CLA Isomers in Milk Fat from Cows Fed Diets with High Levels of Unsaturated Fatty Acids

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ABSTRACT: The concentrations of CLA isomers were determined by Ag^+-HPLC in the milk fat of cows fed a control diet consisting of hay *ad libitum* and 15 kg of fodder beets or this diet supplemented with oilseeds containing either high levels of oleic acid (rapeseed), linoleic acid (sunflower seed), or α-linolenic acid (linseed). Highly significant ($P \leq 0.001$) correlations were found between the daily intakes of oleic acid and the concentration of the CLA isomer *trans*-7,*cis*-9 in milk fat; of linoleic acid and the CLA isomers *trans*-10,*trans*-12, *trans*-9,*trans*-11, *trans*-8,*trans*-10, *trans*-7,*trans*-9, *trans*-10,*cis*-12, *cis*-9,*trans*-11, *trans*-8,*cis*-10, and *trans*-7,*cis*-9; and of α-linolenic acid and the CLA isomers *trans*-12,*trans*-14, *trans*-11,*trans*-13, *cis,trans*/*trans,cis*-12,14, *trans*-11,*cis*-13, and *cis*-11,*trans*-13. CLA concentrations were also determined in the milk fat of cows grazing in the lowlands (600–650 m), the mountains (900–1210 m), and the highlands (1275–2120 m). The concentrations of many isomers were highest in milk fat from the highlands, but only three CLA isomers (*cis*-9,*trans*-11, *trans*-11,*cis*-13, and *trans*-8,*cis*-10) showed a nearly linear increase with elevation. Therefore, these three CLA isomers, and particularly the CLA isomer *trans*-11,*cis*-13, the second-most important CLA in milk fat from cows grazing at the three altitudes, could be useful indicators of milk products of Alpine origin.

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Unsaturated FA (UFA) are generally converted in the rumen of the cow to saturated FA within a short time through ruminal biohydrogenation, but the extent of lipolysis and biohydrogenation in the rumen decreases with increasing amounts of substrate (1). Many *trans*-FA are found in the ruminal fluid (2) and milk fat (3), and these are believed to originate from double-bond migration or alternative pathways of biohydrogenation (4). These *trans* FA are precursors of CLA, a collective term for several conjugated isomers of linoleic acid (5–7).

CLA consists of a collection of positional and geometrical isomers of octadecadienoic acid, with conjugated double bonds ranging from 6,8 to 12,14. For every positional isomer, four geometric pairs of isomers are possible (i.e., *cis*,*trans*; *trans*,*cis*; *cis*,*cis*; and *trans*,*trans*). CLA therefore includes 28 positional and geometrical isomers, of which only *cis*-9, *trans*-11, *trans*-10,*cis*-12, and *trans*-9,*trans*-11 have thus far been proven to have biological activities (8–12). Individual CLA isomers have exhibited different biological activities in animal and cancer cell studies. For example, Corl *et al.* (9) showed that the *cis*-9,*trans*-11 CLA isomer can reduce cancer risk in rats, and Park *et al.* (10) reported that the *trans*-10,*cis*-12 isomer was more effective for the reduction of mouse body fat than the *cis*-9,*trans*-11 CLA isomer. The *trans*-10,*cis*-12 CLA isomer was also found to inhibit cell growth and secretion of insulin-like growth factor-II in Caco-2 cells (11). In addition, current studies show that a mixture of *trans,trans* CLA isomers, mainly composed of *trans*-10,*cis*-12 and *trans*-9,*trans*-11 isomers, exhibited stronger cytotoxicity against NCI-N87 gastric cancer cells than *cis,trans/trans,cis* CLA isomers by inhibiting proliferation and modulating arachidonic acid metabolism (12). These suggest that the biological activities of all individual CLA isomers must be evaluated.

The content of CLA in milk fat can vary widely (about 3 to 25 mg g^{-1} fat) (8,13–15). The underlying factors resulting in this variation are predominantly related to the diet, to the methods of raising ruminants (16), and also to animal variation (17,18). Feeding of rapeseed, soybean, or linseed oils (19,20); rapeseed press cake; full-fat rapeseed or oil-rich rapeseed cake (21); extruded soybeans and fish oil, fed alone or in combination (22); marine oils (23); or a patented high-fat diet (24) has been shown to increase the concentration of CLA in milk fat. Also, the milk fat of cows grazing in the Alps is extraordinarily rich in total CLA (19.20 to 28.70 mg g^{-1} fat) (13), which is also true for Alpine cheese (25). Collomb *et al*. (26) correlated the FA in milk fat with botanical families and individual plant species. The percentage of three species [*Leontodon hispidus, Lotus corniculatus (*and *alpina),* and *Trifolium pratense*] correlated positively with the concentrations of CLA and monounsaturated *trans* 18:1 FA in milk fat. In the majority of studies, only the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA isomers have been used, and only a few publications have dealt with the occurrence of other CLA isomers in milk (27–29). Our knowledge of the variation of isomer distribution in ruminant fat is therefore limited.

The aim of the present experiments was to evaluate variations in the distribution of CLA isomers in milk fat from cows fed either a control diet (CD) consisting of hay *ad libitum* and 15 kg of fodder beets or the CD supplemented with ground

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Abbreviations: Ag^+-HPLC, silver-ion HPLC; CD, control diet (hay *ad libitum* and 15 kg fodder beets); DM, dry matter; LIN1 diet, CD supplemented with 1.0 kg of ground linseed; LIN1.4 diet, CD supplemented with 1.4 kg of ground linseed; RAP1 diet, CD supplemented with 1.0 kg of ground rapeseed; SFA, saturated FA; SUN1 diet, CD supplemented with 1.0 kg of ground sunflower seed; SUN1.4 diet, CD supplemented with 1.4 kg of ground sunflower seed; *t*VA, *trans* vaccenic acid; UFA, unsaturated FA.

oilseeds containing a high concentration of either oleic (rapeseed), linoleic (sunflower seed), or α-linolenic acid (linseed). In a second study, we analyzed the CLA isomer distribution in milk fat from cows grazing at three altitudes. Comparisons were made between the results obtained from the two studies.

MATERIALS AND METHODS

Aim and approach. The composition of the CLA isomers in deep-frozen milk fats (–20°C) originating from two previous studies (13,14,30,31) was analyzed using silver-ion HPLC (Ag^+-HPLC). GC results of the FA composition of these milk fats has already been published (13,14).

Briefly, the first study (14) dealt with the impact of a CD consisting of hay fed *ad libitum* and 15 kg of fodder beets supplemented with each of three ground oilseeds on the FA composition of the milk fat. Thirty-three cows of the breeds Red Holstein, Holstein, and Brown Swiss were included. All animals were fed the CD for 2 wk. From week 3 to 4, the cows were divided into three groups, and the CD was supplemented daily with either 1.0 kg of ground rapeseed (RAP1 diet) [0.92 kg dry matter (DM)], sunflower seed (SUN1 diet) (0.95 kg DM), or linseed (LIN1 diet) (0.87 kg DM). From week 5 to 6, the amount of either sunflower seed or linseed oil increased by 0.4 kg to 1.27 (SUN1.4 diet) or 1.24 kg DM d^{-1} (LIN1.4 diet), respectively. The diets were supplemented with a cereal mix and a protein concentrate based on the average milk yield, milk content, animal body weight, and feed intake of the previous week. Table 1 presents the concentrations of fat, the predominant UFA in the three oilseeds, and the daily intake of UFA in the diets of cows.

The second study (13,30,31) dealt with the impact on the FA composition of milk fat of the fodder plants on which cows grazed in the lowlands (600–650 m), mountains (900–1210 m), and highlands (1275–2120 m) of Switzerland (13). In this investigation, Simmental × Red Holstein cows (45 to 50 cows in the lowlands, four to six herds of 10 to 30 cows in the mountains, and 57 to 88 cows in the highlands) were included. Twelve observations per site were carried out on the three vegetation sites: two sites in the highlands (pooled into a single zone, since their FA compositions did not differ significantly), one site in the mountains, and one site in the lowlands over a period of 3.5 mon (from June to mid-September).

Sample selection. From the first study, we analyzed 10 milk fats from individual cows fed the CD and 10 milk fats from individual cows fed the CD supplemented with oilseeds (five variants) by Ag^+-HPLC. From the second study, 10 summer mixed milk fats from each of three altitudes were analyzed by the same method. In total, 90 milk fats were analyzed.

Sample treatment. The milk samples were centrifuged, and the resulting creams were churned at *ca.* 5°C (32). After the resulting molten butter had been filtered through a hydrophobic filter (Schleicher Schuell no. 597 HY 1/2), the pure milk fat was collected. Fat from cheese samples was extracted in accordance with an IDF standard. All milk fats were frozen and stored at −20°C until analysis.

Methods of analysis. (i) Methylation. The milk fat was dissolved in hexane, and the glycerides were transesterified to the corresponding FAME by a solution of potassium hydroxide in methanol in accordance with an ISO standard (33).

(ii) GC analysis. FAME were analyzed according to Collomb and Bühler (34) using an Agilent 6890 gas chromatograph equipped with an on-column injector and an FID. The FA were separated on a CP-Sil 88 capillary column (100 m × 0.25 mm i.d. × 0.20 μm; Varian BV, Middelburg, The Netherlands) and quantified using nonanoic acid as an internal standard. The results were expressed in absolute values, as milligrams of FA (and not as esters) per gram of fat.

(iii) Ag^+-HPLC analysis. The methyl esters of *cis*-9,*trans*-11 (98%), *trans*-10,*cis*-12 (98%), and technical-grade *cis*-9, *trans*-11 (75–78%) were obtained from Matreya Inc. (Pleasant Gap, PA). Other CLA isomers were synthesized by isomerization of the commercially available reference (technical grade) with I_2 (35). Identification of the CLA isomers was based on co-injection with a commercial reference material and synthesized CLA as well as by comparing the elution order of CLA isomers with the existing literature (27,35). CLA were analyzed by Ag^+-HPLC according to Rickert *et al.*

TABLE 1
Concentrations of Fat and of the Predominant Unsaturated FA of the Three Oilseeds, and Daily Intake in the Diets of Cows

	Ground rapeseed	Ground sunflower seed		Ground linseed	
FA	1.0 kg (RAP1)	1.0 kg (SUN1)	1.4 kg (SUN1.4)	1.0 kg (LIN1)	1.4 kg (LIN1.4)
Fat in oilseeds (g kg^{-1} DM)	513	551		388	
Predominant FA in oilseeds (g kg^{-1} fat)					
Oleic acid	**546.7**[a]	152.0		175.5	
Linoleic acid	179.5	**536.2**		142.1	
α-Linolenic acid	88.8	1.4		**466.3**	
Daily intake (g day^{-1} cow^{-1})					
Oleic acid	**258**	80	106	59	84
Linoleic acid	85	**281**	**375**	48	68
α-Linolenic acid	42	1	1	**157**	**224**

[a]Boldface type indicates the most important FA. RAP1, control diet (hay fed *ad libitum* and 15 kg of fodder beets) supplemented with 1.0 kg of ground rapeseed; SUN1, control diet supplemented with 1.0 kg of ground sunflower seed; SUN1.4, control diet supplemented with 1.4 kg of ground sunflower seed; LIN1, control diet supplemented with 1.0 kg of ground linseed; LIN1.4, control diet supplemented with 1.4 kg of ground linseed; DM, dry matter.

(36), as modified by Kraft *et al.* (27). The analysis was performed on an Agilent LC series 1100 equipped with a photodiode array detector using three ChromSpher 5 Lipids columns in series (stainless steel, 250 × 4.6 mm, 5 μm particle size; Chrompack, Middleburg, The Netherlands). The solvent consisted of UV-grade hexane with 0.1% acetonitrile and 0.5% ethyl ether (flow rate, 1 mL min^{-1}), prepared fresh daily. Ethyl ether was used to reduce the analysis time and minimize retention volume drift, which is a well-known problem encountered in working with Ag^+-HPLC. The column was pretreated daily by eluting it with 1% acetonitrile/hexane for 30–60 min prior to sample analysis. The usual injection volumes were 10–20 μL, representing <250 μg of lipids. The HPLC areas for *trans*-7,*cis*-9 + *trans*-8,*cis*-10 + *cis*-9,*trans*-11 were added and used for comparison with the peak area of the three isomers from the GC chromatogram. The results were expressed as absolute values in mg g^{-1} fat. The Ag^+-HPLC chromatogram is presented in Figure 1.

Statistical analysis. Principal component analysis, ANOVA, and pairwise comparisons of mean values with Fisher's LSD test were performed with Systat for Windows, version 9.0 (37).

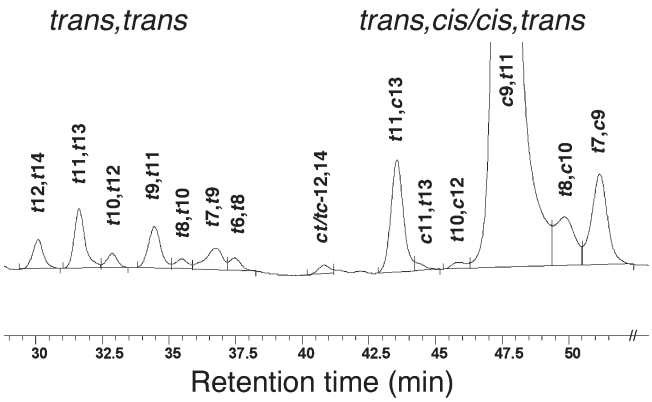

FIG. 1. Silver-ion-HPLC (Ag^+-HPLC) separation of CLA methyl esters of a milk fat using three columns in series.

RESULTS

Oleic, linoleic, α-linolenic and trans *FA in milk fat from cows fed CD or the CD supplemented with oilseeds.* The concentrations of oleic, linoleic, and α-linolenic acids and *trans*-FA in milk depended on the fat source fed (Table 2). The concentrations of most of the *trans*-FA generally increased in parallel with the daily intake of oleic, linoleic, or α-linolenic acid in the LIN1, RAP1, SUN1, LIN1.4, or SUN1.4 diet. At oilseed intakes of 1 kg, the highest concentration of *trans* 6–8 18:1 FA (3.00 mg g^{-1} fat) was found in milk fat from cows fed the oleic acid-rich RAP1 diet. Among the *trans* 18:1 isomers, the highest concentration was found for the combined *trans*-10/*trans*-11 FA (41.91 mg g^{-1} fat) in milk fat from cows fed the linoleic acid-rich SUN1.4 diet. Among the *trans* 18:2 isomers, the highest concentration was found for the combined *trans*-11,*cis*-15/*trans*-9,*cis*-12 18:2 FA (5.00 mg g^{-1} fat) in milk fat from cows fed the α-linolenic acid-rich LIN1.4 diet.

CLA isomers in milk fat from cows fed the CD or the CD supplemented with oilseeds. In milk fat from cows fed the α-linolenic acid-rich LIN1.4 diet, the highest concentrations (Table 3, bold) were found for the CLA isomers *trans*-12, *trans*-14 (0.31 mg g^{-1} fat), *trans*-11,*trans*-13 (0.48 mg g^{-1} fat), *cis,trans*/*trans,cis*-12,14 (0.34 mg g^{-1} fat), *trans*-11, *cis*-13 (0.47 mg g^{-1} fat), and *cis*-11,*trans*-13 (0.03 mg g^{-1} fat). From the LIN1 to the LIN1.4 diet, the concentrations of all these CLA isomers increased significantly ($P \leq 0.05$). The concentrations of the CLA isomers *trans*-12,*trans*-14, *cis,trans*/*trans,cis*-12,14 and *cis*-11,*trans*-13 did not change when the CD was supplemented with each of the other oilseeds rich in either oleic acid (RAP1) or linoleic acid (SUN1 or SUN1.4); no increases in the concentrations of the CLA isomers *trans*-11,*trans*-13 and *trans*-11,*cis*-13 were found when the RAP1 and SUN1 diets were fed.

In milk fat from cows fed the linoleic acid-rich SUN1.4 diet, the highest concentrations of CLA (Table 3, bold) were found for the isomers *trans*-10,*trans*-12 (0.17 mg g^{-1} fat), *trans*-9,*trans*-11 (0.17 mg g^{-1} fat), *trans*-8,*trans*-10 (0.05 mg g^{-1} fat), *trans*-7,*trans*-9 (0.08 mg g^{-1} fat), *trans*-10,*cis*-12

TABLE 2
Oleic, Linoleic, and α-Linolenic Acids and the Most Important *trans* FA in Milk Fat from Cows Fed the Control Diet (CD) or CD Supplemented with Oilseeds[a]

FA	CD	RAP1	SUN1	SUN1.4	LIN1	LIN1.4
18:1 *cis*-9	110.13 ± 16.25^{c}	**166.12** ± 18.79^{a}	160.62 ± 31.85^{a,b}	**175.80** ± 32.24^{a}	143.47 ± 12.73^{b}	**173.13** ± 26.64^{a}
18:2 *cis*-9,*cis*-12	16.19 ± 3.37^{b}	16.08 ± 3.17^{b}	**22.42** ± 4.23^{a}	**25.54** ± 3.98^{a}	14.61 ± 2.83^{b}	15.47 ± 1.83^{b}
18:3 *cis*-9,*cis*-12,*cis*-15	7.02 ± 1.06^{c}	7.29 ± 1.14^{c}	7.04 ± 1.06^{c}	7.19 ± 1.29^{c}	11.80 ± 2.09^{b}	**15.93** ± 2.45^{a}
18:1 *trans*-6–8	0.53 ± 0.14^{e}	3.00 ± 0.49^{b}	2.34 ± 0.55^{c}	**3.66** ± 0.53^{a}	1.45 ± 0.38^{d}	2.14 ± 0.39^{c}
18:1 *trans*-9	1.42 ± 0.14^{d}	3.42 ± 0.40^{b}	3.03 ± 0.53^{b}	**4.40** ± 0.73^{a}	2.24 ± 0.25^{c}	2.93 ± 0.35^{b}
18:1 *trans*-10–11	8.70 ± 1.55^{c}	16.00 ± 3.04^{c}	20.81 ± 4.01^{b}	**41.91** ± 7.71^{a}	13.57 ± 3.63^{c}	21.10 ± 3.40^{b}
18:1 *trans*-12	0.96 ± 0.15^{d}	2.95 ± 0.35^{c}	4.00 ± 1.01^{b}	**5.72** ± 0.83^{a}	2.54 ± 0.59^{c}	3.83 ± 0.61^{b}
18:1 *trans*-13–14 + *cis*-6–8	2.72 ± 0.33^{d}	6.63 ± 0.73^{c}	7.93 ± 1.72^{c}	10.48 ± 1.44^{b}	8.24 ± 1.85^{c}	**13.19** ± 2.25^{a}
18:1 *trans*-16+ *cis*-14	1.26 ± 0.22^{d}	3.06 ± 0.42^{c}	3.77 ± 0.82^{b}	**4.93** ± 0.83^{a}	3.65 ± 0.78^{b,c}	**5.29** ± 0.73^{a}
18:2 *cis*-9,*trans*-12 + *trans*-8,*cis*-13	1.78 ± 0.29^{d}	2.67 ± 0.18^{c}	3.09 ± 0.41^{b,c}	3.45 ± 0.53^{a,b}	2.90 ± 0.45^{c}	**3.75** ± 0.51^{a}
18:2 *cis*-9,*trans*-13 + *trans*-8,*cis*-12	0.98 ± 0.24^{c}	2.13 ± 0.30^{b}	2.48 ± 0.59^{b}	3.19 ± 0.79^{a}	2.39 ± 0.52^{b}	**3.77** ± 0.79^{a}
18:2 *trans*-11,*cis*-15 + *trans*-9,*cis*-12	0.87 ± 0.19^{c}	1.18 ± 0.18^{c}	1.25 ± 0.34^{c}	1.59 ± 0.26^{c}	2.47 ± 0.73^{b}	**5.00** ± 0.81^{a}

[a]Mean ± SD; n = 10 per treatment (values in mg g^{-1} fat). For diet descriptions, see Table 1. Boldface type indicates the highest mean values. $^{a–d}$Values in a row not sharing a common superscript roman letter differ significantly ($P \leq 0.05$).

TABLE 3
CLA Isomer Composition in Milk Fat from Cows Fed the Control Diet (CD) Supplemented with Rapeseed, Linseed, or Sunflower Seed[a]

18:2 CLA	CD	RAP1	SUN1	SUN1.4	LIN1	LIN1.4
Σ*trans/trans*	0.28 ± 0.04^{c}	0.35 ± 0.05^{c}	0.40 ± 0.06^{c}	0.65 ± 0.16^{b}	0.58 ± 0.11^{b}	**1.09** ± 0.21^{a}
12,14	0.03 ± 0.01^{c}	0.04 ± 0.01^{c}	0.03 ± 0.01^{c}	0.05 ± 0.02^{c}	0.13 ± 0.04^{b}	**0.31** ± 0.08^{a}
11,13	0.05 ± 0.01^{d}	0.09 ± 0.02^{c,d}	0.07 ± 0.02^{c,d}	0.12 ± 0.04^{c}	0.23 ± 0.06^{b}	**0.48** ± 0.11^{a}
10,12	0.05 ± 0.01^{c}	0.05 ± 0.01^{c}	0.09 ± 0.02^{b}	**0.17** ± 0.07^{a}	0.05 ± 0.02^{c}	0.06 ± 0.02^{c}
9,11	0.05 ± 0.01^{e}	0.07 ± 0.01^{d,e}	0.09 ± 0.02^{c}	**0.17** ± 0.05^{a}	0.08 ± 0.02^{c,d}	0.13 ± 0.03^{b}
8,10	0.03 ± 0.01^{b,c}	0.03 ± 0.01^{b,c}	0.04 ± 0.01^{b}	**0.05** ± 0.01^{a}	0.02 ± 0.01^{c}	0.03 ± 0.01^{b,c}
7,9	0.06 ± 0.01^{b}	0.06 ± 0.01^{b}	0.06 ± 0.01^{b}	**0.08** ± 0.02^{a}	0.06 ± 0.01^{b}	0.06 ± 0.01^{b}
6,8	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01
Σ*cis,trans/trans,cis*	4.44 ± 1.16^{d}	6.41 ± 1.08^{c}	8.45 ± 1.65^{b}	**16.98** ± 3.10^{a}	5.68 ± 1.57^{c,d}	8.83 ± 1.99^{b}
12,14	0.02 ± 0.01^{c}	0.03 ± 0.01^{c}	0.03 ± 0.00^{c}	0.04 ± 0.01^{c}	0.12 ± 0.04^{b}	**0.34** ± 0.09^{a}
trans-11,*cis*-13	0.09 ± 0.03^{c}	0.11 ± 0.04^{c}	0.12 ± 0.06^{c}	0.21 ± 0.07^{b}	0.23 ± 0.11^{b}	**0.47** ± 0.11^{a}
cis-11,*trans*-13	0.01 ± 0.00^{c}	0.01 ± 0.00^{c}	0.01 ± 0.01^{c}	0.01 ± 0.01^{c}	0.02 ± 0.01^{b}	**0.03** ± 0.01^{a}
trans-10,*cis*-12	0.02 ± 0.01^{c}	0.03 ± 0.01^{c}	0.06 ± 0.01^{b}	**0.10** ± 0.03^{a}	0.02 ± 0.00^{c}	0.02 ± 0.01^{c}
cis-9,*trans*-11	4.07 ± 1.09^{c}	5.47 ± 0.98^{c}	7.46 ± 1.47^{b}	**15.46** ± 2.84^{a}	4.85 ± 1.35^{c}	7.37 ± 1.77^{b}
trans-8,*cis*-10	0.08 ± 0.02^{c}	0.11 ± 0.02^{c}	0.22 ± 0.06^{b}	**0.29** ± 0.08^{a}	0.12 ± 0.04^{c}	0.13 ± 0.04^{c}
trans-7,*cis*-9	0.16 ± 0.03^{e}	0.66 ± 0.10^{b}	0.56 ± 0.12^{c}	**0.88** ± 0.17^{a}	0.33 ± 0.08^{d}	0.49 ± 0.11^{c}
ΣCLA	4.72 ± 1.16^{d}	6.77 ± 1.10^{c}	8.84 ± 1.69^{b}	**17.63** ± 3.22^{a}	6.26 ± 1.62^{c,d}	9.92 ± 2.06^{b}

[a]Mean ± SD; n = 10 per treatment (values in mg g^{-1} milk fat). CLA are ordered according to increasing retention time. Boldface type indicates the highest mean values. [a–e]Values in a row not sharing a common superscript roman letter differ significantly ($P \leq 0.05$). For diet abbreviations see Table 1.

(0.10 mg g^{-1} fat), *cis*-9,*trans*-11 (15.46 mg g^{-1} fat), *trans*-8, *cis*-10 (0.29 mg g^{-1} fat), and *trans*-7,*cis*-9 (0.88 mg g^{-1} fat). From the SUN1 to the SUN1.4 diet, the concentrations of all these CLA isomers increased significantly ($P \leq 0.05$). Compared with the CD, no significant increases in the concentrations of the CLA isomers *trans*-10,*trans*-12, *trans*-8,*trans*-10, *trans*-10,*cis*-12, and *trans*-8,*cis*-10 were found in milk fat from cows fed each of the other oilseeds.

At oilseed intakes of 1 kg, the concentration of *trans*-7,*cis*-9 CLA was highest (0.66 mg g^{-1} fat) when the oleic acid-rich RAP1 diet was fed.

Compared with the CD, the concentration of the main CLA isomer in milk fat, *cis*-9,*trans*-11, increased significantly by 34% on the RAP1 diet, by 19% on the LIN1 diet, by 81% on the LIN1.4 diet, by 83% on the SUN1 diet, and by 280% on the SUN1.4 diet. When the diet was changed from SUN1 to SUN1.4, a 33% increase in the daily intake of linoleic acid (from 281 to 375 g; α-linolenic acid, 1 g) increased the total CLA content by 107% (from 7.5 to 15.5 mg g^{-1} fat).

Table 4 illustrates the significant, positive Pearson correlation coefficients ($P \leq 0.001$) found between the daily intake of oleic, linoleic, or α-linolenic acid from oilseeds and the concentrations of CLA isomers in milk fat. There were significant positive correlations ($P \leq 0.001$) between the daily intake of linoleic acid and the concentrations of the *trans*,*trans* CLA (*trans*-10,*trans*-12, *trans*-9,*trans*-11, *trans*-8, *trans*-10, and *trans*-7,*trans*-9) (Table 4, Fig. 2) and between the daily intakes of α-linolenic acid and the concentrations of the *trans*-12,*trans*-14 and *trans*-11,*trans*-13 CLA. By contrast, the correlations between the daily intake of oleic acid and the *trans,trans* CLA isomers were not significant.

For the *cis,trans*/*trans,cis* CLA, significant positive correlations ($P \leq 0.001$) were also found between the daily intake of linoleic acid and the concentrations of the *trans*-10,*cis*-12, *cis*-9,*trans*-11, *trans*-8,*cis*-10, and *trans*-7,*cis*-9 isomers in milk fat (Table 4, Fig. 3) and between the daily intake of α-linolenic acid and the concentrations of the *cis,trans*/*trans*, *cis*-12,14, *trans*-11,*cis*-13, and *cis*-11,*trans*-13 isomers in milk fat. Only the correlation between the daily intake of oleic acid and the *trans-7,cis-9* CLA isomer was significant.

The two biplots (Figs. 2 and 3) also show excellent discrimination between the *trans,trans* or *cis,trans*/*trans,cis* CLA in the milk fat of the different diets.

Oleic, linoleic, and α-linolenic acids and trans *FA in the milk fat from cows grazing at three altitudes*. The concentrations of oleic, linoleic, and α-linolenic acids and of most *trans* 18:1 FA were highest in the milk fat from cows grazing in the mountains or the highlands (Table 5). Among the *trans* 18:1 isomers, the *trans*-10–11 were the most abundant FA (50.12

TABLE 4
Significant Positive Pearson Correlation Coefficients ($P \leq 0.001$) Between the Daily Intake of Oleic, Linoleic, or α-Linolenic Acid (values in g day^{-1} cow^{-1}) from an Oilseed and the Concentration of CLA in Milk Fat (values in mg g^{-1} fat)

18:2 CLA	Oleic acid	Linoleic acid	α-Linolenic acid
trans/trans	—	—	—
12,14	—	—	0.88
11,13	—	—	0.89
10,12	—	0.78	—
9,11	—	0.58	—
8,10	—	0.60	—
7,9	—	0.47	—
6,8	—	—	—
cis,trans/trans,cis	—	—	—
12,14	—	—	0.88
trans-11,*cis*-13	—	—	0.76
cis-11,*trans*-13	—	—	0.74
trans-10,*cis*-12	—	0.89	—
cis-9,*trans*-11	—	0.81	—
trans-8,*cis*-10	—	0.85	—
trans-7,*cis*-9	0.57	0.74	—

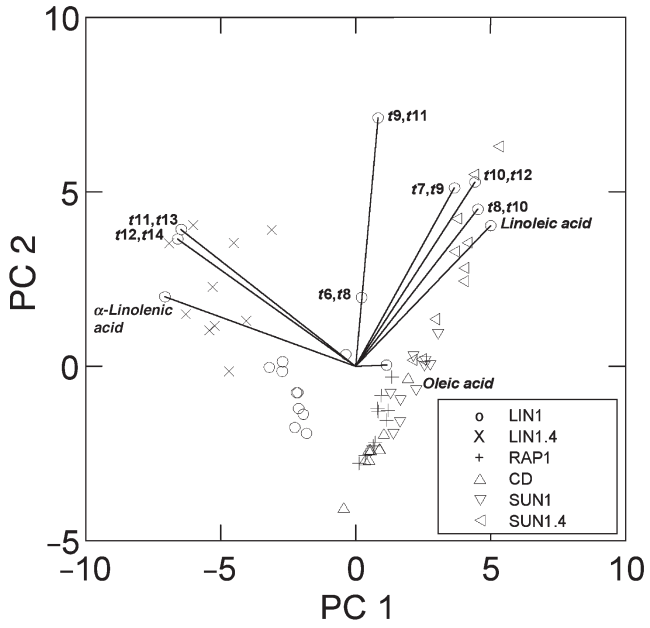

FIG. 2. Correlations between the daily intake of oleic acid, linoleic acid, and α-linolenic acid and the *trans,trans* CLA isomers in milk fat. The first principal component (PC 1) explained 39% of the total variance, and the second principal component (PC 2) explained 28%.

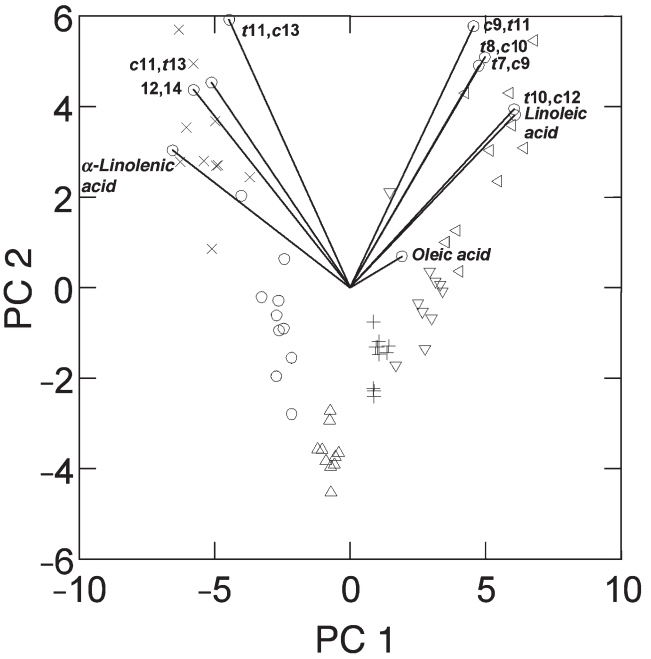

FIG. 3. Correlations between the daily intake of oleic acid, linoleic acid, and α-linolenic acid and the *cis,trans/trans,cis* CLA isomers in milk fat. The first principal component (PC 1) explained 46% of the total variance and the second principal component (PC 2) explained 35%. For symbols see Figure 2.

mg g^{-1} fat) in milk fat from the highlands. The concentrations of all the *trans* 18:2 FA were also highest in milk fat from the highlands. Among the *trans* 18:2 isomers, *trans*-11,*cis*-15/*trans*-9,*cis*-12 was the most abundant (7.32 mg g^{-1} fat) in milk fat from the highlands.

CLA isomers in the milk fat from cows fed grass at different altitudes. The milk fat of cattle at three different altitudes showed many significant differences in the concentrations of CLA isomers. In the milk fat from cows grazing in the mountains, the highest concentrations (Table 6, bold) were found for the CLA isomers *trans*-10,*trans*-12 (0.07 mg g^{-1} fat), *trans*-8,*trans*-10 (0.04 mg g^{-1} fat), *trans*-7,*trans*-9 (0.09 mg g^{-1} fat), *trans*-6,*trans*-8 (0.04 mg g^{-1} fat), *trans*-10,*cis*-12 (0.03 mg g^{-1} fat), and *trans*-7,*cis*-9 (0.51 mg g^{-1} fat). In milk fat from the highlands, the predominant CLA isomers were *trans*-12,*trans*-14 (0.23 mg g^{-1} fat), *trans*-11,*trans*-13 (0.46 mg g^{-1} fat), *trans*-9,*trans*-11 (0.13 mg g^{-1} fat), *trans*-7,*trans*-9 (0.09 mg g^{-1} fat), *cis,trans*/*trans,cis*-12,14 (0.07 mg g^{-1} fat), *trans*-11,*cis*-13 (1.75 mg g^{-1} fat), *cis*-11,*trans*-13 (0.04 mg g^{-1} fat), *cis*-9,*trans*-11 (21.33 mg g^{-1} fat), *trans*-8,*cis*-10 (0.31 mg g^{-1} fat), and *trans*-7,*cis*-9 (0.49 mg g^{-1} fat) (Table 6). The concentrations of the CLA isomers *trans*-7,*trans*-9 and *trans*-7,*cis*-9 were similar in milk fat from the mountains and the highlands and significantly higher than in milk fat from the lowlands. In the milk fat from cows grazing at the three altitudes, the *cis*-9,*trans*-11 and *trans*-11,*cis*-13 CLA isomers were the most abundant among the *cis,trans*/*trans,cis* isomers, and the *trans*-11,*trans*-13 isomer was the most abundant isomer among the *trans,trans* CLA. For the SD of the mean, the values obtained in mixed milks from this study (Table 6) were generally lower than those found in individual milks from the first study (Table 3).

The concentrations of many CLA isomers were highest in milk fat from the mountains or highlands. However, three of these isomers (*cis*-9,*trans*-11, *trans*-11,*cis*-13, and *trans*-8, *cis*-10) exhibited a nearly linear increase in concentration from the lowlands to the highlands as a function of altitude. Compared with the lowlands, the concentration of the *cis*-9, *trans*-11 CLA in milk fat from cows grazing in the mountains increased by 81%; in milk fat from cows grazing in the highlands it increased by 175%. The concentration of the *trans*-11, *cis*-13 CLA (the second-most important CLA isomer in milk fat from cows grazing at different altitudes) increased by 88% in milk fat from the mountains and by 310% in milk fat from the highlands.

DISCUSSION

Different studies have shown the influence of fodder on the concentration of CLA in milk, but only a few have analyzed the different isomers using Ag^+-HPLC (27–29).

Oleic acid in the fodder and CLA in the milk fat. In the first study, with oilseed intakes of 1 kg, the high concentration of the combined *trans*-6–8 18:1 FA (3.00 mg g^{-1} fat) (Table 2) in milk fat from cows fed the oleic acid-rich RAP1 diet (Table 1) was probably due to the increase in the concentration of *trans*-7 18:1 FA. Indeed, the daily intake of oleic acid correlated significantly with the concentration of the CLA isomer *trans*-7,*cis*-9 in milk (Table 4). It is also well known that oleic acid from fodder in the rumen is either not hydrogenated (38), is isomerized to *trans* 18:1 FA with double bonds at positions

TABLE 5
Oleic, Linoleic, and α-Linolenic Acids and the Most Important *trans* FA in Milk Fat from Cows Grazing at Different Altitudes

FA	Lowlands	Mountains	Highlands
18:1 *cis*-9	168.60 ± 15.49[b]	**193.60** ± 9.01[a]	175.34 ± 15.14[b]
18:2 *cis*-9,*cis*-12	11.78 ± 1.39[b]	**13.70** ± 0.88[a]	**13.75** ± 1.23[a]
18:3 *cis*-9,*cis*-12,*cis*-15	8.29 ± 1.63[b]	8.34 ± 0.44[b]	**12.34** ± 1.32[a]
18:1 *trans*-6–8	1.26 ± 0.22[c]	**2.05** ± 0.33[a]	1.85 ± 0.28[b]
18:1 *trans*-9	2.37 ± 0.26[c]	**3.46** ± 0.66[a]	2.92 ± 0.36[b]
18:1 *trans*-10–11	21.50 ± 2.44[c]	36.00 ± 3.08[b]	**50.12** ± 4.94[a]
18:1 *trans*-12	2.27 ± 0.26	2.26 ± 0.24	2.28 ± 0.38
18:1 *trans*-13–14 + *cis*-6–8	**7.51** ± 0.77[a]	5.98 ± 0.67[b]	**6.69** ± 0.96[a]
18:1 *trans*-16 + *cis*-14	3.79 ± 0.46[b]	3.61 ± 0.25[b]	**4.04** ± 0.44[a]
18:2 *cis*-9,*trans*-12 + *trans*-8,*cis*-13	2.75 ± 0.25[b]	2.75 ± 0.11[b]	**3.09** ± 0.21[a]
18:2 *cis*-9,*trans*-13 + *trans*-8,*cis*-12	2.49 ± 0.30[b]	2.35 ± 0.16[b]	**3.01** ± 0.40[a]
18:2 *trans*-11,*cis*-15 + *trans*-9,*cis*-12	3.38 ± 0.36[c]	4.29 ± 0.42[b]	**7.32** ± 1.56[a]

[a]Mean ± SD; $n = 10$ per altitude (values in mg g^{-1} fat). [a–c]Values in a row not sharing a common superscript roman letter differ significantly ($P \leq 0.05$). Boldface type indicates the highest mean values. Lowlands, 600–650 m; mountains, 900–1210 m; highlands, 1275–2120 m.

6–16 of the carbon chain, or is hydrogenated directly to stearic acid (39). Through the use of two different inhibitors of Δ^9-desaturase, Corl *et al.* (5) demonstrated that the *trans*-7, *cis*-9 CLA in milk fat originated almost exclusively *via* endogenous synthesis by Δ^9-desaturase with ruminally derived *trans*-FA; consistent with this result, the CLA isomer *trans*-7, *cis*-9 was not present in the ruminal fluid and was present in only small quantities in the duodenal flow (28). However, Secchiari *et al.* (29) found practically the same high concentrations (1.10 and 1.13 mg g^{-1} fat, respectively) of this CLA isomer when feeding either an olive oil soap that was rich in oleic acid (oleic acid: 38.7 g 100 g^{-1}; linoleic acid: 19.6 g 100 g^{-1} FA) or full-fat extruded soybeans that contained less oleic acid (oleic acid: 22.0 g 100 g^{-1} FA; linoleic acid: 48.9 g 100 g^{-1} FA). The biohydrogenation of linoleic acid in the soybeans into oleic acid in the rumen is certainly the cause of the nondifferentiated concentration of the CLA isomer *trans*-7, *cis*-9. Figure 4 illustrates the metabolic pathway for the formation of the *trans*-7,*cis*-9 CLA and for other CLA.

TABLE 6
CLA Isomer Composition of Milk Fat from Cows Fed Grass at Different Altitudes[a]

18:2 CLA	Lowlands	Mountains	Highlands
Σ *trans/trans*	0.82 ± 0.08[b]	0.83 ± 0.04[b]	**1.03** ± 0.09[a]
12,14	0.15 ± 0.02[b]	0.15 ± 0.01[b]	**0.23** ± 0.04[a]
11,13	0.38 ± 0.06[b]	0.32 ± 0.03[c]	**0.46** ± 0.05[a]
10,12	0.07 ± 0.01[b]	**0.07** ± 0.01[a]	0.06 ± 0.01[c]
9,11	0.11 ± 0.01[b]	0.11 ± 0.08[b]	**0.13** ± 0.01[a]
8,10	0.02 ± 0.00[c]	**0.04** ± 0.00[a]	0.03 ± 0.01[b]
7,9	0.07 ± 0.01[b]	**0.09** ± 0.01[a]	**0.09** ± 0.01[a]
6,8	0.02 ± 0.01[c]	**0.04** ± 0.01[a]	0.03 ± 0.01[b]
Σ *cis,trans/trans,cis*	8.74 ± 1.14[c]	15.68 ± 1.93[b]	**24.01** ± 2.76[a]
12,14	0.07 ± 0.01[a]	0.05 ± 0.01[b]	**0.07** ± 0.01[a]
trans-11,*cis*-13	0.43 ± 0.08[c]	0.80 ± 0.08[b]	**1.75** ± 0.42[a]
cis-11,*trans*-13	0.02 ± 0.00[b]	0.02 ± 0.00[b]	**0.04** ± 0.00[a]
trans-10,*cis*-12	0.03 ± 0.01[b]	**0.03** ± 0.00[a]	0.02 ± 0.01[c]
cis-9,*trans*-11	7.77 ± 1.05[c]	14.06 ± 1.79[b]	**21.33** ± 2.35[a]
trans-8,*cis*-10	0.13 ± 0.02[c]	0.21 ± 0.02[b]	**0.31** ± 0.05[a]
trans-7,*cis*-9	0.31 ± 0.05[b]	**0.51** ± 0.06[a]	**0.49** ± 0.05[a]
Σ CLA	9.57 ± 1.19[c]	16.50 ± 1.94[b]	**25.05** ± 2.78[a]

[a]Mean ± SD; $n = 10$ per altitude (values in mg g^{-1} milk fat). Boldface type indicates the highest mean values. [a–c]Values in a row not sharing a common superscript roman letter differ significantly ($P \leq 0.05$). For description of altitudes, see Table 5.

In the second study, the concentration of the *trans*-6–8 18:1 FA (Table 5) was significantly higher in milk fat from the mountains and highlands (2.05 and 1.85 mg g^{-1} fat, respectively) than in the lowlands (1.26 mg g^{-1} fat), in accordance with a similar increase in the concentration of the CLA isomer *trans*-7,*cis*-9 (Table 6).

Linoleic acid in the fodder and CLA in the milk fat. In the first study, the concentration of the *trans*-10–11 FA was highest in milk fat from cows fed the linoleic acid-rich SUN diets (SUN1 diet: 20.81; SUN1.4 diet: 41.91 mg g^{-1} fat) (Table 2), whereas in the second study, it was highest in milk fat from cows grazing in the highlands (50.12 mg g^{-1} fat) (Table 5). A strong positive correlation between the *trans* isomers of 18:1 [*trans* vaccenic acid (*t*VA), *trans*-13–14, *trans*-15, and *trans*-16] in milk fat and the level of linoleic acid in the diet was first found by Loor *et al.* (40). The latter compound is first isomerized to the CLA *cis*-9,*trans*-11 by *cis*-9,*trans*-11 isomerase and then hydrogenated by *Butyrivibrio fibrisolvens* to *t*VA in the rumen (41) (Fig. 4). These initial steps occur rapidly. The hydrogenation of *t*VA to stearic acid appears to involve a different group of organisms and occurs at a slow rate (42). For this reason, *t*VA typically accumulates in the rumen. This FA is also mainly present in the duodenal flow of lactating cows (28). It is well known that this main *trans* FA is responsible for the formation of the CLA isomer *cis*-9, *trans*-11, which occurs by desaturation of the ruminally derived *t*VA in the mammary gland (7,28). The notable increase in the concentration of the *cis*-9,*trans*-11 isomer when the diet was changed from SUN1 to SUN1.4 (Table 3) was also observed by Secchiari *et al.* (29) and Dhiman *et al.* (19) in milk from cows fed full-fat extruded soybeans or a soybean oil supplement. The latter authors concluded that it may be caused by incomplete biohydrogenation in the rumen and in-

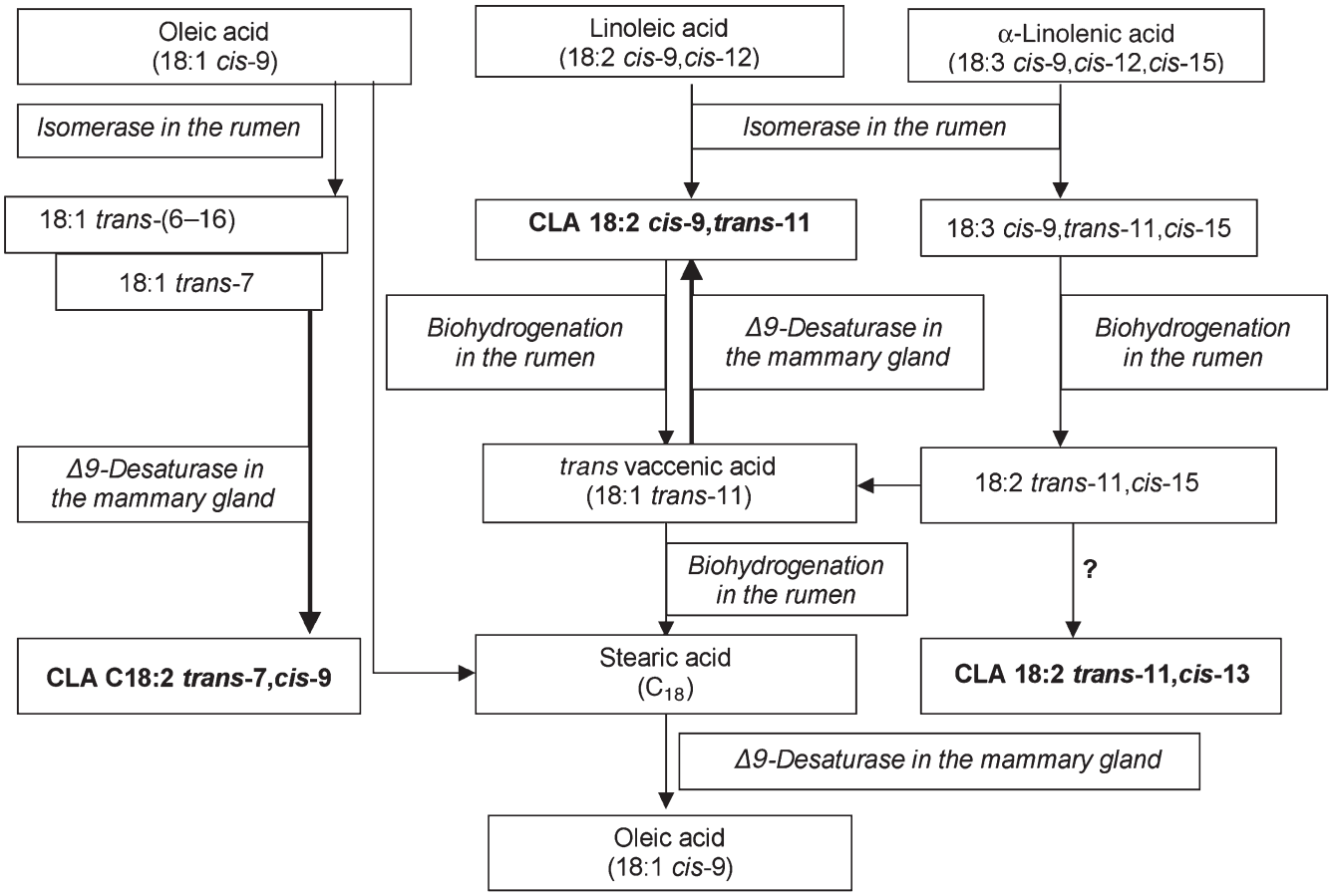

FIG. 4. Known metabolic pathways for the formation of CLA isomers and their precursors.

creased escape of the CLA from the rumen to the lower digestive tract. Piperova *et al.* (28) found much lower CLA concentrations in the duodenal flow of lactating cows fed diets with high (8.6 or 9.1 g d^{-1}) compared with low (1.1 or 1.8 g d^{-1}) proportions of forage than in milk fat. They concluded that the main CLA isomers, excluding *cis*-9,*trans*-11 and *trans*-7,*cis*-9, were essentially formed in the rumen. In the second study, the highest concentration of the *cis*-9,*trans*-11 CLA was found in milk fat from cows grazing in the highlands (21.3 mg g^{-1} fat). French *et al.* (43) also found increased CLA contents in the intramuscular fat from steers grazing on grass compared with grass silage or ingesting concentrate-based diets. It therefore seems that grass provides protection against biohydrogenation in the rumen.

In the first study, the concentration of the CLA isomer *trans*-10,*cis*-12 also correlated strongly with the daily intake of linoleic acid (Table 4). This CLA isomer is a product of ruminal biohydrogenation in which the initial isomerization takes place at the *cis*-9 position of linoleic acid rather than at the *cis*-12 position, as in the more typical pathway (4). Kraft *et al.* (44) found a 40% decrease in milk fat 5 d after an intraduodenal infusion of a CLA mixture, indicating that the *trans*-10,*cis*-12 isomer is responsible for the inhibition of milk fat synthesis. According to Viswanadha *et al.* (45), an intravenous administration of 6 g d^{-1} of this isomer decreased the milk fat percentage from 4.17 to 2.92% on day 5. In a dose–response experiment, Baumgard *et al.* (46,47) confirmed these results when they fed the pure *trans*-10,*cis*-12 isomer. The milk fat from cows supplemented with the highest dose (14 g d^{-1}) contained more *trans*-10,*cis*-12 compared with *cis*-9,*trans*-11, resulting in a dramatically curvilinear reduction in milk fat yield.

α-Linolenic acid in the fodder and CLA in the milk fat. In the first study, the highest concentration of the combined *trans*-11,*cis*-15/*trans*-9,*cis*-12 18:2 FA was found in milk fat from cows fed LIN1 (2.47 mg g^{-1} fat) or LIN1.4 (5.00 mg g^{-1} fat); it was attributed to the high level of *trans*-11,*cis*-15 18:2 with the increase in α-linolenic acid. The correlation coefficient between the concentrations of the combined *trans*-11, *cis*-15/*trans*-9,*cis*-12 18:2 FA in the milk fat and the *trans*-11, *cis*-13 CLA was very high (0.94; $P \leq 0.001$). It is well known that the pathway for the hydrogenation of *cis*-9,*cis*-12,*cis*-15 18:3 FA in the rumen involves an initial isomerization to a conjugated triene (*cis*-9,*trans*-11,*cis*-15 18:3), followed by reduction of double bonds at carbons 9, 15, and 11 to yield the *trans*-11,*cis*-15 18:2, *trans*-11 18:1, and 18:0 FA, respectively, but not *cis*-9,*trans*-11 CLA, as intermediates (48). Kraft *et al.* (27) hypothesized that α-linolenic acid is the indirect precursor of *trans*-11,*cis*-13 CLA. The highest levels of this CLA isomer found in milk fat from cows fed the

α-linolenic acid-rich LIN diets (LIN 1, 0.23 mg g^{-1} fat; LIN1.4, 0.47 mg g^{-1} fat) (Table 3) confirmed that α-linolenic acid was the main indirect precursor of the CLA isomer *trans*-11,*cis*-13 in milk fat. Nevertheless, the increase in the concentration of this CLA isomer in milk fat when the diet was changed from SUN1 (0.12 mg g^{-1}) to SUN1.4 (0.21 mg g^{-1}) (daily intake of α-linolenic acid, 1 g; Table 1) indicated that linoleic acid was the second-most important precursor of this CLA.

Normally, after the overwhelmingly predominant CLA isomer *cis*-9,*trans*-11, the *trans*-7,*cis*-9 is the second-most predominant CLA isomer in ruminant fat (5,28,29,49–51). Piperova *et al.* (52) reported that this isomer represents as much as 40% of the total CLA under special conditions. According to these authors (28), when cows were fed a high- or low-forage diet with or without buffer, most of this isomer may have been produced by the action of Δ^9-desaturase on *trans*-7 18:1 in bovine tissues. By contrast, in milk fat from cows grazing at the three altitudes, the second-most important CLA isomer was the *trans*-11,*cis*-13 CLA (Table 6). The concentration of this CLA in milk fat from cows grazing in the highlands was much higher (about a factor of 4) than in milk fat from cows fed the LIN1.4 diet. Although linseed (14) and fresh grass (53) both contain a high proportion of α-linolenic acid, Wachira *et al.* (54) speculated that the biohydrogenation rate of α-linolenic acid differs depending on the source. α-Linolenic acid is predominantly bound to TAG, whereas in grass the predominant form is glycolipids. The increase in the concentration of this CLA isomer with elevation also could be related to the higher percentage of α-linolenic acid in plants living at lower environmental temperatures, such as in the Alps (53). The pathway from *trans*-11,*cis*-15 FA to the second-most important CLA isomer, *trans*-11,*cis*-13, is as yet unclear (Fig. 4).

CLA are highly correlated with either oleic, linoleic, or α-linolenic acid. The strong positive correlations (Table 4) between the daily intakes of (i) oleic acid or linoleic acid and the concentration of the CLA isomer *trans*-7,*cis*-9 in milk fat; (ii) linoleic acid and the CLA isomers *trans*-10,*trans*-12, *trans*-9,*trans*-11, *trans*-8,*trans*-10, *trans*-7,*trans*-9, *trans*-10, *cis*-12, *cis*-9,*trans*-11, *trans*-8,*cis*-10, and *trans*-7,*cis*-9; and (iii) α-linolenic acid and the CLA isomers *trans*-12,*trans*-14, *trans*-11,*trans*-13, *cis,trans*/*trans*,*cis*-12,14, *trans*-11,*cis*-13, and *cis*-11,*trans*-13 (Table 4) indicated that these FA (oleic, linoleic, and α-linolenic acids) are probably the main indirect precursors of the above-mentioned CLA. Nevertheless, it is well known, for example, that linoleic and α-linolenic acid are both indirect precursors of the CLA isomer *cis*-9,*trans*-11 (55), but the highest concentration of this CLA isomer is generally obtained with linoleic acid-rich diets (14,19).

CLA are potential indicators of Alpine milk products. In the second study, the concentrations of many isomers were the highest in milk fat from the highlands (Table 6), but only three CLA isomers (*cis*-9,*trans*-11, *trans*-11,*cis*-13, and *trans*-8,*cis*-10) showed a nearly linear increase with elevation. The concentration of the isomer *trans*-11,*cis*-13 in milk fat from the highlands (Table 6) was about four times higher than in milk fat from cows fed LIN1.4 (Table 3), which was rich in α-linolenic acid (Table 1). These three CLA isomers, and particularly the CLA isomer *trans*-11,*cis*-13, could therefore be useful indicators of milk products of Alpine origin. Recently, Karoui *et al.* (56) showed that CLA may help to differentiate Alpine from lowlands milk products by using fluorescence spectrometry.

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