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Short Communication

Resolution of lipolysis mixtures from soybean oil by a solid-phase extraction procedure

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ABSTRACT

A solid-phase extraction procedure was developed to rapidly resolve the lipolysis mixtures of free fatty acids, mono- and diacylglycerols from lipase (E.C. 3.1.1.3) cleavage of soybean oil triacylglycerols. Accuracy of the lipolysis procedure against standard mixtures of triacylglycerols showed coefficients of variance of 5% between known and observed fatty acid composition at glycerol carbon 2. An average coefficient of variance of 5% was obtained between results of the lipolysis procedure, fatty acid composition by gas chromatography, and the results by ^{13}C nuclear magnetic resonance stereospecific analysis for fatty acid composition at glycerol carbons 1,3 and 2 of intact triacylglycerol mixtures.

INTRODUCTION

Recently, we conducted a survey of the oxidative stabilities of the triacylglycerol (TAG) obtained from crude oils of many soybeen varieties [1]. Oxidative stability was correlated statistically with triacylglycerol structure or location of particular fatty acids on the glycerol primary and secondary carbons. This survey of many samples required a convenient and rapid TAG structural determination method.

Two methods are available for regiospecific analyses of vegetable oil TAG. One method involves ^{13}C nuclear magnetic resonance (^{13}C NMR) analyses of the chemical shifts of fatty acid carbonyl and olefinic carbons with glycerol carbon location of the acid [2–5]. The ^{13}C NMR procedures are equipment intensive, require lengthy analysis times and do not differentiate between saturated fatty acids on the glycerol moiety of the TAG.

The second method uses lipase (EC 3.1.1.3) directed cleavage of fatty acids at the glycerol 1,3 car-

bons [6–19]. The lipolysis procedures involve treatment of the TAG with lipase in Trizma buffer pH 8.0, bile salts and calcium chloride at 37–40°C [6] or by streaking the TAG solution over a band of lipase solution in Trizma impregnated on a thin-layer chromatography (TLC) plate [7,17]. These procedures give mixtures of free fatty acids (FFAs), 2-mono acylglycerol (MAG), 1,2(2,3)-di-acylglycerol (DAG) and unreacted TAG. Separate procedures of lipolysis have involved TLC [6,7,12,17–19,27], low-pressure liquid chromatography [8,13]; and high-performance liquid chromatography (HPLC) [20,21] and gas chromatography (GC) [22–24].

We report here a simple separation procedure of lipolysis products on a commercially available silica solid-phase extraction cartridge. Further, accuracy of the procedure is compared to lipolysis of standard mixtures of TAG (by weight) and to ^{13}C NMR regiospecific analysis of intact soybean oil TAG.

EXPERIMENTAL

Materials[a]

Soybeans (Glycine Max (L) Merr.) were commercial cultivars or plant introductions (PI). All solvents were HPLC grade. The solid-phase extraction columns (6.5 ml volume, loaded 2.0 g silica) used for removal of non-TAG components from crude soybean oils were purchased from Baxter Health Care (Muskegon, MI, USA). The solid-phase extraction columns (Bond Elut, 3 ml volume, loaded 0.2 g silica) used for resolution of lipolysis mixtures were purchased from Analytichem (Harbor City, CA, USA). Pancreatic lipase (EC 3.1.1.3, type 2, crude from porcine pancreas, activity 220 units/mg protein with olive oil at pH 7.7) and bile salts or sodium cholate were purchased from Sigma (St. Louis, MO, USA). TLC plates (7.5 × 2.5 cm, 250-μm layer of silica gel A, UV 254 nm indicator) were obtained from Whatman (Fairfield, NJ, USA). The GC direct injection column (182.9 × 0.3 cm I.D.) was packed with 1.8 g of 10% SP 2330 on 100/120 mesh Chromosorb W AW, obtained from Supelco (Bellefonte, PA, USA). Standard TAG and GC reference standard mixture 15A were purchased from NuChek Prep (Elysian, MN, USA).

Crude soybean oils (1.2–2.0 g) were obtained by sonification of ground soybeans with hexane for 5 min. Non-TAG components were removed from the crude oil by mixing with activated carbon (weight of carbon equal to 35% of oil weight) in hexane. The oil–carbon mixture was transferred to a 2.0-g silica solid-phase extraction cartridge. Elution of the oil components was as follows: 1.5 ml hexane, fraction 1, non-polar components; 15 ml of diethyl ether–hexane (10:90, v/v), fraction 2, TAG; and 15 ml methanol, fraction 3, polar components. Purity of the TAG fraction was quickly evaluated using TLC with diethyl ether–hexane (20:80, v/v) eluens and visualization by I_2 or sulfuric/chromic acid charring. Confirmation of the TAG purity was by reversed-phase HPLC with UV detection at appropriate wavelengths [1]. Fatty acid composition of the crude and chromatographed oils obtained by GC analysis [1] was within 5% coefficient of variance [25]. Oxidation level of the chromatographed oil was zero as confirmed by analytical reversed-phase HPLC with UV detection at 232 nm, as previously described [23,24].

Procedure

For statistical studies of oxidative stability, the oil was extracted in duplicate from each soybean variety and chromatographed as described above to recover TAG. Duplicate samples from each TAG fraction were lipolyzed by a modification of the procedure of Awl *et al.* [20] for regiospecific analysis. Briefly, 30-mg samples of TAG in 2 ml Trizma buffer (pH 8), containing 70 μl of 0.1% (w/w) calcium cholate and 120 μl of 22% (w/w) calcium chloride in water were vortexed. Lipase (30 mg) was added to the solution. The mixture (in a screw cap test tube) was incubated at 37°C with shaking using an orbital shaker for 10 min. The reaction mixture was extracted with diethyl ether. The ether extracts were washed with distilled water, dried over sodium sulfate, filtered and evaporated, using a stream of nitrogen at 30°C. The lipolysis reaction was 90–98% complete, as estimated, by TLC [diethyl ether–hexane–acetic acid (50:50:1, v/v/v) eluent], when compared to the starting material before lipolysis. The lipolysis products (15–20 mg) were dissolved in

[a] The mention of firm names or trade products does not imply that they are endorsed or recommended by the US Department of Agriculture over other firms or similar products not mentioned.

0.5 ml hexane and transferred to the top of the 0.2-g solid-phase extraction cartridge. Four fractions were eluted from the solid-phase extraction cartridge, as follows (weight applied: 16.1 mg): 5.0 ml diethyl ether–hexane (10:90, v/v), fraction 1, unreacted TAG 0.5 mg; 18:0 ml diethyl ether–hexane (10:90, v/v), fraction 2, fatty acids cleaved from glycerol carbons 1,3, 7.5 mg; 6 ml diethyl ether–hexane–acetic acid (50:50:1, v/v/v), fraction 3, 1,2(2,3)-DAG, incomplete lipolysis products, 2.0 mg; and 4 ml methanol, fraction 4, MAG, complete lipolysis products, 4.5 mg. Completion of the lipolysis was 97% based on wieght of unreacted TAG. Purity and identification of each fraction was determined by TLC against TAG, FFA, DAG and MAG standards. For GC analysis, the MAG and DAG were transmethylated by 0.5 *M* potassium hydroxide methanol at 50°C. GC analysis of each transmethylated sample was performed in triplicate using a Hewlett-Packard Model 5700 gas chromatograph equipped with flame ionization detectors (Avondale, PA, USA). The packed column was operated at 160°C with a helium carrier gas flow-rate of 20 ml/min. Sample injection was at 200°C with a 1-μl sample [0.5% (w/v) solute in diethyl ether]. Methyl ester identification and quantitation was calibrated against NuChek Prep soybean methyl ester standard 15-A. GC peaks were integrated by computer [26]. GC analysis of the transmethylated MAG fraction gave the average fatty acid composition at the 2 position of the TAG. These data plus GC analysis of the transmethylated TAG allowed the

TABLE I

REGIOSPECIFIC ANALYSIS OF CHROMATOGRAPHED SOYBEAN OILS BY LIPOLYSIS

Soybean variety	Glycerol acyl position	Fatty acid composition Area %					Average C.V.[a]
		16:0	18:0	18:1	18:2	18:3	
PI A-16	1,2,3[b]	10.2	4.8	27.0	54.8	3.2	
(Low linolenic acid)	2[b,c]			27.4	69.7	2.9	
	1,2(2,3)[b]	7.5	3.6	25.7	60.0	3.2	
	1,2(2,3)[d]	7.8	3.7	27.2	58.3	3.1	2.6
	1,3[e]	15.1	7.1	26.9	47.5	3.4	
PI 506.722B	1,2,3[b]	9.7	2.7	19.5	59.4	8.7	
(Low oleic acid, high linoleic acid	2[b,c]			19.2	73.6	7.2	
and linolenic acid)	1,2(2,3)[b]	7.0	2.0	17.8	64.1	9.1	
	1,2(2,3)[d]	7.4	2.1	19.4	62.7	8.4	4.1
	1,3[e]	14.4	4.1	19.7	52.3	9.5	
PI 507.319	1,2,3[b]	10.8	4.0	30.7	47.9	6.7	
(Low linoleic acid and	2[b,c]			30.1	63.8	6.1	
high oleic acid)	1,2(2,3)[b]	8.1	2.8	29.3	52.9	6.8	
	1,2(2,3)[d]	8.2	3.0	30.6	51.7	6.6	2.5
	1,3[e]	16.0	6.0	31.0	40.3	7.0	
Century-84							
Average	1,2,3[d]	10.7	3.5	22.8	56.0	7.0	
composition	2[b,c]			23.0	70.2	6.8	
	1,2(2,3)[b]	7.5	2.5	24.0	59.5	6.5	
	1,2(2,3)[d]	8.0	2.6	22.4	60.0	7.0	3.1
	1,3[e]	15.8	5.2	22.9	48.9	7.2	

[a] Coefficient of variance [25] between experimental and calculated 1,2(2,3)-diacylglycerol positions.

[b] Experimentally determined by GC analysis in triplicate. Standard deviation for average of four lipolysis samples per variety ± 0.1–0.6.

[c] Values for saturated acids less than 1%.

[d] Values calculated by [3 (area % triacyl) + (area % monoacyl)]/4 [17].

[e] Values calculated by [3 (area % triacyl) − (area % monoacyl)]/2 [17].

TABLE II

COMPOSITIONAL ANALYSIS OF 2-MONOACYL GLYCEROLS OBTAINED BY LIPOLYSIS FROM SYNTHETIC TRAICYLGLYCEROL MIXTURES

Synthetic triacyl mixture	Fatty acid composition					Average C.V.[a]
	16:0	18:0	18:1	18:2	18:3	
1	21.8[b]	19.3	21.5	19.5	17.9	
	19.6[c]	19.9	21.9	20.5	17.1	3.1
2	5.5[b]	8.4	32.5	29.1	24.5	
	5.4[c]	7.0	33.3	28.6	25.7	5.2
3	9.7[b]	2.1	18.9	51.9	17.4	
	9.2[c]	2.2	18.1	54.7	15.8	4.1
4	11.7[b]	8.4	29.4	46.7	3.8	
	11.1[c]	8.7	30.3	45.6	4.3	3.7

[a] Coefficient of variance [25].
[b] Weight percent fatty acid composition of triacylglycerol mixtures. Mixtures prepared by weight from trihexadecanoin, trioctadecanoin, trioctadecenoin, trioctadecadienoin and trioctadecatrienoin.
[c] Area percent fatty acid composition on carbon-2 of triacylglycerol synthetic mixtures after lipolysis. GC analysis of fatty acid methyl esters in triplicate.

calculation of the average fatty acid composition at the 1,3 positions of the TAG [17]. To determine if the fatty acids of the MAG were representative of the acids at the 2 position of the intact TAG, fatty acid composition determined by GC for the DAG was compared with that obtained by calculation [17]. For representative samples, these fatty acid values should agree within an average co-efficient of vairance (C.V.) [25] of 5% or less for the data to be used in the study of oxidative stability [1]. Accuracy of the above regiospecific method was compared against lipolysis of a standard mixture of TAG prepared by weight. Also, accuracy of the fatty acid composition by glycerol carbon location was compared against ^{13}C NMR regiospecific analysis on intact TAG [5].

RESULTS AND DISCUSSION

This regiospecific analysis procedure was developed for use in statistical studies to evaluate the effect on oxidative stability of unsaturated fatty

TABLE III

STEREOSPECIFIC ANALYSIS OF CHROMATOGRAPHED SOYBEAN OILS

Comparison of ^{13}C NMR to lipolysis (GC) analyses.

Oil	Carbon number		Fatty acid composition			
			16:0+18:0	18:1	18:2	18:3
1	2	NMR[a]	0.0	24.4	65.2	10.4
		GC[b]	0.0	24.6	64.3	11.1
		CV[c]		0.6	1.0	4.6
	1,3	NMR[a]	24.4	22.2	43.8	9.6
		Calculated[d]	24.6	22.9	43.6	8.9
		CV[c]	0.6	2.2	0.3	5.4
2	2	NMR[a]	0.0	31.9	63.5	4.6
		GC[b]	0.0	31.0	65.2	4.4
		CV[c]		2.0	1.9	3.1
	1,3	NMR[a]	27.2	29.3	39.3	4.2
		Calculated[a]	25.9	30.5	39.7	3.9
		CV[c]	3.5	2.8	0.7	5.2
3	2	NMR[a]	0.0	23.4	68.4	8.2
		GC[b]	0.0	22.3	70.0	7.7
		CV[c]		3.4	1.6	4.5
	1,3	NMR[a]	23.9	23.8	43.5	8.8
		Calculated[d]	22.7	24.7	44.4	8.2
		CV[c]	3.6	2.6	1.5	5.0
4	2	NMR[a]	0.0	23.5	68.1	8.4
		GC[b]	0.0	23.0	69.2	7.8
		CV[c]		1.5	1.1	5.4
	1,3	NMR[a]	24.0	24.3	43.2	8.4
		Calculated[d]	23.2	24.9	43.9	8.0
		CV[c]	2.4	1.7	1.1	3.4

[a] ^{13}C NMR analysis of oil [5].
[b] GC analysis of lipolyzed oil monoglycerides.
[c] Coefficient of variance [25].
[d] Fatty acid composition at 1,3 position calculated from GC analysis of lipolyzed oil monoglycerides.

acid location at the 1,3 and 2 positions of the TAG [1].

The regiospecific analysis of duplicate samples of each oil obtained by duplicate extraction of each soybean variety studied, required 11 h. The time phasing of the analysis was: lipolysis and sample work up: 2 h; solid-phase extraction and sample work up: 2 h; transmethylation of MAG and DAG: 1 h; triplicate GC analysis of samples: 6 h.

This method provided a standard deviation of ± 0.1–0.6 for samples, as shown in Table I for the regiospecific analysis for each of several soybean varieties. Fatty acid compositions of the TAG and of the MAG were experimentally determined. The average fatty acid distribution at the 1,3 positions was obtained by calculation [17].

The 1,2(2,3)-DAG fatty acid distributions were obtained experimentally and by calculation [17]. The observed agreement in fatty acid composition obtained by these two methods for the DAG indicates that the MAG and Dag compositions are representative of the composition of the original TAG [7,17]. Lipolysis analyses of the oil samples in Table I shows a C.V. range of 0.9 to 5.9%. For unsaturated fatty acids, the C.V. was usually less than 5%, which was satisfactory for oxidative stability studies.

The data presented in Table I shows that linoleic acid is in higher concentration at the 2 position, while oileic and linolenic acids have a higher concentration at the 1,3 positions. Also, the saturated fatty acids occur almost entirely at the 1,3 positions. These fatty acid distributions are in agreement with other investigations of soybean oil [18–19, 28–29].

Statistical studies on soybean oil stability with respect to fatty acid location require confidence in the accuracy of the solid-phase extraction lipolysis method. As presented in Table II, the average of the fatty acid composition obtaned by GC compared to weight percent, ranged from 3.1 to 5.2% for synthetic triacyl mixtures. Moreover, comparison of results obtained by an independent analytical method, ^{13}C NMR, for regiospecific analysis of intact TAG with those obtained by GC analysis of lipolysis products from the same TAG gave C.V. values of 0.6–3.4%, oleic; 1.0–1.9%, linoleic; and 3.1–5.4%, linolenic acids at the 2 position of TAG as shown in Table III. The calculated composition for the 1,3 positions of TAG, obtained from lipolysis of TAG and GC analysis of MAG, compared to ^{13}C NMR of intact TAG showed CV values of 0.6–3.6%, palmitic + stearic; 1.7–2.8%, oleic; 0.3–1.5%, linoleic; and 3.4–5.4%, linolenic acids.

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