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Identification and Characterization of Conjugated Linolenic Acid Isomers by Ag^+-HPLC and NMR

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Interest in the biological activity of conjugated linolenic acids (CLnA) is growing. Technically, previous methods could not fully characterize and identify CLnA isomers. The present study is the first report on application of silver ion impregnated high-performance liquid chromatography (Ag^+-HPLC) into separation of CLnA methyl ester (CLnAMe) mixture. Compared with the GC and reversed-phase HPLC techniques, Ag^+-HPLC could resolve and separate CLnAMe isomers into three clusters of peaks namely *ttt*, *ctt* and *ctc* geometrical groups. Each positional isomer belonging to the same geometrical group could be further separated by Ag^+-HPLC. Quantitative ^{13}C NMR properties of CLnAMe mixture were subsequently studied. Olefinic signals in the CLnAMe mixture were assigned and used to quantify each isomer in combination with Ag^+-HPLC. The present study provided a detailed analysis of a complex CLnA mixture and may have an important application in future studies dealing with synthesis, oxidative stability, and bioactivity of individual CLnA isomers.

KEYWORDS: Characterization; conjugated linolenic acid; Ag^+-HPLC; NMR

INTRODUCTION

Conjugated linolenic acid (CLnA) is an imprecisely defined term describing a group of positional (8,10,12−18:3; 9,11,13−18:3; 10,12,14−18:3; 11,13,15−18:3, etc.) and geometric (*ttt*, *ctt*, *ctc*, etc.) isomers of octadecatrienoic acids that contain three double bonds in conjugation. CLnAs do not occur to any significant amount in animal lipids. Dietary CLnA is quantitatively minor in the vegetable oils, accounting for up to 0.2% by weight (*1*). However, CLnA in several kinds of seed oils, such as tung oil, pomegranate seed oil, catalpa seed oil, *Momordica charantia* seed oil, and cherry seed oil, is present in large quantities and can account for 40−80% of total fatty acids (*2*, *3*). It is known that CLnA in tung oil is mainly α-eleostearic acid (c9,t11,t13−18:3) and *β*-eleostearic acid (t9,t11,t13−18:3). Pomegranate seed oil mainly contains punicic acid (c9,t11,c13−18:3) (*2*, *3*).

CLnA possesses several biological activities. A CLnA isomer mixture was reported to be a potent suppressor on growth of various human tumor cells (*4*−*11*). Our previous study showed that the supplementation of α-eleostearic acid or punicic acid significantly decreased hepatic cholesterol (*12*). Suzuki et al. reported that the cytotoxicity of c9, t11, t13−18:3; c9, t11, c13−18:3 and t9, t11, c13−18:3 was much higher than that of t8, t10, c12−18:3, suggesting that different CLnA isomers had different biochemical activities (*3*).

Minor structural differences among CLnA isomers make their separation, identification, and quantification extremely difficult. The previous studies on biological activity of CLnA usually tested a CLnA mixture. Chemists want to synthesize only the active CLnA isomer while biologists want to know which CLnA isomer is responsible for the observed biological activities. In this regard, it is imperative to develop a reliable method for separation and characterization of individual CLnA isomers in complex mixtures such as synthetic products and biological materials. However, using IR, UV, GC, and reversed-phase HPLC cannot meet such a purpose. GC-MS has been used to identify and characterize the positional configuration of CLnA DMOX derivatives but it could not separate all CLnA isomers and thus was not useful for a complex CLnA sample. Since it is still impossible to synthesize each single CLnA isomer, GC-MS and NMR data on CLnA isomers are now only limited to α-eleostearic acid, *β*-eleostearic acid, and punicic acid (*8*, *13*). Although ^{13}C NMR properties of α-eleostearic acid, *β*-eleostearic acid, and punicic acid were previously reported, $^{13}C-^{1}H$ COSY correlation and homonuclear decoupling techniques have not yet been used to characterize them, which resulted in the inaccurate assignment of the signals of CLnA olefinic carbons.

The present study had two objectives. First, it was to fully characterize *β*-eleostearic acid, punicic acid, and tung oil-CLnA isomers using NMR followed by assignments of the chemical shifts of proton and carbon atoms by $^{13}C-^{1}H$ COSY correlation

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techniques. The proton−proton coupling constant (J) across the double bonds was subsequently calculated to identify the geometrical configuration. Second, the present study was to separate and quantify a complex CLnA methyl ester (ClnAMe) mixture of pomegranate seed oil using silver ion-impregnated high-performance liquid chromatography (Ag^+-HPLC) and quantitative ^{13}C NMR technique.

MATERIALS AND METHODS

Materials. Tung oil was obtained from Tung Oil Company (Pingding Shan, Henan Province, China). β-Isomerized tung oil was prepared by adding a trace of I_2 to tung oil at room temperature for 10 days until the tung oil completely solidified. CLnA in solidified tung oil was then mainly converted to β-eleostearic acid (*1*). Air-dried seeds of pomegranate were purchased from SanheTian Company (Chengdu, Sichuan Province, China). The pomegranate seed oil was extracted in hexane and concentrated in a rotary evaporator at 30 °C (RE-52 rotary evaporator, Shanghai, China). BF_3/methanol (14%) was purchased from Sigma Chemical Co. (St. Louis, MO). Hexane and acetonitrile were HPLC grade. All other solvents and chemicals were analytical grade.

Preparation of β-Eleostearic Acid, Punicic Acid, and Tung Oil-CLnA. β-Isomerized tung oil (10 g) was bubbled with N_2 for 10 min and then saponified with 150 mL of 0.3 M KOH in 90% ethanol at 25 °C for 24 h. The reaction mixture was acidified by addition of 30 mL of 1.0 M H_2SO_4 and then extracted using hexane (20 mL × 3). The hexane extract containing free fatty acids was washed in distilled water (10 mL × 3). After being dried on anhydrous sodium sulfate, the crude fatty acids were obtained by removal of hexane in a rotary evaporator below 30 °C. Pure β-eleostearic acid was obtained by repeat crystallization in 90% ethanol at −20 °C (*1*). Punicic acid in pomegranate seed oil and tung oil-CLnA were similarly prepared and purified. GC analysis showed that purity of each CLnA reached 99%. $^{13}C-^{1}H$ COSY showed the olefinic carbon signals (ppm) were as follows: β-Eleostearic acid (134.46, 130.87, 130.51, 130.41, 130.73, 134.23), punicic acid (132.69, 128.82, 127.94, 127.79, 128.71, 132.46), and tung oil-CLnA (134.46, 130.87, 130.51, 130.41, 130.73, 134.23; 131.75, 128.72, 132.83, 126.00, 130.53, 135.17). The NMR data were similar to those reported by Tulloch et al. (*13*) except some peak assignments of olefinic carbons were mislabeled.

Preparation of CLnA Methyl Ester Mixture. Pomegranate seed oil (2 g) was weighed in a 250 mL flask and isomerized by addition of 200 mL of 14% BF_3/ methanol reagents. The flask was placed in oil bath at 100 °C for 10 min under a gentle stream of nitrogen gas and then cooled at room temperature. CLnAMe mixture was extracted in hexane (10 mL × 3) and recrystallized twice in hexane at −20 °C. GC analysis showed that purity of total CLnAMe reached 99%. Each isomer in CLnAMe mixture was quantified by Ag^+-HPLC and quantitative ^{13}C NMR.

GC Analysis of Fatty Acids. CLnA isomers were methylated according to the method previously described (*14*). The CLnAMe was analyzed on a flexible silica capillary column (SP 2560, 100 m × 0.25 mm i.d., Supelco, Inc., Bellefonte, PA) in a HP 6890 series gas−liquid chromatograph equipped with a flame-ionization detector and an automated injector (Palo Alto, CA). The column temperature was programmed from 170 to 210 °C at a rate of 2 °C/min and then held for 5 min. The injector and detector temperatures were set at 250 and 300 °C, respectively. Nitrogen gas was used as the carrier gas at a head pressure of 35 psi.

NMR Analysis. NMR spectra were recorded on a Bruker AV-400 Fourier transform NMR spectrometer (Bruker, Fallanden, Switzerland). Deuteriochloroform ($CDCl_3$) (0.2−0.3 mM) in tetramethylsilane was used as an internal reference standard. Chemical shifts are given in δ values in ppm downfield from tetramethylsilane ($\delta_{TMS} = 0$). $^{13}C-^{1}H$ NMR COSY correlation techniques and homonuclear decoupling technique were used to assign the chemical shifts of proton and carbon atoms of β-eleostearic acid, punicic acid, and tung oil-CLN. Similarly, the CLnA mixture derived from pomegranate seed oil was also analyzed using quantitative ^{13}C NMR. The PULPROG of the homonuclear decoupling technique, $^{13}C-^{1}H$ COSY, and quantitative ^{13}C NMR was zg0jd, hmqcgpqf, and zgig30, respectively. The SFO1 of ^{1}H NMR was 400.13 MHz. The SFO1 of ^{13}C NMR was 100.62 MHz.

Ag^+-HPLC Analysis. The individual CLnAMe isomers were separated using a HP-1100 HPLC equipped with a ternary pump delivery system. In brief, 5 μL of CLnAMe samples (5 μg/mL) in hexane were injected onto a silver ion-impregnated column (250 × 4.6 mm i.d., 5 μm, Chrompack, Bridgewater, NJ) via a Rheodyne valve injector. Hexane, containing 0.1% acetonitrile, was chosen as a mobile phase at a flow rate of 1.0 mL/min. The separated individual CLnAMe were monitored at 284 nm. Only the CLnAMe isomers were detected at this wavelength.

Reversed-Phase HPLC Analysis. The CLnAMe isomers were separated using the HP-1100 HPLC equipped with a ternary pump delivery system. In brief, 5 μL of CLnAMe samples (5 μg/mL) in hexane was injected onto an extent C_{18} (250 × 4.6 mm i.d., 5 μm, stainless steel, ZORBAX, Agilent) via a Rheodyne valve injector. Acetonitrile/H_2O (4:1, v/v) was chosen as a mobile phase at a flow rate of 1.4 mL/min. CLnAMe was monitored at 284 nm. Only the CLnAMe isomers were detected at this wavelength.

RESULTS AND DISCUSSION

Comparison of Ag^+-HPLC and Reversed-Phase HPLC. The Ag^+-HPLC and reversed-phase HPLC chromatograms of CLnAMe mixture derived from pomegranate seed oil were shown in **Figure 1a** and **Figure 1b**, respectively. The results clearly demonstrated that Ag^+-HPLC was able to separate the CLnAMe mixture into eight peaks whereas reversed-phase HPLC was only able to resolve the CLnAMe mixture into three peaks (**Figure 1b**), suggesting that Ag^+-HPLC had greater resolution power and was more effective in separation of the CLnAMe isomers. To identify all the eight isomers, β-eleostearic acid, punicic acid, and α-eleostearic acid was characterized by NMR and then chosen as standards in Ag^+-HPLC analysis. Eight CLnA methyl esters in the mixture were subsequently identified by both Ag^+-HPLC and quantitative ^{13}C NMR.

Characterization of β-Eleostearic Acid, Punicic Acid, and α-Eleostearic Acid by NMR. Previous reports only used ^{13}C NMR to characterize these isomers, and the existing NMR data were incomplete. The present study was the first time to use the $^{13}C-^{1}H$ COSY correlation technique to assign the chemical shifts of the proton and carbon atoms of β-eleostearic acid, punicic acid, and α-eleostearic acid. Previous inaccurate assignment of the signals of olefinic carbons was corrected by this technique. In addition, the proton−proton coupling constant (J) across the double bonds was an important parameter and could provide useful information about the geometrical configuration of CLnA. However, the extensive overlap in the ^{1}H NMR spectrum made it difficult to calculate the coupling constant directly. In this regard, we used the homonuclear decoupling technique to simplify the ^{1}H NMR spectrum and calculate coupling constants. The NMR results of three CLnA methyl ester isomers were summarized in **Table 1** and **Table 2**.

Characterization of β-eleostearic acid was used as an example to simplify the representation on the assignment of three CLnA isomers. $^{13}C-^{1}H$ COSY and the homonuclear decoupling spectra of β-eleostearic acid were shown in **Figure 2** and **Figure 3**, respectively. β-Eleostearic acid showed three multiplets at δ_H 6.10 (2H), 6.04 (2H), and 5.66 (2H). They could be separated into inner (11-H, 12-H), middle (10-H, 13-H), and outer (9-H, 14-H) group since β-eleostearic acid was a 9, 11, 13 positional isomer (*13*). Two protons in each group were magnetically equivalent. It has been known that conjugation makes the density of the electron cloud of inner positional olefinic protons decrease compared with that of outer positional olefinic protons. So the chemical shifts of the inner positional olefinic protons are

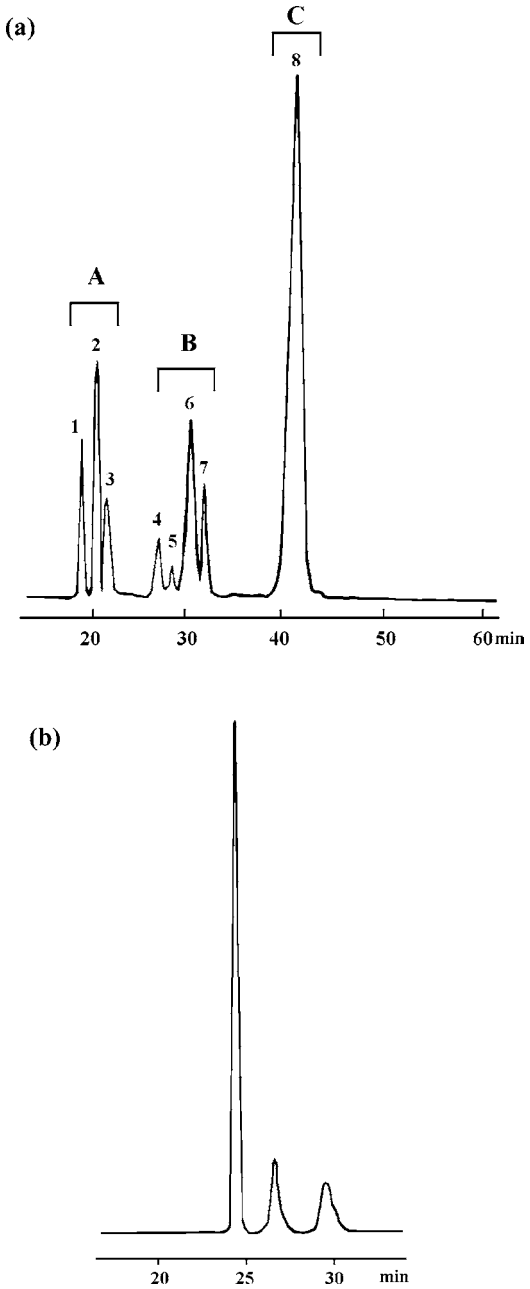

Figure 1. Ag^+-HPLC and reversed-phase HPLC chromatograms of conjugated linolenic acid methyl ester (CLnAMe) mixture derived from pomegranate seed oil. (a) Ag^+-HPLC chromatogram of the CLnAMe mixture. Peaks 1, 2, 3, 4, 5, 6, 7, and 8 were identified as isomer 1 (t10, t12, t14–18:3), isomer 2 (t9, t11, t13–18:3), isomer 3 (t8, t10, t12–18:3), isomer 4 (c11, t13, t15–18:3), isomer 5 (c10, t12, t14–18:3), isomer 6 (c9, t11, t13–18:3), isomer 7 (c8, t10, t12–18:3), isomer 8 (c9, t11, c13–18:3), respectively. (b) Reversed-phase HPLC chromatogram of the CLnAMe mixture.

slightly more downfield than outer protons (*15*). The signal at δ_H 6.10 is then most likely due to the coupling of the two inner positional olefinic protons of the triene system (11-H,12-H). The signals at δ_H 6.04 and 5.66 corresponded to the shifts at the middle (10-H,13-H) and outer (9-H,14-H) position, respectively.

The $^{13}C-^{1}H$ COSY correlation technique was used to assign signals of olefinic carbons (**Figure 2**). The multiple peaks at δ_H 5.66 (9-H,14-H) were connected to the pair of carbon signals at δ_C 134.46 and 134.23. The multiple peaks at δ_H 6.04 (10-H,13-H) were connected to the pair of carbon signals at δ_C 130.87 and 130.73. The signal at δ_H 6.10 (11-H,12-H) was connected to the pair of carbon signals that appeared at δ_C 130.51 and 130.41. These connections supported the assignment of the shifts of the inner carbon atoms (C-11,C-12) at δ_C 130.51 and 130.41, shifts of the middle carbon atoms (C-10,C-13) at δ_C 130.87 and 130.73, and shifts of the outer carbon atoms (C-9,C-14) at δ_C 134.46 and 134.23. It clearly demonstrated that $^{13}C-^{1}H$ COSY correlation technique was straightforward to assign two signals in the same pair to particular carbon compared with the present NMR techniques.

Table 1. Chemical Shift Values of Olefinic Hydrogens of β-Eleostearic Acid, Punicic Acid, and α-Eleostearic Acid (ppm)

	β-eleostearic acid: T9, t11, t13-18:3	punicic acid: c9, t11, c13-18:3	α-eleostearic acid: c9, t11, t13-18:3
9,14-H	5.66, $J = 12.8$ Hz	5.46, $J = 10.8$ Hz	5.40 (9-H, $J = 10.8$ Hz) 5.74 (14-H, $J = 14$ Hz)
10,13-H	6.04, $J = 12.8$ Hz	6.08, $J = 10.8$ Hz	6.01 (10-H, $J = 10.8$ Hz) 6.12 (13-H, $J = 14$ Hz)
11,12-H	6.10, $J = 11.6$ Hz	6.48, $J = 12.8$ Hz	6.40 (12-H, $J = 13.6$ Hz) 6.19 (11-H, $J = 13.6$ Hz)

Table 2. Chemical Shift Values of Olefinic Carbons of β-Eleostearic Acid, Punicic Acid, and α-Eleostearic Acid (ppm)

	β-eleostearic acid: t9, t11, t13-18:3	punicic acid: c9, t11, c13-18:3	α-eleostearic acid: c9, t11, t13-18:3
C-9	134.46	132.69	131.75
C-10	130.87	128.82	128.72
C-11	130.51	127.94	132.83
C-12	130.41	127.79	126.00
C-13	130.73	128.71	130.53
C-14	134.23	132.46	135.17

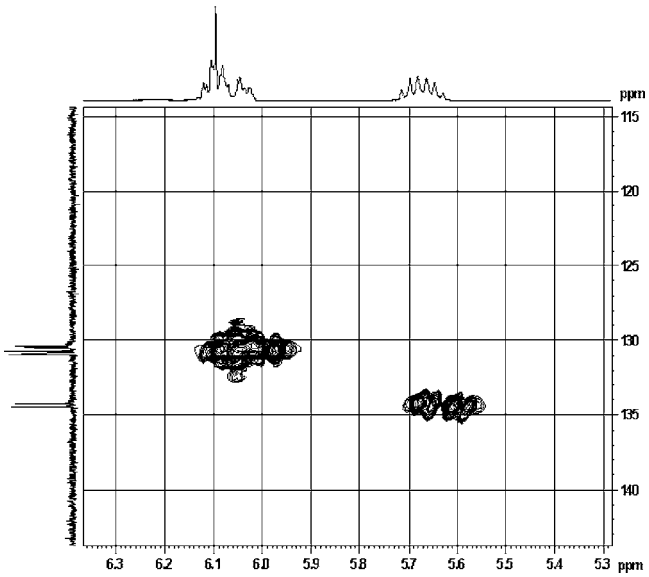

Figure 2. $^{13}C-^{1}H$ COSY spectrum of β-eleostearic acid.

Homonuclear decoupling technique was used to simplify the ^{1}H NMR spectrum and then calculate proton−proton coupling constants. The 8,15-H decoupling spectrum (**Figure 3**) was used as an example to demonstrate this decoupling technique. The coupling of 9,14-H was simplified as dd peak when 8,15-H was irradiated (**Figure 3**). Similarly, irradiating 10,13-H made the coupling of 9,14-H and 11,12-H was simplified as quintet and quartet, respectively. Irradiating 11,12-H made the coupling of 10,13-H and 9,14-H simplified as a doublet and quintet, respectively. It was calculated that $J^{9-10} = J^{13-14} = 12.8$ Hz

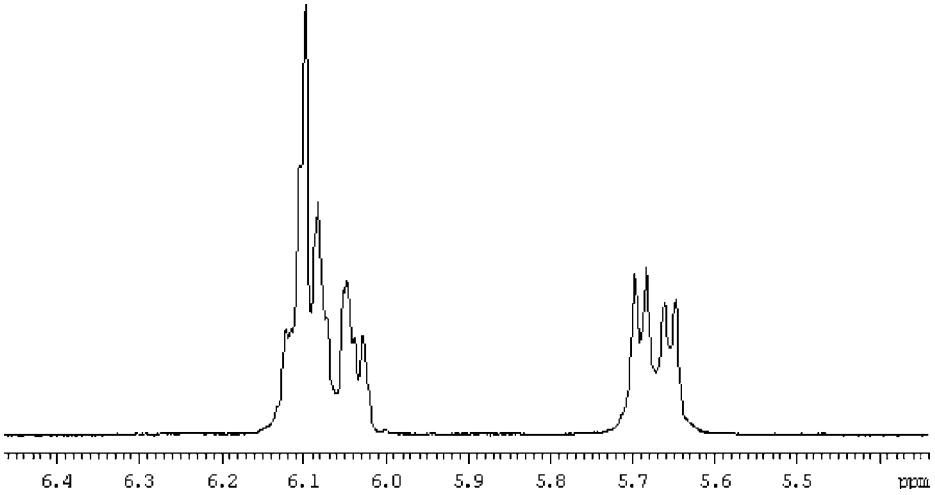

Figure 3. 8,15-H Homonuclear decoupling ^{1}H NMR of β-eleostearic acid. 8,15-H of β-eleostearic acid was irradiated, and the coupling of 9,14-H was simplified as a dd peak.

and $J^{11-12} = 11.6$ Hz. J^{8-9} was further calculated as 6.2 Hz. It was known that the J about 6−11 Hz was for cis and J about 11−15 Hz was for trans configuration (*16*). According to J values described above, 9,14-H, 10,13-H, and 11,12-H were all in trans configuration (**Table 1**).

Similarly, signals of punicic acid and tung oil-CLnA were assigned and proton−proton coupling constants were also calculated (**Table 1**, **Table 2**). It was found that in a previous report, Tulloch et al. (*13*) had mistakenly assigned some of the shifts of the olefinic carbon atoms of α-eleostearic acid, β-eleostearic acid, and punicic acid. In other words, the shifts of the "inner" carbon atoms were assigned in a reversed order.

Identification of β-Eleostearic Acid Methyl Ester, Punicic Acid Methyl Ester, and α-Eleostearic Acid Methyl Ester by Ag$^+$-HPLC. These CLnAMe isomers were analyzed using Ag$^+$-HPLC, and the results showed that both purified β-eleostearic acid and punicic acid was a single CLnA isomer with a retention time of 21.3 min and 40.1 min, respectively, under the present Ag$^+$-HPLC conditions. The results showed that tung oil-CLnA contained two isomers, which eluted at 21.0 and 30.7 min, respectively. The peak at 21.0 min was identified as β-eleostearic acid as it had the same retention time as β-eleostearic acid. The second peak at 30.7 min was then identified as α-eleostearic acid since tung oil contained α- and β-eleostearic acid (*2*). β-Eleostearic acid methyl ester, punicic acid methyl ester, and α-eleostearic acid methyl ester were then used as reference standards to deduce configuration of other isomers in the CLnAMe mixture.

Identification of Eight CLnA Methyl Esters by Ag$^+$-HPLC. Eight peaks separated by Ag$^+$-HPLC were named as isomers 1, 2, 3, 4, 5, 6, 7, and 8. According to their eluting order, they were further separated into three groups, namely Group A (isomer 1, 2, 3), Group B (isomer 4, 5, 6, 7), and Group C (isomer 8). Isomers 2, 6, and 8 were β-eleostearic acid methyl ester (t9, t11, t13−18:3), α-eleostearic acid methyl ester (c9, t11, t13−18:3), and punicic acid methyl ester (c9, t11, c13−18:3), respectively, as they had the same retention times as the reference standards (**Figure 1a**). To further confirm these results, pure β-eleostearic acid methyl ester, tung oil-CLnA methyl ester, and punicic acid methyl ester were added into the isomerized mixture (**Figure 4**). It clearly demonstrated that peaks 2, 6, and 8 were spiked after these standards were added. Ag$^+$-HPLC quantitative analysis showed that the amount of isomers 2, 6, and 8 increased and accounted for 23.53%, 21.50%, and 40.28%, respectively. In contrast, they accounted only for 17.75%, 10.56%, and 32.93%, respectively, before standards were added (**Table 3**). As peak 2, 6, and 8 belonged to group A, group B, and group C, respectively, three groups could be assigned as t,t,t, c,t,t, and c,t,c geometrical isomers, respectively. This assignment was also consistent with the separation principle of Ag$^+$-HPLC. The silver ion-impregnated column, as a polar column, separated organic substances according to their polarity with compounds having a lower polarity being eluted earlier. In fact, the order of polarity of CLnAMe geometrical isomers was t,t,t < c,t,t < c,t,c.

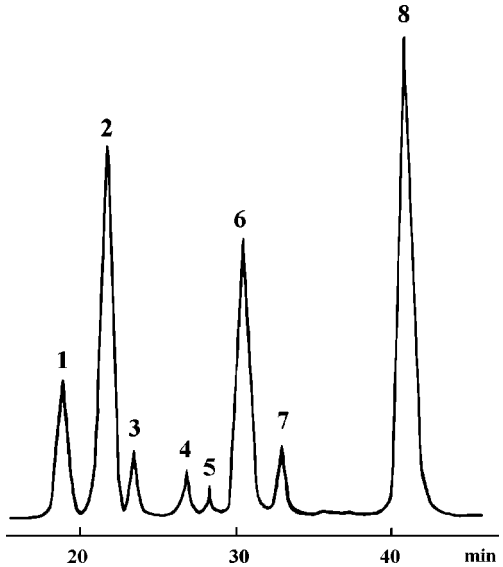

Figure 4. Ag$^+$-HPLC chromatogram of the CLnAMe mixture after β-eleostearic acid methyl ester, punicic acid methyl ester, and tung oil-CLnA methyl ester were added.

Geometrical configuration of different positional isomers within the same group could be deduced based on their different retention time. Ag$^+$-HPLC has a specific separation principle, i.e., the farther the double bonds are from $COOCH_3$, the earlier the isomer elutes. In fact, it had already been reported that CLA (conjugated linoleic acid) methyl ester isomers eluted in the order of 11,13; 10,12; 9,11; 8,10; 7,9 within each geometrical group (*17*). The eluting order of positional isomers of CLnAMe should be similar to that of CLA since they had the similar conjugated configuration. Therefore, eight isomers were deduced

Table 3. Quantitative ^{13}C NMR Chemical Shift Values and Ag^+-HPLC Quantitative Results of CLnA Mixture Derived from Pomegranate Seed Oil

	^{13}C-NMR chemical shift values						amounts in CLnA mixtures (%)	
CLnA isomers	OL-1	OL-2	OL-3	OL-4	OL-5	OL-6	quantitative ^{13}C NMR	Ag^+-HP LC
t10, t12, t14-18:3 (Isomer 1)	134.5 2	130.9 1	130.5 9	130.5 7	130.6 3	134.0 9	10.21	13.19
t9, t11, t13-18:3 (Isomer 2)	134.4 6	130.8 7	130.5 1	130.4 1	130.7 3	134.2 3	15.35	17.75
t8, t10, t12-18:3 (Isomer 3)	134.3 4	130.8 1	130.5 5	130.4 6	130.7 0	134.1 7	9.85	12.29
c11, t13, t15-18:3 (Isomer 4)	131.6 2	128.6 1	132.6 7	125.8 9	130.5 0	134.9 4	5.86	4.78
c10, t12, t14-18:3 (Isomer 5)	131.6 9	128.6 7	132.7 8	125.9 2	130.5 9	135.0 4	1.93	1.65
c9, t11, t13-18:3 (Isomer 6)	131.7 5	128.7 2	132.8 3	126.0 0	130.5 3	135.1 7	9.84	10.56
c8, t10, t12-18:3 (Isomer 7)	131.9 5	128.7 7	132.8 6	126.0 4	130.5 5	135.2 3	7.92	6.86
c9, t11, c13-18:3 (Isomer 8)	132.6 9	128.8 2	127.9 4	127.7 9	128.7 1	132.4 6	39.04	32.93

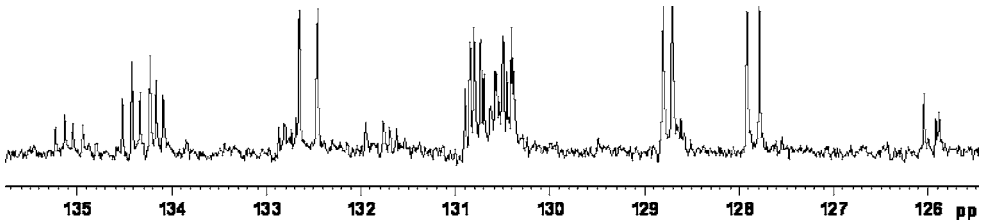

Figure 5. Quantitative ^{13}C NMR spectrum of a purified CLnA mixture derived from pomegranate seed oil.

and identified as t10, t12, t14−18:3 (isomer 1), t9, t11, t13−18:3 (isomer 2), t8, t10, t12−18:3 (isomer 3), c11, t13, t15−18:3 (isomer 4), c10, t12, t14−18:3 (isomer 5), c9, t11, t13−18:3 (isomer 6), c8, t10, t12−18:3 (isomer 7), and c9, t11, c13−18:3 (isomer 8), respectively (**Figure 1a**). Quantification results of each isomer was shown in **Table 3**.

^{13}C NMR Quantification of Eight CLnAMe Isomers. To further confirm the characterization made by Ag^+-HPLC, the CLnAMe mixture was analyzed by quantitative ^{13}C NMR (**Figure 5**). The mixture showed similar olefinic carbon signals to that of β-eleostearic acid, punicic acid, and α-eleostearic acid, demonstrating clearly that this mixture contained t9, t11, t13−18:3, c9, t11, t13−18:3, and c9, t11, c13−18:3 isomers. The result was also consistent with that of Ag^+-HPLC analysis. It was observed that there were two new signals around each signal of β-eleostearic acid and three new signals around each signal of α-eleostearic acid, but no new signal around each signal of punicic acid was observed. Accordingly, it was deduced that this mixture contained two additional t,t,t-18:3 positional isomers and three c,t,t-18:3 positional isomers but there was no additional c,t,c-18:3 positional isomer. For example, there were three new signals (131.62, 131.69, 131.95) near the signal at 131.75, which had already been assigned as C-9 signal of c9, t11, t13−18:3. C-9 is named as OL-1 since it is the olefinic carbon closest to the COOMe group in c9, t11, t13−18:3 isomer. Thus, these new signals were assigned as OL-1 signals of other three c,t,t-18:3 positional isomers since they occurred near the OL-1 signals of c9,t11,t13−18:3 isomer. They could be deduced as signals of C-9, C-10, and C-11 of c8, t10, t12−18:3, c10, t12, t14−18:3 and c11,t13, t15−18:3 isomers. Similarly, all other new signals could be deduced similarly (**Table 3**).

To further confirm above assignment, quantitative ^{13}C NMR technique was used. Under ideal conditions, the integral of the NMR signals is proportional to the concentration of the isomer that produced it. Therefore, olefin signals from the ^{13}C NMR spectrum are integrated to quantify the different CLnAMe isomers in a sample. In proton-deduced ^{13}C NMR experiments, it is necessary to employ inverse gated decoupling and relaxation delays of $>5T1$ to obtain strictly quantitative data. Because the CLnAMe isomers are structurally very similar and only olefin signals are quantified, factors potentially leading to imperfect quantification are expected to be almost identical for each peak. Thus, quantitative ^{13}C NMR gives the correct relative amount for each CLnAMe regardless of the experimental conditions.

Each CLnAMe isomer showed six olefinic signals, and quantitative technique disclosed that their integrals were similar. Accordingly, signals with a similar integral in **Figure 5** was selected and assigned to an isomer. All the signals were separated into eight groups and the percentage of them was calculated according to the quantitative ^{13}C NMR integral results (**Table 3**). In fact, the percentage of each isomer calculated by quantitative ^{13}C NMR was similar with that calculated by Ag^+-HPLC, confirming that deduction on geometrical configuration and quantification of eight isomers in mixture by combination use of Ag^+-HPLC and NMR was correct.

This is the first research on the detailed NMR correlation spectrum characterization of three CLnA isomers, including β-eleostearic acid, punicic acid, and α-eleostearic acid. The results confirmed that β-eleostearic acid, punicic acid, and α-eleostearic acid were t9, t11, t13−18:3, c9, t11, c13−18:3, and c9, t11, t13−18:3, respectively. Signals of olefinic carbon of them were assigned correctly and J was also calculated. The present NMR data were important for future NMR characterization of other CLnA isomers.

The present study was also the first report on use of Ag^+-HPLC to analyze the CLnAMe mixture qualitatively and quantitatively. Ag^+-HPLC resolved well and separated the CLnAMe mixture into ttt, ctt, and ctc geometric groups. Within each group, the positional isomers were separated according to the position of double bonds. Compared with previous GC and reversed-phase HPLC methods, the present Ag^+-HPLC had greater resolution power and had an advantage in separation and quantification of individual CLnAMe isomers in a complex mixture and biological materials. To our best knowledge, this study was the first time to apply quantitative ^{13}C NMR technique into identification and quantification of CLnAMe isomers. Most CLnA isomers are commercially unavailable at the present time although some of them can be isolated from natural sources. The present result can be amplified further to isolate other pure CLnA isomers in a large quantity if a semipreparative Ag^+-HPLC column is used. Currently, we are in attempt to synthesize other CLnA isomers and investigate the oxidative stability and bioactivity of each CLnA isomer.

LITERATURE CITED

(1) Yurawecz, M. P.; Molina, A. A.; Mossoba, M.; Ku, Y. Estimation of conjugated octadecatrienes in edible fats and oils. *J. Am. Oil Chem. Soc.* **1993,** *70*, 1093−1099.

(2) Takagi, T.; Itabashi, Y. Occurrence of mixtures of geometrical isomers of conjugated octadecatrienoic acids in some seed oils: analysis by open tubular gas liquid chromatography and high performance liquid chromatography. *Lipids* **1981**, *16*, 546−551.

(3) Suzuki, R.; Noguchi, R.; Ota, T.; Abe. M.; Miyashita, K.; Kawada, T. Cytotoxic effect of conjugated trienoic fatty acids on mouse tumor and human monocytic leukaemia cells. *Lipids* **2001**, *36*, 477−482.

(4) Igarashi, M.; Miyazawa, T. New recognized effect of conjugated trienoic fatty acids on cultured human tumor cells. *Cancer Lett.* **2000**, *148*, 173−179.

(5) Yasui, Y.; Hosokawa, M.; Kohno, H.; Tanaka, T.; Miyashita, K. Troglitazone and 9cis,11trans,13trans-conjugated linolenic acid: comparison of their antiproliferative and apoptosis-inducing effects on different colon cancer cell lines. *Chemotherapy* **2006**, *52* (5), 220−225.

(6) Yasui, Y.; Hosokawa, M.; Kohno, H.; Tanaka, T.; Miyashita, K. Growth inhibition and apoptosis induction by all-trans-conjugated linolenic acids on human colon cancer cells. *Anticancer Res.* **2006**, *26*, 1855−1860.

(7) Yasui, Y.; Hosokawa, M.; Sahara, T.; Suzuki, R.; Ohgiya, S.; Kohno, H.; Tanaka, T.; Miyashita, K. Bitter gourd seed fatty acid rich in 9c,11t,13t-conjugated linolenic acid induces apoptosis and up-regulates the GADD45, p53 and PPARgamma in human colon cancer Caco-2 cells. *Prostaglandins Leukotrienes Essential Fatty Acids* **2005**, *73* (2), 113−119.

(8) Kohno, H.; Suzuki, R.; Yasui, Y.; Hosokawa, M.; Miyashita, K.; Tanaka, T. Pomegranate seed oil rich in conjugated linolenic acid suppresses chemically induced colon carcinogenesis in rats. *Cancer Sci.* **2004**, *95* (6), 481−486.

(9) Kawaii, S.; Lansky, E. P. Differentiation-promoting activity of pomegranate (Punica granatum) fruit extracts in HL-60 human promyelocytic leukemia cells. *J. Med. Food* **2004**, *7* (1), 13−18.

(10) Kohno, H.; Yasui, Y.; Suzuki, R.; Hosokawa, M.; Miyashita, K.; Tanaka, T. Dietary seed oil rich in conjugated linolenic acid from bitter melon inhibits azoxymethane-induced rat colon carcinogenesis through elevation of colonic PPARgamma expression and alteration of lipid composition. *Int. J. Cancer* **2004**, *110* (6), 896−901.

(11) Kim, N. D.; Mehta, R.; Yu, W.; Neeman, I.; Livney, T.; Amichay, A.; Poirier, D.; Nicholls, P.; Kirby, A.; Jiang, W.; Mansel, R.; Ramachandran, C.; Rabi, T.; Kaplan, B.; Lansky, E. Chemopreventive and adjuvant therapeutic potential of pomegranate (Punica granatum) for human breast cancer. *Breast Cancer Res. Treat.* **2002**, *71* (3), 203−217.

(12) Yang, L.; Leung, K. Y.; Ying, C.; Huang, Y.; Ratnayake, W. M. N.; Chen, Z. Y. α-Linolenic acid but not conjugated linolenic acid is hypocholesterolaemic in hamsters. *Br. J. Nutri.* **2005**, *93*, 433−438.

(13) Tulloch, A. P.; Bergter, L. Analysis of the conjugated triene acid containing oil from *Fevillea trilobata* by ^{13}C nuclear magnetic resonance spectroscopy. *Lipids* **1979**, *14* (12), 996−1002.

(14) Igarashi, M.; Tsuzuki, T.; Kambe, T.; Miyazawa, T. Recommended methods of fatty acid methylester preparation for conjugated dienes and trienes in food and biological samples. *J. Nutr. Sci. Vitaminol.* **2004**, *50*, 121−128.

(15) Lie Ken Jie, M. S. F.; Pasha, M. K.; Alam, M. S. Synthesis and Nuclear Magnetic Resonance Properties of All Geometric Isomers of Conjugated Linoleic Acids. *Lipids* **1997**, *32*, 1041−1044.

(16) Davis, A. L.; McNeill, G. P.; Caswell, D. C. Identification and quantification of conjugated linoleic acid isomers in fatty acid mixture by ^{13}C-NMR spectroscopy. *Advances in conjugated linoleic acid research*; Yurawecz, M. P., Mossoba, M. M., Kramer, J. K. G., Pariza, M. W., Nelson, G. J., Eds.; A publication of AOCS Press: Champaign, IL, 1999; Vol. 1, p 166.

(17) Sehat, N.; Yurawecz, M. P.; Roach, J. A. G.; Mossoba, M. M.; Kramer, J. K. G.; Ku, Y. Silver-ion high-performance liquid chromatographic separation and identification of conjugated linoleic acid isomers. *Lipids* **1998**, *33*, 217−221.

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