Occurrence of Conjugated Linolenic Acids in Purified Soybean Oil

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ABSTRACT: A high performance liquid chromatographic (HPLC) method is described for the determination of conjugated linoleic acids (CLA) and conjugated linolenic acids (CLN). Methyl esters prepared from purified lipid fractions of soybean oil were analyzed using HPLC system equipped with photodiode-array detector to detect peaks having maximum absorption around 233 nm and 275 nm. These peaks were concentrated by $AgNO_3$-silicic acid column chromatography and reversed-phase HPLC. The structural analysis, of dimethyloxazoline (DMOX) derivatized methyl esters, using GC-MS showed the occurrence of 9,11- and 10,12-conjugated linoleic acids (CLA) and 8,10,13-, 8,10,12- and 9,11,13-conjugated linolenic acids (CLN). The comparison of these conjugated fatty acids with authentic isomers by HPLC revealed the presence of isomeric mixtures of CLA (*cis*(c),*trans*(t) or t,c and t,t) and CLN (c,t,t or t,t,c and t,t,t). Traces of 9,11- and 10,12-CLA (c,t or t,c) was found in crude oil. CLN isomers (8,10,12-18:3 and 9,11,13-18:3) were found to be forming during bleaching of soybean oil processing. 8,10,13-CLN and 9,11- and 10,12-CLA (t,t) were only found in soybean oil after deodrization step. CLN contents in commercial soybean oil varied from 387 mg/kg oil to 1316 mg/kg oil. Decreased level of bleaching earth and temperature resulted in reduced CLN content. Possibly, CLN would be derived from linoleate hydroperoxides formed during processing and storage of soybean oil.

KEY WORDS: HPLC, CLA, CLN, soybean oil, lipid oxidation.

Conjugated fatty acids have evoked increased interest due to the beneficial effects they afford in terms of human health. Among them, conjugated linoleic acid (CLA) has been researched and reviewed extensively in relation to its occurrence (1), metabolism and physiological effects (1-4). On the other hand, several researchers have reported the occurrence of various conjugated fatty acids including trienes, tetraenes and pentaenes in different plant resources from both terrestrial and aquatic enviroments.

Conjugated linolenic acids (CLN) have been reported to occur in terrestrial plant lipids, especially seed oils. Important CLN from plant sources include α-eleostearic acid (9*cis*(c),11*trans*(t),13t-18:3), catalpic acid (9t,11t,13c-18:3), punicic acid (9c,11t,13c-18:3), calendic acid (8t,10t,12c-18:3) and jacaric acid (8c,10t,12c-18:3) (5). On another hand, Yurawecz et al. (6) only could find traces (up to 0.2%) of CLN in vegetable oils in their study of 27 oils for CLN content by UV measurement. The isomers were identified as α-eleostearic acid (9c,11t,13t-18:3), β-eleostearic acid (9t,11t,13t-18:3), and 8t,10t,12t-18:3. The possible mechanism for the formation of these CLN isomers involves linoleate oxidation, reduction of hydroperoxide to hydroxide, and dehydration (7,8). CLN exhibits less oxidative stability as reported by our previous study (9), indicating that CLN may influence oxidative deterioration of vegetable oils, in spite of being present in trace.

Crude oil is refined by a series of processes to remove impurities that affect taste, smell, appearance and stability of the oil. The refining processes involve de-gumming, alkali refining, bleaching, and deodorization. Polyunsaturated fatty acids, such as linoleic acid and α-linolenic acid, found in vegetable oils are more susceptible to oxidation during storage and refining of crude oils resulting in oxidation products, mainly hydroperoxides. Hydroperoxides may also be formed by enzymatic oxidation of

linoleate and linolenate by lipoxygenase during storage of oil seeds. Any oxidation is injurious to the flavor and oxidative stability of refined oils as it results in the formation and accumulation of oxidation products. The quality of a processed vegetable oil always depends on the quality of the crude oil used and the processing parameters selected. Indicators such as peroxide value in the final products can be reduced by lowering storage temperature and minimizing exposure to pro-oxidants like air, light and heat during processing. However, complete removal of all oxidation products from oils is impossible. Linoleate hydroperoxides formed during storage and refining of oils may get converted to 9,11,13-CLN or 8,10,12-CLN because of heat and/or acid treatments involved in processing (7,8).

In the present study, we analyzed CLN in crude soybean oil and processed soybean oil by high performance liquid chromatography (HPLC) with photodiode-array detector. This detection system enabled us to determine c,t,t (or t,t,c) and t,t,t isomers of 9,11,13-CLN and 8,10,12-CLN in these soybean oils.

MATERIALS AND METHODS

Materials. Crude soybean oil, purified soybean oil, soybean oil at different processing stage were prepared by Nisshin Oillio, Tokyo, Japan. Commercial vegetable oils were obtained from oil companies in Japan or from local grocerly stores. CLA (9c,11t- and 10t,12c-isomers) were purchased from Matreya, Inc. (State College, PA). Each CLA was converted to its methyl ester with H_2SO_4-methanol solution (10). Standard CLN (α-eleostearic acid (9c,11t,13t-18:3), β-eleostearic acid (9t,11t,13t-18:3), catalpic acid (9t,11t,13c-18:3), and calendic acid (8t,10t,12c-18:4)) was separated by HPLC from methyl esters of CLN containing seed oils as described previously (11,12). Seeds were

powdered in an electric mill and extracted twice with *n*-hexane at room temperature. The seed oil was transesterified to its methyl esters with 0.5 M sodium methoxide in methanol. Each CLN isomer was separated from the mixed methyl esters by reversed-phase HPLC using a column packed with C30 (Develosil C30 UG-5, 250 x 8.0 mm i.d., 5.0 μm particle size, Nomura Chem. Co., Seto, Aichi, Japan).

Preparation of methyl esters from vegetable oils. Thin-layer chromatographic (TLC) analysis of vegetable oils used in this study revealed that these vegetable oils mainly consisted of triacylglycerol with a trace of free fatty acid, monoacylglycerol, and diacylglycerol. TLC was carried out on 0.25 mm silica gel plates (Merck, Darmstadt, Germany) with diethyl ether/*n*-hexane/acetic acid (40:60:1, by vol) as developing solvent. Triolein, oleic acid, monoolein, and diolein were used as standards. Anhydrous HCl/methanol or BF_3/methanol is often used for the methylation of vegetable oils; but this acid-catalyzed condition caused extensive isomerization of original CLA or CLN and artifact formation (13-15). Methyl esters from vegetable oils were, therefore, prepared by transesterification using 0.5M sodium methoxide in methanol (9). Methyl esters thus prepared were subjected to gas chromatography (GC) to determine the fatty acid composition of vegetable oils under study. Shimadzu GC-14B (Shimadzu Seisakusho, Kyoto, Japan) equipped with a flame-ionization detector and a capillary column (Omegawax 320, 30 m × 0.32 mm i.d., Supelco, Bellefonte, PA) was employed for the GC analysis. The column temperature was set at 200°C while the injector and detector were held at 250 and 260°C, respectively, for the analysis. Helium with flow of 50 kpa was used as the carrier gas.

Purification of soybean oil methyl esters. Methyl esters from purified soybean oil (*ca*. 10g) were refined on a silicic acid column (40 x 2.7 cm i.d.) (Silicagel 60, Merck)

by successive elution with *n*-hexane (300 mL) and a solution of diethyl ether/*n*-hexane (5:95, 800 mL; 10:90, 600 mL, vol/vol). Refined methyl esters (>9.5g) eluted with diethyl ether/*n*-hexane (5:95, vol/vol) was used for analytical HPLC separation of esters of conjugated fatty acids.

Analytical and preparative HPLC. Analytical HPLC was done with an analytical reversed-phase column (Develosil C30 UG-5, 250 x 4.6 mm i.d., 5.0 μm particle size, Nomura Chem. Co.) protected with a 10 x 4.0 mm i.d. guard column containing the same stationary phase. A mixture of methanol and water (85:15, vol/vol) at a flow rate of 1.0 mL/min was used as a mobile phase. All the HPLC analysis was carried out on a Hitachi L-7000 system equipped with pump (L-7100) and an auto-sampler (L-7200). The instrument also housed a photodiode-array spectrophotometric detector (Hitachi L-7455) and an online analysis software (Hitachi HPLC system-5-manager; Model D-7000). For the separation of conjugated fatty acid containing fraction obtained by $AgNO_3$-silicic acid column chromatography, a preparative HPLC was carried out. The HPLC conditions were the same as those mentioned in the analytical HPLC except that the column size was 250 x 8.0 mm i.d. and solvent flow was 3.0 mL/min.

Fractionation and identification of conjugated fatty acids in purified soybean oil. Conjugated fatty acid methyl esters were concentrated initially by argentation column chromatography. The soybean oil methyl esters (*ca*. 20g) was refined on a 20% $AgNO_3$-silicic acid column (50 cm x 4 cm i.d.) by eluting with *n*-hexane (500 mL) and a mixture of diethyl ether/*n*-hexane solution (2:98 (500 mL), 5:95 (500 mL), 10:90 (500 mL), 20:80 (500 mL), 30:70 (500 mL), and 40:60 (500 mL), v/v). Fatty acid profile of each fraction was monitored by an analytical HPLC. Most of conjugated fatty acids were eluted with diethyl ether/*n*-hexane 5:95 (vol/vol), although this fraction (>1g)

mainly consisted of oleic acid and linoleic acid. Preparative HPLC was used for the fractionation of conjugated fatty acid containing fraction eluted with diethyl ether/*n*-hexane solution (5:95, vol/vol). The fraction separated by the reversed-phase HPLC still contained non-conjugated fatty acids such as saturated fatty acids, oleic acid, and linoleic acid. The identification of conjugated fatty acid in the fraction was, therefore, carried out using gas chromatography-mass spectrometry (GC-MS) after conversion of the methyl esters to dimethyloxazoline (DMOX) derivatives (16). GC-MS was performed using a Hewlett-Packard HPG1800A instrument (Hewlett-Packard Co., Palo Alto, CA) under the following conditions: Omegawax-250 column (Supelco, Inc., Bellefonte, PA; 30 m x 0.25 mm i.d.); helium carrier gas (40 mL/min); injector 230°C; detector 240°C; column 198°C. Conjugated fatty acid isomers in the fraction separated were also characterized by comparing with the reference CLA and CLN by HPLC with similar conditions as mentioned in the analysis of conjugated fatty acids.

Quantitative HPLC analysis of CLN content in vegetable oils. A known amount (100-1000 μg) of standard CLN methyl esters (9c,11t,13t; 9t,11t,13t; 9t,11t,13t; 8t,10t,12c) was diluted in 1 mL *n*-hexane solution containing 500 μg *n*-butyl benzoate as an internal standard. Ten μL of the solution was injected to HPLC system equipped with an ODS column (Cosmosil 5C18-AR, 4.6 x 150 mm i.d., 5 μm particle size, Nacalai Tesque) connected to a guard column (Develosis ODS-UG, 4.0 x 10 mm, 5 μm particle size, Nomura Chem. Co.). The analysis was carried out at 20°C using methanol/water (85:15, vol/vol) at a flow rate of 1.0 mL/min as the mobile phase. Peaks were monitored with a Hitachi L-7400 UV detector set at 275 nm. HPLC was carried out in a Hitachi L-7000 system as mentioned before in this section. A calibration curve for each CLN isomer was made from the peak ratio of the CLN to internal standard and

an amount of CLN injected. For the quantification of CLN in vegetable oil, methyl esters of the oil (*ca*. 5.0 mg) was dissolved in 1 mL *n*-hexane solution containing 500 μg internal standard, and then 10 μL of the solution was injected to HPLC. CLN in the oil was quantified using calibration curves for CLN isomers. The analysis was duplicated and there was little difference in the CLN content for each determination.

RESULTS AND DISCUSSION

The major fatty acids of purified soybean oil from Nisshin Oillio were linoleic acid (18:2n-6; 52.1%), oleic acid (18:1n-9; 23.3%), palmitic acid (16:0; 11.2%), α-linolenic acid (18:3n-3; 5.2%), stearic acid (18:0; 3.8%), and 18:1n-7 (2.1%). *Trans* isomers of linoleic acid (<1.0%) and α-linolenic acid (<1.0%) were also detected by GC analysis. HPLC chromatogram of the soybean oil methyl esters (Fig. 1) indicates the presence of unsaturated fatty acids (18:2n-6, 18:3n-3, 18:1n-9) as shown by UV detection at 210 nm. Two peaks near 18:2n-6 and 18:3n-3 could be due to the presence of their *trans* isomers. Saturated fatty acids were not observed as indicated by UV detection due to the absence of double bond in the molecule. Some other peaks were observed through UV detection at 233 nm or 274 nm. These peaks showed maximum absorption at 229-240 nm and 266-270 nm, respectively, on the UV spectrum as indicated by photodiode-array detection. The absorbance spectrum of these two peaks was almost identical to that of CLA and CLN standards. HPLC chromatogram of the soybean oil methyl esters fraction obtained by elution with diethyl ether/*n*-hexane (5:95, vol/vol) on $AgNO_3$-silicic acid column is presented in Fig. 2. Peaks showing maximum absorption around 233 nm and 275 nm could be concentrated in this fraction, although GC analysis revealed 18:2n-6, 18:1n-9, 18:0, and 16:0 to be the main fatty acids of this fraction. Most of linoleic acid

and α-linolenic acid were eluted with diethyl ether/*n*-hexane solution (10:90 and 20:80, v/v) and with diethyl ether/*n*-hexane solution (30:70 and 40:60, v/v), respectively. As shown in Fig. 2, the conjugated fatty acid concentrated-fraction was further fractionated by reversed-phase HPLC to F_1 – F_5. These HPLC fractions still contained non-conjugated fatty acid esters. Therefore, the structural analysis of conjugated fatty acid of HPLC fraction (F_1-F_5) was done by GC-MS after conversion of methyl esters to DMOX derivatives and by comparison with authentic CLA and CLN isomers on HPLC (16,17).

GC-MS analysis of DMOX derivatives of F_1 showed the peaks which gave a parent ion at *m*/*z* 331 and characteristic fragmentation ions at 260 and 248, 220 and 208, 194 and 182, showing that the double bonds were located at 8-9, 10-11, and 13-14 positions. GC-MS analysis also indicated presence of two another CLN isomers, 9,11,13-18:3 and 8,10,12-18:3, in both F_2 and F_4 fractions. Double bond positions could be identified by the characteristic loss of 12 daltons; 260 and 248, 234 and 222, 208 and 196 for 9,11,13-isomer, 246 and 234, 220 and 208, 194 and 182 for 8,10,12-isomer. Conjugated fatty acids in F_3 and F_5 were identified by GC-MS to be 9,11- and 10,12-18:2. The DMOX derivatives of the CLA isomers gave a characteristic loss of 12 daltons; 234 and 222, 208 and 196 for 9,11-18:2, 234 and 222, 208 and 196 for 10,12-18:2.

A comparison of F_2 and F_4 fractions with that of authentic CLN isomers on reversed-phase HPLC showed that CLN peaks in F_2 eluted on the same retention time as 9c,11t,13t-18:3 and 9t,11t,13c-18:3, while those in F_4 corresponded to that of 9t,11t,13t-18:3. Fatty acid with *cis* double bonds elutes faster than that with *trans* double bonds. CLN peak in F_2 possibly be consisting of either c,t,t- or t,t,c-isomers of 8,10,12- or 9,11,13-18:3. Similarly those in F_4 might be t,t,t-isomers of 8,10,12- or

9,11,13-18:3. Absorption maxima of CLN peaks in F_2 and F_4 were 268.8 nm and 266.6 nm, respectively. The wavelength and the spectrum of CLN in F_2 and F_4 were identical to those of 9c,11t,13t-18:3 or 9t,11t,13c-18:3 and 9t,11t,13t-18:3, respectively. Consequently, in the purified soybean oil 8,10,12- and 9,11,13-18:3 (c,t,t or t,t,c and t,t,t) were found as CLN isomers with conjugated trienes (Table 1). Geometrical isomers of 8,10,13-18:3 were found in the oil as CLN isomers with conjugated diene. CLA isomers detected in the purified soybean oil were c,t- or t,c- and t,t-isomers of 9,11- and 10,12-18:2.

HPLC chromatogram of crude soybean oil and processed soybean oil at different stages is shown in Fig. 3. Traces of 9,11- and 10,12-CLA isomers (c,t or t,c) were found in crude oil. Sources of these c,t- or t,c-CLA isomers are not clear. One possibility is that these might be forming during oil and meal separation processes involving heating. Another CLA isomers (t,t) appeared after deodorizing. Peak areas of c,t- or t,c-CLA isomers increased after deodorization. The presence of CLA in vegetable oils has been reported (18). The formation of conjugated fatty acids has been observed during heating and acid treatment of linoleate (19,20). The present study also showed the increase in CLA content during heat treatment involved in the deodorization step. CLN isomers with conjugated trienes were observed during bleaching, but not found after de-gumming and alkali refining (Fig. 3). However, CLN with conjugated diene (8,10,12-18:3) was detected after deodorization. Yurawecz et al. (6) have reported the presence of CLN (9,11,13-18:3 and 8,10,12-18:3) in edible oils. The HPLC analysis employing the photodiode-array detector in the present study gives the overall perspective of the formation of conjugated fatty acids during refining of soybean oil. Linoleate hydroperoxides would be the source for 9,11,13- and 8,10,12-CLN isomers.

CLN with conjugated diene (8,10,12-18:3) might be resulting from CLN with conjugated trienes. Only one positional isomer (8,10,13-18:3) could be found by GC-MS analysis of DMOX derivatives of F_1 (Fig. 2), although many peaks appeared on chromatogram of F_1. Further studies are required to identify the structure of conjugated dienoic acids in F_1.

We identified c,t,t- or t,t,c-CLN isomers (8,10,12 and 9,11,13) in F_2 and t,t,t-CLN isomers (8,10,12 and 9,11,13) in F_4 of purified soybean oil methyl esters (Fig. 1 and 2). The quantification of these CLN was carried out by quantitative HPLC using a calibration curve for CLN (9c,11t,13t-18:3; 9t,11t,13c-18:3; 9t,11t,13t-18:3; 8t,10t,12c-18:3). The calibration curve for 9t,11t,13c-18:3, 9c,11t,13t-18:3, and 8t,10t,12c-18:3 was almost the same, but slightly different from that for 9t,11t,13t-18:3, indicating that molecular extinction coefficient of CLN would be dependent on their number of trans configuration. Thus, contents of both types of geometrical isomers (c,t,t or t,t,c and t,t,t) found in soybean oil were determined by HPLC using calibration curves for CLN standard with c,t,t (or t,t,c) and t,t,t configurations.

Table 2 shows CLN contents in crude soybean oil and processed soybean oil. CLN could be hardly detected in crude soybean oil or in the oil after de-gumming and alkali refining; however, a significant amount of CLN (8,10,12 or 9,11,13) was found in soybean oil after bleaching. A little decrease in CLN after deodrization may be due to the isomerization of the CLN to CLN with conjugated dienes (8,10,13-18:3). CLN contents in purified soybean oil from different company in Japan are shown in Table 3. It varied from 387 mg/kg oil to 1316 mg/kg oil, which corresponds to 0.039 to 0.13 % (w/w). These values are agreement with the results of Yurawecz et al. (6). CLN content was affected by bleaching conditions (Table 4). Combinations of higher percentage of

bleaching earth and lower bleaching temperature resulted in reduced CLN content. Similar effects of bleaching temperature and earth combinations have been reported by Bosch (19,20). Table 5 shows CLN content in commercial vegetable oils in Japan. High levels of CLN were found in soybean and corn oils, while moderate levels were noticed in safflower, sesame, rapeseed oils. On the other hand, no CLN was detected in olive oil. The CLN level was strongly associated with linoleic acid content in these oils (Table 6), suggesting the possible source of 18:2n-6 for CLN (7,8). There were no difference in the content of linoleic acid between soybean oil and corn oil. Soybean oil contained 9.5 % 18:3n-3, while corn oil had less than 1%. 18:3n-3 is more easily oxidized than 18:2n-6. The oxidation products from 18:3n-3 can promote the oxidation of 18:2n-6 to produce linoleate hydroperoxides. Hence, CLN might possibly originate from linoleate hydroperoxides. The higher level of CLN in soybean oil as compared to corn oil might be due to the difference in content of 18:3n-3 among them.

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Legends to figures

FIG. 1. Analysis of purified soybean oil methyl esters by analytical HPLC with photodiode-array detector. Procedures for analytical HPLC are described in materials and methods.

FIG. 2. Analytical HPLC of conjugated fatty acid-concentrated fraction obtained by $AgNO_3$-silicic acid column chromatography. Procedures for analytical HPLC are described in materials and methods.

FIG. 3. Analytical HPLC of methyl esters from crude soybean oil (A) and soybean oil after degumming (B), alkali refining (C), bleaching (D), and deodorization (E). Procedures for analytical HPLC are described in materials and methods.

TABLE 1
Identification of Conjugated Fatty Acids of F_1 –F_5

Fraction	GC-MS analysis		Conjugation	Geometrical structure
	Fatty acid	Double bond position		
F_1	18:3	8,10,13	Conjugated diene	-
F_2	18:3	8,10,12 9,11,13	Conjugated triene	c,t,t or t,t,c
F_3	18:2	9,11 10,12	Conjugated diene	c,t or t,c
F_4	18:3	8,10,12 9,11,13	Conjugated triene	t,t,t
F_5	18:2	9,11 10,12	Conjugated diene	t,t

TABLE 2
Content of CLN (8,10,12 or 9,11,13) with Conjugated Triene at Different Stage of Soybean Oil Production

Soybean oil	CLN (mg/kg oil)		
	c,t,t or t,t,c	t,t,t	Total
Crude	ND	ND	ND
Degumming	10	ND	10
Alkali refining	20	ND	20
Bleaching	250	358	610
Deodorization	217	315	530

TABLE 3
CLN Content in Commercial Soybean Oil from Different Japanese Oil Company

Company	CLN (mg/kg oil)		
	c,t,t or t,t,c	t,t,t	Total
A	295	367	662
B	408	618	1026
C	198	230	427
D	753	559	1312
E	181	205	387
F	945	370	1316

TABLE 4
CLN Content of Soybean Oil Obtained by Different Bleaching Conditions

Bleaching condition		CLN (mg/kg oil)		
Activated earth	Temperature	c,t,t or t,t,c	t,t,t	Total
1.5%	110ºC	173	284	457
0.5%	110ºC	122	166	288
1.5%	50ºC	117	186	303
0.5%	50ºC	64	69	133
0%	110ºC	47	ND*	47

*Not detected.

TABLE 5
Content of CLN in Commercial Vegetable Oils

Oil	CLN (mg/kg oil)		
	c,t,t or t,t,c	t,t,t	Total
Soybean	343	423	766
Corn	263	391	654
Sesame	38	85	122
Rapeseed	34	45	79
Safflower	95	165	260
Olive	ND*	ND*	ND*

*Not detected.

TABLE 6
Fatty Acid Profile of Vegetable Oils

Fatty acid (wt%)	Soybean	Corn	Sesame	Rapeseed	Safflower	Olive
16:0	11.4	11.3	9.0	3.6	4.8	10.6
18:0	3.9	2.0	5.9	1.8	2.0	3.1
18:1n-9	18.9	29.8	38.5	70.6	77.1	77.0
18:2n-6	53.2	53.7	43.3	14.7	13.2	4.3
18:3n-3	9.5	0.8	0.3	1.9	0.3	0.6

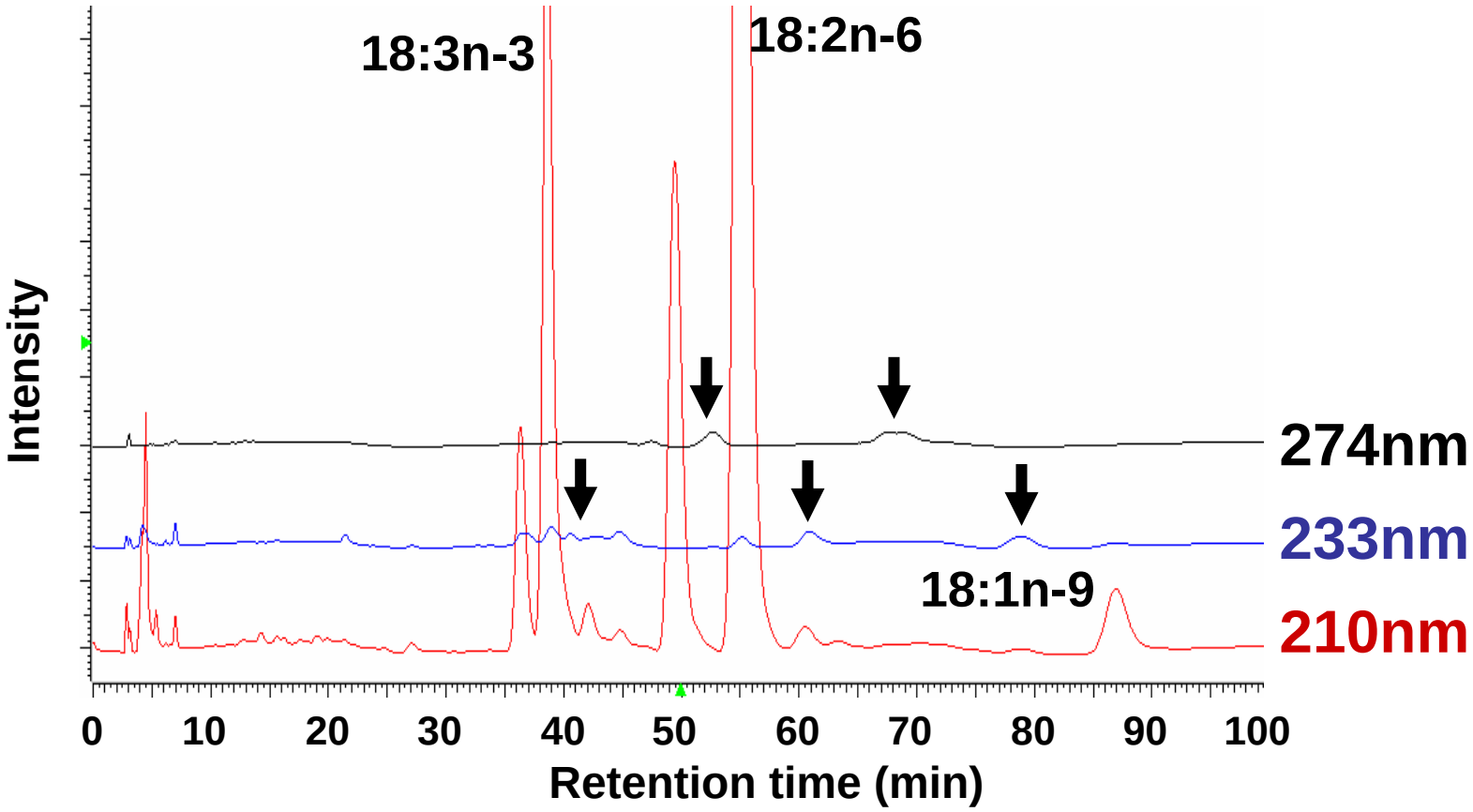

Fig. 1
Kinami et al.

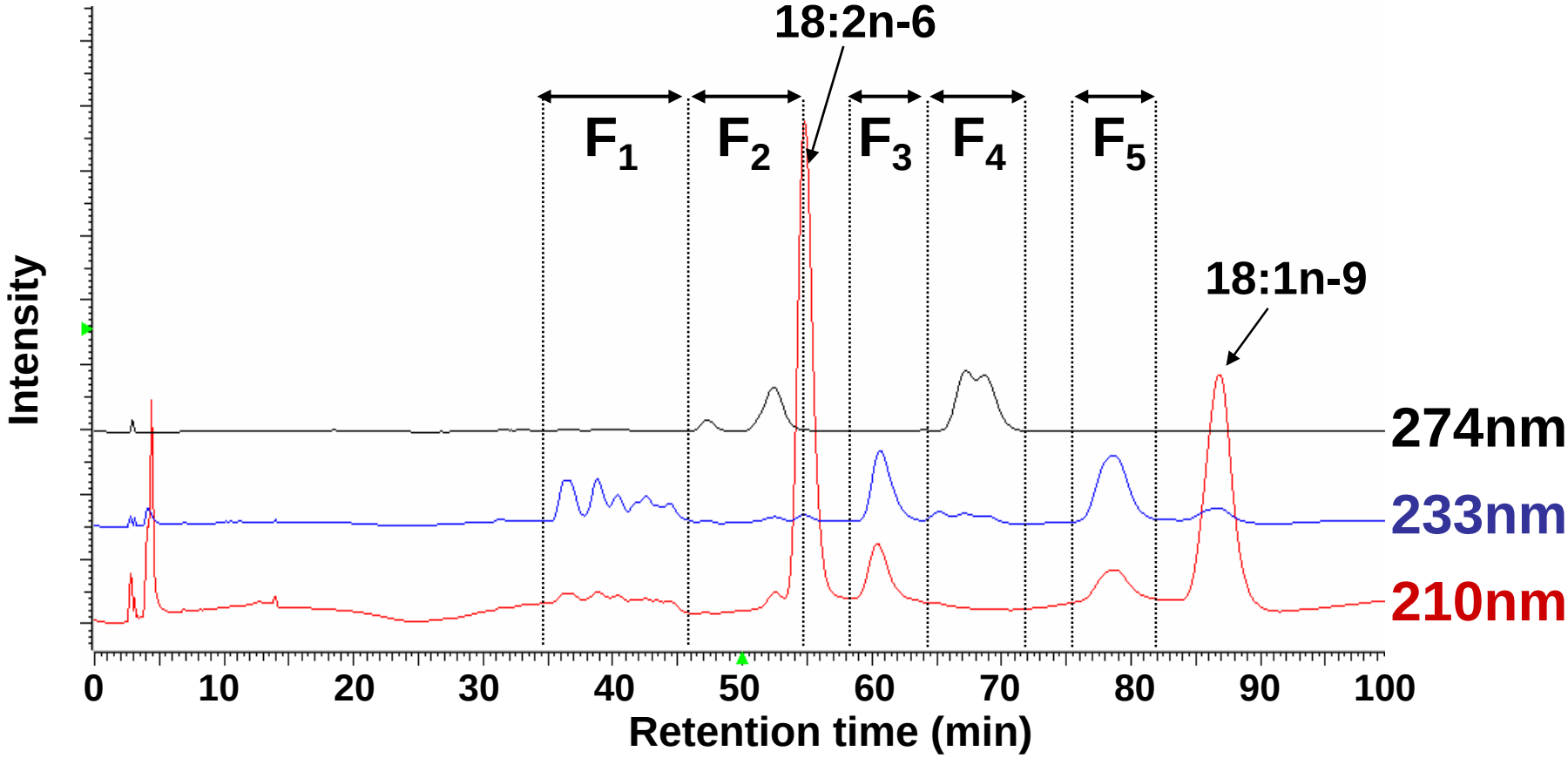

Fig. 2
Kinami et al.

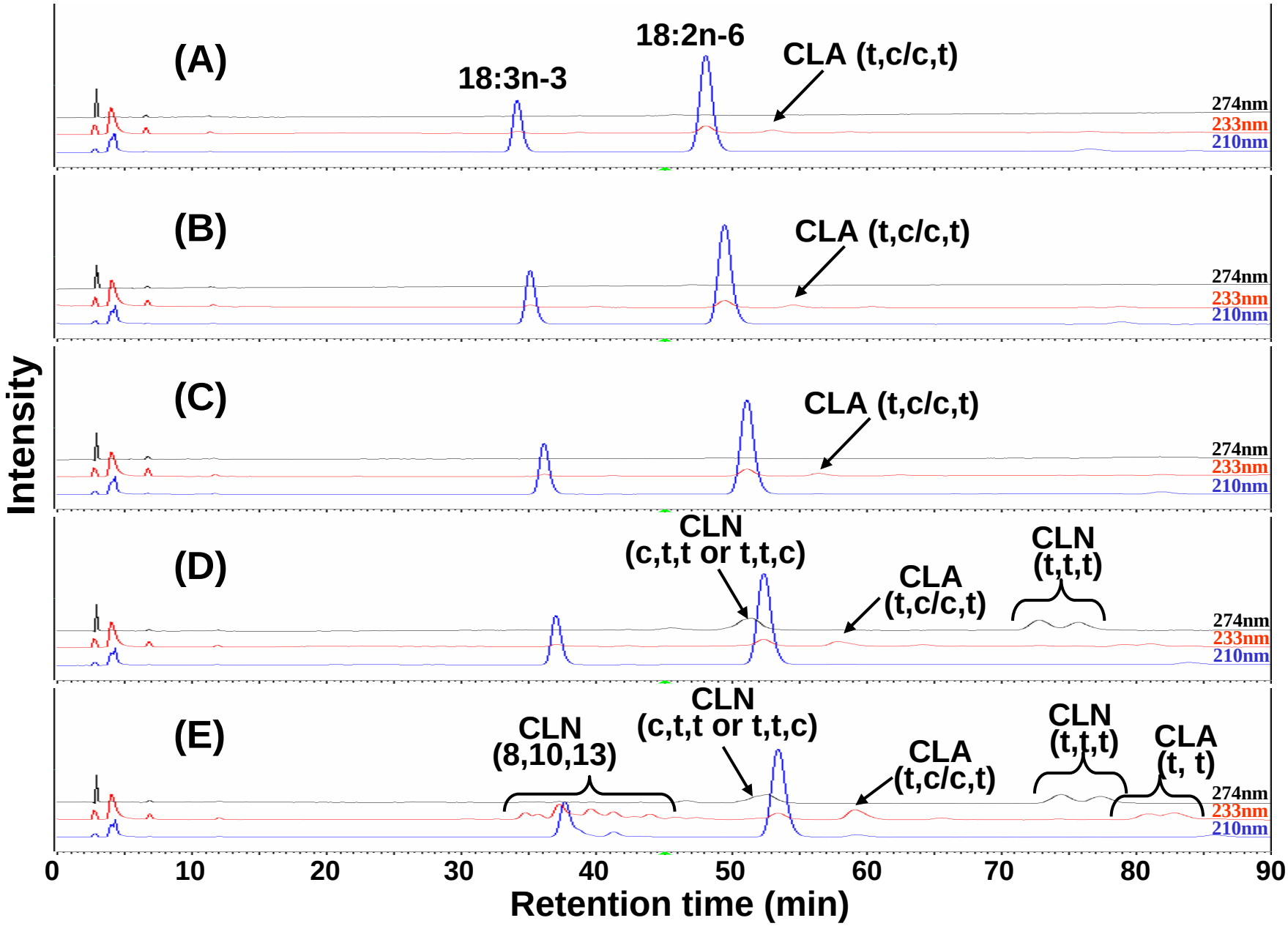

Fig. 3
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