Analysis of Seed Oil from *Ricinus communis* and *Dimorphoteca pluvialis* by Gas and Supercritical Fluid Chromatography

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ABSTRACT: The seed oils from *Dimorphoteca pluvialis* and *Ricinus communis* contain hydroxy fatty acids. *Dimorphoteca pluvialis* contains Δ-9-hydroxy-10*t*,12*t*-octadecadienoic acid (dimorphecolic acid) and *R. communis* contains Δ-12-hydroxy-9*c*-octadecenoic acid (ricinoleic acid). The oils were derivatized and analyzed to determine the content of hydroxy fatty acids. The trimethylsilyl fatty acid methyl ester (TMS–FAME) derivatives were analyzed by capillary gas chromatography (GC), and the free fatty acid (FFA) derivatives and the oils were analyzed by capillary supercritical fluid chromatography (SFC). Further, mass spectroscopy of the TMS–FAME derivatives was performed to check the purity of the derivatives. The results from the GC analyses of TMS–FAME corresponded to the results found by SFC analysis of the FFA. The content of ricinoleic acid in the glycerolipids of *R. communis* was 87.7 wt%, and the content of dimorphecolic acid in *D. pluvialis* was 54.0 wt%. The methods were evaluated with respect to the cost, ease, and time needed for sample preparation and analysis.
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KEY WORDS: Derivatization, *Dimorphoteca pluvialis,* gas chromatography, hydroxy fatty acid, mass spectrometry, method evaluation, *Ricinus communis,* supercritical fluid chromatography, trimethyl silyl derivatives.

Seed oils containing hydroxy fatty acids are important raw materials. Hydroxy fatty acids are used in the manufacture of polymers, such as Nylon-66, coatings, and paints (1). Lately, the natural flavor and fragrance component, 2-nonenal, has been synthesized from *Ricinus communis* oil (2). Oil from *R. communis* (castor oil) is at present the most important source of hydroxy fatty acids because it contains up to 90% Δ-12-hydroxy-9*c*-octadecenoic acid (ricinoleic acid) (3). In search for more sources of hydroxy fatty acids, *Dimorphoteca pluvialis* (cape marigold) has been investigated as a new crop (4). The seeds of *D. pluvialis* contain Δ-9-hydroxy-10*t*,12*t*-octadecadienoic acid (dimorphecolic acid), which differs in structure from ricinoleic acid by having two conjugated double bonds. Dimorphecolic acid is also present in the seeds of *D. sinuata*. Dimorphecolic acid is highly reactive, and *D. pluvialis* seeds are difficult to process by ordinary means, such as pressing (4), but because of possible new applications, work is in progress to find new ways to process the seeds (5). A recent paper (6) described the production of hydroxy fatty acids by epoxidation, followed by catalytic ring opening.

Analysis of lipids that contain hydroxy fatty acids has been performed for many years by different methods. Ahmad *et al.* (7) have used ^{14}C-acetic anhydride to acetylate the hydroxyacyl moieties of several oils, including *D. sinuata* and *R. communis*. They fractionated the ^{14}C-acetylated oil by thin-layer chromatography (TLC), and the content of fatty acids with one, two or three hydroxy groups was determined by measuring the radioactivity of each fraction. Gas chromatography (GC), followed by titrimetric analysis, of fatty acids from *D. sinuata* has been used to determine the total amount of mono- and polyhydroxy acids in the oil (8). Alkali-catalyzed transesterification of glycerides by means of anion-exchange resin columns, followed by GC analysis, has been used to determine the fatty acid composition in *D. sinuata* oil (8,9). GC analysis of methyl esters of hydroxy fatty acids is, however, not quantitative because the long retention times and high temperatures required often cause some thermal degradation of hydroxy fatty acids. Trimethylsilyl (TMS) derivatization of hydroxy fatty acids has been applied to lower the polarity and to increase the volatility of the analytes (5,10,11). Muuse *et al.* (5) found that silylation of the hydroxy groups of *D. pluvialis* oil prior to transmethylation could eliminate losses of dimorphecolic acid. Analysis of silylated triacylglycerols of hydroxy fatty acid-containing lipids has been performed on inactivated metal columns at 320–365°C with both polar and nonpolar stationary phases (5). All these methods are time-consuming because of the complicated sample preparation involved. However, when the samples are analyzed by supercritical fluid chromatography (SFC), sample preparation is less complicated, and the column temperature is typically much lower than in GC, which allows hydroxy fatty acid-containing oils to be analyzed without the risk of thermal degradation. Analysis of *R. communis* oil by SFC separates com-

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ponents according to carbon number on nonpolar columns (12,13) and according to degree of saturation (12) in silverion SFC. SFC has also been applied for determination of *R. communis* oil in lipstick (14). Demirbücker *et al*. (12) have also used SFC to analyze methyl esters of hydroxy fatty acids from *R. communis* oil at a column temperature of 100°C. All components were eluted within approximately 17 min.

Lipids that contain hydroxy fatty acids have been separated from other lipids in samples of various oils, including *R. communis* (15,16) and *D. sinuata* (15), by TLC. The method is fast and qualitative, but not suitable as a quantitative technique. Liquid chromatography (LC) has been used to separate the acylglycerols in the oil from *D. sinuata* with a fair resolution and with the possibility to analyze the collected fractions separately (17).

In this work, we have analyzed the composition of oils from *D. pluvialis* and *R. communis* by different methods. The TMS–fatty acid methyl ester (FAME) derivatives were analyzed by GC, and the oil and free fatty acids (FFA) recovered from the hydrolyzed oil were analyzed by SFC. The results from the various methods were compared and corresponded well. GC–mass spectroscopy (MS) was used to check the purity of the TMS–FAME derivatives. In our literature search, we found no publications on SFC analyses of dimorphecolic acid-containing seed oils, though the unstable nature of this oil makes SFC a well-suited technique. The ease and time of sample preparation were evaluated for all methods.

EXPERIMENTAL PROCEDURES

Materials. Dimorphoteca pluvialis seed oil was obtained by supercritical carbon dioxide extraction of winged seeds at 350 bar and 40°C. The oil was kindly supplied by Ole Henriksen, FLS Miljø, Valby, Denmark. The oil from *R. communis* was purchased in a local pharmacy, and both oils were stored at −15°C. A Δ-9-hydroxy-10*t*,12*c*-octadecadienoic acid standard was purchased from Larodan Fine Chemicals AB (Malmö, Sweden), and the Δ-12-hydroxy-9*c*-octadecenoic acid (ricinoleic acid) standard was purchased from Sigma Chemical Company (St. Louis, MO). The silylating reagent, *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Pierce Chemical Company (Oud-Beijerland, The Netherlands). Triacylglycerol standards were purchased from Nu-Check-Prep Inc. (Elysian, MN). *n*-Heptane LiChrosolv, trichloromethane LiChrosolv, methanol 99.9%, ethanol 99.9%, potassium hydroxide pellets, 5 M hydrochloric acid, and diethyl ether 99.9% were purchased from Merck (Darmstadt, Germany). Helium with a purity of 99.996%, hydrogen with a purity of 99.8%, and atmospheric air were purchased from Hede Nielsen AS (Ballerup, Denmark), and carbon dioxide with a purity of 99.995% was purchased from Linde Gase AG (München, Germany).

Preparation of the FFA. Fatty acid derivatives were prepared by hydrolysis of the oils. Care was taken when acidifying the hydrolysis product from *D. pluvialis* oil because the hydroxy fatty acids may deteriorate under acidic conditions. The raw oil (100 mg) was placed in a screw-cap test tube, and 2 mL 1 M KOH in 95% ethanol was added. The mixture was heated on a waterbath to 45°C and allowed to react for 2 min. After cooling, the unsaponifiable components were extracted twice with 5 mL diethyl ether. Another 5 mL diethyl ether was added to the remaining water phase. The aqueous layer was titrated to pH 6 by dropwise addition of 5 M HCl. The tube was carefully swirled after each drop to prevent excessive local acidic conditions. The FFA were extracted twice with 5 mL diethyl ether. The solvent was removed by evaporation under nitrogen. The FFA were redissolved in 1.5 mL diethyl ether and analyzed by SFC as described in SFC method 1.

Preparation of TMS–FAME derivatives of R. communis *oil.* The method for preparation of TMS–FAME of the oil from *R. communis* has previously been described by Muuse *et al*. (5). After the silylation step, excess silylating reagent was destroyed by the addition of water. The silylated FAME were extracted from the water phase by *n*-heptane and analyzed by GC and GC–MS.

Preparation of TMS–FAME derivatives of the raw D. pluvialis *oil*. Because the method used for the oil from *R. communis* was not successful for the oil from *D. pluvialis*, we used the following method, partly based on the method described by Husek (18). The oil (45 mg) was weighed into a screw-cap vial, and 1 mL of 0.5 M NaOH in dry methanol was added. The mixture was heated to 60°C for 5 min. After addition of 1 mL saturated aqueous NaCl, the methyl esters obtained by the transesterification process were extracted with 1 mL diethyl ether. One mL of diethyl ether was added to the remaining water phase, and the water phase was acidified by dropwise addition of 5 M HCl. After each addition of acid, the solution was carefully mixed to avoid excessive local acidic conditions. The ether phase was added to the first ether extract. The combined ether extracts contained both FAME and FFA. The ether was evaporated, and then the method for silylation and methylation of hydroxy fatty acids, described in the next section, was followed. By this method, the fatty acids in the mixture were converted to silyl derivatives of methyl esters. The mixture was analyzed by GC and GC–MS.

Preparation of TMS–FAME derivatives of hydroxy FFA. This method was used for the transmethylation of the Δ-9-hydroxy-10*t*,12*c*-octadecadienoic acid standard. Because this component is sensitive to acid, the more common acid-catalyzed transmethylation methods for FFA were replaced by the following method (18). The Δ-9-hydroxy-10*t*,12*c*-octadecadienoic acid standard (25 µg) was placed in a screw-cap test tube and 100 µL of acetonitrile/water/methanol/pyridine (7:1:1:1, by vol) was added together with 100 µL methylchloroformate. The mixture was shaken for 10 s, and 100 µL trichloromethane and 100 µL 1M $NaHCO_3$ in water were added. The mixture was shaken, and the trichloromethane layer was recovered. The solvent was removed by evaporation under nitrogen. The remaining solids were redissolved in 100 µL acetonitrile, 100 µL BSTFA was added, and

the mixture was allowed to react for 20 min at room temperature. Excess silylating reagent was removed by evaporation under nitrogen. The TMS–FAME were redissolved in *n*-heptane and analyzed by GC and GC–MS.

GC–flame-ionization detector (FID) analysis of silylated FAME. An HP-GC 5890 (Hewlett-Packard, Avondale, CA), equipped with an FID, was used with helium as carrier gas. The injection method was split injection with a split ratio of 1:50. The gas flow at the outlet of the column was 1.1 mL min^{-1} at an oven temperature of 160°C, which corresponded to a linear velocity of 25 cm s^{-1}. The column was an Omegawax 320 (Supelco Inc., Bellefonte, PA), 30 m × 0.32 mm i.d. × 0.25 µm film thickness. The detector and injector temperatures were 240 and 250°C, respectively. The oven temperature was programmed from an initial temperature of 160°C with an increase by 3°C/min to 200°C, where it was maintained for 1 min, followed by an increase at the rate of 3°C/min to 220°C, and held for 12 min. The total analysis time was 33 min.

GC–MS analysis of silylated FAME (GC–MS method 1). A Carlo Erba (Carlo Erba, Milan, Italy) 8035 GC, equipped with a Fisons MD800 mass spectroscopy detector (Poole, UK) was used with helium as carrier gas. The injection technique was splitless for the standards, and split injections with a split ratio of 1:40 or 1:123 for oil samples. The carrier gas flow was 0.73 mL min^{-1} at 70°C, corresponding to a linear velocity of 23.4 cm s^{-1}. The column was a BPX70 (SGE International, Ringwood Victoria, Australia), 30 m × 0.22 mm i.d. × 0.25 µm film thickness. The injector temperature was 250°C. The oven temperature was programmed from 70 to 155°C at a rate of 15°C/min, from 155 to 196°C at a rate of 3°C/min, from 196 to 240°C at a rate of 15°C/min, and then kept at 240°C for 5 min. The total analysis time was 29.3 min. The MS detector was used with ionization at 70 eV and 200 µA emission, and the scans were performed in the range from 30 to 450 amu. The scan time was 0.9 s and the interscan time was 0.1 s.

GC-MS analysis of silylated FAME (GC–MS method 2). An HP-5890 IIA GC (Hewlett-Packard), equipped with an HP 5972A mass-selective detector, was used with helium as carrier gas. The injection technique was split injections with a split ratio of 1:20. The carrier gas flow was 0.90 mL min^{-1}, corresponding to a linear velocity of 29.6 cm s^{-1}. The column was a DB 1701 (J&W Scientific, Folsom, CA), 30 m × 0.25 mm i.d. × 1.0 µm film thickness. The injector temperature was 250°C. The oven temperature was programmed from 150 to 250°C at a rate of 5°C/min and then held at 250°C for 15 min. The total analysis time was 35 min. The MS detector was used with ionization at 70 eV and 50 µA emission, and the scans were performed in the range from 30 to 450 amu with 1.7 scans/s.

SFC analysis of FFA (SFC method 1). An HP-SFC (Hewlett-Packard), equipped with an FID and an HP-7376 automatic sampler, was used with carbon dioxide as mobile phase. The SFC was run in the upstream mode, meaning that pressure control was performed upstream to the chromatographic column. The pressure drop was maintained by frit restrictors (Dionex Corp., Sunnyvale, CA). The injection technique was split injection with a split ratio of 1:100. The flow of expanded CO_2 at the outlet of the column was measured to 1 mL min^{-1}, corresponding to a linear velocity of 2 cm s^{-1}. The column was a polar DB-225 (J&W Scientific), 25 m × 0.1 mm i.d. × 0.2 µm film thickness. The oven temperature was 110°C, and the FID temperature was 350°C. The density was programmed from 0.4 to 0.52 g mL^{-1} at a rate of 0.002 g $mL^{-1}min^{-1}$. The total analysis time was 60 min.

Polar column SFC analysis of the oils (SFC method 2). The apparatus and column are described in SFC method 1. The oven temperature was 110°C, and the FID temperature was 350°C. The density was programmed from 0.4 to 0.64 g mL^{-1} at a rate of 0.002 g mL^{-1} min^{-1}. The total analysis time was 120 min.

Peak identification and quantitation. Identification of the peaks in the SFC methods was performed by analysis of standards, followed by comparison of retention times. In the GC methods, the mass spectra of the peaks were compared to those of standards. Conversion from area percentage to weight percentage was performed by means of theoretical response factors.

RESULTS AND DISCUSSION

GC analysis of TMS–FAME. The preparation of TMS-FAME of hydroxy fatty acids is not so straightforward as was first assumed. The first experiments, performed according to the method proposed by Muuse *et al.* (5), gave reproducible results for the oil from *R. communis* but not from *D. pluvialis.* This could be due to the presence of FFA in *D. pluvialis*. The purchased oil from *R. communis* was refined and did not contain FFA. Acid-catalyzed methylation could not be used because of the risk of addition of methanol to the conjugated double bond systems, as described by Christie (19). Alternative methylation methods, found in the literature, include diazomethane methylation (20) and those where the methylation reaction takes place in the injection port of the GC (21,22). However, the method adopted was initial hydrolysis of the oil to obtain the FFA, which were then methylated with methylchloroformate according to Hušek (18). This method was fast and gave reproducible results for *D. pluvialis*.

Table 1 presents the results from the GC analyses of TMS–FAME. The content of 87.7% ricinoleic acid in the oil from *R. communis* corresponds well to what others have reported (1,2). The information on dimorphecolic acid content in seed oils from *D. pluvialis* is sparse. Muuse *et al.* have reported contents in the range from 54.2 to 61.8% (4,5), depending on the method for processing the seeds. These figures compare well with our result of 53.6%. The investigation of *D. pluvialis* seed is recent. Only a few years of breeding have been done; therefore, the dimorphecolic acid content may differ from harvest to harvest. The hydroxy fatty acid content in seeds of *R. communis* is more uniform because this species has been bred for many years.

TABLE 1
Fatty Acid Compositions of Oils from *Ricinus communis* and *Dimorphoteca pluvialis* Determined by Gas Chromatographic Analyses of Trimethylsilyl–Fatty Acid Methyl Esters (FAME)

	Weight percentage in the acylglycerol part of	
FAME	*Ricinus communis*	*Dimorphoteca pluvialis*
$C_{16:0}$	1.1	1.8
$C_{18:0}$	0.9	1.5
$C_{18:1\omega9}$	2.9	16.0
$C_{18:1\omega7}$	0.5	0.5
$C_{18:2\omega6}$	4.9	12.4
$C_{18:3\omega3}$		0.6
Ricinoleic	87.7	
Dimorphecolic		53.6
$C_{20:0}$		0.9
$C_{20:1}$		1.1
$C_{22:0}$	2.1	

GC–MS analysis of TMS derivatives of the standards. The TMS derivatives of the hydroxy fatty acid standards were used for the identification of hydroxy FAME on the GC–FID chromatograms, but GC–MS analyses were also performed to check whether the mass spectra of the standards were identical to those of the natural mixtures. The GC retention time of the TMS–FAME of Δ-9-hydroxy-10*t*,12*c*-octadecadienoic acid was slightly different from that of the major peak of the TMS–FAME derivatives of the oil from *D. pluvialis*. GC–MS analysis was carried out according to Method 1 (Experimental Procedures), and the mass spectrum of the TMS–FAME derivatives of the standard and of the major peak from the oil were compared. Figure 1 is the mass spectrum of the TMS derivative of the major peak from the oil from *D. pluvialis*. In spite of the difference in retention times the spectra appeared identical. This observation was corroborated by carrying out another GC–MS analysis with a column of different polarity (Method 2; Experimental Procedures). Scrutiny of the GC–MS chromatograms of the derivatives of the oil from *D. pluvialis* revealed a minor peak in the vicinity of the major peak with an identical mass spectrum, but with a retention time identical to that of the derivative of the standard. These observations can be explained by *cis-trans* isomerism. The minor peak is the Δ-9-hydroxy-10*t*,12*c*-octadecadienoic acid methyl ester that corresponds to the standard, whereas the major peak must be Δ-9-hydroxy-10*t*,12*t*-octadecadienoic acid methyl ester.

SFC analysis of the oils. SFC of the oils was performed to determine the composition of the FFA and triacylglycerols in the oils. This information is of course lost when the lipids in the oils are transmethylated without prior lipid class separation. Figures 2 and 3 present the SFC chromatograms of the oils from *R. communis* and *D. pluvialis,* and Table 2 shows the derived results. The oil from *R. communis* contains only

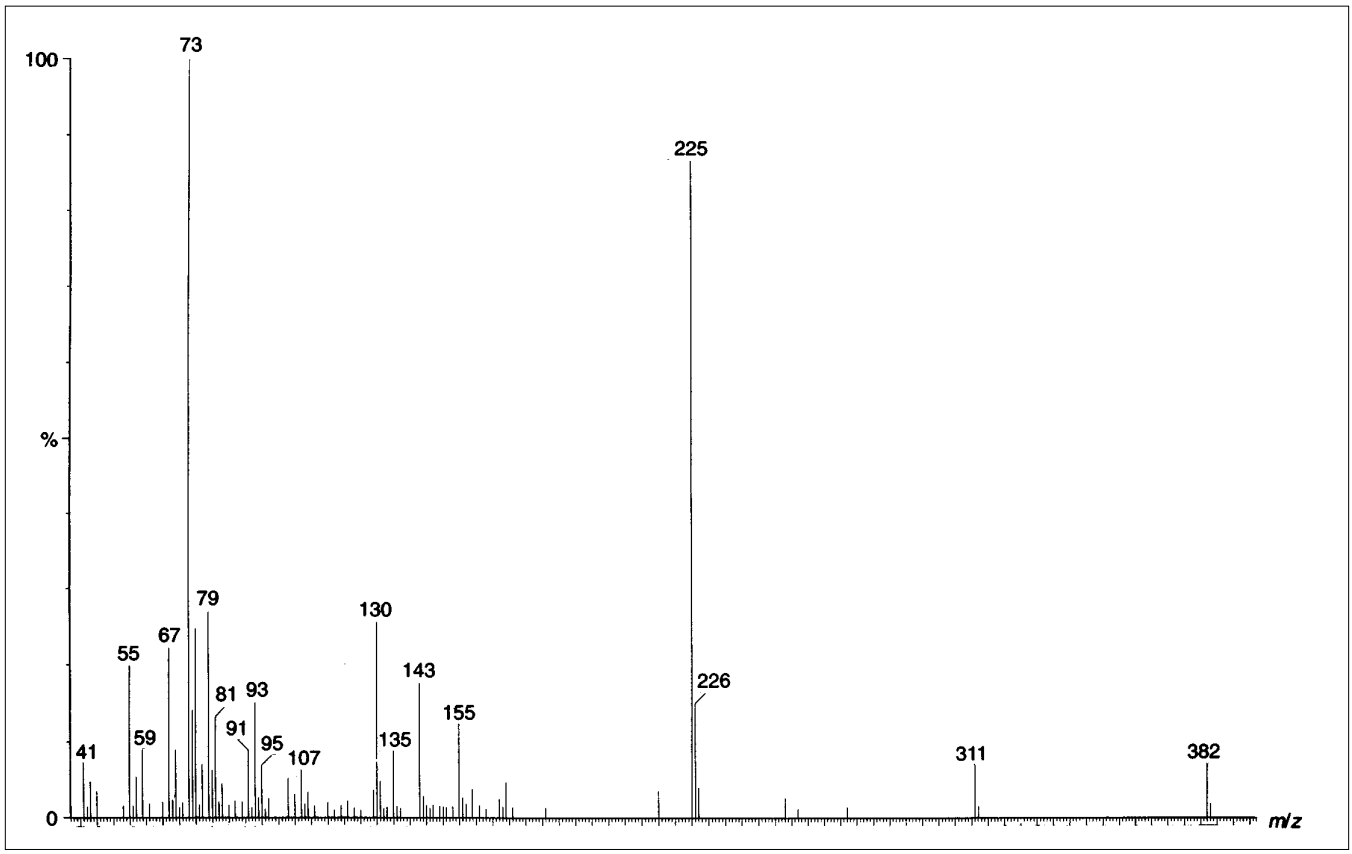

FIG. 1. Mass spectrum of the major peak in the silylated and methylated oil from *Dimorphoteca pluvialis.*

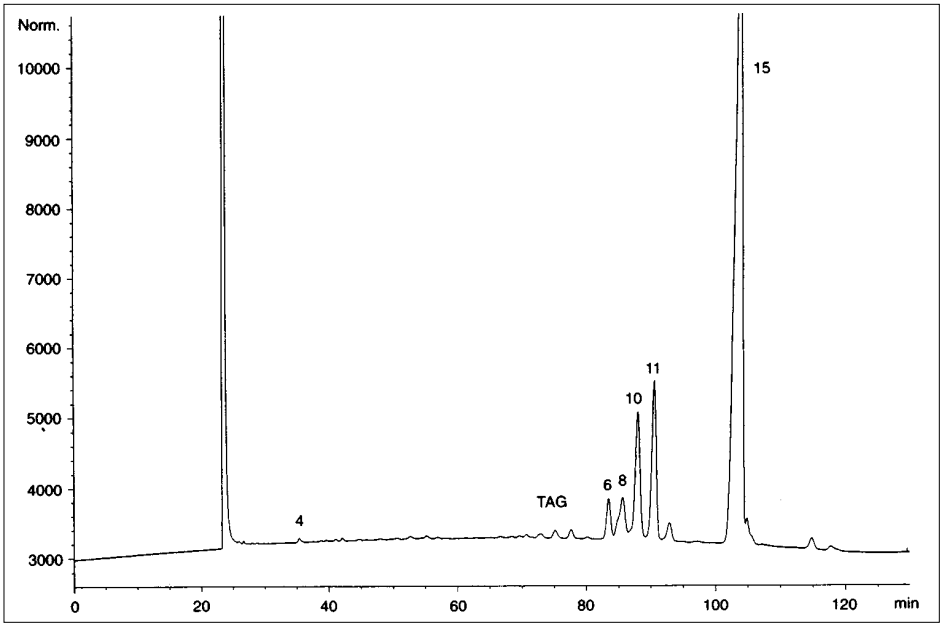

FIG. 2. Chromatogram of polar column supercritical fluid chromatography of oil from *Ricinus communis*. Peak identification and quantitation are reported in Table 2. Norm., normalized detector response; TAG, triacylglycerols.

triacylglycerols, whereas the raw oil from *D. pluvialis* contains both FFA and triacylglycerols. This difference is due to the fact that the oil from *R. communis* was refined, whereby free acids have been removed, whereas the oil from *D. pluvialis* was obtained by supercritical fluid extraction of the seeds and analyzed without further purification. Once the identification and quantitation of the components in the sample have been performed, it is possible to calculate the approximate content of the hydroxy fatty acid in the oils by adding the contents of hydroxy fatty acid from each triacylglycerol species and from the free acids. The FFA that contains the hydroxy group will count as one, triacylglycerols with one hydroxy group will count as one-third, triacylglycerols with two hydroxy groups will count as two-thirds, and so on. From Table 2, the content of ricinoleic acid in the oil from *R. communis* can be calculated as follows:

$$\text{Ricinoleic acid} = 0.2 + \tfrac{1}{3}(2.5 + 3.9) + \tfrac{2}{3}(9.2+11.5) + 68.9 = 85 \text{ wt\%} \quad [1]$$

Similarly, the content of dimorphecolic acid in the oil from *D. pluvialis* is 53 wt%. These approximate values correspond to the results from the GC analysis of TMS–FAME in Table 1. That is, the SFC analysis of the oil gives the composition of FFA and triacylglycerols present in the oil, but one can also calculate the approximate content of the hydroxy fatty acid. The time used for sample preparation is minimal, and an analysis time of 120 min is reasonable.

SFC analysis of FFA from the oils. The content of hydroxy fatty acids determined by SFC analysis of the oil is only approximate, but the total acid composition of the oil can easily be determined more accurately by hydrolyzing it, followed by extracting of the acids and analyzing them by SFC directly without further derivatization. The SFC chromatograms of the FFA from the oils from *R. communis* and *D. pluvialis* appear in Figures 4 and 5, and the peak identification and quantitation are shown in Table 3. The results can be compared to the results of GC analyses of TMS–FAME in Table 1, which shows that the numbers correspond. The advantages of SFC analysis of FFA are the rather short time for sample preparation, the inexpensive chemicals used, and the analysis time of

TABLE 2
Peak Identifications and Compositions of *Ricinus communis* and *Dimorphoteca pluvialis* Oils Determined by Supercritical Fluid Chromatography Analyses

		Weight percentage in the acylglycerol part of	
Peak number	Component[a]	*R. communis*	*D. pluvialis*
1	P		0.4
2	O		1.3
3	L		1.2
4	R	0.2	
5	D		10.7
6	ROO	2.5	
7	DOO		3.8
8	RLL	3.9	
9	DLL		2.6
10	RRO	9.2	
11	RRL	11.5	
12	DDP		6.0
13	DDO		27.8
14	DDL		21.4
15	RRR	68.9	
16	DDD		3.3

[a]P, palmitic acid; O, oleic acid; L, linoleic acid; D, dimorphecolic acid; R, ricinoleic acid.

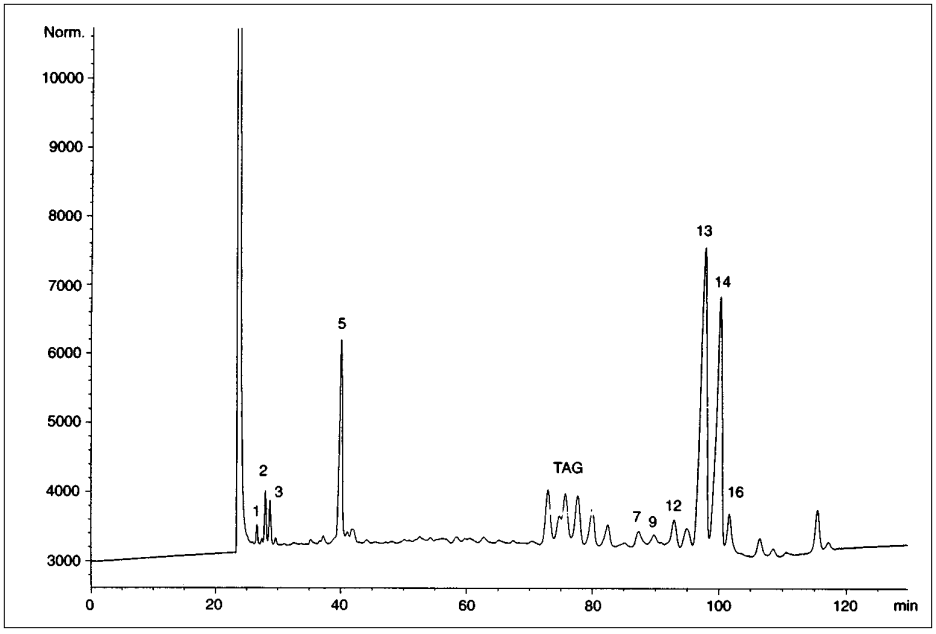

FIG. 3. Chromatogram of polar column supercritical fluid chromatography of raw oil from *Dimorphoteca pluvialis.* Peak identification and quantitation are reported in Table 2. For abbreviations see Figure 2.

only 40 min. However, as mentioned in the methods section, the hydrolysis step requires some skill owing to the acidification step, where care should be taken not to get excessively low pH in the reaction mixture. The result of a low pH would be addition of methanol to the conjugated double-bond system. The oil from *D. pluvialis* is more complicated to derivatize than the oil from *R. communis*, which has no conjugated double bonds.

In this work, we have evaluated three techniques to determine the hydroxy fatty acid content in the seed oils from *R. communis* and *D. pluvialis* with respect to the ease, cost, and time of sample preparation, and different information has

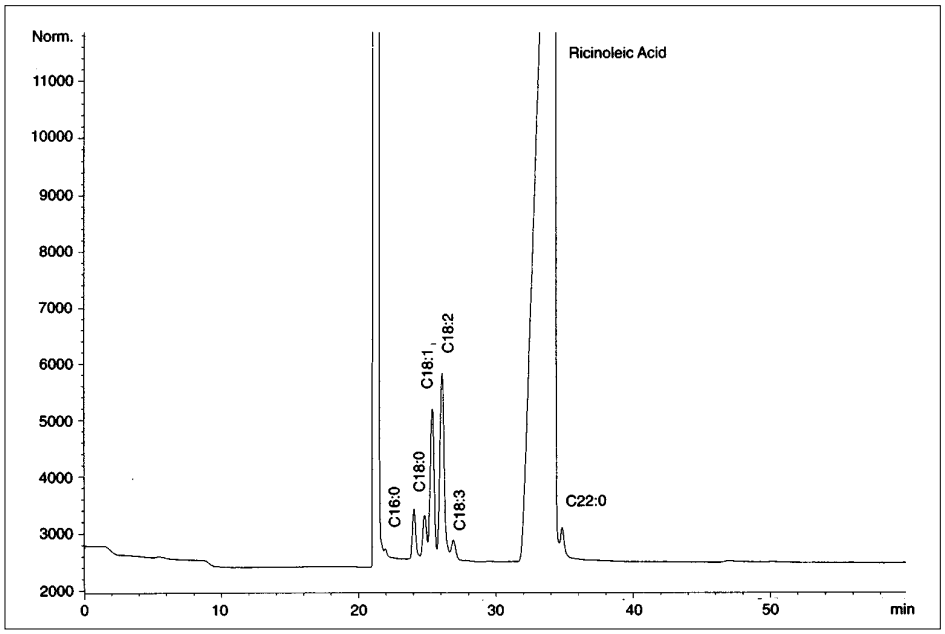

FIG. 4. Polar column supercritical fluid chromatography chromatogram of free fatty acids derivatives of oil from *Ricinus communis.* For quantitation, see Table 3. For abbreviation see Figure 2.

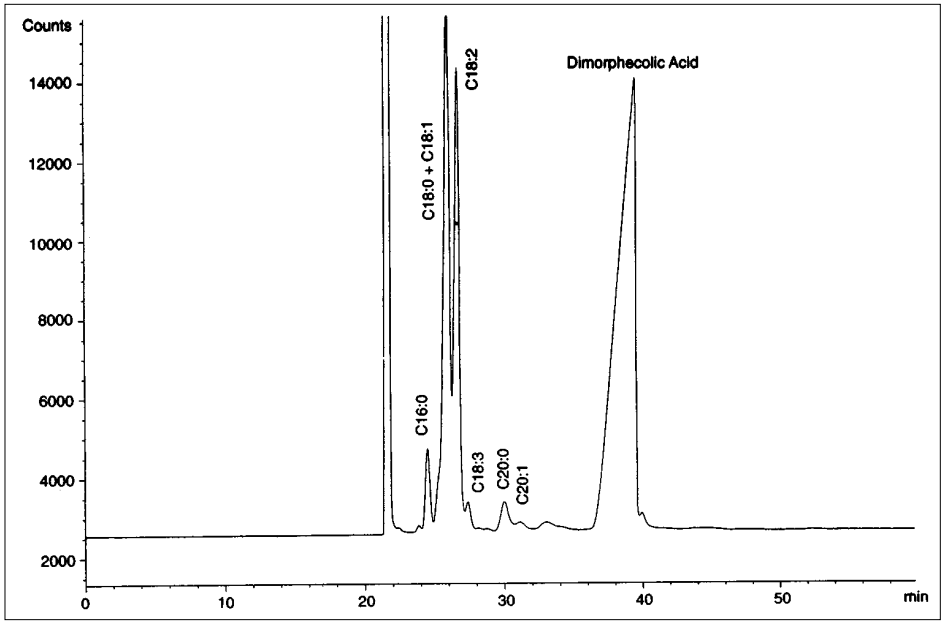

FIG. 5. Polar column supercritical fluid chromatography chromatogram of free fatty acids derivatives of oil from *Dimorphoteca pluvialis*. For quantitation, see Table 3.

been derived from each method. The three methods were: GC of the TMS–FAME, SFC of the oils, and SFC of the FFA. Of the three methods, SFC analysis of the oils offered the shortest time for sample preparation and provided an overall composition of the oils. However, the hydroxy fatty acid content determined by this method was only approximate. The overall composition of the oils cannot be determined as readily by GC, which requires TMS derivatization to elute hydroxy fatty acid components.

A direct determination of the total fatty acid composition was achieved by two other methods, GC of the TMS–FAME and SFC of the FFA. These methods required about the same time for sample preparation, but both methods have advantages and disadvantages. GC of TMS–FAME is straightforward when a reproducible method for transmethylation is available. The TMS derivatization step, however, requires silylating agent, which is expensive. After the silylation step has been performed, the excess silylating reagent must be removed by evaporation to protect the GC column and the injection liner. The SFC method for FFA requires only inexpensive chemicals, but because of the acidification step this method requires extreme care. This care is more crucial with the oil from *D. pluvialis* than with the oil from *R. communis* because of the presence of conjugated double bonds in the former. Because both methods have advantages and disadvantages when it comes to ease and cost of sample preparation, the choice of method will depend on the chromatographic equipment at hand. The hydroxy fatty acid content was determined indirectly by SFC analysis of the oil, and although the result corresponded to the two direct methods, the results can only be regarded as approximate. On the other hand, it gives the overall composition of the oil.

TABLE 3
Fatty Acid Compositions of Oils from *Ricinus communis* and *Dimorphoteca pluvialis* Determined from Supercritical Fluid Chromatography Analyses of Free Fatty Acid Derivatives

	Weight percentage in the acylglycerol part of	
FAME[a]	*Ricinus communis*	*Dimorphoteca pluvialis*
$C_{16:0}$	1.0	2.5
$C_{18:0}$	1.0	n.d.
$C_{18:1\omega9} + C_{18:1\omega7}$	3.6	21.4[b]
$C_{18:2\omega6}$	4.7	15.1
$C_{18:3\omega3}$	n.d.	0.9
Ricinoleic	87.5	n.d.
Dimorphecolic	n.d.	53.4
$C_{20:0}$	n.d.	1.6
$C_{20:1}$	n.d.	0.6
$C_{22:0}$	1.2	n.d.

[a]n.d., not detected. See Table 1 for other abbreviation.
[b]Coelution between $C_{18:0}$ and $C_{18:1}$ isomers.

GC–MS has been performed on the TMS derivatives of the hydroxy fatty acid standards, ricinoleic acid, and Δ-9-hydroxy-10*t*,12*c*-octadecadienoic acid. The spectra provide useful information for the identification of the TMS–FAME from the oils. In some of the early experiments, we used acid-catalyzed transmethylation on the oil from *D. pluvialis,* and the results obtained from the GC–FID seemed correct. However, a GC–MS analysis revealed that the spectrum of the major peak did not match the spectrum of the TMS derivative of Δ-9-hydroxy-10*t*,12*c*-octadecadienoic acid methyl ester. This was probably due to the addition of methanol to the conjugated double bonds in the hydroxy fatty acids. Therefore, we decided to use alkali-catalyzed transmethylation, followed by methylation with methylchloroformate. Further, GC–MS

showed the presence of two isomers of the Δ-9-hydroxy fatty acid in the oil from *D. pluvialis*, that is, Δ-9-hydroxy-10*t*,12*c*-octadecadienoic acid methyl ester and Δ-9-hydroxy-10*t*,12*t*-octadecadienoic acid methyl ester, the latter being dimorphecolic acid.

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