figure 2.

DISCUSSION

One of the goals of this work was to improve the analysis of CLA in food products. Indeed, GC analysis did not individually resolve all CLA isomers. Moreover, there is no available commercial standard for each CLA isomer that may help to identify the composition of a CLA mixture by GC. Structural studies conducted by Ha *et al.* (1) with GC–MS used methyl ester derivatives of CLA. Such derivatives are inappropriate for the determination of the double-bond position because the double bond is unstable upon fragmentation and can migrate along the carbon chain (23). We combined two analytical methods, namely, GC–FTIR and GC–MS of the DMOX derivatives, to obtain both geometry and double-bond localization.

Examination of the spectra of DMOX derivatives revealed the presence of four positional isomers in cheese (18:2Δ8,10; 18:2Δ9,11; 18:2Δ10,12; and 18:2Δ11,13), whereas only three had been identified previously (5). GC–FTIR analysis conducted on these dienes enabled the identification of a configuration for each double bond. According to Hopkins (24), the conjugated double bonds are characterized by an absorption at 986 cm^{-1} for the *trans-trans* isomers, and at 980 and 950 cm^{-1} for the *cis-trans* isomers. If detectable, the *cis-cis* conjugated double bond might be observed around 730 cm^{-1}. CLA detection by GC–FTIR indicated that the *cis-trans* and the *trans-cis* dienes eluted first, then the *cis-cis,* and then the *trans-trans*.

To confirm this result further and to determine whether the *cis-trans* eluted ahead the *trans-cis* isomers, we used repre-

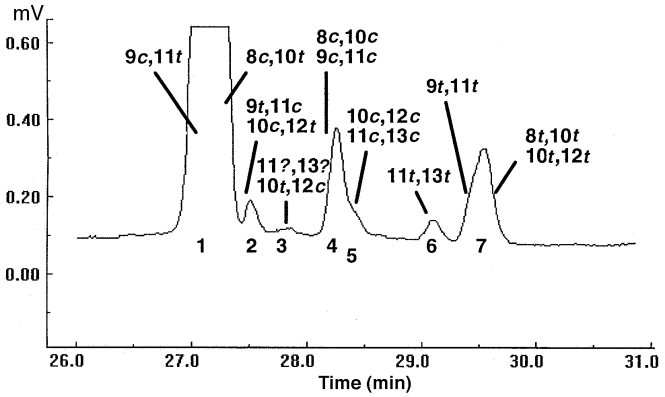

FIG. 4. Gas chromatography (GC) profile of conjugated peaks eluted on a BPX 70 column (SGE, Villeneuve St. Georges, France). Peak 1: 18:2Δ9*c*,11*t* and 18:2Δ8*c*,10*t*; peak 2: 18:2Δ9*t*,11*c* and 18:2Δ10*c*,12*t*; peak 3: 18:2Δ10*t*,12*c* and 18:2Δ11*?*,13*?*; peak 4: 18:2Δ8*c*,10*c* and 18:2Δ9*c*,11*c*; peak 5: 18:2Δ10*c*,12*c* and 18:2Δ11*c*,13*c*; peak 6: 18:2Δ11*t*,13*t*; peak 7: 18:2Δ8*t*,10*t*, 18:2Δ9*t*,11*t*, and 18:2Δ10*t*,12*t*.

sentative monoenes of CLA produced after hydrazine reduction. These monoenes are representative of the dienes, both with regard to double-bond position and configuration, and are also easier to analyze than their parent dienes. GC–MS carried out with these monoenes as DMOX derivatives confirmed the double-bond localization obtained with the original dienes. Moreover, GC–FTIR and GC analyses, conducted with the FAME monoenes isolated by $AgNO_3$-TLC, indicated a larger peak for both 18:1Δ9*c* and 18:1Δ11*t* than for 18:1Δ9*t* and 18:1Δ11*c* (data not shown). Thus, by deduction, 18:2Δ9*c*,11*t* eluted ahead of the 18:2Δ9*t*,11*c* in GC, and by extrapolation, the *cis-trans* isomers eluted ahead of the *trans-cis* isomers. This was further confirmed by using a pure standard of 18:2Δ9,11 that contained all geometrical isomers (20). By combining all these methods, we can propose the sequence of elution of CLA as depicted in Figure 4. However, it was not possible to determine the CLA content of each of the individual isomers.

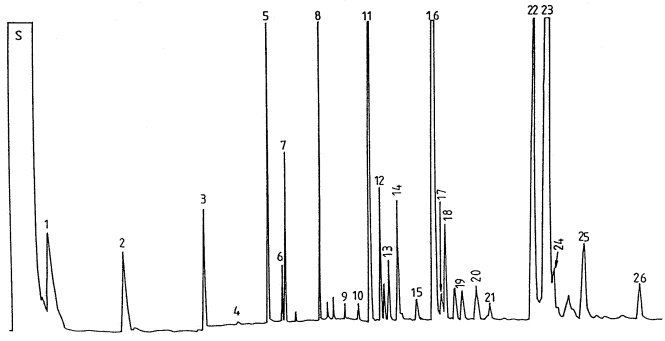

FIG. 5. GC elution profile of total fatty acid isopropyl esters of cheese I (Comté). S: solvent; peak 1 = 4:0; 2 = 6:0; 3 = 8:0; 4 = 9:0; 5 = 10:0; 6 = 10:1; 7 = 11:0; 8 = 12:0; 9 = 13:0; 10 = *iso*14:0; 11 = 14:0; 12 = 14:1; 13 = *iso*15:0; 14 = 15:0; 15 = *iso*16:0; 16 = 16:0; 17 = 16:1Δ7*c*; 18 = 16:1Δ7*c*; 19 = *iso*17:0; 20 = 17:0; 21 = 17:1; 22 = 18:0; 23 = 18:1Δ9*c*; 24 = 18:1Δ11*c*; 25 = 18:2Δ9*c*,12*c*; 26 = 18:3Δ9*c*,12*c*,15*c*.

By using cheeses produced from milk of different origins (seasons, pasteurized or raw), geography, processes as well as variable maturation, the present study allowed a better understanding of the origin of CLA in cheeses. Conditions of process and aging have been reported to modulate CLA formation. For instance, Lin *et al.* (12) reported low values of CLA in certain dairy products, such as nonfat yogurt (0.2% of total fatty acids), and higher values in processed cheeses (0.4 to 0.8%). Chin *et al.* (25) also found relatively low values in nonprocessed dairy products. Additionally, we found a broad variation of CLA content in cheeses with a long ripening time (>4 mon) compared with cheeses ripened for a shorter duration (<1 mon) (9.9 to 15.8 mg/g of fat and 5.3 to 6.0 mg/g of fat, respectively) (Tables 1 and 4). Long ripening time is also associated with other side-processing activities, such as mechanical action and heat treatment, which allow longer air exposure and greater proteolysis. These two factors are known to increase the radical-mediated oxidation of lipids, which is involved in the nonbacterial formation of CLA. Nevertheless, it is unlikely that a moderate heat treatment of cheeses alone leads directly to a substantial isomerization of linoleic acid to CLA. Mechanical and thermal treatments probably modulate in synergy the extent of linoleic acid conversion into CLA. However, some discrepancies might be observed between our findings and the study of Ha *et al.* (5) on one side, and the data reported by Chin *et al.* (25) on the other side. For instance, in our study, cheese G had the longer ripening time and also had the higher CLA content. Even if lower values for CLA were reported by Ha *et al.* (5), the CLA content in parmesan (10 mon) was increased 1.5- and 2-fold compared to Cheddar (6 mon) or Romano cheese (5 mon). Conversely, Chin *et al.* (25) found lower values for CLA in the most-ripened cheeses. They concluded that lipolysis by bacterial enzymes, occurring during ripening, increases CLA oxidation and indirectly reduces CLA content in aged cheeses. However, neither Ha *et al.* (5) nor Chin *et al.* (25) reported the time of year when the milk was collected to make the cheeses. It is likely that the discrepancies between all these studies, including ours, could be explained by the seasonal variation of CLA in milk. This factor should be taken into account because it has been demonstrated that the CLA content in cow's milk is noticeably influenced by diet, and therefore by season. For instance, compared to forage feeding in winter, pasture feeding in summer considerably increased the CLA content in milk, ranging from 3.9 mg/g of fat in winter to 22.7 mg/g of fat in summer (26). Based on the seasons, our set of cheeses may be divided into two groups: one group made with winter milk (W in Table 1), ripened for a short period and low in CLA; the other group made with summer milk (S in Table 1), ripened for a longer time and high in CLA. The CLA contents ranged from as low as 5.3 mg/g of fat in the former group up to 15.8 mg/g of fat in the latter group. The CLA content found by Jiang *et al.* (26) in milk was the same range, and one may surmise that the variation of CLA contents observed in cheeses is due mainly to the variation of CLA content in milk from different seasons.

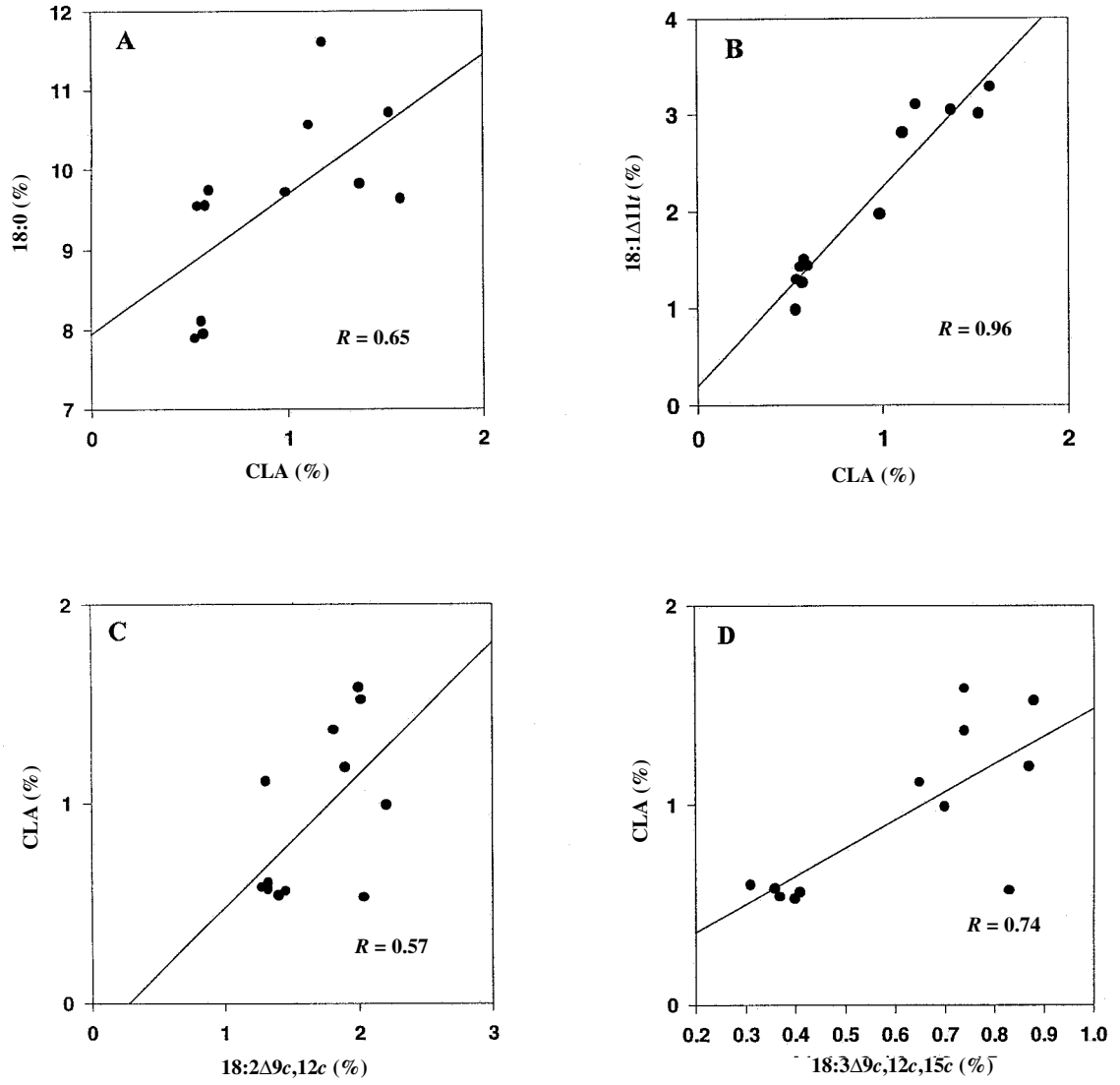

FIG. 6. Linear regression fit (A) between 18:0 content and CLA content in cheeses; (B) between 18:1Δ11*t* and CLA content; (C) between 18:2Δ9*c*,12*c* content and CLA content; (D) between 18:3Δ9*c*,12*c*,15*c* content and CLA content. The correlation coefficient (*R*) is indicated. Abscissa: precursor of biohydrogenation; ordinate: product of biohydrogenation.

The experiment carried out with unripened Comté cheeses seems to confirm this hypothesis and additionally excludes the large influence of the ripening time over CLA content in cheeses. For instance, the CLA content in unripened Comté (20 h after manufacture) was similar to the values found for Comté matured for several months to 1 yr (Table 5). In that latter situation, unripened and 1-yr matured cheeses were made with milk of the same season (September). The experiment carried out with Comté cheeses produced from cow's milk of different pastures also indicates an influence of the geographic factor on the CLA content in milk and thereby in cheeses (Table 5). All Comté cheeses are produced in the same area of France (Jura massif) and from milk of the same cow strain (Montbéliard). The only difference among the three unripened Comté cheeses for this experiment was the site of pasture. Plants change with altitude, and the kind of pasture changes from the plain to medium altitude and up to the high plateau. As for the finding of Jiang *et al.* (26), this dietary factor would explain the different CLA contents between the "plain" and "medium-altitude" Comté on one side, and the "high-altitude" Comté on the other side. Conversely, the CLA profiles were identical for all cheeses (Tables 2 and 5). This suggests that, if the origin of the milk and thereby the dietary status of the lactating cows widely influence the total CLA content of cheeses, it does not influence isomer distribution. In addition, we did not find any heterogeneous distribution of CLA according to the site of sampling within a cheese with regard to CLA content (mg/g of fat) or CLA peaks (Table 5). These results again point out that CLA originate from the milk and that their formation is probably low in cheeses. Additionally, the correlations calculated between the different fatty acids involved in biohydrogenation in the rumen (Fig. 6) also support that CLA in cheese originate mainly from the microorganisms found in the rumen of lac-

TABLE 5
CLA Content and Isomer Distribution of Unripened (20 h) and Matured Comtés (5 mon and 1 yr)[a]

	Unripened Comté (wt% of total CLA content)[b]			Matured Comté (wt% of total CLA content)[b]	
Isomers[c]	Plain	Medium altitude	High plateau	5 Months	1 Year
Peak 1	88.8 ± 0.8	88.3 ± 1.4	85.1 ± 2.2	89.3 ± 0.5	87.7 ± 0.1
Peak 2	1.3 ± 0.3	1.4 ± 0.3	1.7 ± 0.5	1.4 ± 0.1	1.3 ± 0.1
Peak 3	0.4 ± 0.1	0.4 ± 0.0	0.5 ± 0.2	0.4 ± 0.1	0.4 ± 0.1
Peak 4	4.6 ± 0.2	4.3 ± 0.4	5.3 ± 0.3	2.6 ± 0.1	4.5 ± 0.1
Peak 5	0.5 ± 0.2	0.5 ± 0.2	0.5 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
Peak 6	0.8 ± 0.2	0.9 ± 0.0	1.2 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
Peak 7	3.5 ± 0.3	4.1 ± 0.6	5.5 ± 1.1	4.5 ± 0.3	5.0 ± 0.2
CLA (mg/g of fat)	20.8 ± 4.0	20.6 ± 1.1	15.0 ± 0.7	16.1 ± 0.7	17.2 ± 0.2

[a]Mean values ± standard deviations obtained from three determinations for each cheese.
[b]Mean values ± standard deviations expressed as weight percentage of total CLA content in gas chromatographic analysis.
[c]Peak 1: 18:2Δ9*c*,11*t* + 18:2Δ8*c*,10*t*; Peak 2: 18:2Δ9*t*,11*c* + 18:2Δ10*c*,12*t*; Peak 3: 18:2Δ10*t*,12*c* + 18:2Δ11*?*,13*?*; Peak 4: 18:2Δ8*c*,10*c* + 18:2Δ9*c*,11*c*; Peak 5: 18:2Δ10*c*,12*c* + 18:2Δ11*c*,13*c*; Peak 6: 18:2Δ11*t*,13*t*; Peak 7: 18:2Δ9*t*,11*t* + 18:2Δ8*t*,10*t* + 18:2Δ10*t*,12*t*.

tating cows, such as *Butyrivibrio fibrisolvens*, which produce CLA as an intermediate of the biohydrogenation of the 18:2Δ9*c*,12*c* (22). Especially, we found positive correlations between CLA and 18:0, 18:1Δ11*t*, 18:2Δ9*c*,12*c*, and 18:3Δ9*c*,12*c*,15*c* (Fig. 6). The correlation was strong between 18:1Δ11*t* and CLA content. Jiang *et al.* (26) also found a strong linear correlation between 18:1Δ11*t* and 18:2Δ9*c*,11*t* content in cheese fat. They explained the relationship between these two fatty acids by the microbial biohydrogenation activity occurring in the rumen of the lactating cow. Indeed, complete biohydrogenation of linoleic acid to stearic acid involves at least two steps: the conversion of the dienoic acid to a monoenoic acid (21) of *trans* configuration (27) and the conversion of the monoenoic acid to the saturated acid. Lin *et al.* (12) found the same positive correlation and also reported a negative correlation between the CLA content and 18:2Δ9*c*,12*c*, which is not confirmed by our observations in French cheeses. In addition, we found a positive relationship between the α-linolenic acid content and the CLA content in cheeses (Fig. 6D). Such a relationship was not found in milk by others (26). Kepler and Tove (22) showed that biohydrogenation of linolenic acid was the first step involved in the formation of dienoic acids. As Viviani (4) did, we can hypothesize that biohydrogenation of linolenic acid in cheese leads to the formation of CLA as intermediates. Thus, CLA in cheeses could have two microbial origins: the biohydrogenation of linoleic acid in the rumen and the biohydrogenation of linolenic acid in cheeses. Stable isotope or labeled precursor studies may confirm these hypotheses.

In conclusion, this study established that up to 15 mg of CLA/g of cheese fat are present in French cheeses. The CLA content seems to be influenced by the origin of the milk and, in minor proportions, by the conditions of processing and ripening. Although we have not been able to assess their individual content, we precisely identified 14 isomers present in cheeses and confirmed that 18:2Δ9*c*,11*t* was the main CLA isomer in this dairy product. We also identified 18:2Δ8,10 acids as new isomers in cheese.

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