Characterization of α- and γ-Linolenic Acid Oils by Reversed-Phase High-Performance Liquid Chromatography–Atmospheric Pressure Chemical Ionization Mass Spectrometry

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ABSTRACT: Triacylglycerols of oils rich in α- and/or γ-linolenic acids were analyzed by reversed-phase high-performance liquid chromatography (HPLC) coupled with atmospheric pressure chemical ionization mass spectrometry [(APCI)MS]. The (APCI)MS spectra of most triacylglycerols exhibited $[M + H]^+$ and $[M - RCOO]^+$ ions, which defined the molecular weight and the molecular association of fatty acyl residues, respectively. Reversed-phase HPLC resulted in, at least, partial separation of triacylglycerols containing α- and/or γ-linolenic acid moieties. Molecules containing α-linolenic acid residues exhibited a relatively weaker retention by the stationary phase than the corresponding molecules containing γ-linolenic acid residues. Good separation of triacylglycerols of cloudberry seed oil, evening primrose oil, borage oil, and blackcurrant seed oil was achieved. The molecular species identification of separated components was based on the (APCI)MS data as well as on the elution properties of triacylglycerols on reversed-phase HPLC. This study demonstrated the efficiency of HPLC–(APCI)MS in determining the molecular species compositions of triacylglycerols in complex natural mixtures. Good quality mass spectra could be extracted even from minor chromatographic peaks representing 0.2% or less of the total triacylglycerols.

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KEY WORDS: Atmospheric pressure chemical ionization mass spectrometry, blackcurrant, borage, cloudberry, evening primrose, α-linolenic acid, γ-linolenic acid, reversed-phase high-performance liquid chromatography, triacylglycerols.

Interest on 18:3n-6 oils has increased during the last decade owing to the potential use of γ-linolenic acid for nutritional and medical purposes (1). The occurrence and properties, as well as nutritional and medical importance, of γ-linolenic acid have been reviewed recently (2,3). γ-Linolenic acid is the Δ 6-desaturation product of linoleic acid (18:2n-6) in the metabolic pathway of essential n-6 fatty acids. Depression of the Δ 6-desaturase activity may result in functional deficiency of long-chain polyunsaturated n-6 metabolites of linoleic acid, a state which may be alleviated by dietary supplementation of γ-linolenic acid.

The most studied natural sources of γ-linolenic acid have been evening primrose oil and borage oil. In addition, blackcurrant seed oil has been studied, as plants belonging to the genus *Ribes* are good sources of γ-linolenic acid. Triacylglycerols of evening primrose oil and borage oil have been determined by high-performance liquid chromatography (HPLC) both on reversed-phase (4–7) and silver-ion mode (8). Reversed-phase HPLC also has been utilized for the determination of the triacylglycerol composition of blackcurrant seed oil (5,9,10). Both Perrin *et al.* (9) and Aitzetmüller and Grönheim (5) have shown the potential of reversed-phase HPLC to separate γ-linolenic acid containing triacylglycerols from the corresponding α-linolenic acid containing molecules. In addition to HPLC, supercritical fluid chromatography (SFC) has been applied for studying oils containing γ-linolenic acid (11,12). Capillary SFC on a cyanopropyl stationary phase has resulted in excellent separation of triacylglycerols having only an α-γ-difference in one linolenic acid moiety (12). Triacylglycerols of currant seed oils also have been analyzed by mass spectrometry (MS) using direct probe sample introduction and chemical ionization (10,12). However, α- and γ-linolenic acids cannot be differentiated by MS; therefore, chromatographic separation of molecules containing linolenic acid isomers is essential. Although both HPLC and SFC can be used for separation of γ-linolenic acid containing molecules, the identification of the components can be difficult. Most often the components separated by HPLC have been detected by ultraviolet (UV), refractive index, or evaporative light-scattering detection. Capillary SFC typically is combined with flame-ionization detection.

Byrdwell and coworkers (13–15) were the first to apply atmospheric pressure chemical ionization MS [(APCI)MS] to the detection and identification of triacylglycerols in combination with reversed-phase HPLC separation. The advantage of (APCI)MS is that in most cases it provides information on both the molecular weight and the fatty acyl residues of a triacylglycerol. Recently, oils rich in α-linolenic acid (cloudberry seed oil), γ-linolenic acid (evening primrose oil and borage oil), or both α- and γ-linolenic acids (alpine currant and blackcurrant seed oils) have been analyzed by silver-ion

HPLC combined with (APCI)MS (16). In the present study, α- and/or γ-linolenic acids containing oils were separated by reversed-phase HPLC and detected by (APCI)MS for identification purposes.

EXPERIMENTAL PROCEDURES

Materials. The oil of cloudberry (*Rubus chamaemorus*) seeds was extracted with chloroform/methanol (2:1, vol/vol) (17). Blackcurrant (*Ribes nigrum*) seed oil was purchased from a local health-food shop. The samples of evening primrose (*Oenothera biennis*) oil and borage (*Borago officinalis*) oil were the same as analyzed in our previous study (16). The oils were dissolved in *n*-hexane after which triacylglycerols were eluted through a Florisil™ (Fluka Chemie AG, Buchs, Switzerland) column with 10 mL *n*-hexane/diethylether (4:1, vol/vol). Triacylglycerols were dissolved in 1,2-dichloroethane to a concentration of ~10 mg/mL and stored under nitrogen at –20°C. The triacylglycerol standards tristearoylglycerol (18:0-18:0-18:0), trioleoylglycerol (18:1-18:1-18:1), trilinoleoylglycerol (18:2-18:2-18:2), tri-α-linolenoylglycerol (18:3n-3-18:3n-3-18:3n-3), *rac*-1,2-distearoyl-3-oleoyl-*sn*-glycerol (*rac*-18:0-18:0-18:1), and *rac*-1,2-dilinoleoyl-3-oleoyl-*sn*-glycerol (*rac*-18:2-18:2-18:1) were purchased from Sigma (St. Louis, MO). The reference compounds 1,3-dioleoyl-2-α-linolenoyl-*sn*-glycerol (*sn*-18:1-18:3n-3-18:1) and 1,3-dioleoyl-2-γ-linolenoyl-*sn*-glycerol (*sn*-18:1-18:3n-6-18:1) were custom-synthesized by Larodan (Malmö, Sweden). Triacylglycerol standards were dissolved in 1,2-dichloroethane. All solvents used were HPLC-grade supplied by Rathburn (Walkerburn, Scotland) or Merck (Darmstadt, Germany).

Reversed-phase HPLC. The HPLC system consisted of a Merck Hitachi L-6200A Intelligent pump (Hitachi, Tokyo, Japan) equipped with an SSI Model LP-21 lo-pulse unit (Scientific Systems Inc., State College, PA). Separations were achieved using two columns (250 mm × 4.6 mm i.d., spherical 5 μm particles) with ODS phase (Zorbax™, DuPont, Wilmington, DE; Spheri-5™, Brownlee Labs, Santa Clara, CA) in series. Triacylglycerols in 1,2-dichloroethane (~2–4 μL) were injected onto the column and separated at ambient temperature using a binary solvent gradient consisting of A) acetonitrile and B) dichloromethane-1,2-dichloroethane (4:1, vol/vol). The program steps of the linear gradient were: 0 min 70% A - 30% B; 50 min 55% A - 45% B; 70 min 25% A - 75% B; 75 min 25% A - 75% B. The flow rate was 0.8 mL/min. The column was equilibrated for 20 min at the initial conditions before each analysis.

MS. The mass spectrometric determinations were conducted with a Finnigan MAT TSQ-700 instrument (Finnigan MAT, San Jose, CA) equipped with an APCI source and an ICIS II data system. The values for the APCI parameters were: the vaporizer temperature 400°C, capillary heater temperature 200°C, corona current 5 μA, sheath gas (nitrogen) pressure 50 psi, and auxiliary gas (nitrogen) flow 5 mL/min. Positively-charged ions with *m/z* values of 400–1100 were scanned with a scan time of 0.8 s. The HPLC flow of 0.8 mL/min was introduced into the APCI source without any splitting.

RESULTS

The samples chosen for this study were selected to represent natural oils being rich in α-linolenic acid and/or γ-linolenic acid. Cloudberry seed oil contained 30 mol% α-linolenic acid and no γ-linolenic acid. Evening primrose and borage oil contained 10 mol% and 22 mol% γ-linolenic acid, respectively, and a negligible amount of α-linolenic acid. Blackcurrant seed oil was rich in both isomeric forms of linolenic acid: 13 mol% α- and 16 mol% γ-linolenic acid; and contained 3 mol% stearidonic acid (18:4n-3). The complete fatty acid compositions of the studied evening primrose oil and borage oil are presented elsewhere (16). The compositions of cloudberry seed oil and blackcurrant seed oil were comparable to those reported earlier (16).

Reversed-phase HPLC–(APCI)MS of reference components. It is well documented, that the separation of triacylglycerols by reversed-phase HPLC is affected by both the combined number of carbon atoms and the number of double bonds in the acyl chains of a molecule (18,19). In general, the molecules elute in ascending order of the combined number of acyl carbons, each of the double bonds reducing the retention of the molecule to the stationary phase. The retention time-reducing effect of double bonds depends on the chromatographic system applied. The components of the reference mixture contained 54 acyl carbons each, with varying numbers of double bonds in the acyl chains. The components eluted mainly based on the number of double bonds in the acyl chains (Fig. 1). In addition, triacylglycerols with the same equivalent carbon number (ECN; ECN = ACN − 2*n*, where ACN is the combined number of acyl carbons and *n* is the combined number of double bonds in the acyl chains of a triacylglycerol) were partially separated. The elution order was *rac*-1,2-dilinoleoyl-3-oleoyl-*sn*-glycerol followed by 1,3-dioleoyl-2-α-linolenoyl-*sn*-glycerol and 1,3-dioleoyl-2-γ-linolenoyl-*sn*-glycerol. Thus, the distribution of double bonds between the fatty acyl residues as well as the positions of double bonds had an effect on the retention of the molecules. The chromatographic system used in the present study separated α- and γ-linolenic acids containing triacylglycerols. The characteristic ion species of triacylglycerols produced by reversed-phase HPLC–(APCI)MS are listed in Table 1.

Reversed-phase HPLC–(APCI)MS of seed oils. The reconstructed ion chromatograms (RIC) of the seed oils studied are presented in Figures 2 and 3. No smoothing has been applied for the RIC. The identifications of the components are listed in Tables 2 and 3. This study was focused on the identification of the components; thus, the proportions of the separated triacylglycerol peaks in each RIC were estimated based on the peak areas without any corrections according to the molecular response factors. The identification of the molecular species was based on the mass spectral data as well as on the elution properties of triacylglycerols. Figure 4 shows as an

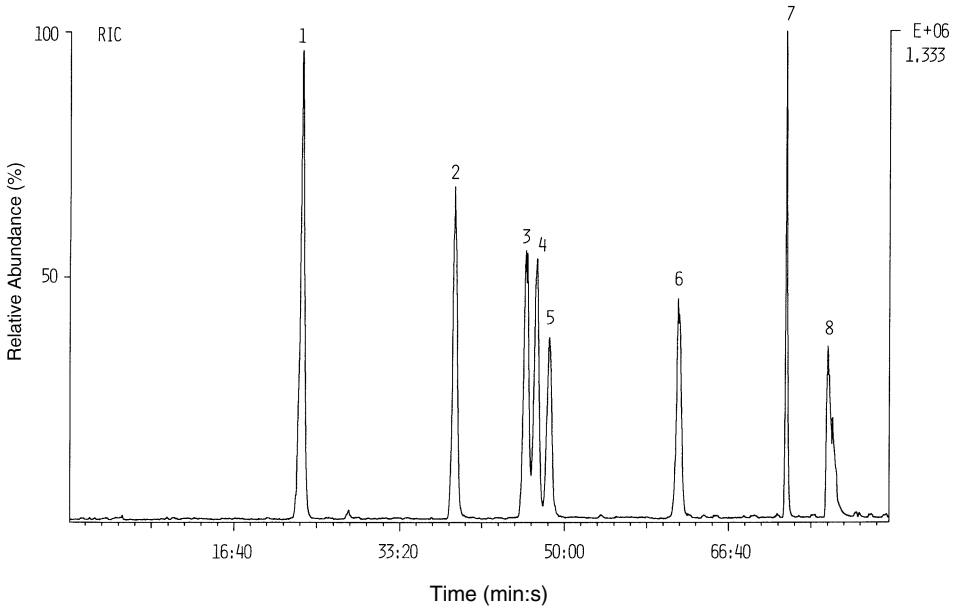

FIG. 1. The reconstructed ion chromatogram (RIC) of a mixture of triacylglycerol reference components achieved by reversed-phase high-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry [HPLC–(APCI)MS]. Peak identification: 1 = tri-α-linolenoylglycerol, 2 = trilinoleoylglycerol, 3 = *rac*-1,2-dilinoleoyl-3-oleoyl-*sn*-glycerol, 4 = 1,3-dioleoyl-2-α-linolenoyl-*sn*-glycerol, 5 = 1,3-dioleoyl-2-γ-linolenoyl-*sn*-glycerol, 6 = trioleoylglycerol, 7 = *rac*-1,2-distearoyl-3-oleoyl-*sn*-glycerol, and 8 = tristearoylglycerol. The analytical conditions are described in the Experimental Procedures section. Peak numbers refer to those presented in Table 1.

example APCI mass spectra obtained from four chromatographic peaks of the triacylglycerols of cloudberry seed oil. Most spectra consisted of $[M + H]^+$ ions and $[M - RCOO]^+$ ions, which defined the molecular weight (i.e., the combined number of acyl carbons and double bonds in the acyl chains) and the fatty acyl residues of a triacylglycerol. Single component chromatographic peaks produced easily interpretable mass spectra of triacylglycerols [Fig. 4 (A and D)]. Multicomponent chromatographic peaks resulted in more complex mass spectra; however, reliable identification of molecular species was possible in most cases [Fig. 4 (B and C)]. Good quality mass spectra of triacylglycerols were obtained from both the major and minor chromatographic peaks.

Cloudberry seed oil. The RIC of cloudberry seed oil presents an efficient separation of triacylglycerols with the chromatographic system used (Fig. 2A). The identification of the components is listed in Table 2. A total of 31 peaks were separated from cloudberry seed oil which represented molecules containing 50 to 60 acyl carbons and varying numbers of double bonds in their acyl chains. Although several critical pairs of triacylglycerols, i.e. molecules having the same ECN, were separated, some critical pairs remained unresolved. For example, peak number 8 was a mixture of 16:0/18:2/18:3n-3 and 18:0/18:3n-3/18:3n-3 (the distribution of fatty acyl residues between the *sn*-1, *sn*-2, and *sn*-3 positions is not differentiated), and peak number 10 consisted of 18:1/18:2/18:2 and 20:1/18:2/18:3n-3. A total of 52 molecular species of triacylglycerols were identified from cloudberry seed oil. The most abundant molecular species were 18:2/18:2/18:3n-3, 18:1/18:2/18:3n-3, 18:2/18:3n-3/18:3n-3, 18:2/18:2/18:2, and

TABLE 1
Ion Species of Triacylglycerol Standards Produced by Reversed-Phase HPLC–(APCI)MS

Peak[a] number	Triacylglycerol	t_r[b] (min:s)	$[M + H]^+$ *m/z* (abundance)[c]	$[M - R_1COO]^+$ *m/z* (abundance) [ion]	$[M - R_2COO]^+$ *m/z* (abundance) [ion]
1	18:3n-3–18:3n-3–18:3n-3	23:32	873.9 (100.0)	595.6 (33.7) $[M - 18:3]^+$	
2	18:2–18:2–18:2	38:50	879.9 (100.0)	599.6 (40.3) $[M - 18:2]^+$	
3	*rac*-18:2–18:2–18:1	46:03	881.9 (100.0)	599.7 (50.3) $[M - 18:1]^+$	601.7 (40.6) $[M - 18:2]^+$
4	*sn*-18:1–18:3n-3–18:1	47:10	881.9 (100.0)	599.6 (94.8) $[M - 18:1]^+$	603.8 (15.8) $[M - 18:3]^+$
5	*sn*-18:1–18:3n-6–18:1	48:25	881.9 (100.0)	599.6 (94.5) $[M - 18:1]^+$	603.7 (60.3) $[M - 18:3]^+$
6	18:1–18:1–18:1	61:28	885.9 (11.7)	603.7 (100.0) $[M - 18:1]^+$	
7	*rac*-18:0–18:0–18:1	72:26	889.9 (4.6)	605.7 (75.1) $[M - 18:0]^+$	607.7 (100.0) $[M - 18:1]^+$
8	18:0–18:0–18:0	76:39	891.8 (n.d.)[d]	607.7 (100.0) $[M - 18:0]^+$	

[a]Peak numbers refer to those presented in the reconstructed ion chromatogram in Figure 1.
[b]Retention time of the compound.
[c]Relative ion abundance in a normalized mass spectrum.
[d]Not detected; HPLC–(APCI)MS, high-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry.

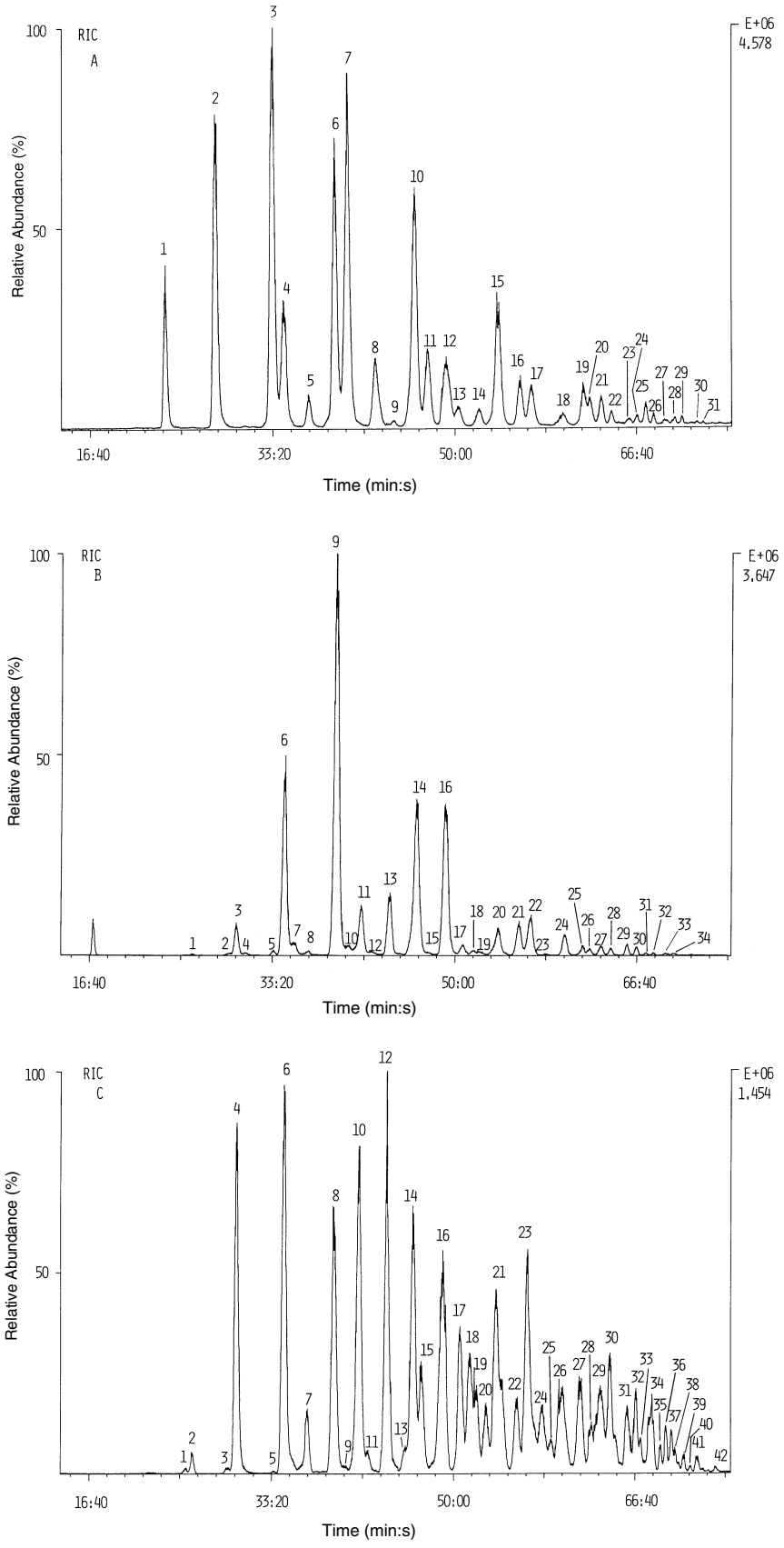

FIG. 2. The RIC of triacylglycerols achieved by reversed-phase HPLC–(APCI)MS. (A) Cloudberry seed oil, (B) evening primrose oil, and (C) borage oil. The analytical conditions are described in the Experimental Procedures section. Peak numbers refer to those in Table 2. See Figure 1 for abbreviations.

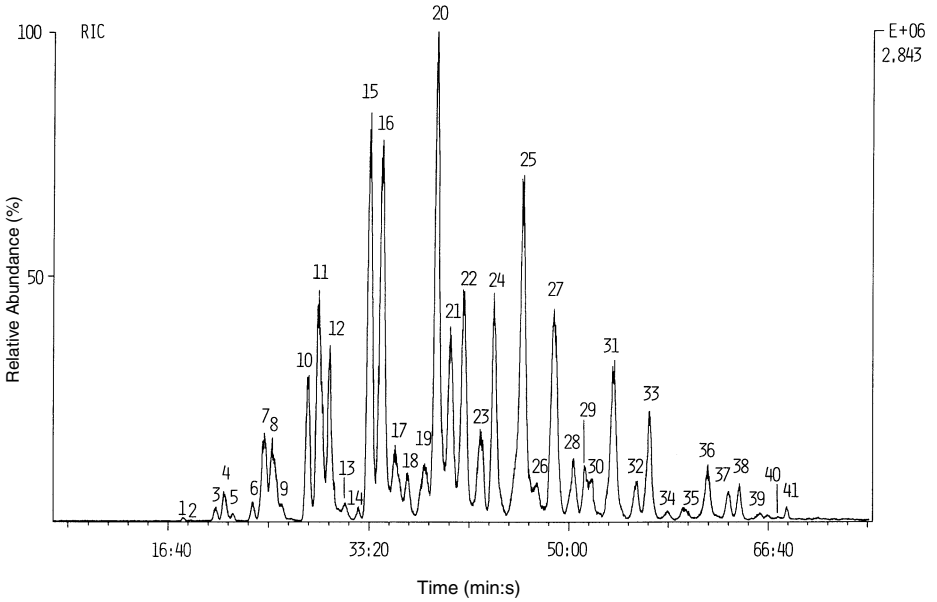

FIG. 3. The RIC of the triacylglycerols of blackcurrant seed oil, achieved by reversed-phase HPLC–(APCI)MS. The analytical conditions are described in the Experimental Procedures section. Peak numbers refer to those in Table 3. See Figure 1 for abbreviations.

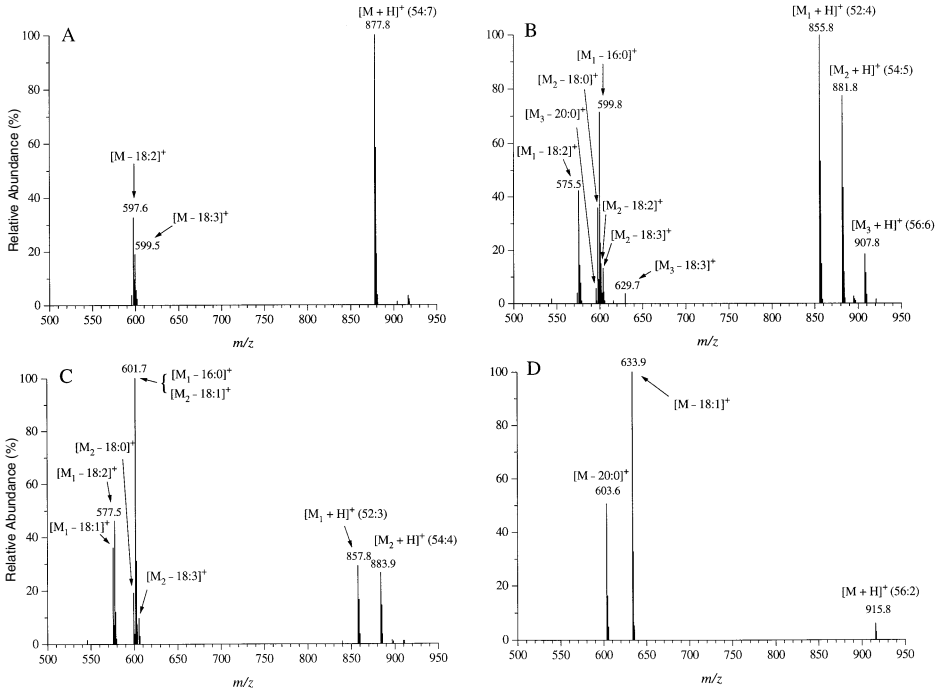

FIG. 4. Examples of atmospheric pressure chemical ionization mass spectra of triacylglycerols of cloudberry seed oil separated by reversed-phase HPLC. The mass spectra were extracted from (A) peak number 3 (M = 18:2/18:2/18:3), (B) peak number 12 (M_1 = 16:0/18:2/18:2, M_2 = 18:0/18:2/18:3, M_3 = 20:0/18:3/18:3), (C) peak number 17 (M_1 = 16:0/18:1/18:2, M_2 = 18:0/18:1/18:3), and (D) peak number 29 (M = 20:0/18:1/18:1). Peak numbers refer to those presented in Figure 2A and in Table 2. The *m/z* value of an $[M + H]^+$ ion defined the number of acyl carbons (ACN) and the number of double bonds (*n*) in the acyl chains of a triacylglycerol (the ACN:*n* identifications are presented in parentheses, e.g., 54:7). The loss of a fatty acyl residue from the molecule has been shown as $[M – 18:2]^+$. See Figure 1 for abbreviations.

TABLE 2
Molecular Species Identification of the Triacylglycerols of Cloudberry Seed Oil, Evening Primrose Oil, and Borage Oil Analyzed by Reversed-Phase HPLC–(APCI)MS

Cloudberry seed oil					Evening primrose oil					Borage oil				
Peak number[a]	ACN:*n*[b]	Triacylglycerol[c]	ECN[d]	MS[e] (%)	Peak number	ACN:*n*	Triacylglycerol	ECN	MS (%)	Peak number	ACN:*n*	Triacylglycerol	ECN	MS (%)
1	54:9	α18:3/α18:3/α18:3	36	4.1	1	54:9	γ18:3/γ18:3/γ18:3	36	0.1	1	54:9	α18:3/γ18:3/γ18:3, 18:2/γ18:3/18:4	36	0.1
2	54:8	18:2/α18:3/α18:3	38	10.8	2	54:8	18:2/α18:3/γ18:3, 18:2/18:2/18:4	38	0.1	2	54:9	γ18:3/γ18:3/γ18:3	36	0.3
3	54:7	18:2/18:2/α18:3	40	14.7	3	54:8	18:2/γ18:3/γ18:3	38	1.9	3	54:8	18:2/α18:3/γ18:3, 18:2/18:2/18:4	38	0.1
4	54:7	18:1/α18:3/α18:3	40	5.1	4	54:8	18:2/18:3/18:3[f]	38	0.2	4	54:8	18:2/γ18:3/γ18:3	38	6.2
5	52:6	16:0/α18:3/α18:3	40	1.1	5	54:7	18:2/18:2/α18:3	40	0.2	5	52:7	16:0/γ18:3/18:4	38	<0.1
6	54:6	**18:2/18:2/18:2**	42	10.6	6	54:7	18:2/18:2/γ18:3	40	13.1		54:7	**18:2/18:2/α18:3**	40	
	56:7	18:2/20:2/α18:3	42		7	54:7	18:2/18:2/18:3[f]	40	1.1	6	52:6	16:1/18:2/18:3[f]	40	8.1
7	54:6	**18:1/18:2/α18:3**	42	13.5	8	54:7	**18:1/γ18:3/γ18:3**	40	0.3		54:7	**18:2/18:2/γ18:3**	40	
	56:7	20:1/α18:3/α18:3	42			53:6	17:2/18:2/18:2	41		7	54:7	18:1/γ18:3/γ18:3	40	1.4
8	52:5	**16:0/18:2/α18:3**	42	3.0	9	54:6	18:2/18:2/18:2	42	30.4	8	52:6	16:0/γ18:3/γ18:3	40	5.9
	54:6	18:0/α18:3/α18:3	42		10	52:5	16:1/18:2/18:2	42	1.0		54:6	**18:2/18:2/18:2**	42	
9	56:6	18:2/18:2/20:2	44	0.2	11	54:6	18:1/18:2/γ18:3	42	4.1	9	52:5	**16:1/18:2/18:2**	42	0.2
10	54:5	**18:1/18:2/18:2**	44	10.8	12	50:4	16:0/16:2/18:2	42	0.3		56:7	20:1/18:3/18:3[f]	42	
	56:6	20:1/18:2/α18:3	44		13	52:5	16:0/18:2/γ18:3	42	4.5	10	54:6	18:1/18:2/γ18:3	42	7.5
11	54:5	18:1/18:1/α18:3	44	3.5	14	54:5	18:1/18:2/18:2	44	14.0	11	50:4	16:0/16:2/18:2	42	0.4
12	52:4	**16:0/18:2/18:2**	44	3.7	15	56:6	20:1/18:2/γ18:3	44	0.3		56:7	**20:1/γ18:3/γ18:3**	42	
	54:5	18:0/18:2/α18:3	44		16	52:4	16:0/18:2/18:2	44	13.3	12	52:5	**16:0/18:2/γ18:3**	42	7.1
	56:6	20:0/α18:3/α18:3	44		17	54:5	18:0/18:2/γ18:3	44	0.8		56:6	18:2/18:2/20:2	44	
13	52:4	16:0/18:1/α18:3	44	0.8	18	52:4	16:0/18:1/γ18:3	44	0.3	13	54:6	18:0/γ18:3/γ18:3	42	0.5
14	56:5	20:1/18:2/18:2	46	0.9	19	53:4	17:0/18:2/18:2	45	0.2	14	54:5	18:1/18:2/18:2	44	6.3
15	50:3	16:0/16:0/α18:3	44	6.1		56:5	**20:1/18:2/18:2**	46		15	56:6	20:1/18:2/γ18:3	44	2.2
	54:4	**18:1/18:1/18:2**	46		20	50:3	16:0/16:0/γ18:3	44	2.6	16	52:4	**16:0/18:2/18:2**	44	6.5
	56:5	18:1/20:1/α18:3	46			54:4	**18:1/18:1/18:2**	46			54:5	18:1/18:1/γ18:3	44	
16	54:4	**18:0/18:2/18:2**	46	2.0	21	54:4	18:0/18:2/18:2	46	2.6	17	54:5	18:0/18:2/γ18:3	44	3.3
	56:5	20:0/18:2/α18:3	46		22	52:3	16:0/18:1/18:2	46	3.3	18	52:4	16:0/18:1/γ18:3	44	2.8
	58:6	22:0/α18:3/α18:3	46		23	54:4	18:0/18:1/γ18:3	46	0.1	19	56:5	20:1/18:2/18:2	46	2.1
17	52:3	**16:0/18:1/18:2**	46	1.9	24	50:2	16:0/16:0/18:2	46	1.6	20	58:6	22:1/18:2/γ18:3	46	1.6
	54:4	18:0/18:1/α18:3	46		25	54:3	18:1/18:1/18:1	48	0.6	21	50:3	16:0/16:0/γ18:3	44	6.2
18	52:3	16:0/18:0/α18:3	46	0.9	26	56:4	20:0/18:2/18:2	48	0.4		54:4	**18:1/18:1/18:2**	46	
	56:4	**18:1/20:1/18:2**	48		27	54:3	18:0/18:1/18:2	48	0.7		56:5	18:1/20:1/γ18:3	46	
19	54:3	18:1/18:1/18:1	48	1.6	28	52:2	16:0/18:1/18:1	48	0.4	22	54:4	18:0/18:2/18:2	46	1.8
20	56:4	**20:0/18:2/18:2**	48	1.0	29	52:2	16:0/18:0/18:2	48	0.6	23	52:3	**16:0/18:1/18:2**	46	6.1
	58:5	22:0/18:2/α18:3	48		30	50:1	16:0/16:0/18:1	48	0.4		54:4	16:0/20:1/γ18:3	46	
21	54:3	**18:0/18:1/18:2**	48	1.1		58:4	**22:0/18:2/18:2**	50		24	54:4	18:0/18:1/γ18:3	46	1.9
	56:4	20:0/18:1/α18:3	48		31	56:3	20:0/18:1/18:2	50	0.1		58:5	**22:1/18:2/18:2**	48	
22	52:2	16:0/18:1/18:1	48	0.5	32	54:2	18:0/18:1/18:1	50	0.1	25	60:6	24:1/18:2/γ18:3	48	0.8
23	52:2	16:0/18:0/18:2	48	0.2	33	54:2	18:0/18:0/18:2, 16:0/20:0/18:2	50	0.1	26	50:2	16:0/16:0/18:2	46	3.1
	54:3	18:0/18:0/α18:3	48			60:4	**24:0/18:2/18:2**	52			56:4	**18:1/20:1/18:2**	48	
	56:3	**18:1/18:1/20:1**	50		34	52:1	16:0/18:0/18:1	50	0.1		58:5	20:1/20:1/γ18:3, 18:1/22:1/γ18:3	48	
24	50:1	16:0/16:0/18:1	48	0.3		58:3	**22:0/18:1/18:2**	52		27	52:3	**16:0/18:0/γ18:3**	46	2.5
	58:4	**22:0/18:2/18:2**	50								54:3	18:1/18:1/18:1	48	
	60:5	24:0/18:2/α18:3	50							28	54:3	**16:0/20:1/18:2**	48	1.0
25	56:3	**20:0/18:1/18:2**	50	0.6							56:4	18:1/18:1/20:2, 20:0/18:2/18:2	48	
	58:4	22:0/18:1/α18:3	50							29	54:3	**18:0/18:1/18:2**	48	2.9
26	54:2	18:0/18:1/18:1	50	0.2							56:4	20:0/18:1/γ18:3, 16:0/22:1/γ18:3	48	
27	54:2	18:0/18:0/18:2	50	0.2							58:5	22:0/18:2/γ18:3	48	
	56:3	20:0/18:0/α18:3	50								60:5	24:1/18:2/18:2	50	
	60:4	**24:0/18:2/18:2**	52							30	52:2	16:0/18:1/18:1	48	3.0
28	58:3	22:0/18:1/18:2	52	0.2							58:4	**18:1/22:1/18:2, 20:1/20:1/18:2**	50	
29	56:2	20:0/18:1/18:1	52	0.2						31	52:2	16:0/18:0/18:2	48	1.5
30	60:3	24:0/18:1/18:2[g]	54	0.1							56:3	**18:1/18:1/20:1**	50	
31	58:2	22:0/18:1/18:1	54	<0.1						32	50:1	16:0/16:0/18:1	48	1.4
											56:3	**16:0/22:1/18:2**	50	
										33	56:3	18:0/20:1/18:2	50	0.4
											58:4	**18:0/22:1/γ18:3**	50	
											60:5	18:1/24:1/γ18:3[g]	50	

(*continued on next page*)

TABLE 2 (continued)

Cloudberry seed oil					Evening primrose oil					Borage oil				
Peak[a] number	ACN:*n*[b]	Triacylglycerol[c]	ECN[d]	MS[e] (%)	Peak number	ACN:*n*	Triacylglycerol	ECN	MS (%)	Peak number	ACN:*n*	Triacylglycerol	ECN	MS (%)
										34	54:2	16:0/18:1/20:1, 18:0/18:1/18:1	50	1.8
											60:4	**18:1/24:1/18:2, 20:1/22:1/18:2**	52	
										35	58:3	18:1/20:1/22:1	52	0.4
										36	54:2	18:0/18:0/18:2	50	0.7
											58:3	**18:0/22:1/18:2, 16:0/24:1/18:2**	52	
										37	52:1	16:0/18:0/18:1	50	0.6
											60:4	**18:0/24:1/γ18:3**	52	
										38	56:2	**16:0/18:1/22:1, 18:0/18:1/20:1, 16:0/20:1/20:1**	52	0.4
											62:4	20:1/24:1/18:2, 22:1/22:1/18:2, 20:1/20:1/22:2[g]	54	
										39	60:3	18:1/18:1/24:1, 18:1/20:1/22:1	54	0.3
										40	60:3	18:0/24:1/18:2[g]	54	0.1
										41	54:1	18:0/18:0/18:1	52	0.3
											58:2	**16:0/20:1/22:1, 16:0/18:1/24:1, 18:0/18:1/22:1[g]**	54	
										42	60:2	18:0/20:1/22:1, 18:0/18:1/24:1, 16:0/18:1/26:1[g]	56	0.1

[a]Peak numbers refer to those presented in corresponding reconstructed ion chromatograms in Figure 2.
[b]ACN is the combined number of acyl carbons and *n* the combined number of double bonds in the acyl chains of a triacylglycerol.
[c]The distribution of fatty acids between the *sn*-1, *sn*-2, and *sn*-3 positions is not differentiated. The triacylglycerol exhibiting the most abundant $[M + H]^+$ ion has been typed in bold font. Identification of triacylglycerols was based on the presence of $[M + H]^+$ and $[M - RCOO]^+$ ions in the mass spectra. α18:3 = 18:3n-3 and γ18:3 = 18:3n-6.
[d]Equivalent carbon number (ECN) = ACN – 2*n*.
[e]Proportions of triacylglycerols based on peak areas in the reconstructed ion chromatograms obtained by (APCI)MS.
[f]Differentiation between 18:3n-3 and 18:3n-6 was not possible.
[g]Other components may be present in this peak. See Table 1 for other abbreviation.

18:1/18:2/18:2, representing together approximately 60% of the total triacylglycerols.

Evening primrose oil. Compared with cloudberry seed oil, the RIC of evening primrose oil looks relatively simple (Fig. 2B). Similar chromatographic profiles of the major components of evening primrose oil have been reported elsewhere (5–7). In the present study, 34 peaks were separated and identified, representing 40 molecular species of triacylglycerols (Table 2). Although the chromatographic resolution was relatively good, a few triacylglycerols were unresolved. On the other hand, 18:2/18:3/18:3 and 18:2/18:2/18:3 were present in more than one chromatographic peak, which may be partially explained by the small amount of α-linolenic acid present in evening primrose oil. The general elution order of triacylglycerols was similar to that of cloudberry seed oil with few exceptions concerning linolenic acid-containing molecules: for example, 16:0/18:2/18:2 coeluted with 18:0/18:2/18:3n-3 in cloudberry and separated well from 18:0/18:2/18:3n-6 in evening primrose oil. The most abundant molecular species of triacylglycerols in evening primrose oil were 18:2/18:2/18:2, 18:1/18:2/18:2, 16:0/18:2/18:2, and 18:2/18:2/18:3n-6. These species accounted for approximately 71% of the total triacylglycerols. Trilinoleoylglycerol alone was 30% of all components. Redden *et al*. (6) and Huang *et al*. (7) have reported very similar results on the triacylglycerol composition of evening primrose oil. Their identification of the major components was comparable to our results. However, reversed-phase HPLC–(APCI)MS offers improved sensitivity, thus, making the identification of relatively minor components possible. In addition, two identifications of triacylglycerols having an odd-number of acyl carbons were made in the present study.

Borage oil. The RIC of the triacylglycerols of borage oil (Fig. 2C) is very complex compared with those of cloudberry and evening primrose oils. This was expected based on the fatty acid compositions of the studied oils: in addition to C_{16} and C_{18} fatty acids, borage oil contained substantial amounts of 20:1n-9, 22:1n-9, and 24:1n-9 fatty acids (16). An improved separation of borage oil components, especially the late-eluting ones, was achieved in this study compared with related separations reported elsewhere (5–7). Borage oil triacylglycerols were separated into 42 chromatographic peaks which together represented over 80 molecular species of triacylglycerols (Table 2). In addition to the molecular species with ACN 50–54, borage oil consisted of several molecules with ACN 56–62. Owing to the complexity of the sample, several chromatographic peaks contained more than one triacylglycerol molecular species. Borage oil demonstrates more clearly the effect of γ-linolenic acid on the retention and thus on the elution order of triacylglycerols than evening primrose oil. Several pairs of triacylglycerols which coeluted in cloudberry seed oil (where 18:3 = 18:3n-3) were at least partially separated in borage oil (where 18:3 = 18:3n-6), for example pairs 16:0/18:2/18:3–18:0/18:3/18:3, 16:0/18:2/18:2–18:0/18:2/18:3, and 16:0/18:1/18:2–18:0/18:1/18:3. On the contrary, some triacylglycerols which were well separated in cloudberry seed oil coeluted in borage oil, for example pairs 16:0/18:3/18:3–18:2/18:2/18:2 and 18:1/18:1/18:3–16:0/18:2/18:2. The most abundant molecular species of borage

TABLE 3
Molecular Species Identification of the Triacylglycerols of Blackcurrant Seed Oil Analyzed by Reversed-Phase HPLC–(APCI)MS

Peak[a] number	ACN:n[b]	$[M + H]^+$[c] m/z (abund.)	Triacylglycerol[d]	ECN[e]	MS[f] (%)
1	54:11	869.7 (100.0)	α18:3/18:4/18:4	32	<0.1
2	54:11	869.8 (100.0)	γ18:3/18:4/18:4	32	<0.1
3	54:10	871.8 (100.0)	α18:3/α18:3/18:4	34	0.2
4	54:10	871.8 (100.0)	α18:3/γ18:3/18:4, 18:2/18:4/18:4	34	0.5
5	54:10	871.8 (100.0)	γ18:3/γ18:3/18:4	34	0.1
6	54:9	873.8 (100.0)	α18:3/α18:3/α18:3	36	0.3
7	54:9	873.8 (100.0)	α18:3/α18:3/γ18:3, 18:2/α18:3/18:4	36	2.0
8	54:9	873.8 (100.0)	α18:3/γ18:3/γ18:3, 18:2/γ18:3/18:4	36	2.0
9	54:9	873.8 (100.0)	γ18:3/γ18:3/γ18:3	36	0.3
10	54:8	875.8 (100.0)	18:2/α18:3/α18:3	38	2.7
11	54:8	875.9 (100.0)	18:2/α18:3/γ18:3, 18:2/18:2/18:4	38	5.4
12	54:8	875.8 (100.0)	18:2/γ18:3/γ18:3, 18:1/α18:3/18:4	38	3.3
13	54:8	875.8 (100.0)	18:1/γ18:3/18:4	38	0.4
14	52:7	849.8 (100.0)	16:0/α18:3/18:4	38	0.1
15	52:7	849.8 (2.5)	16:0/γ18:3/18:4	38	8.3
	54:7	877.8 (100.0)	18:2/18:2/α18:3	40	
16	54:7	877.8 (100.0)	18:2/18:2/γ18:3, 18:1/α18:3/α18:3	40	9.2
17	54:7	877.8 (100.0)	18:1/α18:3/γ18:3, 18:1/18:2/18:4	40	2.2
18	52:6	851.8 (33.9)	16:0/α18:3/α18:3	40	1.1
	54:7	877.8 (100.0)	18:1/γ18:3/γ18:3	40	
19	52:6	851.9 (100.0)	16:0/α18:3/γ18:3, 16:0/18:2/18:4	40	1.6
20	52:6	851.8 (7.2)	16:0/γ18:3/γ18:3	40	10.9
	54:6	879.8 (100.0)	18:2/18:2/18:2	42	
21	54:6	879.8 (100.0)	18:1/18:2/α18:3	42	4.6
22	54:6	879.8 (100.0)	18:1/18:2/γ18:3	42	5.3
23	52:5	853.8 (100.0)	16:0/18:2/α18:3	42	2.4
	54:6	879.8 (1.8)	18:1/18:1/18:4	42	
24	52:5	853.8 (100.0)	16:0/18:2/γ18:3	42	4.8
	54:6	879.9 (2.8)	18:0/α18:3/γ18:3, 18:0/18:2/18:4	42	
	56:6	907.9 (4.6)	18:2/18:2/20:2	44	
25	52:5	853.9 (n.d.)[g]	16:0/18:1/18:4	42	9.8
	54:5	881.9 (100.0)	18:1/18:2/18:2	44	
	56:6	907.9 (6.4)	20:1/18:2/α18:3	44	
26	54:5	882.0 (100.0)	18:1/18:1/α18:3	44	1.0
	56:6	907.9 (54.5)	20:1/18:2/γ18:3	44	
27	52:4	855.9 (100.0)	16:0/18:2/18:2	44	6.3
	54:5	881.9 (24.3)	18:1/18:1/γ18:3, 18:0/18:2/α18:3	44	
28	52:4	855.8 (23.8)	16:0/18:1/α18:3	44	1.6
	54:5	881.9 (100.0)	18:0/18:2/γ18:3	44	
29	52:4	855.8 (56.7)	16:0/18:1/γ18:3	44	1.0
30	56:5	909.9 (100.0)	20:1/18:2/18:2	46	0.9
31	54:4	883.9 (62.6)	18:1/18:1/18:2	46	4.4
32	54:4	883.8 (100.0)	18:0/18:2/18:2	46	1.0
33	52:3	857.9 (37.4)	16:0/18:1/18:2	46	2.5
34	54:4	883.9 (75.6)	18:0/18:1/γ18:3	46	0.3
35	50:2	831.9 (<1.0)	16:0/16:0/18:2	46	0.5
	56:4	911.9 (100.0)	18:1/20:1/18:2	48	
36	54:3	885.9 (9.8)	18:1/18:1/18:1	48	1.4
37	54:3	885.9 (37.7)	18:0/18:1/18:2	48	0.6
38	52:2	860.0 (7.0)	16:0/18:1/18:1	48	0.6
39	52:2	860.0 (n.d.)	16:0/18:0/18:2	50	0.2
	56:3	913.9 (13.5)	18:1/18:1/20:1	50	
40	56:3	913.9 (40.9)	20:0/18:1/18:2	50	<0.1
41	54:2	887.9 (4.8)	18:0/18:1/18:1	50	0.2

[a]Peak numbers refer to those presented in the reconstructed ion chromatogram in Figure 3.
[b]ACN is the combined number of acyl carbons and n the combined number of double bonds in the acyl chains of a triacylglycerol.
[c]The relative abundance of an $[M + H]^+$ in the normalized mass spectrum is presented in the parentheses.
[d]Molecular species composition of a triacylglycerol. The distribution of fatty acids between the *sn*-1, *sn*-2, and *sn*-3 positions has not been differentiated. Identification of triacylglycerols was based on the presence on $[M + H]^+$ and $[M - RCOO]^+$ ions in the mass spectra. α18:3 = 18:3n-3 and γ18:3 = 18:3n-6.
[e]Equivalent carbon number (ECN) = ACN − 2n.
[f]Proportions of triacylglycerols are based on the peak areas in the reconstructed ion chromatogram obtained by (APCI)MS.
[g]n.d., not detected. See Table 1 for other abbreviation.

oil triacylglycerols were 18:2/18:2/18:3n-6, 18:1/18:2/18:3n-6, 16:0/18:2/18:3n-6, 18:1/18:2/18:2, 18:2/18:3n-6/18:3n-6, 16:0/18:2/18:2, 16:0/18:1/18:2, and 18:2/18:2/18:2, corresponding to approximately 54% of the total triacylglycerols. Similar identification of the major components has been reported by Huang *et al.* (7), whereas more differences were observed in comparison to the results of Redden *et al.* (6).

Blackcurrant seed oil. The RIC of blackcurrant seed oil is presented in Figure 3, and the identification of the components is presented in Table 3. The triacylglycerol elution profile of blackcurrant was quite different from the other oils studied, which contained only α- or γ-isomer of linolenic acid. Currant seed oil contained both α- and γ-linolenic acids as well as stearidonic acid (16). Triacylglycerols of blackcurrant seed oil were separated into 41 chromatographic peaks. Most of the triacylglycerols consisted of 52 or 54 acyl carbons with 2 to 11 double bonds in the acyl chains. In addition, some molecular species with ACN 56 were identified.The chromatographic system separated α- and/or γ-linolenic acids containing isomeric triacylglycerols into several peaks. Since it was not possible to distinguish α- and/or γ-linolenic acids containing molecules by MS, the elution properties of triacylglycerols containing only α- or γ-linolenic acid were taken into account. Trilinolenoylglycerols were partially separated into four peaks, and the elution order of the isomers was 18:3n-3/18:3n-3/18:3n-3 followed by 18:3n-3/18:3n-3/18:3n-6, 18:3n-3/18:3n-6/18:3n-6, and finally 18:3n-6/18:3n-6/18:3n-6. Isomeric molecules containing two linolenic acid moieties (e.g., 18:3/18:3/18:4, 18:2/18:3/18:3, 18:1/18:3/18:3) were separated into three peaks. The elution order was *X*/18:3n-3/18:3n-3 > *X*/18:3n-3/18:3n-6 > *X*/18:3n-6/18:3n-6, where *X* is a fatty acid different from linolenic acid. Isomeric triacylglycerols consisting of one linolenic acid moiety (e.g., 18:2/18:2/18:3, 18:1/18:2/18:3, 16:0/18:2/18:3) were separated into two peaks; *X*/*Y*/18:3n-3 eluted earlier than *X*/*Y*/18:3n-6, where *X* and *Y* are fatty acids different from linolenic acid. In addition to α- and γ-linolenic acids, information on the molecular distribution of stearidonic acid moieties in the triacylglycerols was achieved. The most abundant components of blackcurrant seed oil were 18:2/18:2/18:2, 18:2/18:2/18:3n-6, 18:1/18:2/18:2, 18:2/18:2/18:3n-3, 18:2/18:3n-3/18:3n-6, 18:1/18:2/18:3n-6, and 16:0/18:2/18:2. These species represented approximately 52% of the total triacylglycerols. Excellent separation of the triacylglycerols of blackcurrant seed oil has been reported earlier by Perrin *et al.* (9) and Aitzetmüller and Grönheim (5). Their tentative identification of the main components was comparable to our study, although it was done without mass spectrometric detection.

DISCUSSION

HPLC in reversed-phase mode has been the most popular chromatographic technique used for the separation of triacylglycerols. Most often the components have been detected by UV, refractive index, evaporative light-scattering, or flame-ionization detection. In addition, the dual nature of separation, i.e., according to the number of acyl carbons and the number of double bonds in the acyl chains of a triacylglycerol, complicates identification of the eluted components. Thus, on-line coupling of HPLC with a system providing information on molecular composition is most valuable for identification purposes.

As shown in the present study, structural information of complex natural mixtures of triacylglycerols can be achieved by reversed-phase HPLC–(APCI)MS. The (APCI)MS spectra of most triacylglycerols exhibited abundant $[M + H]^+$ and $[M - RCOO]^+$ ions, which defined the molecular weight and the fatty acyl residues of a triacylglycerol. Fully saturated triacylglycerols did not yield any $[M + H]^+$ ions with the eluent composition used; thus, their identification would be more uncertain, if present in the sample. The chromatographic separation of blackcurrant triacylglycerols, containing both α- and γ-linolenic acid moieties, was essential because they could not be differentiated according to their (APCI)MS spectra. In addition, the triacylglycerol separations of cloudberry seed oil, evening primrose oil, and borage oil were of great value in studying the general elution order of molecules. Comparison of the elution order of triacylglycerols, for example by superimposing the chromatographic peaks representing trilinoleoylglycerol, showed good agreement between different seed oils. Comparable oils studied here recently have been analyzed by using silver-ion HPLC for separation in combination with (APCI)MS detection (16). Both techniques gave similar results. The observed differences concerned only the minor components and were of negligible value. Both HPLC modes can be used for separation of α- and/or γ-linolenic acids containing molecules; the main difference is that the elution order of isomeric triacylglycerols is reversed in silver-ion HPLC compared with reversed-phase HPLC. The separation mechanism of triacylglycerols on silver-ion HPLC differs from that on reversed-phase HPLC. Therefore, components which coelute in reversed-phase HPLC may be separated in silver-ion HPLC, and *vice versa*.

Information on the molecular weight of a triacylglycerol is of primary importance in the identification of compounds. The (APCI)MS spectra of most triacylglycerols yielded an $[M + H]^+$ ion except those that were fully saturated. The *m/z* value of the protonated molecular ion defined nearly unambiguously the number of acyl carbons and the number of double bonds in the acyl chains of a triacylglycerol. In addition, the (APCI)MS spectra of triacylglycerols provided data on the fatty acyl residues ($[M - RCOO]^+$ ions) for molecular species identification. Information on the regiospecific distribution of acyl moieties could not be determined in the oils studied. However, differences in the relative abundance of $[M - RCOO]^+$ ions formed by the loss of a fatty acyl residue from the *sn*-2 and *sn*-1/3 positions have been reported with reference components (16). The HPLC–(APCI)MS technique allowed a reliable identification of components eluting in a chromatographic peak consisting of one or few components. Coelution of components having the same molecular weight

but different fatty acid composition made the identification more difficult and uncertain, especially if one of the components is minor. Quite often, one $[M - RCOO]^+$ ion may be a fragment of two or more triacylglycerols which differ in molecular weight. In such cases it is nearly impossible to deduce the origin of the $[M - RCOO]^+$ ion. Slight differences in the chromatographic elution order of the components may substantially facilitate the identification. In general, HPLC–(APCI)MS provided valuable data for structure elucidation of triacylglycerols. Sensitivity of the technique was good, and high-quality mass spectra could be extracted even from chromatographic peaks representing 0.2% or less of the total triacylglycerols.

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REFERENCES

1. Huang, Y.-S., and D.E. Mills, γ-*Linolenic Acid, Metabolism and Its Roles in Nutrition and Medicine*, AOCS Press, Champaign, 1996.
2. Gunstone, F.D., γ-Linolenic Acid—Occurrence and Physical and Chemical Properties, *Prog. Lipid Res. 31*:145–161 (1992).
3. Horrobin, D.F., Nutritional and Medical Importance of γ-Linolenic Acid, *Ibid. 31*:163–194 (1992).
4. Ratnayake, W.M.N., D.G. Matthews, and R.G. Ackman, Triacylglycerols of Evening Primrose (*Oenothera biennis*) Seed Oil, *J. Am. Oil Chem. Soc. 66*:966–969 (1989).
5. Aitzetmüller, K., and M. Grönheim, Separation of Highly Unsaturated Triacylglycerols by Reversed-Phase HPLC with Short Wavelength UV Detection, *J. High Resol. Chromatogr. 15*:219–226 (1992).
6. Redden, P.R., Y.-S. Huang, X. Lin, and D.F. Horrobin, Separation and Quantification of the Triacylglycerols in Evening Primrose and Borage Oils by Reversed-Phase High-Performance Liquid Chromatography, *J. Chromatogr. A 694*:381–389 (1995).
7. Huang, Y.-S., X. Lin, P.R. Redden, and D.F. Horrobin, *In Vitro* Hydrolysis of Natural and Synthetic γ-Linolenic Acid-Containing Triacylglycerols by Pancreatic Lipase, *J. Am. Oil Chem. Soc. 72*:625–631 (1995).
8. Christie, W.W., Fractionation of the Triacylglycerols of Evening Primrose Oil by High-Performance Liquid Chromatography in the Silver-Ion Mode, *Fat Sci. Technol. 93*:65–66 (1991).
9. Perrin, J.-L., A. Prevot, H. Traitler, and U. Bracco, Analysis of Triglyceride Species of Blackcurrant Seed Oil by HPLC *via* a Laser Light-Scattering Detector, *Rev. Fr. Corps Gras 34*:221–223 (1987).
10. Rezanka, T., and P. Mares, Determination of Plant Triacylglycerols Using Capillary Gas Chromatography, High-Performance Liquid Chromatography and Mass Spectrometry, *J. Chromatogr. 542*:145–159 (1991).
11. Blomberg, L.G., M. Demirbüker, and P.E. Andersson, Argentation Supercritical Fluid Chromatography for Quantitative Analysis of Triacylglycerols, *J. Am. Oil Chem Soc. 70*:939–946 (1993).
12. Manninen, P., P. Laakso, and H. Kallio, Separation of γ- and α-Linolenic Acid Containing Triacylglycerols by Capillary Supercritical Fluid Chromatography, *Lipids 30*:665–671 (1995).
13. Byrdwell, W.C., and E.A. Emken, Analysis of Triglycerides Using Atmospheric Pressure Chemical Ionization–Mass Spectrometry, *Ibid. 30*:173–175 (1995).
14. Neff, W.E., and W.C. Byrdwell, Soybean Oil Triacylglycerol Analysis by Reversed-Phase High-Performance Liquid Chromatography Coupled with Atmospheric Pressure Chemical Ionization Mass Spectrometry, *J. Am. Oil Chem. Soc. 72*:1185–1191 (1995).
15. Byrdwell, W.C., E.A. Emken, W.E. Neff, and R.O. Adlof, Quantitative Analysis of Triglycerides Using Atmospheric Pressure Chemical Ionization–Mass Spectrometry, *Lipids 31*:919–935 (1996).
16. Laakso, P., and P. Voutilainen, Analysis of Triacylglycerols by Silver-Ion High-Performance Liquid Chromatography–Atmospheric Pressure Chemical Ionization Mass Spectrometry, *Ibid. 31*:1311–1322 (1996).
17. Christie, W.W., *Lipid Analysis*, Pergamon Press, Oxford, 1982, p. 22.
18. Christie, W.W., *High-Performance Liquid Chromatography and Lipids*, Pergamon Press, Oxford, 1987, pp. 169–210.
19. Ruiz-Gutierrez, V., and L.J.R. Barron, Methods for the Analysis of Triacylglycerols, *J. Chromatogr. B 671*:133–168 (1995).

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