Analysis of Conjugated Linoleic Acids as 9-Anthrylmethyl Esters by Reversed-Phase High-Performance Liquid Chromatography with Fluorescence Detection

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Abstract

A simple and highly sensitive method for determining the fatty acid composition of food lipids containing conjugated linoleic acid (CLA) is described. The method is based on the separation of the 9-anthrylmethyl ester derivatives of saturated and unsaturated (conjugated and non-conjugated) fatty acids by reversed-phase high-performance liquid chromatography with fluorescence detection. Just like the other fatty acids, CLA reacts readily with 9-anthryldiazomethane at room temperature to produce 9-anthrylmethyl esters without isomerization and decomposition of the conjugated double bonds. Clear resolution of the individual fatty acids as their 9-anthrylmethyl esters is achieved on a highly efficient octadecylsilylated silica column (150- × 3-mm i.d., 3-µm particle size) using a stepwise gradient elution with methanol–water. The method is standardized with commercially available CLA isomers (*cis*-9, *trans*-11 and *trans*-10, *cis*-12-octadecadienoic acids, and their *cis,cis* and *trans,trans* isomers) and applied for determination of the fatty acid compositions of milk and sdairy products.

Introduction

Conjugated linoleic acid (CLA), which is a collective name for several geometrical and positional isomers of octadecadienoic acid with conjugated double bonds, has attracted attention in recent years because of its many biological activities, including anticarcinogenic, antiatherogenic, and lean body mass-enhancing properties (1–4). CLA is a minor component in nature present mainly in the ruminant tissue fat and products such as milk, cheese, and butter. The CLA amount in dairy products ranges from 2 to 37 mg/g fat and the major CLA isomer, *cis*-9,*trans*-11-octadecadienoic acid, constitutes approximately 90% of the total isomers (5).

Silver ion high-performance liquid chromatography (Ag+-HPLC), reversed-phase (RP) HPLC, gas chromatography (GC), Fourier transform IR spectroscopy (FTIR), NMR spectroscopy, some hyphenated techniques such as GC–MS or GC–FTIR, and, mostly, several of these methods in conjunction are used for separation and identification of the geometrical and positional CLA isomers (6,7). RP-HPLC has been used exclusively as a preliminary method for concentration of CLA in foodstuffs as well as for analysis of CLA metabolites (7,8). Until now, analysis of CLA by RP-HPLC has been carried out only as free acids, methyl esters, or *p*-methoxyphenacyl esters, using for detection the characteristic absorption of the conjugated double bonds (230–235 nm) (8) or *p*-methoxyphenacyl (270 nm) groups (9).

Usually, the detailed fatty acid composition of food lipids including CLA is determined by polar capillary GC in combination with other methods for preliminary fractionation of the isomers or their identification (or both) (6,7). A simpler and very sensitive method using RP-HPLC with fluorescence detection for the detection of 9-anthrylmethyl esters of CLA has been developed. These derivatives are often used for highly sensitive analysis of fatty acids from biological materials (10–15), but they have never been applied for analysis of CLA. This study has established that the 9-anthryldiazomethane (ADAM) is a quite suitable reagent for the preparation of 9-anthrylmethyl esters, reacting with CLA readily at room temperature without causing isomerization or degradation of the conjugated double bonds. Moreover, this reaction is nonselective to the number and type of the double bonds, so all fatty acids in the sample could be detected in equal sensitivity.

The present paper describes a simple and highly sensitive method for determining the free and esterified fatty acids including CLA in dairy products as 9-anthrylmethyl ester derivatives by RP-HPLC with fluorescence detection. The method was standardized with authentic saturated and unsaturated noncon-

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jugated and conjugated fatty acids and was applied for determination of the fatty acid composition of cow's milk, cheese, and butter, which are the main source of CLA in human nutrition.

Experimental

Materials

Pure fatty acids, butyric (4:0), caproic (6:0), caprylic (8:0), capric (10:0), lauric (12:0), tridecanoic (13:0), myristic (14:0), palmitic (16:0), heptadecanoic (17:0), stearic (18:0), palmitoleic (16:1n-9), oleic (18:1n-9), linoleic (18:2n-6), and α-linolenic (18:3n-3), were obtained from Wako Pure Chemicals (Osaka, Japan). Pentadecanoic acid (15:0); myristoleic acid (14:1n-9); docosapentaenoic acid (22:5n-3); and a CLA mixture, *cis*- and *trans*-9,11- and -10,12-octadecadienoic acids (18:2), were purchased from Sigma (St. Louis, MO). Pure CLA isomers of *cis*-9, *trans*-11-18:2 (> 96%) and *trans*-10, *cis*-12-18:2 (> 98%) from Cayman Chemical (Ann Arbor, MI) and *cis*-9, *cis*-11-18:2 (> 96%) and *trans*-9, *trans*-11-18:2 (> 98%) from Matrea, Inc. (Pleasant Gap, PA) were also used. ADAM was obtained from Funakoshi (Tokyo, Japan). Fresh cow's milk, butter, and cheese were purchased in July of 2002 from a convenience store in Sapporo, Japan.

Preparation of 9-anthrylmethyl ester derivatives

Individual fatty acids were dissolved in methanol to give 2-mg/mL standard solutions (A). A mixture of 0.5 µg/mL of CLA and 1.0 µg/mL of the other fatty acids was prepared by diluting A with methanol (B). Similarly, 1 mg ADAM was dissolved in 1 mL methanol. The 9-anthrylmethyl ester derivatives were prepared by adding 100 µL of B to the same volume of the ADAM–methanol solution. After storage for 1 h in the dark at room temperature, 10 µL of the reaction mixture (without further purification) was injected into the HPLC column as described later.

RP-HPLC

Analytical mode

RP-HPLC was carried out on a Hitachi L-6200/L-6000 gradient system (Hitachi, Tokyo, Japan) equipped with an octadecylsilylated silica (ODS) column (Cadenza CD-C18, 150- × 3.0-mm i.d., 3-µm particle size) (Imtakt, Kyoto, Japan) connected to a guard column containing the same material (5- × 2-mm i.d.) (14). The analysis was carried out at 50°C using a stepwise gradient elution with water–methanol and a flow rate of 0.5 mL/min. The following gradient was used: step 1, 80% methanol for 20 min; step 2, initial conditions change to 90% methanol over 5 min; step 3, 90% methanol for 40 min; step 4, initial condition change to 100% methanol over 5 min; step 5, 100% methanol for 30 min; and step 6, 80% methanol for 10 min for column re-equilibrium. Peaks were monitored with a Hitachi F-1050 fluorescence detector. The excitation and emission wavelengths were set at 365 and 412 nm, respectively. A column containing cyanopropyl-bonded phase (Capcell Pak CN SG 120, 250- × 4.6-mm i.d., 5-µm particle size) (Shiseido, Tokyo, Japan) was also used for resolution of the unresolved peaks on the ODS column. This separation was carried out by isocratic elution at 50°C with acetonitrile–water (6:4, v/v) as the mobile phase and 1.5 mL/min flow rate.

Preparative mode

Preparative RP-HPLC on ODS was applied for purification of the 9-anthrylmethyl esters before their analysis by mass spectrometry (MS) and HPLC on a cyanopropyl-bonded phase. The HPLC conditions were the same as those employed for the analytical mode described previously. The CLA fractions from several injections were pooled for the subsequent experiments.

MS

MS was performed on a Finnigan MAT SSQ-7000 MS equipped with an atmospheric pressure chemical ionization (APCI) interface and an ICIS data system (Finnigan MAT, San Jose, CA). Purified 9-anthrylmethyl esters of the CLA standards and the CLA fraction from butter fat fatty acids (each ca. 500 ng) were dissolved in acetonitrile-methanol–water (8:1:1, v/v/v) and then introduced into the MS instrument at a flow rate of 0.1 mL/min using a syringe pump. The corona voltage was set at 4.5 kV. The heated capillary temperature was 150°C. High-purity nitrogen gas was used as the sheath gas (nebulizer gas) at an operating pressure of 70 psi and an auxiliary gas at 10 units. The positive and negative APCI–MS spectra were taken in the mass range of *m/z* 100–700.

Analysis of milk and dairy products

Butter fat was obtained by dissolving 1 g of butter in 10 mL of hexane and removing the insoluble matter by centrifugation for 5 min at 3000 rpm. Lipids from milk (1 mL) and cheese (1 g) were extracted with chloroform–methanol (2:1, v/v), according to the methods reported previously (15,16). An aliquot of the total lipid extracts (mainly triacylglycerols) was subjected to alkaline hydrolysis (8), and the resulting fatty acids (50–100 µg) were esterified in 100 µL of a 0.1% (w/v) ADAM–methanol solution, as described in the previous paragraph. After the addition of 900 µL of methanol to the reaction products, 2 µL of the solution were introduced into the HPLC column using an autoinjector. The alkaline hydrolysis is necessary because the derivatization reaction is esterification between free fatty acids and ADAM, which is unreactive to triacylglycerls.

Results and Discussion

In order to establish a nonspecific and highly sensitive method for determining the composition of saturated, conjugated, and nonconjugated fatty acids in foodstuffs, a conversion of the carboxyl groups of fatty acids into fluorescent derivatives and the RP-HPLC separation of the derivatives were investigated using an ADAM reagent and an ODS stationary phase, respectively.

Derivatization

As with saturated and unsaturated non-conjugated fatty acids (10–14), CLA readily reacted with ADAM without any degradation of the acyl chains during the preparation of 9-anthrylmethyl esters. The correlation factor of the calibration plot of the peak areas of the 9-anthrylmethyl esters versus the amounts of CLA

(*cis*-9,*trans*-11-18:2) in the range of 1 to 200 ng was greater than 0.99, indicating quantitative reaction of CLA with ADAM. No *trans,trans* isomers were observed by RP-HPLC (chromatograms not shown). This is an indication that no appreciable isomerization of the double bonds from *cis* to *trans* configuration occurs during the derivatization procedure and the HPLC analysis under the conditions employed.

Figure 1 shows the positive and negative APCI–MS spectra of the 9-anthrylmethyl ester derivative of the CLA standard (*cis*-9,*trans*-11-18:2). In addition to the molecular related $[M+H]^+$ (*m/z* 471) and $[M+NH_4]^+$ (*m/z* 488) ions, the positive ion spectrum gave a characteristic $[M–RCOO]^+$ (*m/z* 191) ion, but the negative ion spectrum showed a prominent carboxylate anion $[RCOO]^-$ (*m/z* 279). The $[M+NH_4]^+$ ion probably resulted from residual ammonium salts in the system from other applications. Essentially, the same spectra were obtained for the derivatives of another CLA standard (*trans*-10,*cis*-12-18:2) and non-conjugated linoleic acid (spectra not shown). These results confirm the identity of 9-anthrylmethyl esters of CLA. The prominent $[M–RCOO]^+$ (*m/z* 191) and $[RCOO]^+$ (*m/z* 279) ions in positive and negative ion modes, respectively, have also been observed in RP-HPLC–APCI–MS of the anthrylmethyl ester derivatives of common saturated and unsaturated fatty acids using methanol as the mobile phase (17). RP-HPLC–MS with belt-transport interface also gave the $[M–RCOO]^+$ (*m/z* 191) ion for the 9-anthrylmethyl esters of saturated and unsaturated non-conjugated fatty acids (18).

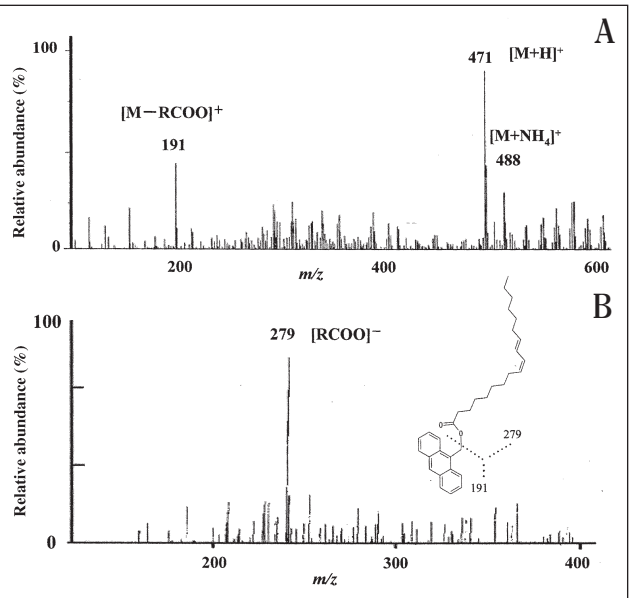

Figure 1. APCI–MS of the 9-anthrylmethyl ester of CLA (*cis*-9,*trans*-11-18:2): positive ion spectrum (A) and negative ion spectrum (B). APCI–MS conditions were as given in the text.

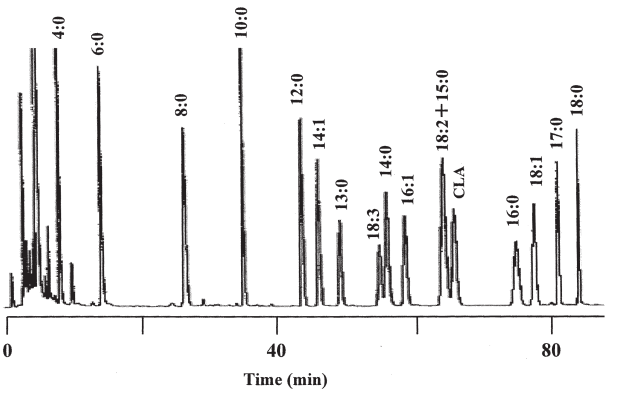

Figure 2. RP-HPLC separation of a standard mixture of fatty acids as 9-anthrylmethyl esters on an ODS column, Cadenza CD-C18. HPLC conditions were as given in the text.

Separation

Figure 2 illustrates the RP-HPLC separation of the 9-anthrylmethyl esters of a standard mixture of 18 fatty acids, including two CLA isomers (a mixture of *cis*-9,*trans*-11-18:2 and *trans*-10,*cis*-12-18:2) on an ODS column, Cadenza CD-C18. These fatty acids are common constituents of the lipids of cow's milk and dairy products (5). All fatty acids, except for 18:2 and 15:0, were clearly resolved in 90 min by gradient elution using a simple solvent system consisting of water and methanol. Like the methyl esters (8), the 9-anthrylmethyl esters gave no resolution between

Table I. Chromatographic Parameters of Fatty Acid 9-Anthrylmethyl Esters on an ODS Column, Cadenza CD-C18*

Fatty acid		Parameter†			
Acyl group	ECN‡	Vr (mL)	*k′*	α	R_s
4:0	4	4.01	7.2		
				1.75	9.7
6:0	6	7.02	12.5		
				1.86	17.5
8:0	8	13.08	23.4		
				1.30	14.0
10:0	10	17.03	30.4		
				1.28	13.5
12:0	12	21.78	38.9		
				1.06	3.1
14:1	12	22.98	41.0		
				1.07	3.6
13:0	13	24.55	43.8		
				1.12	7.7
18:3	12	27.42	49.0		
				1.02	1.3
14:0	14	27.96	49.9		
				1.05	2.6
16:1	14	29.28	52.3		
				1.10	5.1
15:0, 18:2	14	32.11	57.3		
				1.02	1.2
CLA§	14	32.88	58.7		
				1.14	7.6
16:0	16	37.48	66.9		
				1.04	2.1
18:1	16	38.82	69.3		
				1.04	4.2
17:0	17	40.55	72.4		
				1.04	5.1
18:0	18	42.02	75.0		

* HPLC conditions as given in the text.
† Vr = retention volume corrected by subtracting the column void volume (0.56 mL); *k′* = capacity factor; α = separation coefficient between neighboring fatty acids; and R_s = peak resolution of neighboring fatty acids: $R_s = 2(t_2 - t_1)/(w_1 + w_2)$, where *t* = retention time (min) and *w* = peak width (min).
‡ ECN = equivalent carbon number (total acyl carbon number – 2 × number of double bonds.
§ A mixture of *cis*-9, *trans*-11-18:2 and *trans*-10,*cis*-12-18:2.

cis-9,*trans*-11 and *trans*-10,*cis*-12-18:2, but the geometrical isomers of *cis*-9,*trans*-11, *cis*-9,*cis*-11, and *trans*-9,*trans*-11-18:2 eluted from the column in this order. Although some overlapping between the *cis*,*trans*- (*trans*,*cis*-) and *cis*,*cis*-isomer peaks occurred, the *trans*,*trans*-isomers were completely resolved from the others (chromatograms not shown). A similar elution pattern of these geometrical CLA isomers has been observed for nonesterified CLA by RP-HPLC on an ODS column using acetonitrile–water–acetic acid (70:30:0.12, v/v/v) as the mobile phase (8).

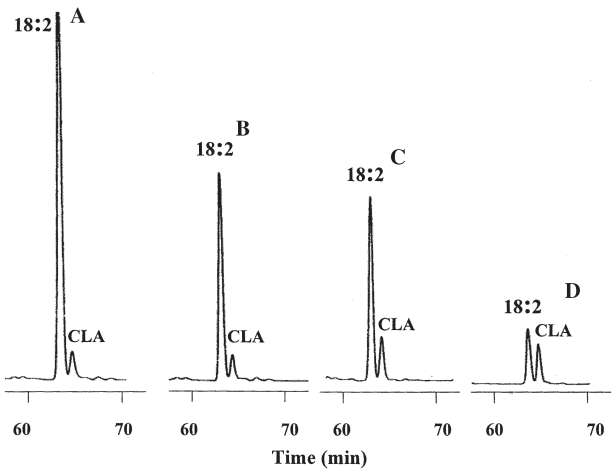

Figure 3. RP-HPLC separation of the 9-anthrylmethyl esters of 18:2 and CLA (*cis*-9,*trans*-11-18:2) in different proportions. The 18:2/CLA ratios: 20:1 (200 ng:10 ng) (A); 10:1 (100 ng:10 ng) (B); 5:1 (50 ng:10 ng) (C); and 1:1 (10 ng:10 ng) (D). HPLC conditions were the same as those in Figure 3.

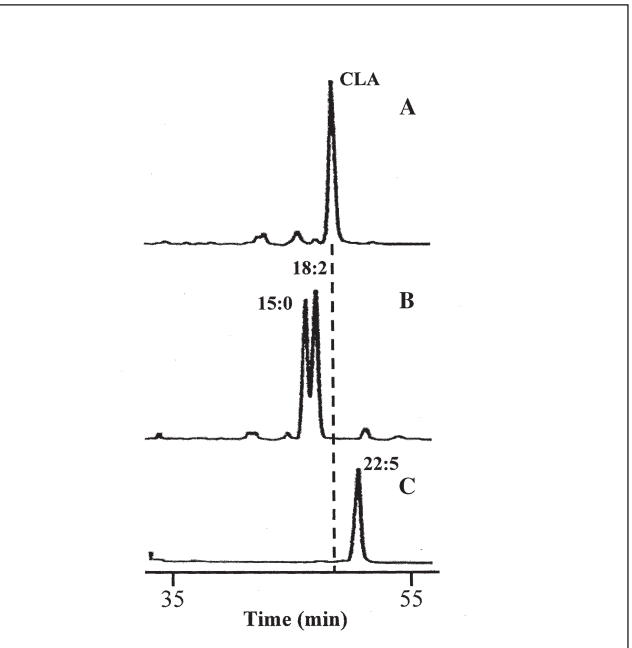

Figure 4. RP-HPLC resolution of 18:2, 15:0, 22:5, and CLA as 9-anthrylmethyl esters on a cyanopropyl-bonded phase, Capcell Pak CN SG 120. A CLA fraction isolated from butter fat fatty acids using Cadenza CD-C18 ODS column (A); mixture of 15:0 and 18:2 standards (B); and 22:5 standard (C). HPLC conditions as given in the text.

The chromatographic parameters obtained for the derivatives of all fatty acids are given in Table I. Like the methyl esters, the 9-anthrylmethyl esters of fatty acids with the same equivalent carbon numbers (ECN) have close retention times on ODS columns (13,17). Thus, the two CLA isomers (*cis*-9,*trans*-11- and *trans*-10,*cis*-12-18:2) appeared together just after 18:2 (ECN = 14) with a separation coefficient (α) of 1.02 and peak resolution of 1.16 under gradient elution (Table I).

Figure 3 shows the HPLC resolution of the 9-anthrylmethyl esters of CLA and non-conjugated 18:2 in different proportions. In all these chromatograms, the CLA peaks were clearly separated from the 18:2 peaks, even if the blend ratio of CLA to 18:2 was 1:20. These results demonstrate that RP-HPLC on the ODS column used in this study permits accurate measurement of CLA and the other fatty acids in various kinds of foodstuffs. In addition to the unresolved 15:0 and 18:2 (Figure 2), docosapentaenoic acid (22:5), which is a common fish oil component, coeluted with CLA on the ODS column. These critical pairs, however, could be resolved clearly by HPLC on a cyanopropyl-bonded (CN) phase (Figure 4), which showed different elution characteristics than ODS for the 9-anthrylmethyl esters, although some other saturated and unsaturated species were poorly separated (chromatograms not shown). Therefore, when the RP-HPLC method is applied to fish oil samples containing CLA (19), the use of the two different types of columns (ODS and CN) would give an unambiguous identification of CLA.

Application

Under the HPLC conditions employed for the fatty acid standards (Figure 2), the fatty acids of the total lipids from cow's milk, butter, and cheese were analyzed. Figure 5 shows the typical HPLC chromatogram of the 9-anthrylmethyl esters of milk fat fatty acids on the ODS column, Cadenza CD-C18. Like the standard CLA (Figure 2), the CLA in the milk fat was clearly separated from the other saturated and unsaturated fatty acids. Similar chromatograms were obtained for the fatty acids from butter and cheese fats (chromatograms not shown). A CLA fraction collected from the butter fat fatty acids by preparative HPLC on the ODS column was rechromatographed on the CN column (Figure 4A).

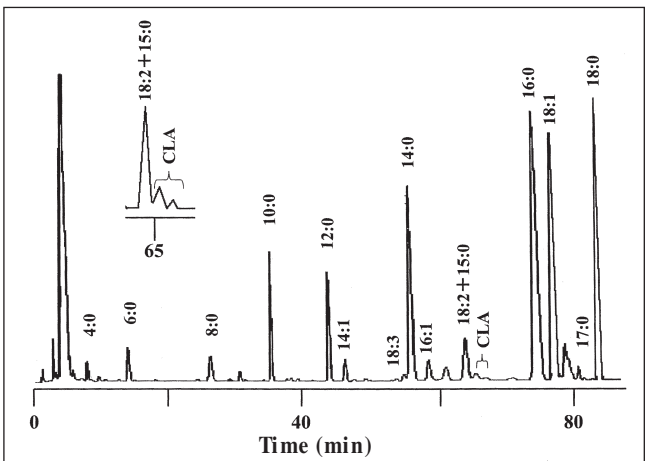

Figure 5. RP-HPLC separation of the 9-anthrylmethyl esters of milk fat fatty acids on an ODS column, Cadenza CD-C18. HPLC conditions are the same as those in Figure 3.

Table II. Fatty Acid Compositions of Cow's Milk, Butter, and Cheese (mol%)

	Cow's milk			Butter			Cheese		
Fatty acid	mol%	Fat (mg/g)	Product (mg/mL)	mol%	Fat (mg/g)	Product (mg/g)	mol%	Fat (mg/g)	Product (mg/g)
4:0	2.3	5.1	0.18	3.2	9.1	7.3	2.8	6.4	0.85
6:0	1.8	4.0	0.14	1.7	4.9	3.9	1.9	4.3	0.57
8:0	1.9	4.2	0.15	1.7	4.9	3.9	1.8	4.1	0.55
10:0	4.9	10.9	0.39	4.7	13.4	10.7	4.5	10.3	1.37
12:0	5.3	11.8	0.43	5.3	15.1	12.1	4.9	11.2	1.49
14:1	1.2	2.7	0.10	1.3	3.7	3.0	4.9	11.2	1.49
18:3	0.5	1.1	0.04	0.7	2.0	1.6	0.7	1.6	0.21
14:0	14.4	32.0	1.15	15.6	44.6	35.7	14.9	34.0	4.53
16:1	1.8	4.0	0.14	1.9	5.4	4.3	1.9	4.3	0.57
20:4	1.6	3.6	0.13	1.8	5.1	4.1	2.0	4.6	0.61
18:2+15:0	4.2	9.3	0.33	3.5	10.0	8.0	3.4	7.8	1.04
CLA*	0.9	2.0	0.07	1.4	4.0	3.2	2.5	5.7	0.76
16:0	30.3	67.3	2.42	28.7	82.0	65.6	34.1	77.8	10.37
18:1	18.5	41.1	1.48	18.2	52.4	41.6	17.8	40.6	5.41
17:0	0.7	1.6	0.06	0.8	2.3	1.8	0.8	1.8	0.24
18:0	9.8	21.8	0.79	9.5	27.1	21.7	1.1	2.5	0.33

* Mainly *cis*-9,*trans*-11-18:2.

The retention time of the main peak obtained was in agreement with that of the CLA standard (a mixture of *cis*-9,*trans*-11-18:2 and *trans*-10,*cis*-12-18:2). The positive and negative APCI–MS spectra also gave essentially the same fragmentation patterns as those of the reference CLA isomers (Figure 1).

Table II presents the fatty acid compositions of the lipids from cow's milk, cheese, and butter examined in this study. The CLA content in the total fatty acids from milk, butter, and cheese was 0.9% (2.2 mg/g fat), 1.4% (4.0 mg/g fat), and 2.5% (5.7 mg/g fat), respectively. Cow's milk fat is the richest natural source of CLA, and it has been reported that the levels are in range from 2 to 37 mg/g fat (5). It has also been reported that the average values of the CLA contents of various butter and cheese samples are 5.0 and 9.3 mg/g fat, respectively, which are influenced almost entirely by the CLA content of the milk fat (5). The lower CLA content in the butter and cheese lipids analyzed in this study may be reflected by the lower CLA level in the milk fat.

Conclusion

A simple and highly sensitive HPLC method for determination of the fatty acid composition of food lipids containing CLA was developed. For this purpose, saturated and unsaturated fatty acids including CLA were converted into their 9-anthrylmethyl esters at room temperature without any catalysts, and then, without further purification, they were subjected to RP-HPLC with fluorescence detection. Clear separations of saturated, conjugated, and non-conjugated fatty acids were achieved on a highly efficient C18 column using a stepwise gradient elution system. The derivatives give essentially the same sensitivity to saturated, conjugated and non-conjugated fatty acids, and can be detected at nanogram levels. Thus, the method presented could be utilized widely for determining the composition of free and esterified fatty acids containing CLA in food lipids.

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