Extraction and Characterization of *Dimorphotheca pluvialis* Seed Oil

Bart G. Muuse*, F. Petrus Cuperus and **Johannes T.P. Derksen**
Agrotechnological Research Institute (ATO-DLO), Wageningen 6700 AA, The Netherlands

Dimorphotheca pluvialis **is increasingly recognized as an interesting industrial new oilseed crop because it contains up to 60% of the unusual fatty acid dimorphecolic acid (9-hydroxy,10*t*,12*t*-18:2) (DA), for which new applications are being developed. In this paper, the yield, composition and quality are evaluated for dimorphotheca oils (DMO) which were recovered by pressing, conventional solvent extraction and supercritical carbon dioxide extraction (SCE). Mechanical pressing of the seeds required high temperatures and resulted in an oil recovery of only 40%, whereas the extraction protocols yielded more than 95%. Oil recovery by pressing of winged seed was even more difficult than that of unwinged seeds; hence, solvent extraction of winged seeds was preferred. The dark-colored DMO, recovered by expelling or by extraction with organic solvents, needed further refining to remove pigments and gums, whereas the light yellow-colored SCE DMO did not require further refining. SCE oil had a low phospholipid content (11 mg P/kg). Pressed oil (95 mg P/kg) and hexane- or pentane-extracted DMO (200 mgP/kg) had much higher phospholipid contents. Peroxide and *p*-anisidine values were low for freshly recovered oils, but increased after storage, especially in the SCE oil, due to the low concentration of natural antioxidants in SCE DMO, such as tocopherols. The DA content of the oils recovered by the various techniques showed only minor differences, except that supercritical carbon dioxide had slightly decreased solubilizing power for tri- and di-dimorphecolin as compared to hexane and pentane.**

KEY WORDS: Dimorphecolic acid, *Dimorphotheca pluvialis*, expelling, hydroxy fatty acid, oil recovery, solvent extraction, supercritical fluid extraction, triacylglycerol.

Dimorphotheca pluvialis (L.) Munch seed oil (DMO) contains an interesting, unusual fatty acid with many potential oleochemical applications (1-6). The major fatty acid, β-dimorphecolic acid (DA), has been identified as the optically active *S*(+) 9-hydroxy-*trans,trans*-10,12-octadecadienoic acid (7-14). Its highly reactive conjugated hydroxydiene structure, containing a chiral center, is a versatile raw material for specialty applications in, e.g., the oleochemical, pharmaceutical and flavor and fragrance industries. The fatty acid can be dehydrated to conjugated *all-trans* trienoic acids (11), modified by addition, polymerized or rearranged to yield a wide variety of new chemicals that are useful as compounds in lubricants, surface coatings, foam plastics and nylons (15,16). Selected chemical and physical characteristics of DMO and other new seed oils have recently been reported (17).

Due to industrial interest, agricultural production of *Dimorphotheca* seeds is rising, and it is increasingly important to define an oil recovery protocol that yields high-quality oil. Information on the processing of *Dimorphotheca* seeds is virtually nonexistent, so we studied the recovery of DMO by mechanical expelling, hexane and pentane solvent extraction and supercritical carbon dioxide extraction (SCE). Here we report the composition and quality of the oils recovered with the different methods.

*To whom correspondence should be addressed at Agrotechnological Research Institute (ATO-DLO), P.O. Box 17, NL-6700 AA Wageningen, The Netherlands.

EXPERIMENTAL PROCEDURES

Materials. Dimorphotheca pluvialis seeds were obtained from Cebeco Handelsraad (Dutch National Agricultural Co-Operative Whole Sale Society, Rotterdam, The Netherlands). The seeds were stored in 60-L gunny bags at 20°C and 70% relative humidity. The unwinged type of seed (cones) from the flower heads was used (22.8% oil) for the oil recovery experiments.

Oil recovery. Press oil was obtained directly from whole seeds by mechanical expelling in a continuous-flow screw-press (Komet single-screw oil expeller, model SS 87G; IBG Monforts + Reiners Gmbh, Mönchengladbach, Germany), which was operated at varying conditions of feeding speed (10–50 rpm of 25-cm feeding screw with 8 coils), choke setting (10–15 mm) and temperature of the press head (up to 140°C). The expelled oil was centrifuged at 2000 × *g* for 30 min to remove fines.

Extraction of the seeds was performed after prior milling of the seeds with a hammer mill with a sieve aperture of 2 mm, followed by a continuous Soxhlet extraction on 5-L pilot scale during 3–4 h with hexane or with petroleum ether (b.p. 30–40°C). Residual solvent in the oil was removed at 50°C with a rotatory evaporator.

Supercritical carbon dioxide-extracted oil was obtained from 0.05–0.25 mm flaked seeds by batch extraction of 150 g seeds with carbon dioxide at 300 bar and 45°C in a pilot-scale SCE apparatus (Sitec-Sieber Engineering AG, Zürich, Switzerland). The oil fraction was dried with sodium sulfate and centrifuged for 20 min at 2000 × *g*.

Fatty acid analysis. Thirty mg oil was taken for analysis for capillary gas chromatographic (GC) analysis of fatty acids and triacylglycerols (TAG). To analyze the oil in the seed as such, 1 g of seed was thoroughly ground in 20 mL petroleum ether (b.p. 40–60°C) with an 18-mm Ultra Turrax T25 high-speed mill (IKA Labortechnik, Heitersheim, Germany) at 24,000 rpm for 1 min. The extract was centrifuged, and 3 mL of the clear extract was dried under nitrogen at 50°C. Silylating reagent (200 μL) N,O-*bis*-(trimethylsilyl)-trifluoroacetamide (BSTFA; Pierce, Oud-Beijerland, The Netherlands) was added to the dry oil in a 4-mL vial and heated for 30–45 min at 70°C. After the silylation, the surplus silyl reactants were evaporated completely under nitrogen at 50°C, and the residual silylated oil was dissolved in 2 mL petroleum ether. This sample was used directly for TAG analysis and for subsequent transmethylation reactions for fatty acid methyl ester (FAME) analysis. TAG transmethylation was performed by the addition of 200 μL 2N KOH in dry MeOH, followed by vigorous shaking for 30–50 s (18,19) and immediate removal of the alkali by slightly swirling with 1 mL water and removing the lower phase by suction. The petroleum ether phase was dried with sodium sulfate.

GC analyses of FAME were carried out with a Carlo Erba (Milano, Italy) instrument model MEGA 5360, equipped with a cold on-column injector, AS-550 autosampler and hydrogen carrier gas. The sample (0.4 μL) was separated on a fused-silica column coated with CP-SIL-5 chemically bonded phase (25 m × 0.25 mm i.d., 0.12 μm df) and installed with a 1 m × 0.53 mm uncoated GAP precolumn (Chrompack, Middelburg, The Netherlands). The detector temperature was kept at 275°C. After injection, the temperature of the oven was raised from 80 to 220°C at a rate of 25°C/min and was maintained at 220°C for 15 min. For separation of C18:1 and C18:2 and of silylated and nonsilylated DA, a Carlo Erba GC VEGA model was equipped with a splitter injector and an analytical column coated with CP-WAX-58-CB (25 m × 32 mm, 0.2 μm df; Chrompack). Helium was used as carrier gas, and the oven was set isothermally at 210°C. The fatty acid identification was carried out by GC–mass spectrometry (MS) with a Carlo Erba MEGA/QMD-1000 Quadrupole instrument in electron impact (EI) mode.

TAG analysis. The TAG were analyzed on a Carlo Erba model MEGA 5360 GC with split injector and A200S autosampler. A WCOT stainless steel column coated with HT-SIMDIST-CB apolar phase (20) was used (5 m × 0.5 mm, 0.1 μm df; Chrompack). For more detailed analysis, a slightly more polar column of 25 m × 0.32 mm HT-TAP (phenyl-methyl-polysiloxane) was used. The linear gas velocity of the hydrogen carrier gas through the column was about 65 cm/s. The temperature of the oven was raised 1 min after injection at a rate of 10°C/min from 320 to 360°C, and held at 360°C for 15 min. Detector and injector temperature were set at 400°C.

Other methods. The positional distribution of DA in silylated TAG was assessed according to International Union of Pure and Applied Chemistry (IUPAC) method 2.210 (21). The amount of DA in the 2-position and in the 1- and 3-positions is expressed as percentage of the total fatty acids. To assess oil color, the AOCS Cc 13b-45 Lovibond tintometer method (22) was used, resulting in values for red, yellow and blue. Phosphorus content was determined according to IUPAC method 2.423 (23) by atomic absorption spectrometry with a graphite furnace oven. Free fatty acids were determined titrimetrically according to IUPAC method 2.201 (21), and expressed as percentages of oleic acid. The peroxide value was determined according to IUPAC 2.501 (21) in which the liberated iodine is titrated with thiosulfate. Oil *para*-anisidine values were assessed by IUPAC method 2.504 (21), measuring aldehyde and ketone oxidation products.

RESULTS AND DISCUSSION

GC method for analysis of DA. One of the problems was the nonreproducibility of the standard FAME methods of analysis for quantitation of DMO fatty acid content. A quantitative methylation of the fatty acids was not possible with routine alkaline-catalyzed transmethylation methods, because of the inordinate sensitivity of the dienol group in DA to alkali. Serious and variable losses of up to 60% of the DA content were found. The conversion of DA to trienoic acids during the GC analysis, presumed by Freedman *et al.* (24) and stated by Meijer zu Beerentrup *et al.* (4,5), should have been noticed in the chromatograms by a significant increase of the trienoic C18 isomers just before the DA peak or by a broad-tailing DA peak. However, none of these phenomena were observed to such an extent that the loss of DA could be explained. GC experiments with cold, on-column vs. hot split injections (25) showed no differences. Subsequent methylation experiments with reduced alkali concentrations, reduced incubation time or alternative methylation procedures with alkaline sodium methoxide or acid-catalyzed procedures, combined with succeeding silylations, all failed to improve the DA analysis results. The most successful procedure was silylation of the dimorphecolic hydroxyl group prior to transmethylation. This led us to conclude that DA or DA-methyl ester was lost prior to the GC separation during transmethylation with alkali in methanol. It was essential to remove the by-products and excess silylating reagent before methylation, by either evaporation at 50°C under nitrogen or by washing with water. Because the silylated DA seems to be not completely stable under alkaline transmethylation conditions, the time of the methylation reaction should not exceed 50 s. The neutralized and dried petroleum ether phase containing the silylated FAMEs was stable over a period of several weeks when stored at 4°C. The precision of the method (internal reproducibility 2.6% and repeatability 1.1%) was similar to commonly used methods of fatty acid analysis at the concentration level of DA.

Composition of the oils. Chromatograms of silylated FAMEs of DMO, separated on a CP-SIL-5-CB column and on a CP-WAX-58-CB, are shown in Figures 1 and 2. The CP-SIL column does not separate the silylated and nonsilylated DA, but the CP-WAX column does. The chromatograms show a group of minor peaks (3%) preceding the DA peak. These minor peaks include 2% C20:0 and C20:1. In addition, two peaks are identified by GC–MS as trienoic acid isomers of C18, one having a similar retention time as calendic acid (8*t*,10*t*,12*c*-18:3). It is possible

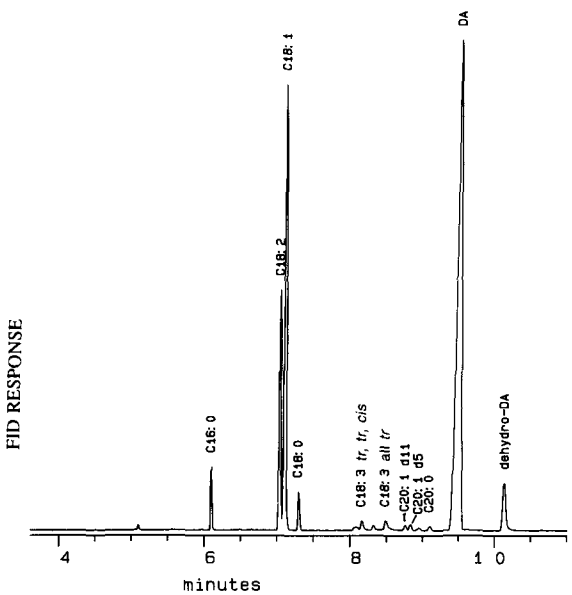

FIG. 1. Fatty acid profile of silylated and methylated dimorphotheca oil of good quality, with low content of trienoic acids, separated on a 25 m × 0.25 mm CP-SIL-5-CB apolar phase (cold on-column injection, oven temperature 80–220°C; see Experimental Procedures section). The dimorphecolic acid peak (DA) contains the silylated and nonsilylated DA. FID, flame-ionization detector; *tr, trans.*

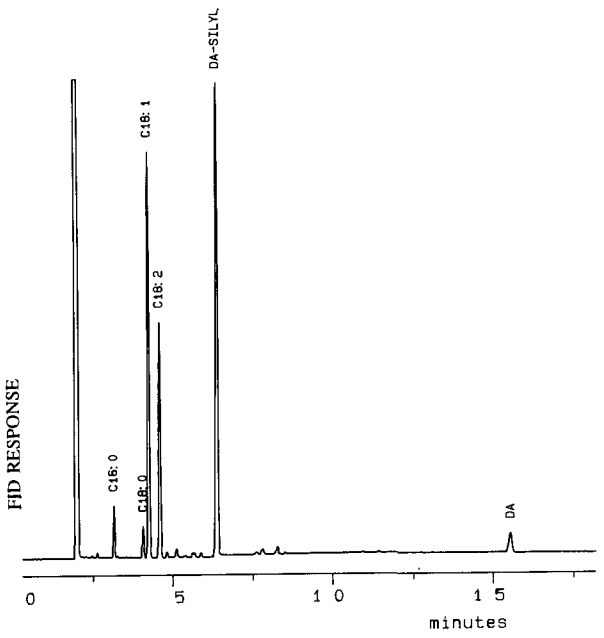

FIG. 2. Fatty acid profile of silylated and methylated dimorphotheca oil, separated on a 25 m × 0.32 mm CP-WAX-58-CB mid polar phase (splitter injection, oven temperature 210°C; see Experimental Procedures section). Separation of silylated dimorphecolic acid methyl ester (DA-silyl) from nonsilylated dimorphelolic acid (DA). FID, flame-ionization detector.

that part of the minor C18:3 peaks were naturally present within the seed and part may have been produced by dehydration of the DA. Of the natural representatives beside α-linolenic acid, Earle *et al.* (8) and Hopkins and Chisholm (9) found two C18:3 isomers in DMO, which were the conjugated 8*t*,10*t*,12*c*-18:3 (calendic acid) and *all-trans* 9,11,13-18:3 fatty acid. Characteristic of DMO virgin oils is the low content of other conjugated C18:3 isomers because DMO degradation is apparent by increase of these isomers due to dehydration of DA.

A dehydro-DA peak is found (0.5–1%), which contained a keto or α-pyran structure according to GC–MS. Binder *et al.* (26) and Freedman *et al.* (24) also reported such a compound in DMO fatty acid analyses. This compound is thought to be naturally related to DA, and not an artifact, because it is found to the same extent in the seeds and in the recovered oil. Moreover, it was not produced by flushing the oil with oxygen for 2 h at room temperature, and therefore it is not likely to be an oxidative degradation product of DA. GC analysis of the SCE-A extract shows an increased amount of dehydro-DA of 2.5%, which is ascribed to the selectivity of the solvent (Table 1).

From results with the CP-WAX-58-CB column (Fig. 2), we concluded that more than 95% of DA is silylated, or that up to 5% is desilylated during the transmethylation procedure. To prepare a fully silylated DA, a second silylation after the transmethylation is required. However, for quantitative results, this is not necessary because silylated and nonsilylated DA have similar retention times on a CP-SIL-5-CB column (Fig. 1), and can simply be summed.

Figure 3 illustrates the composition of the TAG species in DMO. The mono-, di- and tridimorphecolyl glycerols are separated due to the differing number of trimethylsilyl groups bound to the DAs. Because the analysis of these high-molecular weight TAG requires high-temperature GC, a short inactivated metal column with a thin film phase was used. The results are expressed as area percentages and are representative for the mass percentages. A slightly more polar HT-TAP column phase of phenyl-methyl-polysiloxane in a WCOT stainless steel column, developed by Chrompack, separated the TAG into subgroups (Fig. 4). Of the 6% DA found at the 2-position of the TAG (Table 2), 1/3 originates from C54-3DA (Table 3). This implies that in the C54-1DA and C54-2DA triglycerides, about 15% contain DA at the 2-position.

Mechanical oil expelling. Mechanical expelling of *Dimorphotheca* seeds resulted in an oil with a dark green color and a high viscosity (Table 2). Press conditions were optimized by varying temperature, choke size and feeding speed. An optimal yield was found at a low production rate with a screw speed of 10 rpm and a 10-mm choke at 65°C. Under these conditions, a recovery of 60% was found. A high production rate could be obtained with 50 rpm and 15-mm choke at 140°C, but the oil recovery then decreased to only 40%. Extraction of the press cake with pentane produced a residual oil fraction with the same characteristics as the pentane-extracted seeds (Table 2), except for slightly higher phosphorus content (230 mg/kg) and much higher viscosity (750 cPoises). Apart from slightly better resistance to oxidation and phosphorus content of 93 mg/kg, the quality of the press oil was much worse than DMO recovered by the other techniques, when

TABLE 1

Effect of Oil Recovery Technique on Fatty Acid Composition of *Dimorphotheca pluvialis* Seed Oil (results expressed as percentage from the total of fatty acids)

	C16:0	C18:0	C18:1 Oleic	C18:2 Linoleic	C18:3 Linolenic	DA	Dehydro-DA	Minors[a]
Press oil	1.9	1.5	17.5	12.3	0.7	61.7	1.0	3.4
Pentane press cake[b]	2.3	1.6	17.5	13.8	1.0	59.5	0.9	3.4
Pentane	2.1	1.6	17.9	13.2	0.8	60.1	0.9	3.4
Hexane	2.1	1.6	18.0	13.2	0.8	60.4	0.9	3.0
SCE A[c]	2.5	1.9	22.1	12.5	0.8	54.2	2.5	3.5
SCE B[d]	2.4	1.8	20.4	14.9	0.7	55.2	1.2	3.4

[a]Minor fatty acids = *ca.* 2% (C20:0 + C20:1), *ca.* 1% C18:3 isomers. DA, dimorphecolic acid.
[b]Pentane press cake = pentane extract from prepressed whole seeds.
[c]SCE A, oil from a short (single pass) supercritical carbon dioxide extraction.
[d]SCE B, oil from a continuous supercritical carbon dioxide extraction for 1.5 h.

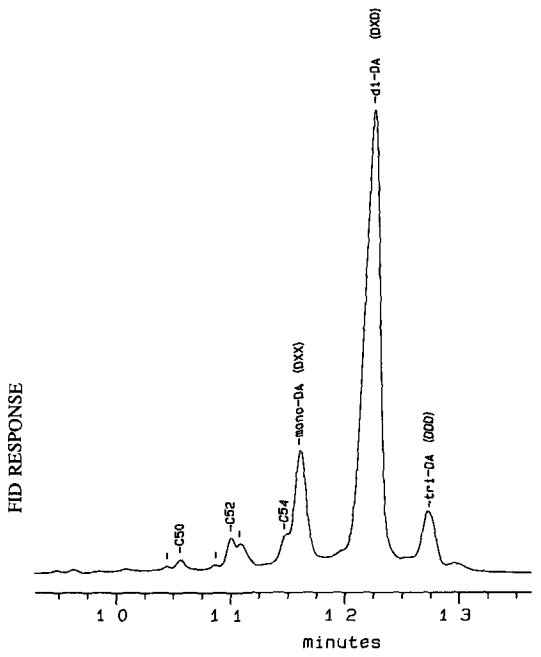

FIG. 3. Triacylglycerol pattern of silylated dimorphotheca oil, separated by carbon number (Cn) on a 5 m × 0.5 mm stainless-steel column with HT-SIMDIST apolar phase (oven temperature 320–360°C; see Experimental Procedures section). Separation of mono-, di- and tridimorphecolin. Abbreviations as in Figure 1.

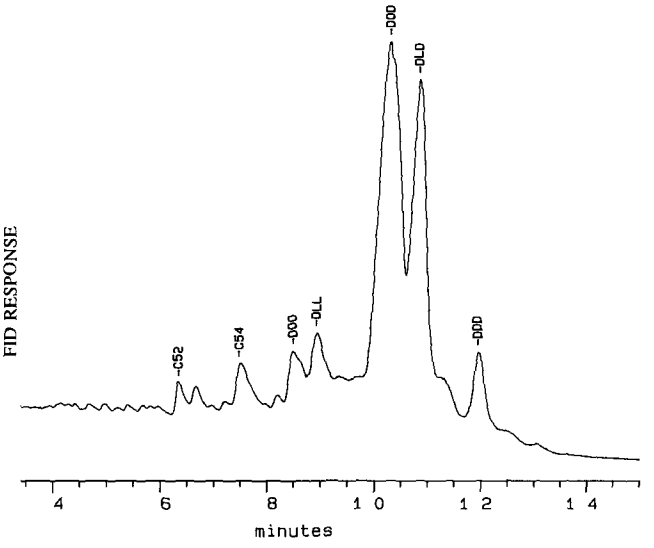

FIG. 4. Triacylglycerol pattern with subseparation of the dimorphecolyl triglycerols of silylated dimorphotheca oil. Separation on a 25 m × 0.32 mm stainless-steel column with a slightly polar HT-TAP phase (oven temperature 320–365°C; see Experimental Procedures section).

taking into account the high viscosity and dark color caused by unsaponifiable matter, such as carotenes, chlorophyll and sediments.

For comparison only, one batch of winged *Dimorphotheca* seeds, from the same accession as the nonwinged seeds, was pressed. These seeds had an oil content (18.5%) slightly below that of the nonwinged type (22.8%). Oil yield from the winged seed was much lower (30–45%) because of the more spongy material with high fiber content that retained much oil. Because *Dimorphotheca* seeds typically contain about 65% winged material (3), this fact is a major determinant for the total oil yield and must be optimized further.

SCE. Oil was recovered from two SCE experiments, one run with a single pass of CO_2 (A) and the other in which carbon dioxide was recycled for 1.5 h (B). The results were not significantly different (Table 1). The color of both oils, after drying, varied from light yellow to light brown. The oil recovery was over 95% for all SCE runs. The SCE technique has the advantage over conventional solvent-extracted oil in that little or no further refining is needed; only co-extracted water has to be removed. The phosphorus content was only 11 mg/kg, whereas for the other techniques up to 200 mg/kg was found. Solvent extraction with hexane or pentane is both hazardous and increasingly incompatible with environmental concerns. In contrast, SCE uses innocuous CO_2. However, the investment costs are rather high for SCE. The observation that DA in general (Table 1), and especially di- and tridimorphecolin (Table 3), are found in SCE oil at significantly lower concentrations than in the other recovery methods,

TABLE 2

Physicochemical Quality Parameters of *Dimorphotheca pluvialis* Seed Oils

	Press oil	Hexane	Pentane	SCE A[a]	SCE B[b]
Free fatty acids (% oleic acid)	4	6	6	n.d.	3
Peroxide value (meq/kg)	13(5)[c]	18(15)[c]	21	15(72)[c]	25(52)[c]
p-Anisidine value	10(18)[c]	8(18)[c]	9	9	7(29)[c]
Color (red/yellow/blue)	0/600/20	0/400/2	0/700/20	1/35/0	1/35/0
P-lipids (mg/kg)	95	210	190	n.d.[e]	11
DA%	7	5	4	6	10
DA% in 1-3 Position[d]	55	55	56	48	45
Viscosity (cPoises 25°C)	960	750	430	n.d.	815
Recovery yield %	42	96	94	n.d.	>95

[a]SCE A, oil from a short (single pass) supercritical carbon dioxide extraction.
[b]SCE B, oil from a continuous supercritical carbon dioxide extraction for 1.5 h.
[c]Number in parentheses indicates the value for oil, stored for four months at room temperature in the dark.
[d]The differences of dimorphecolic acid (DA) in 2- and 1-3- positions are indicative.
[e]n.d., Not determined.

TABLE 3

Effect of Oil Recovery Technique on DMO Triacylglycerol Composition of *Dimorphotheca pluvialis* Seed Oil (results expressed as percentage from the total of triacylglycerols)

	C_n[a]					
	C50	C52	C54	C54-1DA[b]	C54-2DA[b]	C54-3DA[b]
Press oil	0.6	1.9	4.2	15.8	70.7	4.6
Pentane press cake[c]	1.6	2.1	2.9	13.2	72.5	4.6
Pentane	1.0	2.1	2.8	13.4	73.9	4.7
Hexane	0.9	2.0	2.8	12.1	74.9	5.6
SCE A[d]	1.2	2.5	7.7	15.9	69.6	3.2
SCE B[e]	1.3	2.3	4.1	19.7	69.0	3.0

[a]Cn, carbon number of the total carbon atoms from the fatty acids.
[b]C54-xDA, mono-, di- and tridimorphecolyl glycerol of C54.
[c]Pentane press cake, pentane extract from prepressed whole seeds.
[d]SCE A, oil from a short (single pass) supercritical carbon dioxide extraction.
[e]SCE B, oil from a continuous supercritical carbon dioxide extraction for 1.5 h.

suggests that the DA-containing TAG are less soluble in supercritical carbon dioxide than are TAG with fewer or no hydroxyl groups, because of the low polarity of supercritical carbon dioxide.

In Table 2, the peroxide and *p*-anisidine values were initially low and at similar levels for the different oils. However, after storage for four months, the peroxide value increased notably in SCE DMO. This may be due to a lower concentration of the natural antioxidants (e.g., tocopherols) and phospholipids than in expelled or solvent-extracted oils. DMO has low oxidative stability (17); therefore, stabilizing SCE DMO by addition of antioxidant seems to be necessary to increase storage stability.

Organic solvent extraction. The oils from organic solvent extractions were dark-colored, similar to press oil and much darker than SCE DMO. After one hour of extraction, the recovery was 95%, equal to SCE recovery. The oxidative values, free fatty acid and phosphorus contents did not differ much. Pentane was preferred over hexane because of its lower boiling point (36 vs. 69°C). As evident from Table 2, the low viscosity of DMO recovered by pentane extraction could arise from a lower degree of polymerization of the oil due to a lower extraction temperature. Surprisingly, the viscosity of the low temperature-recovered SCE oil is also high. These aspects are currently under further investigation.

Alternative options to increase oil yield from *Dimorphotheca* seeds are vacuum extraction with solvents at room temperature; cellulolytic and lipolytic enzyme treatment prior to the recovery of the fatty acids from the seeds; and the use of expanders as a seed pretreatment for conventional solvent extraction. These options are currently under investigation.

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