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

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ABSTRACT

Analytical methods to obtain the detailed compositons of the fatty acids in oils containing more than one conjugated octadecatrienoic acid by open-tubular gas liquid chromatography (GLC) and by reversed-phase high performance liquid chromatography (HPLC) were established. Effective GLC separations of *cis,trans,trans*-9,11,13-octadecatrienoic acid (*ctt*-9,11,13-18:3), *ctc*-9,11,13-18:3, *ttc*-9,11,13-18:3, *ttt*-9,11,13-18:3, *ttc*-8,10,12-18:3, and *ttt*-8,10,12-18:3 were obtained with an open-tubular column coated with the nonpolar liquid phase OV-1 using an instrument having all-glass carrier gas pathways. The HPLC method also gave satisfactory separations for the isomeric conjugated octadecatrienoates on the basis of number of the *cis* and *trans* double bonds. Two or three minor conjugated trienoic acids were found along with the principal conjugated trienoic acid in tung oil, and seed oils of cherry, *Prunus sp.*, *Momordica charantia*, *Trichosanthes anguina*, *Punica granatum*, *Catalpa ovata*, and *Calendula officinalis*. The mechanism for the formation of the conjugated trienoic acid mixtures in the seed oils is discussed. The *C. ovata* seed oil also contained *ct* and *tt*-9,12-octadecadienoic acids. The *tt* isomer is presumed to be a precursor of *ttc*-9,11,13-18:3, the main conjugated trienoic acid in this oil.

INTRODUCTION

Various geometrical isomers of conjugated octadecatrienoic acids are known: α-eleostearic acid (*cis,trans,trans*-9,11,13-octadecatrienoic acid (*ctt*-9,11,13-18:3)), punicic (*ctc*-9,11,13-18:3), catalpic (*ttc*-9,11,13-18:3), calendic (*ttc*-8,10,12-18:3), and jacaric (*ctc*-8,10,12-18:3) acids have been reported as single conjugated trienoic components in some seed oils (1-3). Recently, a ^{13}C NMR method for determining the composition of seed oils which contain more than one conjugated trienoic acid was presented by Tulloch and Bergter (4), and it was shown that the fatty acids of *Fevillea triobata* contained 30% punicic acid and 9% α-eleostearic acid (4). This seems to be the only paper which reports the occurrence of a geometrical isomer mixture of conjugated trienoic acids in the fatty acids of a natural seed oil, although it has been shown that in tung oil, β-eleostearic acid is an alteration product of and coexists with α-eleostearic acid (5).

In this study, the geometrical isomers of conjugated octadecatrienoic acids have been successfully separated by reversed-phse high performance liquid chromatography (HPLC) and open-tubular gas liquid chromatography (GLC), and practical methods for determining the compositions of fatty oils which contain more than one conjugated trienoic acid have been established. These methods were applied to the fatty acid analysis of some seed oils containing conjugated octadecatrienoic acids, and minor geometrical isomers of conjugated trienes have been satisfactorily determined.

MATERIALS AND METHODS

Materials

Air-dried seeds of *Trichosanthes anguina* (snake gourd), *Calendula officinalis* (pot marigold), *Momordica charantia* (litchi, Japanese name Futo-reishi) were the products of a nursery company (Sakata Shubyō Co., Ltd., Yokohama). Intact air-dried pods of *Catalpa ovata* (Japanese name Ki-sasage) were obtained at a pharmacy for Chinese drugs. Fresh cherry kernels were obtained from cherries *Prunus* sp. labeled as Hood River Cherries, Weyerhaeuser Co., Yakima, WA USA, and *Punica granatum* seeds were obtained from the fruits labeled as California pomegranate, USA. Tung oil was a bottled commercial product. The seeds were ground to a powder with an electric mill, and extracted with hexane 3 times at room temperature. Each extraction consisted of standing with occasional shaking for about 3 hr. After drying with anhydrous sodium sulfate, hexane was evaporated below 30 C in a rotary evaporator. The residual oil was directly converted to methyl esters by addition of 0.5 M sodium methoxide/methanol reagent. The mixture was

allowed to stand overnight at room temperature and the methyl esters were recovered by hexane extraction. All these procedures were done in the dark under nitrogen.

Preparation of α-eleostearate (94% purity) was carried out with an urea adduct method from the mixture of monoenoates and conjugated octadecatrienoates separated by $AgNO_3$-silica gel column chromatography of tung oil methyl esters. Eleostearate was extracted from the nonurea-adduct fraction after acidification with dilute hydrochloric acid (6). Geometrical isomerization of tung oil and *C. officinalis* oil methyl esters was effected by addition of a trace amount of iodine to a CS_2 solution of the methyl esters (7), and keeping for 10 hr at room temperature. Palmitic acid (99% purity) was a product of P.L. Biochemicals, Inc., Milwaukee, WI.

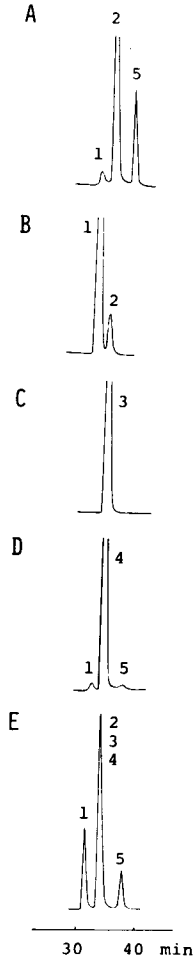

FIG. 1. HPLC resolution of conjugated octadecatrienoates on a Zorbax ODS column. (1) *ctc*-9,11,13-18:3; (2) *ctt*-9,11,13-18:3; (3) *ttc*-8,10,12-18:3; (4) *ttc*-9,11,13-18:3; (5) *ttt*-9,11,13-18:3. (A) Tung oil; (B) *T. anguina*; (C) *C. officinalis*; (D) *C. ovata*; (E) A mixture of A, B, C, D and *trans* isomerized A. HPLC conditions are given in Materials and Methods.

HPLC Analysis

A mixture of the conjugated octadecatrienoates was separated according to the configuration of the conjugated double bonds by a HPLC instrument, Shimadzu-Du Pont LC-1 (Shimadzu Seisakusho Co. Ltd., Kyoto, Japan), including a PCP-1 Model constant pressure liquid pump, a vacuum degassing-nitrogen sealing attachment, a GRE-1 solvent programmer for gradient elution, and a SPD-1 spectrophotometric detector for detection in wavelength from 190 to 750 nm. HPLC analysis was done on a 25 cm × 4.6 mm Zorbax ODS column connected to a Permaphase ODS precolumn (5 cm × 2 mm) by elution with acetonitrile/H_2O (4:1, v/v) at a flow rate of 1.4 mL/min and a pressure 50 kg/cm^2. The column temperature was 42 C. Usually 0.5 μL of 1% (w/v) sample solution of methyl esters in hexane was injected at sensitivity 0.32 AUFS.

GLC Analysis

Open-tubular GLC of the methyl esters was done with a Shimadzu GC-6AM instrument equipped with a dual FID detector on a wall-coated open-tubular (WCOT) glass column, 54 m × 0.28 mm, coated with OV-1 (Nippon Kuromato Kōgyō Co. Ltd., Tokyo). The carrier gas was N_2 at a flow rate of 0.4 mL/min and split ratio 1/155. The column temperature was 190 C, and injector and detector were 200 C. All carrier gas pathways including the splitter consisted of glass tubes, and the vaporized sample was kept completely out of contact with any metal surface. Usually, 1 μL of 1% (w/v) sample solution in hexane was injected.

RESULTS AND DISCUSSION

HPLC Separation

In HPLC, under the conditions of this study, the conjugated octadecatrienoate peaks emerged in the order (*cis,trans,cis*), (*cis,trans, trans*, and *trans,trans,cis*), and (*trans,trans, trans*). Each peak was completely separated from the others. Three positional isomers, catalpic (*ttc*-9,11,13), calendic (*ttc*-8,10,12) and α-eleostearic (*ctt*-9,11,13) acids could not be separated to any degree (Fig. 1). The methyl esters obtained from the seed oils showed, as expected, a symmetrical large peak for the main conjugated trienoate component, and most of them additionally showed small peaks for

minor components, thought to be geometrical isomers of the conjugated trienoates. The recorder chart area ratios of the peaks were dependent on the wavelength of the detector, since each conjugated trienoate has absorption maxima of different intensity and wavelengths: α-eleostearic acid nm ($E^{1\%}_{1\ cm}$) 272 (1766) in cyclohexane, β-eleostearic acid 268 (2190) in ethanol, and punicic acid 275 (1694) in cyclohexane (3). In this study, the detector was operated at 284 nm for quantitative anlayses. Since the absorption of α- and β-eleostearic acids and punicic acid shows almost the same intensities at 284 nm (8), the area percent of each peak is essentially equal to mole or weight percent. The deviations of the peak area percents relative to the wavelength selection for the detector are shown in Table 1. The ratio of α- and β-eleostearic acids in tung oil has been calculated indirectly from the absorption of UV spectra at the 3 wavelengths using an equation for the calibration (9). In the method, small instrumental deviations have a large influence on the accuracy of the results, since the interval of α- and β-eleostearate peak maxima is only about 4 nm, and most parts of the 2 peaks overlap. The deviation can be corrected with pure standard reference samples uncontaminated with other isomers. However, preparation of such specimens is very difficult. The HPLC method presented in this paper enables the separation of more than one geometrical isomer of the conjugated trienoates and it gives more accurate results directly without any pure reference specimen. Determination of conjugated trienoates is possible with an HPLC instrument having only a simple UV detector for an absorption at a fixed wavelength (e.g., 254 nm) using calibration factors.

Recrystallization of the esters or the acids has frequently been used for the refining and preparation of conjugated trienoates (4), but the efficiency of this procedure is poor, judging by the results of HPLC and GLC analyses of the recrystallized products in our experiments. This study showed that the HPLC method gave better results in the preparation and purification of the conjugated trienoates on a small scale. The separated sample showed only one peak in HPLC, and the results of the open-tubular GLC also gave the evidence for the effective separation.

GLC Separation

GLC analysis of the methyl esters of conjugated trienoic acids has been generally recognized as giving unsatisfactory results because of decomposition and isomerization (4,10,11). Thus, there have been no reports of identification of particular conjugated trienoate isomers by GLC, though the total contents of the conjugated octadecatrienoate isomers obtained by GLC were reported to be in fair agreement with the contents obtained by UV analysis (12), and conjugated dienoates were determined by GLC without isomerization (13).

In this study, open-tubular GLC effectively separated the geometrical isomers of conjugated octadecatrienoates, and an almost pure single peak was observed for the conjugated trienoates in the open-tubular GLC of C and D in Figure 2 and for cherry methyl esters. The compositions of the conjugated trienoates determined by GLC were in accord with those obtained from the HPLC analysis (Table 2). These results show that isomerization and decomposition of the conjugated trienoates were not caused by GLC under the conditions of this experiment. The conjugated trienoate peaks were sharp and their theoretical plate numbers were nearly equal to those of the ordinary fatty acid methyl esters. This supports the view that no decomposition

TABLE 1

Comparison of Composition of Conjugated Octadecatrienoic Acid Isomers Obtained by GLC and HPLC (Peak Area %)[a]

Isomer[b]	GLC	HPLC (detector, wavelength nm)					
		254	273	283	284	285	290
ctc	32.14 ± 0.28[c]	25.11	27.92	31.22 ± 0.65	31.31 ± 0.33	31.48 ± 0.70	43.64
ctt + *ttc*[d]	38.49 ± 0.36	38.49	39.36	37.93 ± 0.22	38.89 ± 0.26	39.96 ± 0.21	41.73
ttt	28.58 ± 0.15	36.40	32.71	30.86 ± 0.54	29.80 ± 0.11	28.56 ± 0.64	16.54

[a]A mixture of methyl esters of *T. anguina* seed oil, tung oil and *trans* isomerized tung oil was used. GLC and HPLC conditions are given in Materials and Methods.

[b]*C* and *t* denote *cis* and *trans* double bonds in order in the 9-, 11- and 13-positions in octadecatrienoic acids.

[c]Mean ± SD of 5 analyses.

[d]Source of *ttc* isomer is tung oil, see Table 3.

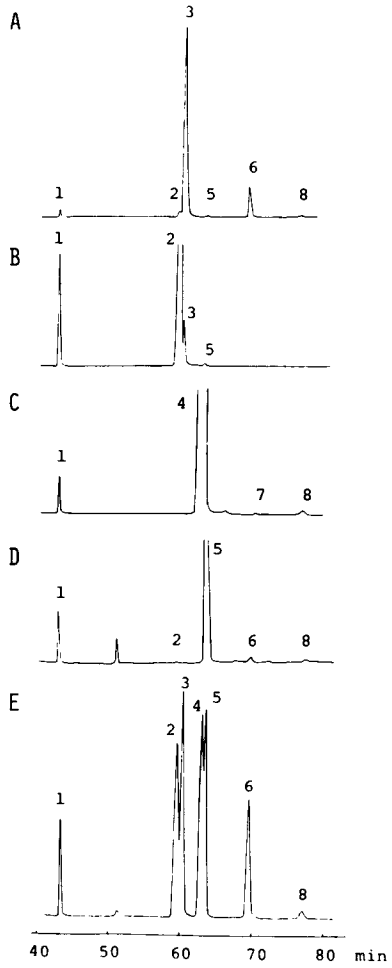

FIG. 2. GLC resolution of conjugated octadecatrienoates on a WCOT OV-1 column. (1) 16:0; (2) *ctc*-9,11,13-18:3; (3) *ctt*-9,11,13-18:3; (4) *ttc*-8,10,12-18:3; (5) *ttc*-9,11,13-18:3; (6) *ttt*-9,11,13-18:3; (7) *ttt*-8,10,12-18:3; (8) 20:1. (A) Tung oil; (B) *T. anguina*; (C) *C. officinalis*; (D) *C. ovata*; (E) A mixture of A, B, C, D and *trans* isomerized A. GLC conditions are given in Materials and Methods.

and no isomerization occur on the conjugated trienoates in the GLC. GLC of the mixtures of methyl palmitate and α-eleostearate in known weight ratios gave a weight response factor of 1.06 for the eleostearate relative to palmitate. The respective response factors for oleate, linoleate, linolenate and docosahexaenoate were 0.99, 0.99, 1.01 and 1.07 under the same conditions.

To establish the degree of loss of the conjugated octadecatrienoates in the esterification procedure, the methyl esters of tung oil were treated with the reagents CH_3ONa-CH_3OH, BF_3-CH_3OH, and HCl-CH_3OH under various conditions, and the products were analyzed by GLC before and after the treatment. The CH_3ONa-CH_3OH reagent gave reproducible results without the loss of the conjugated octadecatrienoates as shown in Table 3. To avoid the reported loss of conjugated trienoates (14), the preparation of the methyl esters for this study was carried out with 0.5 M CH_3ONa-CH_3OH for 10-12 hr at room temperature under nitrogen for the analysis of the seed oils containing the conjugated trienoates.

The peaks of punicate and α-eleostearate were completely coincident in GLC on Silar 5CP and 10C open-tubular columns (67 and 56 m × 0.28 mm, respectively), but they were effectively separated on the OV-1 open-tubular column (54 m × 0.28 mm) with a peak separation 80% in the analysis of 1:1 (w/w) mixture. The open-tubular GLC on OV-1 generally showed the much better separation than HPLC on Zorbax ODS, except for the separation between punicate and α-eleostearate which gave 100% peak separation in the HPLC.

In the GLC on OV-1, the peaks appeared in the following order, as shown in Figure 2 and Table 4, *ctc*-9,11,13; *ctt*-9,11,13; *ttc*-8,10,12; *ttc*-9,11,13; *ttt*-9,11,13; *ttt*-8,10,12. Under the conditions of this analysis, the two peaks having nearly the same height and a difference of 0.01 in the ECL were separated to the extent

TABLE 2

Comparison of Compositions of Conjugated Octadecatrienoic Acids in Some Seed Oils by GLC and HPLC (Peak Area %)[a]

	Tung oil		*Prunus* sp.		*M. charantia*		*T. anguina*		*P. granatum*		*C. ovata*		*C. officinalis*	
Isomer[b]	GLC	HPLC	GLC	HPLC	GLC	HPLC	GLC	HPLC	GLC	HPLC	GLC	HPLC	GLC	HPLC
ctc	1.7	2.3	–	–	0.9	1.2	92.7	94.1	96.1	96.5	0.1	2.5	–	–
ctt + *ttc*	84.3	83.8	99.8	99.8	98.6	98.6	7.3	5.9	3.9	3.5	98.6	96.3	99.6	99.8
ttt	14.0	13.9	0.2	0.2	0.5	0.2	–	–	–	–	1.3	1.2	0.4	0.2

[a]GLC and HPLC conditions are given in Materials and Methods.

[b]*C* and *t* denote *cis* and *trans* double bonds in order in the 9-, 11- and 13- (8-, 10- and 12- for *C. officinalis*) positions in octadecatrienoic acids.

of about 30-40% (e.g., the *ttc*-9,11,13 and *ttc*-8,10,12-18:3 peaks).

Composition of Conjugated Trienoates

The fatty acid compositions of the seed oils reported in this paper have been reported in a previous paper (12), but the data were obtained by packed column GLC. The occurrence of *ctt*-9,11,13-18:3 in the fatty acids of cherry kernel oil has been reported with UV spectrophotometric and GLC data (15,16). In this study, the fatty acid composition of some seed oils was obtained in more detail using open-tubular GLC (Table 4). The percentages of main components are generally in harmony with those described in the previous paper (12).

It is notable that *ttc*-9,11,13-18:3 was found in the fatty acids from tung oil, *T. angina* oil, and *P. granatum* oil, and *ctc*-9,11,13-18:3 was found in the fatty acids from tung oil, *M. charantia* oil, and *C. ovata* oil, all as minor components.

A considerable amount of *ttt*-9,11,13-18:3 was found in the fatty acids of tung oil, *M. charantia* oil, and *C. ovata* oil, but it was only a trace in the methyl esters from fresh cherry kernels (Table 4). The presence of the *ttt* isomer in these oils has been recognized due to isomerization with light and other factors after extraction. The isomerization of the conjugated trienoates in the seeds may be possible, since various amounts of the *trans* isomers of the main conjugated trienoates have been found in the seed oils as minor components, though the extraction of the seeds was done carefully in the dark under nitrogen. It may be safely said that *ttt*-9,11,13-18:3 and *ttt*-8,10,12-18:3 are both natural fatty acids.

It is plausible that the biosynthetic pathway

TABLE 3

Influence of the Esterification Procedures on the Compositions of Conjugated Trienoates[a]

Isomer[b]	Control	CH_3ONa-CH_3OH 0.05 M	CH_3ONa-CH_3OH 0.5 M
ctc	1.4	1.4	1.4
ctt	67.8	68.4	67.4
ttt	11.3	11.1	11.2
Usual acids[c]	19.5	19.1	20.0

[a]Peak area % by GLC (conditions are given in Materials and Methods). Tung oil methyl esters (control) were treated with the reagents for 10 hr at 20-28 C.

[b]*C* and *t* denote *cis* and *trans* double bonds in order in the 9-, 11- and 13-positions in octadecatrienoic acids.

[c]Sum of usual fatty acid components.

TABLE 4

Fatty Acid Compositions of Some Seed Oils Containing Conjugated Octadecatrienoic Acids by GLC (Peak Area %)

Acid	ECL[a]	Tung oil	*Prunus* sp.	*M. charantia*	*T. anguina*	*P. granatum*	*C. ovata*	*C. officinalis*
14:0		tr	0.05	tr	tr	0.04	tr	0.05
15:0		–	0.03	tr	tr	tr	tr	tr
16:1	15.79	–	0.49	tr	tr	0.06	0.04	tr
16:0		2.61	7.56	1.46	5.31	2.25	2.77	2.40
17:1	16.71	–	0.09	–	–	–	tr	–
17:0		0.06	0.09	0.09	0.09	–	tr	tr
18:2ω6	17.62	7.07	38.01	8.60	17.22	4.23	39.95[b]	27.92
18:3ω3	17.66	0.11	tr	tr	tr	tr	0.56	0.70
18:1ω9	17.72	6.57	38.56	14.64	16.40	3.98	7.71	3.95
ω7	17.76	0.36	0.71	0.11	0.50	0.35	0.97	0.47
18:0		2.13	2.48	17.40	8.15	1.77	2.65	1.22
18:3 *ctc*-9,11,13[c]	18.95	1.33	–	0.50	48.48	82.99	tr	–
ctt-9,11,13	18.99	67.69	10.63	56.24	3.43	3.16	–	–
ttc-8,10,12	19.09	–	–	–	–	–	–	62.17
ttc-9,11,13	19.10	0.17	–	–	0.41	0.20	42.25	–
ttt-9,11,13	19.36	11.27	tr	0.32	–	–	0.55	–
ttt-8,10,12	19.39	–	–	–	–	–	–	0.24
20:1	19.63	0.54	0.32	0.34	tr	0.54	0.42	0.33
20:0		tr	0.99	0.29	–	0.42	0.23	0.24
Others		0.15	–	–	–	–	1.89	0.77

[a]ECL = equivalent chain length. GLC conditions are given in Materials and Methods.

[b]Includes 9,12-18:2 isomers: *cc* 70.8%, *ct* 3.2%, and *tt* 26.0% (ECL *cc* 19.43, *ct* 19.20, *tc* 19.28, and *tt* 19.02 on Silar 10C open-tubular column (56 m × 0.28 mm) at 170 C.

[c]18:3 *ctc*-9,11,13 = *cis,trans,cis*-9,11,13-octadecatrienoic acid.

of the conjugated trienoates could include a mechanism for elimination of the hydroperoxide group from linoleate hydroperoxide formed by peroxidase, rather than the dehydration of conjugated hydroxy dienoates presented previously by Gunstone and coworkers (17,18). Thus, *ctt*- and *ctc*-9,11,13-18:3 found in the seeds might be formed from linoleate through specific oxidation at position 13 (which is frequently observed in enzymatic oxidation [19]).

Occurrence of *ttc*-9,11,13-18:3 in *C. ovata* indicated in Table 4 is similarly explainable by the peroxidation of *tt*-9,12-18:2 at position 13. In practice, the presence of *tt*-9,12-18:2 in *C. ovata* seeds was established by coinjection with reference material prepared by *trans* isomerization of linoleate with nitrous acid (20). The ECL values of the *trans* isomers are in harmony with those reported previously (21). The results of the GLC analysis are shown in Table 4 (footnote b gives the retention data). The *tt*-9,12-18:2 acid has been also found in seed oil of *Chilopsis linearis*, which contains *ttc*-9,11,13-18:3 (22). Enzymatically specific conversion of the *tt*-9,12-18:2 component of the 9,12-18:2 isomers to *ttc*-9,11,13-18:3 will occur in both of these seeds. Formation of *ttc*-8,10,12-18:3 in *C. officinalis* will occur through 9-oxidized-*tc*-10,12-18:2 formed from linoleate by the similar mechanism. In conclusion, the pathway to the conjugated trienoates shown in Table 4 could include the following sequence. The underlined compounds are main components.

T. anguina and *P. granatum*
cc-9,12-18:2 → 13-oxidized-*ct*-9,11-18:2 →
ctc-9,11,13-18:3 and *ctt*-9,11,13-18:3
ctc-9,11,13-18:3 → *ttc*-9,11,13-18:3

M. charantia
cc-9,12-18:2 → 13-oxidized-*ct*-9,11-18:2 →
ctt-9,11,13-18:3 and *ctc*-9,11,13-18:3
ctt-9,11,13-18:3 → *ttt*-9,11,13-18:3

C. ovata
tt-9,12-18:2 → 13-oxidized-*tt*-9,11-18:2 →
ttc-9,11,13-18:3 and *ttt*-9,11,13-18:3

C. officinalis
cc-9,12-18:2 → 9-oxidized-*tc*-10,12-18:2 →
ttc-8,10,12-18:3
ttc-8,10,12-18:3 → *ttt*-8,10,12-18:3

Further study on the component fatty acids in the natural lipids containing conjugated fatty acids by GLC and HPLC methods proposed in this paper can be expected to give the detailed information which will be useful in understanding the mechansim for the biosynthesis of conjugated fatty acids in plants.

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