Improved HRGC Separation of cis,trans CLA Isomers as Diels-Alder Adducts of Alkyl Esters

F. Blasi, L. Giua, G. Lombardi, M. Codini, M.S. Simonetti, P. Damiani, and **L. Cossignani***

Università degli Studi di Perugia, Dipartimento di Scienze Economico-Estimative e degli Alimenti, Sezione di Chimica Bromatologica, Biochimica, Fisiologia e Nutrizione

Abstract

This paper reports the separation of four isomers of conjugated linoleic acid (CLA), *c,t/t,c*-8,10; *c,t/t,c*-9,11; *c,t/t,c*-10,12; *c,t/t,c*-11,13, after reaction of esterification with aliphatic alcohols of different chain length and adduct formation with 4-methyl-1,2,4-triazoline-3,5-dione (MTAD). The high resolution gas chromatographic analyses were carried out using a simple 50-m cyanopropyl polysiloxane capillary column both with a flame ionization detector and a mass spectrometer. The resolution between the two pair of isomers: *c,t/t,c*-9,11 and *c,t/t,c*-10,12 and between *c,t/t,c*-10,12 and *c,t/t,c*-11,13 isomers were good for all the investigated alkyl esters and increased with the chain length of alcohol esterified to carboxylic moiety of CLA isomers. The most interesting result was relative to the *c,t/t,c*-8,10 and *c,t/t,c*-9,11 isomers, critical pair of isomers also when analyzed with a 120-m cyanopropyl polysiloxane capillary column; their resolution also increased from methyl to hexyl esters of CLA isomers and reached an acceptable value (0.8) in the case of hexyl esters. The best resolutions of the four considered CLA isomers were obtained with the hexyl esters of MTAD adducts of the isomers, without excessive analysis time. This method was useful and simple to evaluate the profile of the four main *c,t* isomers in commercial CLA samples.

Introduction

In recent years there has been great interest towards the positional and geometric isomers of linoleic acid (*cis*-9,*cis*-12 octadecadienoic acid) with conjugated double bonds.

Conjugated linoleic acid (CLA) has been reported to have several beneficial effects in health-related disorders using animal models and cell cultures derived from humans and animals. Thus, CLAs have been shown to have antiadipogenic (1), anticarcinogenic (2), antiatherogenic (3), antidiabetogenic (4), and anti-inflammatory properties (5).

Recent studies suggest that the effects of CLA may be isomer dependent and that 9*c*,11*t* CLA and 10*t*,12c CLA have different effects on blood lipids and on metabolism in adipocytes (6–8). In relation to this, it is therefore important to have reliable and precise techniques for identification and quantification of particular CLA isomers in food.

*Author to whom correspondence should be addressed: email coslina@unipg.it.

Because of the multiplicity of geometrical and positional CLA isomers in foodstuffs and commercial CLA products, complete separation and accurate analysis of complex mixtures is demanding. A combination of high resolution gas chromatography (HRGC) and silver-ion high-performance liquid chromatography (Ag^+-HPLC) was found to be necessary to resolve all CLA isomers (9,10).

HRGC analysis is by far the most common method for fatty acid (FA) analysis comprising the isomers of CLA. The 100-m highly polar cyanosilicone capillary columns are the best for trying to resolve most of the closely related isomers of CLA (CP-Sil88, Supelco SP-2560, BPX 70). The order of elution of the isomers was 9*c*,11*t* followed by other *c,t*-isomers, 11*c*,13*t*, and 10*t*,12*c*, then the *c,c*-isomers and then the *t,t*-isomers. The 8*t*,10*c* isomer has a slightly longer retention time than the 9*c*,11t isomers but at best occurs as a shoulder on the 9*c*,11t peak and could not be quantified separately. The resolution of the two isomers improves on a very long polar column (120-m BPX 70) (11).

HRGC with flame ionization detector (FID) has been a standard analytical tool for lipid analysis and can provide useful information about the number of mixture components, retention times, and elution sequence. However, FID traces do not provide structural information and the gas chromatographic identification of CLA isomers should be based on gas chromatography–mass spectrometry (HRGC–MS) or gas chromatography–fourier transform infrared (HRGC–FTIR).

Methyl ester derivatives of CLA isomers have been successfully used to determine the FA double bond position and geometric configuration by HRGC–MS–MS (12).

Specific derivatization techniques such as dimethyloxazoline (DMOX), picolinyl ester, or pyrrolidide derivatives are useful to determine the double bond position of FAs by HRGC–MS (13,14). DMOX derivatization of CLA geometric isomers retains their conjugated double bond configurations in the products. The GC separation of DMOX CLA derivatives results from the geometry and position of the conjugated diene (15). The reaction of the carboxylic acid functionality with 2-amino-2-methylpropanol forms a DMOX derivative that directs EI (electron ionization) fragmentation by retaining the positive charge on the nitrogen of the derivative (16).

Another approach, that can be applied to commercial CLA, utilized the reactivity of conjugated systems with the dienophile 4-methyl-1,2,4-triazoline-3,5-dione (MTAD) to form Diels-Alder

cycloaddition products (17). Diene-specific adducts and fragmentation-directing derivatives facilitate mass spectrometric identification of individual CLA positional isomers. Diels-Alder adducts of conjugated dienes with MTAD produce diagnostic EI mass spectra that differentiate positional isomers of CLA. The conjugated diene is incorporated into a six-member heterocyclic ring that dominates fragmentation of the molecule. Information about the geometry of the diene system is destroyed, but the location of the diene is easily determined from EI mass spectra of adducts. The most abundant ions are diagnostic of the diene position in the carbon chain. These ions arise as preferential cleavages on either side of the ring and provide complementary data for the location of the conjugated system in the carbon chain. It has been reported that *c,t-* and *t,t-* isomers give MTAD adducts of different stereochemistry (*trans-* and *cis*-isomers of the chains about the ring, respectively) that can be resolved by HRGC–MS (18).

Even if the diagnostic ions are able to identify the presence of a particular CLA isomer also in case of co-elution with other CLA isomers, a good resolution of the CLA mixture components should be achieved. Christie et al. (19) reported that commercial samples of CLA, analyzed by HRGC–MS as MTAD adducts of methyl esters, showed the presence of four unresolved isomers, 8,10; 9,11; 10,12, 11,13 dienes; the cited authors also reported that the same CLA mixtures showed two major peaks well resolved from each other along with minor peaks (all *cis-* and all *trans*-dienes) when analyzed as FAME with a 25-m Carbowax capillary column.

To the best of our knowledge, no prior investigations on the separation of MTAD adducts of CLA isomers as different alkyl esters have been carried out. In this paper, for the first time, the HRGC separation of a commercial mixture of four CLA isomers (*c,t/t,c*-8,10; *c,t/t,c*-9,11; *c,t/t,c*-10,12; *c,t/t,c*-11,13) as MTAD adducts of alkyl esters on a 50-m cyanopropyl polysiloxane column has been reported.

Experimental

Materials

CLA standard mixture, 4-methyl-1,2,4-triazoline-3,5-dione, *n*-propanol, *n*-pentanol, *n*-hexanol, and 1,3-hexadiene were purchased from Sigma (St. Louis, MO). Dichloromethane was from BDH (Poole, England). Methanol, ethanol absolute, diethyl ether, and *n*-hexane were purchased from Panreac (Barcelona, Spain). *n*-Butanol, *n*-pentane, petroleum ether, and sulfuric acid were from J.T. Baker (Deventer, Holland). Sodium sulfate anhydrous was purchased from Riedel-de Haën (Sellze, Germany). Formic acid was purchased from Carlo Erba (Milan, Italy).

Esterification of CLA mixture

Six aliquots of CLA standard mixture (100 µg) were subjected to sulfuric acid esterification by reaction with 100 µL of methanol, ethanol absolute, *n*-propanol, *n*-butanol, *n*-pentanol, and *n*-hexanol in the presence of 10 µL of sulfuric acid. The reactions were carried out in pentane, at 50°C for 1 h to obtain methyl, ethyl and propyl CLA esters and at 65°C for 15 min to obtain butyl, pentyl, and hexyl CLA esters. After the esterification reaction, the samples were washed five times with water to remove acid residues. Samples were anhydrificated with anhydrous sodium sulfate, dried, and diluted in *n*-hexane. The completion of esterification of CLA samples was determined by a precoated silica gel TLC (thin-layer chromatography) analysis. Samples were developed with a petroleum ether–diethyl ether–formic acid (70:30:1, v/v/v) mixture, then the TLC plates were visualized with iodine vapor.

Synthesis of alkyl ester MTAD adducts

The MTAD derivatives of CLA alkyl esters were obtained by Dobson's procedure (16). In brief, a solution of each FA alkyl esters (40 µg) was mixed with MTAD (65 µg) in dichloromethane (100 µL) at 0°C under magnetic stirring for 40 s. Then the reactions were immediately stopped by addition of 1,3-hexadiene (0.5 µL), followed by agitation for a few seconds. The mixtures were dried under nitrogen stream and solved in dichloromethane.

HRGC analyses

Chromatograms were obtained with a Dani GC1000 gas chromatograph, (Norwalk, CT) equipped with a split/splitless injector port and an FID. The fused silica WCOT capillary column CP-Select CB for FAME (50 m × 0.25 mm i.d., 0.25 µm f.t., Varian, Superchrom, Milan, Italy) was used. The chromatograms were acquired and processed using Clarity integration software. The chromatographic conditions were the following: the injector and detector temperature was 275°C; the oven temperature was 200°C, then increased to 275°C at 3°C/min; the final temperature was held for 40 min. The carrier gas (He) flow rate was 1.2 mL/min.

HRGC-MS was performed on a Shimadzu GCMS-QP2010 gas chromatograph equipped with a quadrupole mass spectrometer (Shimadzu, Milan, Italy) equipped with a split-splitless injector maintained at 275°C. The same column and the same chromatographic conditions already described for HRGC–FID analyses were used. The following MS parameters were used: interface temperature, 270°C; MS ionization mode, electron ionization; detector voltage, 0.9 kV; acquisition mass range, 50–500 u; scan speed, 1,000 u/s; acquisition mode, full scan; scan interval, 0.5 s; solvent delay, 6 min. Data were collected by the GCMS Solution software (Shimadzu).

Results and Discussion

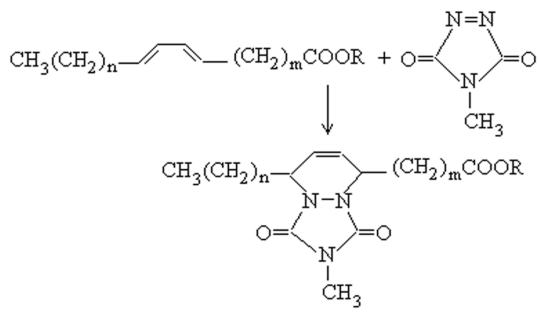
Reaction of CLA isomers with triazoline dione

The reaction of conjugated diene with triazoline dione has been investigated, and it remains useful for determining the position of conjugation in FAs; Figure 1 shows the Diels-Alder cycloaddition products, formed in the reaction of CLA isomers with MTAD. The diagnostic ions of MS spectra deriving from analyte fragmentation on either sides of the adduct ring permit the identification of the presence of positional CLA isomers without the need of their chromatographic separation. It has been reported that adducts of isomer mixtures occur as a single chromatographic peak, with only a small degree of separation within

the peak, and that it is possible to estimate the different positional isomers semi-quantitatively using the areas of the $[M\text{-}R_1]^+$ and $[M\text{-}R_2]^+$ ions, where R_1 corresponds to the alkyl moiety and R_2 the methyl ester part of the chain (20).

HRGC separation of *cis,trans*-CLA isomers as Diels-Alder adducts of alkyl esters

The attempt to achieve some degree of chromatographic separation of the four main *c,t*-CLA isomers as Diels-Alder adducts represents the objective of the present investigation. The starting compounds, the methyl esters of a commercial CLA preparation, were subjected to reaction with MTAD, then the adducts were analyzed by HRGC on a 50-m cyanopropyl polysiloxane capillary column. Figure 2A shows the relative chromatogram with the separation of three peaks; the MS spectra of the first eluting peak revealed the fragments of *c,t/t,c*-8,10 and *c,t/t,c*-9,11 isomers, while the second and third eluting peak resulted the *c,t/t,c*-10,12 and the *c,t/t,c*-11,13 isomers, respectively. In order to improve the resolution of the critical pair of isomers, the CLA mixture was subjected to esterification reaction with longer chain alcohols; ethyl, *n*-propyl, *n*-butyl, *n*-pentyl, *n*-hexyl CLA esters were prepared and then reacted with MTAD. The adducts were analyzed by HRGC in the same experimental conditions already used for methyl ester adducts and the chromatograms are shown in Figures 2B–2F, respectively. It is possible to observe that the separation of the critical pair of isomers, the *c,t/t,c*-8,10 and *c,t/t,c*-9,11, improved as the ester alkyl chain increased and that the resolution degree of the other isomers improved as well. In Table I, the retention times of the CLA isomers esterified with different chain length alcohols and reacted with MTAD have been reported, as average values of four determinations; in the same Table, the repeatability of the procedure, reported as intra-day and inter-day relative standard deviations, are shown. These last values show a good precision, even if generally slightly higher standard deviations have been obtained for inter-day analyses.



MTAD adduct	n	m
c,t/t,c-8,10	6	6
c,t/t,c-9,11	7	5
c,t/t,c-10,12	8	4
c,t/t,c-11,13	9	3

R =		
	$-CH_3$	Me
	$-CH_2CH_3$	Et
	$-CH_2CH_2CH_3$	Pr
	$-CH_2CH_2CH_2CH_3$	Bu
	$-CH_2CH_2CH_2CH_2CH_3$	Pe
	$-CH_2CH_2CH_2CH_2CH_2CH_3$	He

Figure 1. Reaction of CLA isomer alkyl esters with 4-methyl-1,2,4-triazoline-3,5-dione.

Resolution of *c,t*-CLA isomers

In Table II the resolutions between adjacent CLA isomers have been reported, for the MTAD adducts of the different CLA isomer alkyl esters. It is possible to observe that the resolution values for the critical pair of isomers, *c,t/t,c*-8,10 and *c,t/t,c*-9,11, become higher from the adducts of butyl to the hexyl CLA to reach the acceptable value of 0.8 for the adducts of the longer chain alkyl esters. The resolution degree of the other pair of isomers, *c,t/t,c*-9,11 – *c,t/t,c*-10,12 and *c,t/t,c*-10,12 – *c,t/t,c*-11,13, already good for the adducts of methyl esters shows increasing values as the ester alkylic chain increased. The result obtained with the adducts of *c,t/t,c*-8,10 and *c,t/t,c*-9,11 CLA hexyl esters is considerable, considering the impossibility to separate the cited isomers in form of methyl esters using HRGC, also using 100–120 m column. The presented analytical procedure is simple, considering both the derivatization steps and the HRGC conditions. Moreover the chromatographic resolution of the four *c,t*-CLA isomers permit to perform a quantitative analysis also with a FID. Furthermore, the identity of CLA isomers, as MTAD adducts, can be confirmed by HRGC–MS.

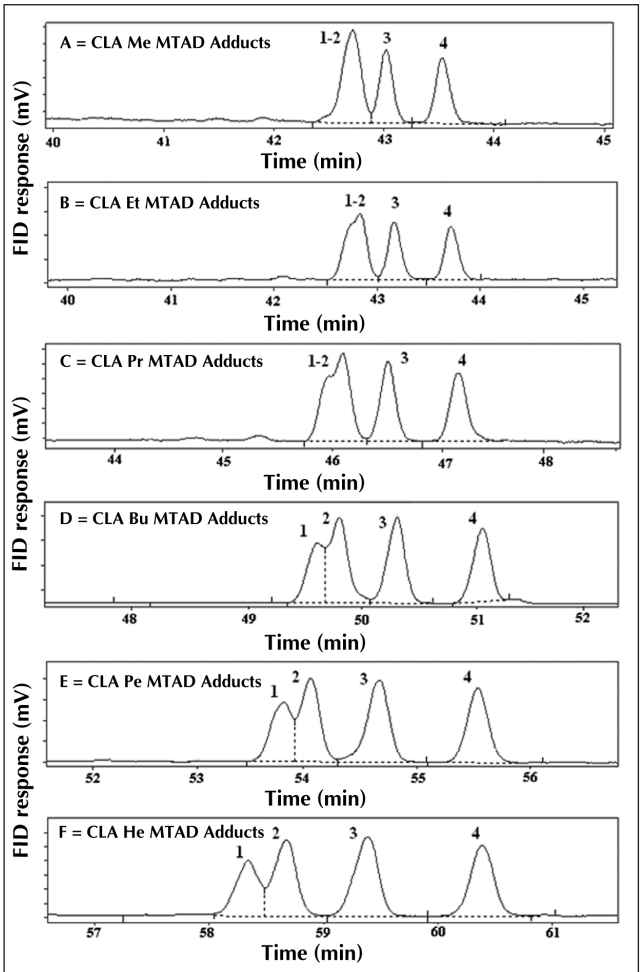


Figure 2. HRGC separations of CLA mixture as Diels-Alder adducts of alkyl esters (1: *c,t/t,c*-8,10; 2: *c,t/t,c*-9,11; 3: *c,t/t,c*-10,12; 4: *c,t/t,c*-11,13).

MS fragmentation of adducts of CLA isomers

In Table III the characteristic MS fragments of the adducts of the different esters of CLA isomers have been reported, in particular the molecular ion and the cleavage fragments that include the ring formed during adduct synthesis. It is possible to observe that, as expected, the different alkyl esters of the same CLA positional isomer present the same ion deriving from the cleavage on the methyl side $[M\text{-}R_1]^+$ and a different one from the cleavage on the ester side of the ring $[M\text{-}R_2]^+$. Considering the acceptable resolution degree for the hexyl esters, the four considered CLA isomers could be analyzed either by HRGC–FID using standard compounds for the identification and by HRGC–MS, to evaluate through the fragmentation patterns the identity of the compounds.

Table I. Retention Times, Intra- and Interday Variability of CLA Isomers as Alkyl Ester Adducts

MTAD adduct	Rt (min)	RSD Intraday (n = 4)	RSD Interday (n = 4)
c,t/t,c-8,10-CLAMe *c,t/t,c*-9,11-CLAMe	42.6	0.18	1.11
c,t/t,c-10,12-CLAMe	42.9	0.17	1.11
c,t/t,c-11,13-CLAMe	43.4	0.18	1.11
c,t/t,c-8,10-CLAEt *c,t/t,c*-9,11-CLAEt	42.7	0.46	0.88
c,t/t,c-10,12-CLAEt	43.1	0.48	0.90
c,t/t,c-11,13-CLAEt	43.6	0.47	0.91
c,t/t,c-8,10-CLAPr *c,t/t,c*-9,11-CLAPr	46.0	0.26	0.58
c,t/t,c-10,12-CLAPr	46.3	0.54	0.49
c,t/t,c-11,13-CLAPr	46.5	0.78	0.66
c,t/t,c-8,10-CLABu	49.5	0.44	0.37
c,t/t,c-9,11-CLABu	49.7	0.44	0.38
c,t/t,c-10,12-CLABu	50.2	0.44	0.38
c,t/t,c-11,13-CLABu	51.0	0.44	0.39
c,t/t,c-8,10-CLAPe	53.8	0.05	0.41
c,t/t,c-9,11-CLAPe	54.0	0.06	0.42
c,t/t,c-10,12-CLAPe	54.6	0.05	0.41
c,t/t,c-11,13-CLAPe	55.5	0.06	0.40
c,t/t,c-8,10-CLAHe	58.3	0.07	0.38
c,t/t,c-9,11-CLAHe	58.5	0.06	0.25
c,t/t,c-10,12-CLAHe	59.3	0.06	0.38
c,t/t,c-11,13-CLAHe	60.4	0.05	0.36

Table II. Resolution of CLA Isomers as MTAD Adducts of Alkyl Esters

	Resolution		
MTAD adduct	*c,t/t,c*-8,10 *c,t/t,c*-9,11	*c,t/t,c*-9,11 *c,t/t,c*-10,12	*c,t/t,c*-10,12 *c,t/t,c*-11,13
CLAMe	–	1.1	2.1
CLAEt	–	1.2	2.2
CLAPr	–	1.3	2.3
CLABu	0.5	1.4	2.5
CLAPe	0.6	1.6	2.5
CLAHe	0.8	1.8	2.6

It should be pointed out that MTAD adducts are especially useful for commercial CLA preparations, containing the main isomers *c,t/t,c*-9,11 and *c,t/t,c*-10,12 CLA in roughly equal amounts, while *c,t/t,c*-8,10 and *c,t/t,c*-11,13 CLA may be either absent or present to varying degrees. The reported analytical methodology presents the advantage of the resolution of the four *c,t* isomers of CLA, obtained with simple conditions and no excessive analysis time, both for derivatization and for chromatographic steps. With regard to the problem arising when other isomers, such as *c,c* or *t,t*-CLA, are present in commercial CLA preparation, the results obtained by Reaney et al. (18) should be cited. They reported that the main product formed from the reaction of methyl-9*c*,11*t*-octadecadienoate and methyl-*c*9,*c*11-octadecadienoate with MTAD was the *t*-Diels-Alder adduct, while the *c*-Diels-Alder adduct derived from the derivatization of methyl-9*t*,11*t*-octadecadienoate and the different adducts were separated in the adopted experimental conditions.

From these considerations, it can be concluded that minor CLA isomers, if present, cannot be evaluated with the proposed procedure; in fact, the *c,t* and *c,c* geometrical isomers of the same positional CLA isomer result not separated, and the *t,t* isomers in any case difficult to resolve in the mixture of the main represented *c,t* isomers. However the presence of *c,c* and *t,t* isomers can be easily detected by analyzing the mixture as alkyl esters on the same 50-m cyanopropyl polysiloxane column, used with success for the separation of the MTAD adducts of the four principal *c,t*-CLA isomers in commercial mixture.

Table III. Characteristic MS Fragments* of CLA Isomers as MTAD Adducts of Alkyl Esters

MTAD adduct	M^+	$[M\text{-}R_1]^+$	$[M\text{-}R_2]^+$
c,t/t,c-8,10-CLAMe	407	264	308
c,t/t,c-9,11-CLAMe	407	250	322
c,t/t,c-10,12-CLAMe	407	236	336
c,t/t,c-11,13-CLAMe	407	222	350
c,t/t,c-8,10-CLABu	449	264	350
c,t/t,c-9,11-CLABu	449	250	364
c,t/t,c-10,12-CLABu	449	236	378
c,t/t,c-11,13-CLABu	449	222	392
c,t/t,c-8,10-CLAEt	421	264	322
c,t/t,c-9,11-CLAEt	421	250	336
c,t/t,c-10,12-CLAEt	421	236	350
c,t/t,c-11,13-CLAEt	421	222	364
c,t/t,c-8,10-CLAPe	463	264	364
c,t/t,c-9,11-CLAPe	463	250	378
c,t/t,c-10,12-CLAPe	463	236	392
c,t/t,c-11,13-CLAPe	463	222	406
c,t/t,c-8,10-CLAPr	435	264	336
c,t/t,c-9,11-CLAPr	435	250	350
c,t/t,c-10,12-CLAPr	435	236	364
c,t/t,c-11,13-CLAPr	435	222	378
c,t/t,c-8,10-CLAHe	477	264	378
c,t/t,c-9,11-CLAHe	477	250	392
c,t/t,c-10,12-CLAHe	477	236	406
c,t/t,c-11,13-CLAHe	477	222	420

* Molecular ion M^+, cleavage on the methyl side $[M\text{-}R_1]^+$ and on the ester side $[M\text{-}R_2]^+$ of the ring.

Conclusions

The reported methodology permits the separation of four *c,t*-CLA isomers, after a two step derivatization: the esterification with different chain length alcohols and the cycloaddition reaction of diene system with MTAD. The best result is relative to the HRGC separation of *c,t/t,c*-8,10 and *c,t/t,c*-9,11 CLA isomers as Diels-Alder adducts of hexyl esters. The improved resolution of the cited CLA isomers assumes relevance considering that in some food products the most represented isomer is the *c,t/t,c*-9,11 and that commercial mixtures of *c,t/t,c*-9,11 and *c,t/t,c*-10,12 CLA isomers can contain also non-trascurabile amounts of *c,t/t,c*-8,10 and *c,t/t,c*-11,13 isomers.

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