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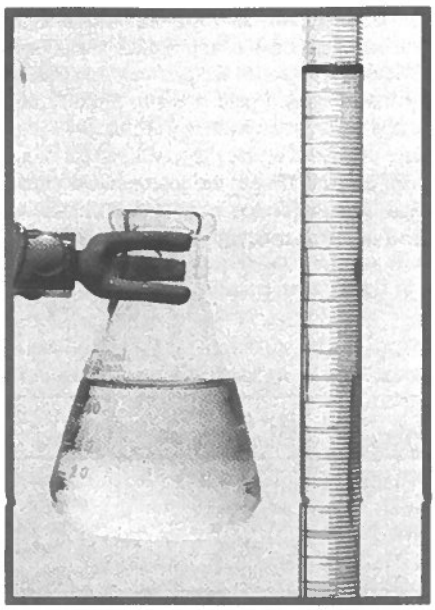

A New Method to Analyze Triacylglycerol Composition of Vegetable Oils[1]

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The ability to characterize the triacylglycerol composition of a potential fat or oil food product is integral to the formulation of a desirable end product. Reversed-phase high-performance liquid chromatography (RP-HPLC) with a flame ionization detector (4,9,10,13), refractive index (4), or evaporative light scattering detector (9) has become common for triacylglycerol analysis. These detectors provide satisfactory qualitative and quantitative composition information for triacylglycerols from a variety of sources. Quantification of triacylglycerol species using a flame ionization detector has two advantages over other two-dimensional detectors in that it is linear over a broad range without the need for response factors (4,10,13) and it is not adversely affected by gradient runs. Flame ionization detectors have proven satisfactory for triacylglycerol analyses in which chromatographic standards are available for the triacylglycerols or in which structures can be identified by theoretical carbon number (4,13) or equivalent carbon length. Unfortunately, however, these detectors do not allow characterization of unidentified triacylglycerol for which no standards are available, and they cannot differentiate between triacylglycerol species that are completely chromatographically overlapped. The failure to resolve overlapped peaks can cause triacylglycerol species to be overestimated, while others go unidentified.

Recently, we demonstrated that in the cases of normal seed oils (1,2,11,12), genetically modified seed oils (2), and synthetically useful seed oils (11,12) for which no commercial standards were available RP-HPLC coupled to a mass spectrometer through an atmospheric pressure chemical ionization (APCI) source could provide excellent qualitative identification of molecular species and differentiation, by mass, of completely overlapped species. Others have demonstrated similar successes with qualitative identification of triacylglycerol species in complex mixtures using APCI-mass spectrometry (MS) with silver-ion HPLC (5) or supercritical fluid chromatography (8). Furthermore, we have shown that response factors that allow quantification of triacylglycerol species (1–3) can be calculated easily. Quantification of triacylglycerols from data obtained by RP-HPLC/APCI-MS exhibits less error in calculated triacylglycerol compositions than corresponding flame ionization detector data compared with theoretically expected compositions. RP-HPLC/APCI-MS data also exhibit less error than HPLC flame ionization detector data on the fatty acid compositions calculated from the triacylglycerol compositions compared with the fatty acid compositions determined from fatty acid methyl esters analyzed by gas chromatography (GC) with flame ionization detection (FID).

In the present study, we use RP-HPLC/APCI-MS compositional analysis of triacylglycerol species in normal and randomized corn and soybean oils to illustrate an application of the new RP-HPLC/APCI-MS methodology.

Experimental Design

Materials. Commercially refined, bleached, and deodorized normal corn and soybean oils were obtained from P.V.O. Foods (Granite City, IL). All solvents were HPLC-grade or the highest available quality and used without further purification.

[1] Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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Randomization of Vegetable Oil Samples. Randomization in the presence of sodium methoxide as a catalyst was performed as previously described (6).

HPLC. For RP-HPLC/APCI-MS, a MS quaternary pump system with membrane degasser (LDC 4100, Thermo Separation Products, Shaumburg, IL) was used. Two 25 cm × 4.6 mm, 5 μm in series, columns were used (Inertsil ODS-80A, GL Sciences, Keystone Scientific, Bellefonte Park, PA). The gradient elution was 70% acetonitrile, 30% dichloromethane held for 40 min; to 65% acetonitrile, 35% dichloromethane at 45 min, held until 55 min; to 60% acetonitrile, 40% dichloromethane at 60 min, held until 70 min; to 55% acetonitrile, 45% dichloromethane at 80 min. The flow rate was 0.85 mL/min throughout. The column effluent was split so ≈720 μL/min went to an evaporative light-scattering detector and ≈130 μL/min went to the APCI interface. A 10-μL (25 μg/μL) sample in dichloromethane was injected.

MS. A quadrupole mass spectrometer (SSQ 710C, Finnigan MAT, San Jose, CA) fitted with an atmospheric pressure chemical ionization source was used to acquire mass spectral data. Conditions have been described previously (1).

GC. Fatty acid methyl esters were prepared by potassium hydroxide catalyzed transmethylation of the triacylglycerol mixtures (10). Fatty acid methyl esters were analyzed using calibrated GC-FID according to the procedure of Neff and coworkers (10). The sample solution (5 μL, 5 mg of sample per milliliter of hexane) was analyzed by direct-injection capillary GC. The capillary column (Supelco SP2380) was 30 m × 0.25 mm i.d. with 0.2-μm thick film. The gas chromatograph (Star model 3400, Varian, Inc., Walnut Creek, CA) was equipped with a flame ionization detector. The GC column was operated at a starting temperature of 150°C. The column was programmed to hold for 150°C for 35 min, then heat at 2 degrees Celsius per minute to 210°C, then to 220°C, and hold at 220°C for 5 min. The helium carrier gas had a column head pressure of 15 psi. The injector and detector were maintained at 240 and 280°C, respectively. The GC calibration mixture was a fatty acid methyl ester mixture (20 A, Nu-Chek Prep, Inc., Elysian, MN).

Response Factor Calculation. Response factors were calculated for the corn oil triacylglycerols by calculating the ratio of the fatty acid (FA) composition based on GC to the fatty acid composition calculated from the raw triacylglycerol composition obtained by APCI-MS:

$$r_{FA} = (FA\%_{GC\text{-}FID})/(FA\%_{APCI\text{-}MS})$$

The calculated response factor was normalized to one of the fatty acids set equal to 1.0, which was usually the fatty acid with the smallest area, by percent (unless it was present at a very low level, in which case the fatty acid with the smallest area over 1% was used). The final fatty acid response factor, R_{FA}, equaled the initial response factor divided by the smallest response factor:

$$R_{FA} = r_{FA}/r_{min}$$

Table I illustrates the response factor calculations for normal corn oil.

Triacylglycerol (TAG) response factors were calculated by multiplying the fatty acid response factors together:

$$R_{TAG} = R_{FA1} \times R_{FA2} \times R_{FA3}$$

For example, the triacylglycerol response factor for tristearin was 1.0000, while that of dioleoylpalmitoylglycerol was 1.9403. The fatty acid percent composition values were placed in an Excel (Microsoft Corp.) spreadsheet, and all resultant values were calculated using the appropriate equations. Calculated response factors were multiplied by the integrated peak areas to produce adjusted peak areas, which were used to produce an adjusted triacylglycerol percent composition. Rounding to the appropriate decimal place was performed at the end to obtain the final adjusted triacylglycerol composition.

Triacylglycerol Quantitation Method

Triacylglycerol compositions based on area, by percent, were calculated from RP-HPLC/APCI-MS data for normal and randomized corn to illustrate the procedure for a nonlinolenic acid oil and for normal and randomized soybean oil to illustrate the application of the procedure to a more complex oil that contains linolenic acid. A RP-HPLC/APCI-MS chromatogram of soybean oil is used to illustrate the identification and elution of individual triacylglycerol species (Fig. 1). The chromatogram shows the results for soybean oil to which cottonseed stearine was added at a ratio of 80% oil to 20% stearine to form a blend. The peak labels identify individual triacylglycerol molecular species. Palmitic, palmoleic, stearic, linoleic, oleic, and linolenic fatty acids are triacylglycerol molecular species. Stearine was added to emphasize the triacylglycerols dipalmitoylstearoyl (PPS), distearoylpalmitoyl (SSP), and tristearoyl (SSS, tristearin) glycerols, which normally occur in low amounts in soybean oil. Figure 1B shows the RP-HPLC/APCI-MS chromatogram. Figure 1A shows the same sample run using RP-HPLC coupled to a HPLC flame ionization detector. Comparison of Fig. 1A and B shows the ability of RP-HPLC/APCI-MS to resolve and identify coeluted and partially resolved triacylglycerol molecular species. This identification is not readily accomplished with the HPLC flame ionization detector, as observed in Fig. 1A. For APCI-MS data, the area for each triacylglycerol was obtained by the summation of the areas under all peaks of fragments arising from a particular triacylglycerol, plus the area under the mass of the protonated molecular ion. The amount of fragmentation in APCI-MS spectra strongly depends on the degree of unsaturation in the triacylglycerol. Because of this, quantitation of triacylglycerols by APCI-MS also depends on the degree of unsaturation in the triacylglycerol. Triacylglycerols that contain a high degree of unsaturation produce more molecular ions and provide less overall response, while those triacylglycerols that are more saturated produce mostly $[M\text{-}RCOO]^+$ fragments and larger peak areas. The saturates tend to be overrepresented in percent compositions, while the unsaturates tend to be underrepresented. To solve this, a method for calculating the response factors for triacylglycerols determined by APCI-MS was developed. We previously showed that the method developed produced less error, compared with known compositions, than results of RP-HPLC-FID (2). This method of quantitative analysis was extended to the present study.

Table I. Response Factor Calculations for Normal Corn Oil Triacylglycerol (TAG)[a]

TAG Fatty Acid	Raw APCI-MS (% Composition)	GC (% Composition)	Ratio[b] (r_{FA})	Response Factor (R_{FA})
Palmitic	15.61	11.69	0.7489	1.1178
Linolenic	1.11	0.89	0.8018	1.1967
Linoleic	51.73	60.19	1.1635	1.7366
Oleic	28.56	25.21	0.8827	1.3175
Stearic	3.00	2.01	0.6700	1.0000

[a] APCI-MS = atmospheric pressure chemical ionization mass spectometry; GC = gas chromatography.
[b] Minimum is 0.670.

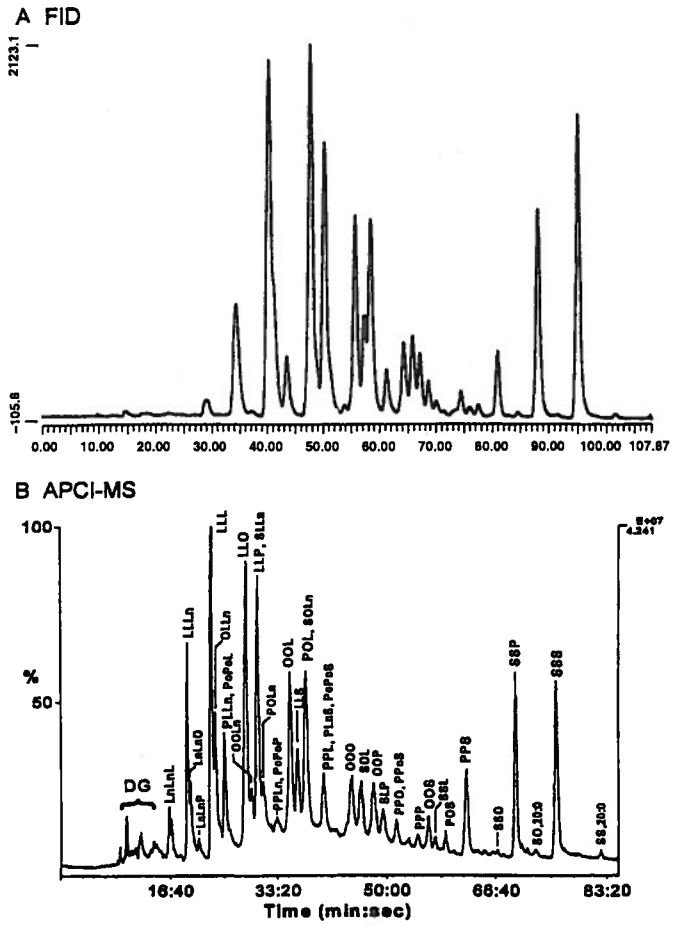

Fig. 1. A, High-performance liquid chromatography-flame ionization detection and B, high-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry chromatograms of a soybean oil-cottonseed stearine blend (80/20%, by weight). Peak labels identify individual triacylglycerol molecular species. P, Ln, L, O, and S = palmitic, linolenic, linoleic, oleic, and stearic fatty acids of triacylglycerol, respectively. Analytical conditions described in text.

Triacylglycerol compositions obtained by RP-HPLC/APCI-MS are presented in Table II for normal and randomized corn oil and soybean oil. Table III lists the fatty acid compositions calculated from each of the triacylglycerol compositions, as well as the fatty acid composition determined as fatty acid methyl esters using calibrated GC-FID. The full set of triacylglycerol response factors applied to the raw APCI-MS data resulted in an adjusted area, by percent (Table II). The fatty acid composition calculated from the triacylglycerol composition determined using APCI-MS (Table II) is given in Table III.

The calculated fatty acid composition data exhibited a low average absolute error of 0.4% for normal corn oil, 0.1% for randomized corn oil, 0.5% for normal soybean oil, and 0.3% for randomized soybean oil with respect to the fatty acid composition determined experimentally by calibrated GC-FID of the same oil sample after transmethylation to the fatty acid methyl esters. This proves good triacylglycerol quantitation is possible even though, as observed in Figure 1B, some triacylglycerols occur only as shoulders on larger peaks for linolenic acid oils in the RP-HPLC/APCI-MS chromatogram, such as linolenoyllinoleoyloleoylglycerol as a shoulder on trilinolein, dioleoyllinolenoylglycerol as a shoulder on dilinoleoyloleoylglycerol, and palmitoyloleoyllinolenoylglycerol as a shoulder on dilinoleoylpalmitoylglycerol. Their peak areas include the area attributable to the overlapping triacylglycerols. As the

APCI-MS results show, these species are easily differentiated by the masses of their different fragments, so the triacylglycerol compositions by APCI-MS are close to the predicted compositions for these samples.

The average absolute error of the area, by percent, of normal and randomized soybean oils (Table III) obtained by APCI-MS was 0.5, and 0.3%, respectively, compared with the respective statistically predicted compositions. This comparison with the statistically predicted composition cannot be made for normal oil or nonrandomized oils because the fatty acid in these oils are not expected to be completely randomly distributed.

The small error associated with the APCI-MS data resulted largely from the ability of the MS data to differentiate chromatographically overlapped triacylglycerol species. Many species can be conclusively identified and readily quantified in amounts as low as 0.01%.

Table II. Triacylglycerol (TAG) Composition (%) of Corn and Soybean Oils by Reversed-Phase High-Performance Liquid Chromatography with Mass Spectrometric (HPLC-MS) Detection[a]

TAG[b]	Corn Oil	Randomized Corn Oil		Soybean Oil	Randomized Soybean Oil	
Fatty Acid	MS[c]	MS	PRE[d]	MS	MS	PRE
LLO	21.5	22.9	27.2	17.7	19.9	20.6
LLL	25.4	22.3	20.1	13.6	13.2	14.7
LLP	14.7	14.2	12.5	11.8	10.4	9.7
OOL	10.7	12.4	12.3	8.5	8.8	9.6
PLO	10.0	10.0	11.2	8.0	9.3	9.1
LLLn	1.2	0.9	0.8	6.7	6.8	5.9
LnLO	0.9	0.9	0.7	6.3	5.8	5.5
LLS	2.2	1.8	2.2	4.9	4.0	3.3
LOS	1.8	2.1	2.0	2.9	2.9	3.1
PLnL	0.5	0.4	0.3	2.5	2.3	2.6
PPL	2.5	2.6	2.6	2.4	2.4	2.2
OOP	2.9	3.1	2.5	2.5	2.2	2.1
OOO	2.8	2.9	1.8	2.5	1.8	1.5
PLS	0.8	0.9	0.9	1.6	1.5	1.5
OOLn	0.1	0.1	0.2	1.2	1.3	1.3
PLnO	0.1	0.1	0.2	1.5	1.3	1.2
PPO	0.9	1.1	1.2	0.6	1.0	1.0
LnLS	0.1	0.1	0.1	0.8	0.8	0.9
LnLnL	0.0	0.0	0.0	0.8	0.8	0.8
OOS	0.6	0.6	0.4	1.0	0.8	0.7
POS	0.3	0.4	0.4	0.5	0.6	0.7
LnOS	0.0	0.0	0.0	0.4	0.4	0.4
LnLnO	0.0	0.0	0.0	0.4	0.4	0.4
PPLn	0.0	0.0	0.0	0.2	0.2	0.3
SSL	0.1	0.1	0.1	0.4	0.3	0.2
PLnS	0.0	0.0	0.0	0.1	0.1	0.2
LnLnP	0.0	0.0	0.0	0.1	0.1	0.2
PPS	0.0	0.1	0.1	0.0	0.1	0.2
PPP	0.0	0.1	0.2	0.0	0.1	0.2
SSO	0.0	0.1	0.0	0.1	0.1	0.1
LnLnS	0.0	0.0	0.0	0.1	0.0	0.1
SSP	0.0	0.0	0.0	0.0	0.0	0.1
SSLn	0.0	0.0	0.0	0.0	0.0	0.0
SSS	0.0	0.0	0.0	0.0	0.0	0.0
Sum[e]	100.1	100.2	100.0	100.1	99.7	100.4

[a] Analytical conditions described in text.
[b] TAG fatty acids: Ln, L, O, S, and P = linolenic, linoleic, oleic, stearic, and palmitic, respectively.
[c] HPLC-MS data has been adjusted using response factors calculated using gas chromatography-flame ionization detector data.
[d] Predicted TAG composition from statistical distribution of fatty acids among glycerol moiety carbons during chemical randomization or interesterification.
[e] Calculated at more decimal places, difference from 100% represents the sum of rounding errors.

Conclusions

The physical properties of food formulation products depend in part on triacylglycerol composition. As a result, it is important to have good analytical procedures with which to analyze the triacylglycerol compositions. Also, to ensure quality control a triacylglycerol analysis should be performed on vegetable oils used in formulations. The adequacy of the triacylglycerol composition analysis can be determined by calculating the fatty acid compositions from the triacylglycerol compositions obtained by RP-HPLC/APCI-MS and comparing them with the fatty acid compositions obtained experimentally from calibrated GC-FID analysis of the methyl esters of the same oil sample. Fatty acid analysis by GC-FID is considered very reliable because each fatty acid is referenced to a standard mixture of fatty acid whose composition is known by weight.

Knowledge of accurate triacylglycerol composition is important for understanding which triacylglycerol composition is responsible for important physical properties of food formulation products, such as margarines. For example, lubricity or mouthfeel of a food product (i.e., how well the food product melts in the mouth to give a pleasant, cooling effect) is an important physical property related to triacylglycerol species and quantity (7). Triacylglycerols that are disaturated or trisaturated are most important for this effect. Normal corn, soybean, and canola oil blends with cottonseed and soybean oil stearine hard stocks have high concentrations of trisaturated triacylglycerols, such as dipalmitoylstearoylglycerol, distearoylpalmitoylglycerol, and tristearin. The interesterified blends have higher concentrations of di- and trisaturated triacylglycerol than the original liquid oils. Selection of products with these triacylglycerol types may assist development of margarines with good lubricity or mouthfeel. Similarly, spreadability, tub stability, and other important characteristics

Table III. Fatty Acid Composition (%) of Corn and Soybean Oils Obtained Experimentally by Gas Chromatography-Flame Ionization Detection (GC-FID) and Calculated from Triacylglycerol (TAG) Composition[a]

TAG	Corn Oil			Randomized Corn Oil			Soybean Oil			Randomized Soybean Oil		
Fatty Acid	MS	AE[b]	GC	MS	AE	GC	MS	AE	GC	MS	AE	GC
Palmitic	12.0	0.3	11.7	12.3	0.2	12.1	11.6	0.7	10.9	11.9	0.3	11.6
Linolenic	0.9	0.0	0.9	0.8	0.0	0.8	7.5	0.2	7.3	7.3	0.3	7.0
Linoleic	60.9	0.7	60.2	58.7	0.1	58.6	52.3	0.7	53.0	52.2	0.5	52.7
Oleic	24.1	1.1	25.2	26.1	0.3	26.4	24.1	0.7	24.8	24.5	0.1	24.6
Stearic	2.0	0.0	2.0	2.1	0.0	2.1	4.4	0.3	4.1	4.1	0.2	3.9
AAE[c]		0.4			0.1			0.5			0.3	

[a] Experimental conditions described in text. Triacylglycerol composition listed for high-performance liquid chromatography with mass spectrometry (HPLC-MS) in Table II.
[b] Absolute error = absolute value of the error compared with GC-FID for each fatty acid.
[c] Average absolute error = sum of the absolute error compared with GC-FID for each fatty acid divided by the number of fatty acids ($n = 5$).

depend on the presence of specific triacylglycerol species. The effort to link texture and flavor characteristics with structural properties is ongoing, and the ability to produce a good estimate of a product's triacylglycerol composition is central to gaining a better understanding of the relationship between triacylglycerol form and function.

The RP-HPLC/APCI-MS method studied here is now available for accurate triacylglycerol analysis to provide necessary information for more thorough characterization of oil products than ever before.

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Gary R. List

Gary R. List is a lead scientist at the National Center for Agricultural Utilization Research in Peoria, IL. his career in fats and oils research spans 37 years. He has published more than 200 technical publications and abstracts. He has contributed to numerous books on fats and oils, including two editions *of Bailey's Industrial Oil and Fat Products*. He is a member of the American Oil Chemists' Society (AOCS) is currently serving as an associate editor for the *Journal of the American Oil Chemists' Society*. His research interests include analytical method development, edible oil processing, oil refining, hydrogenation, interesterification, lecithin, supercritical fluids, and structurally modified oilseed crops. In 1999 he was awarded the Alton E. Bailey Medal by AOCS and has received numerous honors and awards for his research.

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