Silver Ion High-Performance Liquid Chromatography of the Triacylglycerols of *Crepis alpina* Seed Oil

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The triacylglycerols of *Crepis alpina* oil were characterized because this oil has a high concentration of crepenynic (*cis*-9-octadecen-12-ynoic) acid, a fatty acid useful in the chemical synthesis of deuterated fats for human metabolism studies. The triacylglycerols were separated from the crude oil by solid-phase extraction. Resolution, quantitation and isolation of the individual triacylglycerols were performed by silver ion high-performance liquid chromatography on a commercial column, an acetonitrile in hexane isocratic mobile phase and flame-ionization detection. Isolated triacylglycerols were identified by capillary gas chromatography of their fatty acid methyl esters. Of the eleven eluted triacylglycerols of *Crepis alpina* oil, 85% included 35% tricrepenynoyl, 34% linoleoyldicrepenynoyl and 16% dilinoleoylcrepenynoyl glycerols. Triacylglycerols eluted according to the numbers of alkene and alkyne bonds. Elution times, resolution and quantitation were reproducible over a three-month period. The flame-ionization detector response required no response factors for quantitation of the triacylglycerols present in *Crepis alpina* oil. The silver ion chromatography system permitted the identification of 95% of the triacylglycerols compared to 70% of the triacylglycerols previously identified with reversed-phase high-performance liquid chromatography.

KEY WORDS: Alkynoic acids, crepenynic acid, *Crepis alpina* oil, flame-ionization detection, silver ion high-performance liquid chromatography, triacylglycerols, tricrepenynoyl glycerol.

The triacylglycerol (TAG) composition of *Crepis alpina* oil (CrAO) was first obtained by reversed-phase C:18 high-performance liquid chromatography (RP-HPLC) with flame-ionization detection (FID) (1). The CrAO is a rich source (70–80% yield) for crepenynic acid, [(*cis*-9-octadecen-12-ynoic acid; (Cr)] and is a useful intermediate in the chemical synthesis of deuterium-labeled compounds for human metabolism studies (2).

TAG compositions of seed oils have been obtained through the applications of gas chromatography (GC) and RP-HPLC, procedures recently reviewed by Christie (3–5). Aitzetmuller (6) found HPLC to be the major analytical technique for seed oil TAG analysis. Also, applications of supercritical fluid chromatography (SFC) of TAG with capillary columns and FID detection are under investigation (7,8). However, SFC has not obtained the TAG resolution offered by HPLC (8). Thermal conditions may adversely affect GC analysis of TAG with unsaturated fatty acids (FA). RP-HPLC avoided thermal alteration, but TAG resolution, with respect to both FA unsaturation and chainlength, made chromatograms difficult to interpret. On the other hand, silver ion high-performance liquid chromatography (Ag-HPLC) resolved TAG based mainly on FA unsaturation, giving simpler chromatograms to assist in TAG identification (5,9). Previous Ag-HPLC systems for TAG and FA separations have mostly used complex ternary mobile phases (9,10). Further, detectors that lack linear response and thus require response factors, such as the light-scattering detector, have been used for semiquantitative determination of oil TAG compositions (10). The transport FID gives a linear response without the use of response factors for quantitative TAG analysis (1,5,10–12). An FID detector has been used with silver nitrate/silica–HPLC for quantitative determination of cocoa butter, palm and soybean oil TAG (13). Recently, a simple isocratic mobile phase (acetonitrile in hexane) and a commercially available Ag-HPLC column were used to resolve *cis* and *trans* FA methyl ester isomers (14).

In this paper, we applied Ag-HPLC analytical techniques to the separation of TAG of CrAO with FID quantitative detection.

EXPERIMENTAL PROCEDURES

Materials. CrAO was obtained from K. Carlson and R. Kleiman (USDA, ARS, NCAUR, Peoria, IL). Solid-phase extractive purification of CrAO TAG, to avoid interference by non-TAG during Ag-HPLC, was conducted by a previously reported procedure (1). Reference standards were purchased from Nu-Chek-Prep, Inc. (Elysian, MN), and Cr_3 glycerol was obtained by RP-HPLC chromatography of CrAO with subsequent characterization by gas chromatography/mass spectrometry (GC/MS) of the methyl ester.

Ag-HPLC. The Ag-HPLC column (Chromsphere Lipids; 4.6 mm i.d. × 250 mm stainless steel; 5 micron) was purchased from Chrompack International (Middelburg, The Netherlands) and used as received. The TAG standards and CrAO mixtures (0.3 mg in 10 mL hexane) were injected in triplicate. All TAG were eluted in 120 min by an isocratic mobile phase of 0.5% acetonitrile in hexane at a flow rate of 1.0 mL/min. The Tracor Model 945 FID detector was operated as described previously (15), except the block temperature was set at 110°C, the cleaning flame hydrogen at 300 mL/min and the oxygen flow at 175 mL/min. The detector signal was monitored by a real-time computer for calculation of peak areas from solute responses (1). Quantitation of FID response was checked against weighed TAG standards. For TAG collection and identification, the HPLC effluent stream was split between the detector and a collection port. Repeated injections of 30 μL (1 mg) of CrAO solution on the analytical column were conducted, and TAG fractions were collected with respect to recorder response until at least 0.5 mg of each of eleven individual TAG was obtained for characterization. Ag-HPLC TAG retention times were referenced against standard TAG and pure Cr_3 glycerol.

TAG characterization. GC analyses of the transmethylated TAG Ag-HPLC fractions were conducted for TAG identification with respect to standard mixtures of palmitic (P), stearic (St), oleic (O), linoleic (L) and Cr FA methyl esters as reported previously (1).

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RESULTS AND DISCUSSION

The chromatogram of the Ag-HPLC separation of *C. alpina* TAG is presented in Figure 1. Eleven TAG fractions were isolated. The TAG composition of these fractions was determined by transmethylation, by identification and quantitation of the resulting methyl esters by GC-FID. These data are given in Table 1 and support the identification of the TAG resolved in Figure 1 as S_3, CrS_2, CrSO, CrSL, CrOL, CrL_2, Cr_2S, Cr_2O, Cr_2L, Cr_2L and Cr_3. The TAG abbreviations are: S = saturated FA (palmitic, P, and stearic, St); O = monoene FA (oleic); L = dienoic FA (linoleic); and Cr = FA with both alkene and alkyne bonds (crepenynic) (Fig. 1). The Cr to other FA ratios, presented in Table 1, support the identification of the above TAG with a ratio of about 0.5 for those TAG with one Cr and two FA and about 2.0 for those TAG with two Cr and one FA. TAG S_3 contain only palmitic and stearic acids. TAG Cr_3 contains only Cr acid. Some of the above TAG can be further identified: oleoyllinoleoylcrepenynoyl (CrOL); dilinoleoylcrepenynoyl (CrL_2); oleoyldicrepenynoyl (Cr_2O); linoleoyldicrepenynoyl (Cr_2L); and Cr_3. Identification of Cr_3 TAG was confirmed by a retention time match with a Cr_3 glycerol standard. There are two Cr_2L TAG isomers. As shown in Table 1, the identifications of TAG were further supported by comparison of the FA composition, obtained by GC of transmethylated CrAO, with FA composition data calculated from the CrAO TAG data (Table 2) (16).

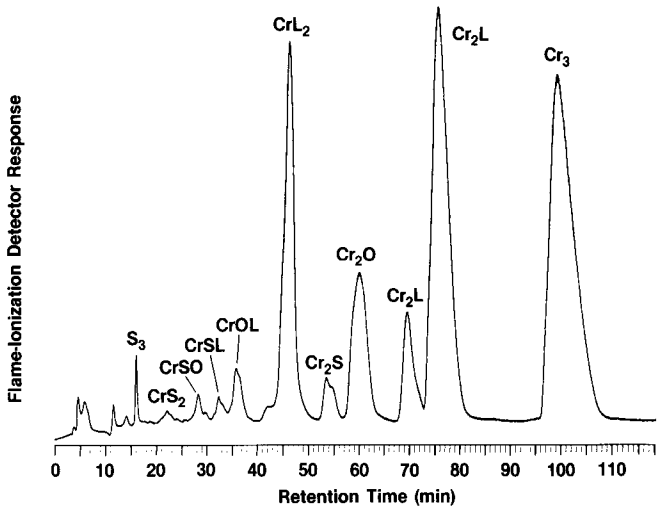

FIG. 1. Silver ion high-performance liquid chromatography (Ag-HPLC-FID) with flame-ionization detection (FID) analysis of the triacylglycerols of chromatographed *Crepis alpina* seed oil. Ag-HPLC-FID conditions: 0.5 mg sample; 5 micron Chromspher Lipids column (Chrompack International, Middelburg, The Netherlands) (4.6 × 250 mm); mobile phase 0.5% acetonitrile in hexane (vol/vol); flow rate 1.0 mL/min; FID. FID conditions given in the Experimental Procedures section. Chromatogram peak triacylglycerol fatty acid abbreviations: S, saturated: palmitic and stearic; O, oleic; L, linoleic; and Cr, crepenynoic fatty acids.

Absolute retention times are listed in Table 2 for the CrAO TAG resolved by Ag-HPLC (Fig. 1). The CrAO TAG retention times can be converted to relative retention times and compared to standard TAG tristearoyl (StStSt), trioleoyl (OOO), trilinoleoyl (LLL), trilinolenoyl (LnLnLn) and triarachidonoyl (ArArAr) glycerol retention times presented in Table 2. These data show that the TAG elute in the order of the increased numbers of alkene and alkyne bonds.

Absolute retention times for the TAG resolved by Ag-HPLC (Table 2) show a standard deviation of less than one minute for triplicate analyses of CrAO. Even after "continuous use," 240 injections for three months, CrAO TAG retention times on the silver column varied by less than two minutes. However, it is probably best to use relative retention data to identify CrAO TAG peaks on the Ag-HPLC chromatogram.

Since the start of our analytical work, some loss in TAG resolution did occur. For example, at the start of column use, the resolution between the Cr_2L TAG isomers was at 3.70 min per cm (17). After three months, the resolution was still satisfactory, but had decreased to 2.66 min per cm for the Cr_2L isomers. Minimum satisfactory resolution is 1.50 min per cm (17). Also, resolution for all the CrAO TAG remained satisfactory after three months of column use because the FID area percentage composition remained within 0.1% for each TAG. Thus, the Ag-HPLC column showed little deterioration with use.

TABLE 1

Fatty Acid (FA) Composition of the Oil of *Crepis alpina* and of the Triacylglycerols (TAG) Resolved by Silver Ion Chromatography[a,b]

	Ag-HPLC fractions: gas chromatography flame-ionization area percentage[c]											*Crepis alpina* oil FA composition (%)	
Acid[d]	S_3	CrS_2	CrSO	CrSL	CrOL	CrL_2	Cr_2S	Cr_2O	Cr_2L	Cr_2L	Cr_3	Experimental[e]	Calculated[f]
St and P	100	71.0	33.5	35.0			32.0					5.6	2.1
O			33.6		34.5			31.0				2.9	4.0
L				35.5	35.5	70.0			29.2	32.4		20.7	22.8
Cr		29.0	32.9	29.5	30.0	30.0	68.0	69.0	70.8	67.6	100	70.8	71.1
Cr/FA[g]		0.4	0.5	0.4	0.7	0.4	2.1	2.2	2.4	2.1			

[a]See Figure 1 for silver ion high-performance liquid chromatography (Ag-HPLC) conditions and resolution of the HPLC fractions.
[b]Ag-HPLC was performed on the pure TAG fraction from *Crepis alpina* oil.
[c]Gas chromatography (GC) was performed on the transmethylated HPLC fractions (Ref. 1).
[d]Abbreviations: St, stearic; P, palmitic; O, oleic; L, linoleic; Cr, crepenynic; FA, fatty acids; S, saturated FA.
[e]GC was performed on the pure *Crepis alpina* oil TAG after transmethylation (Ref. 1).
[f]FA composition was calculated (Ref. 16) from the TAG composition listed in Table 2.
[g]Ratio of methyl crepenynate to other methyl FA.

TABLE 2

Silver Ion High-Performance Liquid Chromatography with Flame-Ionization Detection (Ag-HPLC) of the Triacylglycerols (TAG) of *Crepis alpina* Oil[a]

TAG[c]	Unsaturation: Number double bonds	Unsaturation: Number triple bonds	TAG retention time[b]: Absolute time (min)	Content: Triacylglycerol area percentage
S_3	0	0	15.90 ± 0.50	0.6 ± 0.1
CrS_2	1	1	22.53 ± 0.78	0.4 ± 0.1
CrSO	2	1	28.58 ± 0.64	0.8 ± 0.1
CrSL	3	1	32.88 ± 0.81	0.8 ± 0.1
CrOL	4	1	36.26 ± 0.67	2.0 ± 0.1
CrL_2	5	1	46.27 ± 0.64	16.3 ± 0.1
Cr_2S	2	2	53.91 ± 0.83	1.9 ± 0.2
Cr_2O	3	2	59.83 ± 0.22	8.9 ± 0.1
Cr_2L	4	2	69.50 ± 0.03	4.9 ± 0.1
Cr_2L	4	2	74.85 ± 0.38	28.9 ± 0.2
Cr_3	3	3	98.37 ± 0.98	34.5 ± 0.2

[a]See Figure 1 for Ag-HPLC conditions, TAG resolution and identification. Identification based on TAG fatty acid composition given in Table 1.
[b]Absolute retention times of references: tristearoyl, 15.90 min; trioleoyl, 25.27 min; trilinoleoyl, 45.00 min; trilinolenoyl, 92.30 min; and triarchidonoyl, 129.83 min retention times.
[c]Abbreviations: Cr, monoenoic monoalkynoic = crepenynic acid; see Table 1 for other abbreviations.

Quantitation obtained by the Ag-HPLC system with FID demonstrated accuracy by area percentage without the need for response factors as required for other detectors (light-scattering and ultraviolet absorption). Ag-HPLC analysis of a standard TAG mixture, consisting of StStSt, OOO, LLL and ArArAr glycerols, gave the following compositions (TAG, area percentage, weight percentage, respectively): St_3, 14.8, 14.2; O_3, 44.6, 45.5; L_3, 20.7, 19.2; and Ar_3, 19.9, 21.1.

A linear plot was obtained for FID response vs. sample weight (0.1 to 1.0 mg) for Cr_3 glycerol. Thus, FID linearity, which was previously demonstrated with Ag-HPLC of tripalmitoyl and trilauroyl glycerols (13), has now been observed for alkene-alkyne TAG.

The CrAO TAG composition data are presented in Table 2. The major TAG are Cr_3 (34.5%), Cr_2L (33.8%, two isomers), and CrL_2 glycerols (16.3%), which together account for 85% of the TAG. Further, the Ag-HPLC-FID system allowed 95% of all the TAG to be absolutely identified as opposed to the RP-HPLC-FID system, in which only 70% of the TAG were identified (1). However, quantitation results for the major TAG in CrAO were comparable as determined by either method. The content of Cr_3 glycerol was 34.5 and 36.4%, and that of Cr_2L glycerol isomers was 33.8 and 33.4% by Ag-HPLC-FID and RP-HPLC-FID, respectively (1).

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