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Influence of Specific Fatty Acids on the Asymmetric Distribution of Saturated Fatty Acids in Sunflower (*Helianthus annuus* L.) Triacylglycerols

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The 1,3-random-2-random theory was proposed several years ago to explain the fatty acid distribution in vegetable oil triacylglycerols. However, by demonstrating an asymmetry between positions *sn*-1 and *sn*-3 in olive oil, cocoa butter, sunflower oil, etc., a number of studies have shown that this theory does not hold true for some oils and fatty acids. Accordingly, the distribution of fatty acids in sunflower triacylglycerols has been studied, calculating the α coefficient of asymmetry in several combinations of standard linoleic, high-oleic, and high-stearic sunflower oils. The results obtained from the oils of these lines and from single seed oil samples indicate that the asymmetry for saturated fatty acids is greater in high-oleic than in standard linoleic backgrounds. Hence, the distribution of the fatty acids within the triacylglycerol molecule appears to depend not only on the fatty acid under study but also on the other fatty acids in the oil. Thus, it is demonstrated for the first time that certain fatty acids can influence the distribution of other fatty acids within triacylglycerols.

KEYWORDS: ***Helianthus annuus*; sunflower; triacylglycerols; 1,3-random-2-random theory**

INTRODUCTION

Both the fatty acid composition and the triacylglycerols (TAG) species in oils determine their physical, chemical, and nutritional properties. TAGs are the main components of oils, and they are made up of three fatty acid molecules esterified to a glycerol backbone. These molecules are synthesized in the glycerol-3-P pathway, first through the acylation of glycerol 3-phosphate with acyl-CoA esters at *sn*-1 that is mediated by the glycerol 3-phosphate acyltransferase (GPAT, EC 2.3.1.15) and then at the *sn*-2 position through lysophosphatidate acyltransferase (LPAAT, EC 2.3.1.51) that produces phosphatidate. Significantly, these two enzymes also act in the biosynthesis of phospholipids. This phosphatidate is then hydrolyzed to diacylglycerol by phosphatidate phosphohydrolase and, subsequently, further acylation of diacylglycerol by diacylglycerol acyltransferase (DAGAT, EC 2.3.1.20) yields triacylglycerol. The latter enzyme is specific to TAG biosynthesis.

One of the main chemical properties of vegetable oils is that they have low saturated fatty acid content in the *sn*-2 TAG position, even in oils with high saturated fatty acid content such as cocoa butter and high-stearic sunflower oils (*1*). In addition, the saturated fatty acid distribution at the stereochemical *sn*-1 and *sn*-3 TAG positions in most vegetable oils is not random, as originally proposed (*2*). For example, the oleic acid content is higher at the *sn*-1 than at the *sn*-3 position in cocoa butter TAG (*3, 4*), but it is virtually the same in olive oil (*5, 6*). Furthermore, stearic acid is predominantly found in the *sn*-3 position of sunflower (*7*) and olive oil TAGs (*5*). A coefficient has been proposed to calculate the asymmetry of saturated fatty acids between the *sn*-1 and *sn*-3 TAG positions (*8*), denominated the α coefficient. This coefficient of asymmetry has a value between 0 and 0.5, and when $\alpha = 0.5$ there is a symmetrical distribution of saturated fatty acids in accordance with the Van der Wal theory (*2*). A coefficient smaller than 0.5 implies that there are more saturated fatty acids at *sn*-1 than at the *sn*-3 position, or vice versa. This asymmetric distribution implies that fewer disaturated and more monosaturated TAGs can be found with respect to the expected values calculated by the lipase analysis of the TAG, thus modifying the properties of the oil.

In this study we describe the asymmetric distribution of stearic acid at external TAG positions in a set of standard, high-oleic, and high-stearic oils from sunflower plants and individual seeds. Moreover, we demonstrate that the nature of the unsaturated fatty acids in the oil influences this uneven distribution.

MATERIALS AND METHODS

Plant Materials. The sunflower (*Helianthus annuus* L.) plants used here were from distinct lines with different stearic acid contents in their seed oils (**Table 1**). The plants were cultivated in growth chambers with a 16 h photoperiod associated with a 25/15 °C day/night cycle and a photon flux density of 200 μmol m^{-2} s^{-1}.

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Table 1. Average, Standard Deviation, Maximum and Minimum Fatty Acid Compositions, and Saturated Fatty Acids Content and Distribution (αSAT) of the Set of Sunflower Oils Studied in This Work[a]

	fatty acid composition (%)							
	P	E	O	L	A	B	SAT	αSAT
av	5.8	17.9	58.4	15.4	1.0	1.5	26.1	0.27
SD	1.2	5.7	20.2	15.0	0.3	0.4	5.9	0.05
max	8.2	30.3	83.8	49.6	1.6	2.0	40.2	0.36
min	4.5	6.5	10.2	1.2	0.5	0.7	12.7	0.20

[a] P, palmitic acid; E, stearic acid; O, oleic acid; L, linoleic acid; A, arachidic acid; B, behenic acid. Average and SD values were calculated from 22 different sunflower oils.

Oil Extraction and TAG Purification. Mature seeds (achenes without a hull and seed coat) were ground in a screw-cap glass tube (10 × 13 mm) with the aid of a pestle and sand. The total lipids were extracted (*9*), and the triacylglycerols were purified by thin-layer chromatography (TLC) on silica gel plates (0.25 mm thick), which were then developed with hexane/ethyl ether/formic acid (75:25:1 by vol). To detect the position of the TAGs, the TLC plates were partially covered with a glass plate and exposed to iodine vapors. Unexposed TAG fractions were scraped off the plates and eluted from silica with hexane/ethyl ether (95:5 by vol). The samples of purified TAGs were analyzed by GLC, as described below, and lipase hydrolysis. The data presented are the average of three measurements, the SD being <10% of the mean value.

Half-Seed Oil Extraction. Oil from half-seeds was extracted as described previously (*10*) by heating the samples at 80 °C for 1 h in 3 mL of a mixture containing 1 mL of NaCl (0.17 M) in methanol and 2 mL of heptane. The TAGs were analyzed by GLC as described below, and the remaining TAG in the heptane solution was transmethylated with a solution containing methanol/toluene/dimethoxypropane/H_4SO_2 (39:20:5:2 by vol). FAMES were analyzed by GLC as described below.

Lipase Hydrolysis. For the positional analysis of TAG *sn*-2 fatty acids, 10 mg of purified TAGs were hydrolyzed with 2 mg of pancreatic lipase in 1 mL of 1 M Tris-HCl buffer (pH 8), 0.1 mL of $CaCl_2$ (22%), and 0.25 mL of deoxycholate (0.1%). The reaction was stopped when approximately 60% of the TAGs were hydrolyzed (1−2 min) by adding 0.5 mL of 6 N HCl (*11*). The lipids were extracted three times with 1.5 mL aliquots of ethyl ether, and the reaction products were separated by TLC (see above). Free fatty acids and *sn*-2-monoacylglycerol bands, representing the positions *sn*-1,3 and *sn*-2 of TAGs, respectively, as well as the remaining TAGs, were scraped off the plate and transmethylated (see below).

Fatty Acid Methyl Ester Analysis. Fatty acid methyl esters were obtained from the isolated lipids (*12*) by heating the samples at 80 °C for 1 h in 3 mL of methanol/toluene/H_2SO_4 (88:10:2 by vol). After cooling, 1 mL of heptane was added, and the samples were mixed. The fatty acid methyl esters were recovered from the upper phase, separated, and then quantified using an Agilent 6890 gas chromatography system (Palo Alto, CA) with a Supelco SP-2380 capillary column of fused silica (30 m length, 0.25 mm i.d., 0.20 μm film thickness; Bellefonte, PA). Hydrogen was used as the carrier gas, and the linear gas rate was 28 cm s^{-1}. The detector and injector temperatures were 220 °C, the initial oven temperature was 170 °C, the split ratio was 1:50, and a temperature gradient from 170 to 220 °C at 5 °C min^{-1} was applied. Fatty acids were identified by comparison with known standards (Sigma, St. Louis, MO).

TAG Analysis by GLC. TAG species were determined by GLC using an Agilent 6890 gas chromatography system (Palo Alto, CA) and hydrogen as the carrier gas (*13*). The injector and detector temperatures were 360 and 370 °C, respectively, the oven temperature was maintained at 335 °C, and a head pressure gradient from 100 to 180 kPa was applied. The gas chromatography column was a Quadrex Aluminum-Clad 400-65HT (30 m length, 0.25 mm i.d., 0.1 μm film thickness; Woodbridge, CT), using a linear gas rate of 50 cm s^{-1}, a split ratio 1:80, and a flame ionization detector (FID). The TAGs were identified and the data corrected for the relative response of the FID (*13, 14*).

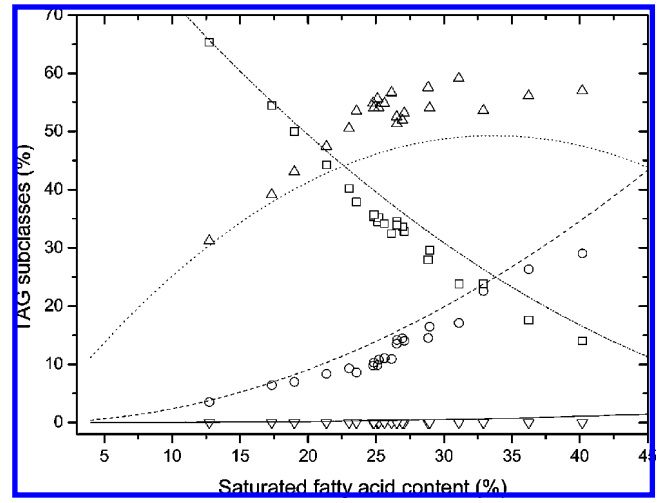

Figure 1. Triacylglycerol subclasses as a function of the total saturated fatty acids content for a set of sunflower oils studied and compared with the theoretical values (represented as lines) when the α coefficient is 0.5. Trisaturated TAG, SSS (▽ and solid line); disaturated TAG, SUS (○ and dashed line); monounsaturated TAG, SUU (△ and dotted line); and fully unsaturated TAG, UUU (□ and dashed-dotted line).

α Coefficient of Asymmetry. The distributions of saturated fatty acids between the *sn*-1 and *sn*-3 external positions of TAGs were calculated using the α coefficient of asymmetry (*8*) This coefficient was determined as the α coefficient of the SatUnsSat/SatUnsUns (αSUU/SUS) TAGs, as recommended for vegetable oils with low saturated fatty acid content in the *sn*-2 position. An α value of 0.5 indicates that saturated fatty acids are distributed equally between the *sn*-1 and *sn*-3 stereochemical positions in TAGs.

RESULTS AND DISCUSSION

Fatty Acid Distribution in a Set of Sunflower Oils. The asymmetric distribution of saturated fatty acids between positions *sn*-1 and *sn*-3 of triacylglycerols has been studied in several oils extracted from a complete set of high-stearic and low-stearic sunflower lines, the stearic acid content ranging between 6.5 and 23.8%, and from lines with different oleic/linoleic ratios ranging from a standard linoleic to high-oleic content (**Table 1**). The content of triacylglycerol subclasses, trisaturated (SSS), disaturated (SUS), monosaturated (SUU), and triunsaturated (UUU) TAGs, was assessed in relation to their saturated fatty acids content (**Figure 1**). In these plots, the lines represent the expected content of these TAG subclasses in a sunflower oil with an α value of 0.5, wherein the saturated fatty acids are evenly distributed between the *sn*-1 and *sn*-3 TAG positions. In all oils the contents of SSS, SUS, and UUU TAGs were lower than expected, whereas the SUU TAG content was higher, thereby resulting in an asymmetric α coefficient of <0.5. As shown, the α value in these oils was between 0.20 and 0.36, with an average of 0.27 (**Table 1**). This asymmetry could be due to the higher affinity of the GPAT for unsaturated acyl-CoAs and a weaker affinity for saturated fatty acids or the opposite (see below). Indeed, the lysophosphatidate acyltransferase in plants, the second enzyme in the glycerol-3P pathway, avoids saturated fatty acids. Hence, saturated acyl-CoAs might accumulate that could be introduced at the *sn*-3 by the DAGAT, therefore producing more SUU and, consequently, a reduction in SUS, UUU, and SSS. By contrast, the GPAT could have a higher affinity for saturated fatty acids, but given that previous data have shown a higher saturated fatty acid content at *sn*-3 in sunflower oils ((*7*), the former situation would appear to be at play.

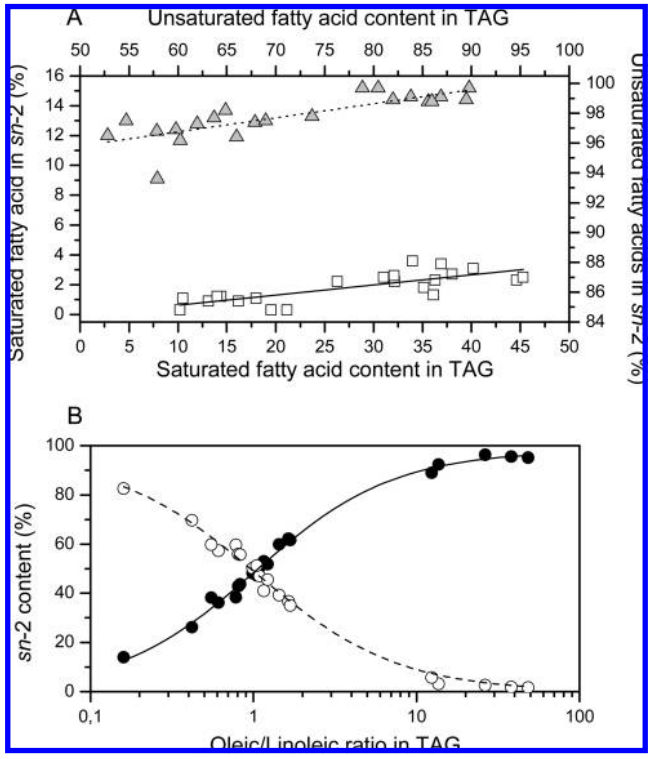

Figure 2. Saturated and unsaturated fatty acids content in the *sn*-2 position of oil TAGs as function of the total saturated fatty acids content in TAGs (**A**); oleic and linoleic acid content at the *sn*-2 position with respect to the oleic/linoleic ratios in TAGs (**B**): saturated fatty acids (□); unsaturated fatty acids (△); oleic acid (●); linoleic acid (○).

Saturated Fatty Acids at *sn*-2 of Sunflower Triacylglycerols. Because it is not possible to determine the fatty acid composition of the oil of many individual seeds analytically, a database was established with the total saturated fatty acid and unsaturated fatty acid contents at *sn*-2 to study the asymmetric fatty acid distribution in a population of seeds. These data come from *sn*-2 fatty acid lipase analysis of sunflower oils from different lines, such as standard; high-oleic oils; high-stearic oils on a standard and high-oleic background; high-palmitic oils on a standard and high-oleic background; and high-palmitoleic oils on standard background (*1*, *8*, *15*−*19*). These data regarding the total unsaturated and saturated fatty acids content versus saturated fatty acid content in *sn*-2 were plotted (**Figure 2**). Whereas the saturated fatty acid content at *sn*-2 of sunflower oils is always low (<4%), even when the total saturated fatty acid content is 45%, the unsaturated fatty acid content was very high (>94%). This database enables us to predict the saturated fatty acid content of the *sn*-2 position in any sunflower TAG oil.

Accordingly, we established a formula to calculate the saturated fatty acid content at *sn*-2:

$$\text{saturated fatty acids in } sn\text{-}2 = \text{total saturated fatty acid content} \times 0.0678 - 0.0550$$

Importantly, it was previously shown that small differences in the value of the saturated fatty acid content in *sn*-2, such as that found in this figure, does not influence on the α coefficient (*8*). Therefore, in this work we use the formula instead of the theoretically more precise analytical data, which was more inaccurate for the sample sizes examined.

Within the TAGs (**Figure 2B**), the distribution of the oleic and linoleic acids in the *sn*-2 position is adjusted to a sigmoid distribution, with some preference for linoleic acid. Indeed, when both fatty acids were at 50%, there was slightly more linoleic acid in the *sn*-2 position (50.1%) than oleic acid (48%).

Table 2. Average, Standard Deviation, Maximum and Minimum Fatty Acid Compositions, Saturated Fatty Acids Content, and Distribution (αSAT) of Individual Sunflower Seeds Obtained from a Cross between the High-Stearic High-Oleic CAS-15 Line and the High-Oleic HA-OL9 Line[a]

	fatty acid composition (mol %)							
	P	E	O	L	A	B	SAT	αSAT
av	6.3	14.6	69.2	6.2	1.3	2.3	24.53	0.26
SD	1.0	3.1	4.8	3.3	0.3	0.6	3.6	0.03
max	9.9	26.2	84.8	27.6	2.5	4.3	35.8	0.30
min	3.9	3.5	50.1	1.6	0.2	0.2	11.8	0.18

[a] P, palmitic acid; E, stearic acid; O, oleic acid; L, linoleic acid; A, arachidic acid; B, behenic acid. Average and SD values were calculated from 670 individual half-seeds.

Table 3. Saturated Fatty Acid Content, Distribution (αSAT) and TAG Subclasses from a Theoretical Oil with Symmetric Saturated Fatty Acids Distribution (αSAT = 0.5) and Seed Oil from High-Stearic Sunflower Lines on Standard Linoleic and High-Oleic Backgrounds

			TAG subclasses (%)			
line	SAT(%)	αSAT	SSS	SUS	SUU	UUU
theoretical	34.0	0.50	0.7	25.3	42.3	24.7
high-stearic	34.4	0.36	0.0	24.9	53.3	21.8
high-stearic, high-oleic	33.5	0.23	0.0	19.3	61.9	18.8

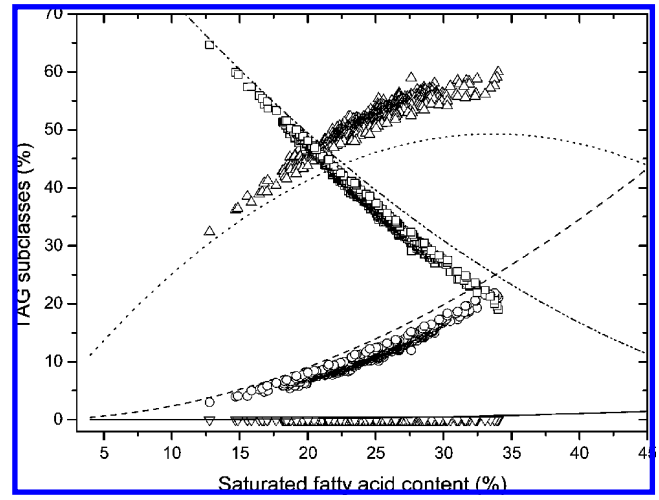

Figure 3. Triacylglycerol subclasses as a function of the total saturated fatty acids content in individual F2 sunflower seeds from a cross of the CAS-15 high-oleic and high-stearic acid line with the HA-OL9 high-oleic line, compared to the theoretical values when the α coefficient is 0.5 (represented as lines): trisaturated TAG, SSS (▽ and solid line); disaturated TAG, SUS (○ and dashed line); monounsaturated TAG, SUU (△ and dotted line); and fully unsaturated TAG, UUU (□ and dashed-dotted line).

Saturated Fatty Acid Distribution in Individual Sunflower Seeds. The distribution of the saturated fatty acids in TAGs from individual seeds was studied in the F2 segregating population of a cross between a high-oleic line and a high-stearic and high-oleic line (**Table 2**). All of the individual seeds studied had a high-oleic content and a range of stearic acid contents; a total of 670 individual half-seeds were analyzed, and the TAG and fatty acid compositions were determined (**Figure 3** and **Table 2**). As before, these seed oils have a higher SUU content and reduced SSS, SUS, and UUU contents with respect to the

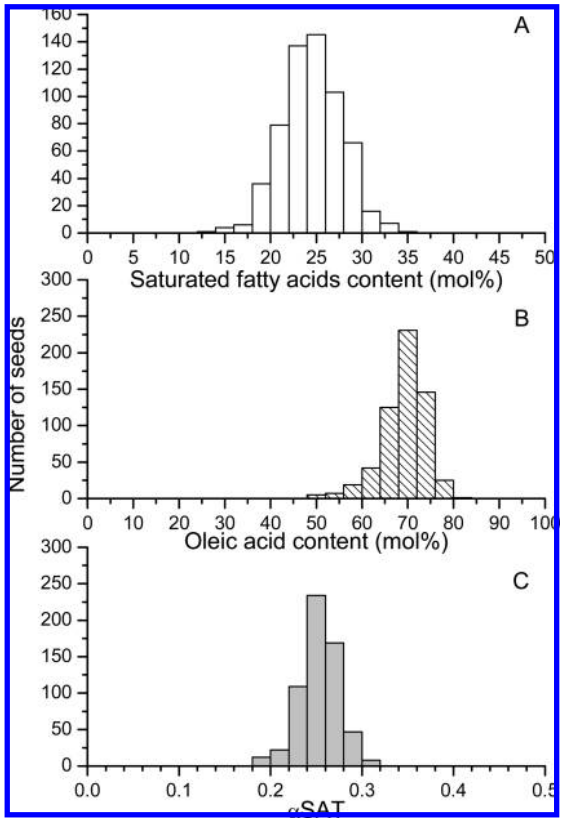

Figure 4. Stacked histograms showing the saturated fatty acids content (**A**), oleic acid content (**B**), and αSAT symmetry coefficient (**C**) in F2 sunflower seeds with variable stearic acid contents on a high-oleic background.

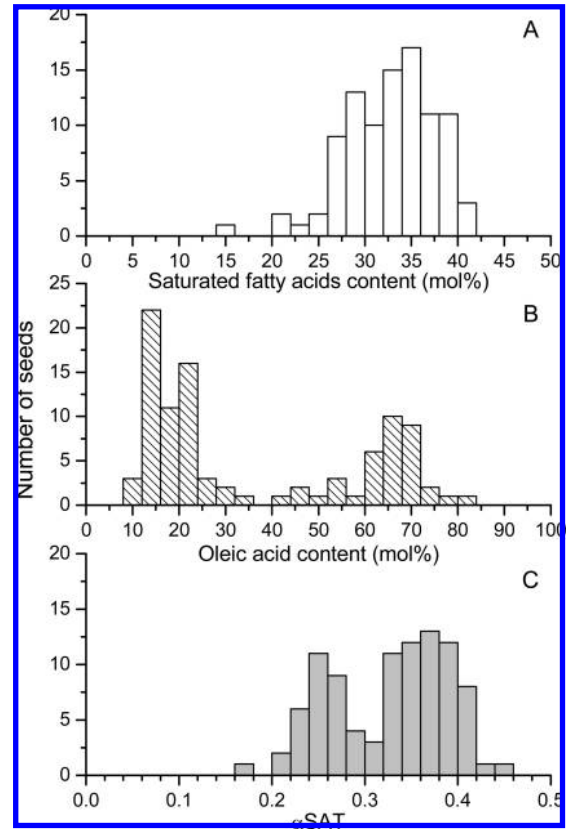

Figure 5. Stacked histograms showing the saturated fatty acids content (**A**), oleic acid content (**B**), and αSAT symmetry coefficient (**C**) in F2 sunflower seeds with variations in their stearic and oleic acid contents.

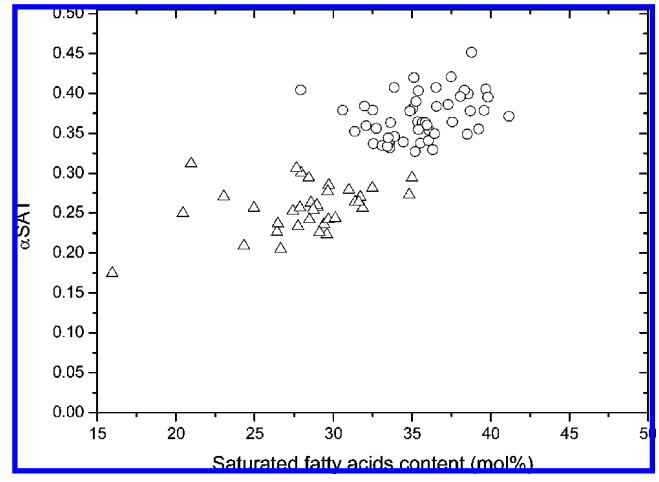

Figure 6. Saturated fatty acids content plotted against the αSAT symmetry coefficient in F2 sunflower seeds with variations in their stearic and low (○) or high (△) oleic acid content.

expected values, and, indeed, there was an asymmetric distribution of saturated fatty acids within the TAG molecules. This distribution followed a pattern similar to a Gaussian curve (**Figure 4**), with a mean αSAT of 0.26 (SD ± 0.03, **Figure 4C**) for a saturated fatty acid content between 11.8 and 35.8% (**Figure 4A**), and all with high-oleic content (**Figure 4B**).

It is important to note that the αSAT asymmetry coefficient is smaller in these high-oleic seeds (**Table 2**) than in the oils from standard and high-oleic lines (**Table 1**). To determine whether these differences were due to the different genetic backgrounds that may be reflected in different acyl transferase alleles, or if they were related to the oleic acid content, the distribution of the saturated fatty acids in the TAGs and the αSAT was determined in a segregating F2 population from a cross of a high-stearic and high-oleic line with a high-stearic line on a standard linoleic background (**Figure 5**). The saturated fatty acid content of these seeds varies from around 20 to 40% (**Figure 5A**), and the oleic acid content was bimodal, with high-oleic seeds accumulating around 65% of oleic acid, whereas linoleic seeds accumulated only around 20% of oleic acid (**Figure 5B**). In addition, there was a peculiar bimodal distribution of the αSAT asymmetric coefficient, with one population displaying a mean αSAT of 0.26 and the other a mean αSAT of 0.35 (**Figure 5C**). Indeed, the seeds with smaller αSAT value corresponded to the high-oleic seeds (**Figure 6**), which also had a lower stearic content as described previously (*20, 21*). These data could be explained if the oleic acid competes with the stearic acid for the external TAG positions to a greater extent than linoleic acid.

Saturated Fatty Acid Distribution in Isogenic Lines. To determine whether the smaller αSAT values observed above were due to the oleic content or to the content of other fatty acids such as stearic acid, the asymmetric αSAT coefficient was determined in two isogenic high-stearic lines with similar saturated fatty acid contents, in either high-oleic or standard background. Both of these high-stearic lines have similar saturated fatty acid contents with averages of 34.4% on the standard linoleic background and 33.5% in the high-oleic line (**Figure 7**). However, the αSAT coefficient was smaller in the high-oleic line, with an average of 0.23 when compared to the mean of 0.36 for the line on the standard linoleic background. This result suggests that the higher degree of saturated fatty acids asymmetry is indeed due to the higher oleic acid content, probably reflecting stronger competitive inhibition by oleic acid

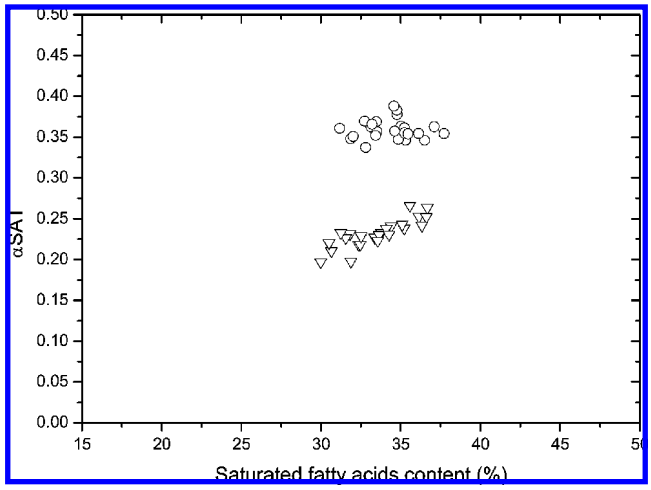

Figure 7. Saturated fatty acids content plotted against the αSAT symmetry coefficient in sunflower seeds from high-stearic isogenic lines on normal (○) or high-oleic (▽) acid backgrounds.

than linoleic acid when compared to stearic acid. Such an effect would reduce the amount of stearic acid at the *sn*-1 position in a high-oleic background and increase it at the *sn*-3 position, raising the SUU TAG content and decreasing that of the other TAG subclasses.

In conclusion, certain asymmetry exists in the distribution of saturated fatty acids in sunflower oils, as reflected in the reduced amounts of SUS, UUU, and SSS TAGs and an increase the amount of SUU TAGs. This asymmetry may be due to the higher affinity of the GPAT for unsaturated acyl-CoAs, leaving a higher proportion of saturated acyl-CoAs that are not favored by the plant LPAT and that therefore become available for the nonspecific DAGAT or vice versa. This hypothesis would explain all of the available data: the decrease in SSS, SUS, and UUU and the increase in SUU content. This asymmetry could be found in oils from different lines and in individual seeds, although it was most evident in lines with high-oleic acid content, probably because there is a stronger competitive inhibition of the GPAT activity between oleic and stearic acids than between linoleic and stearic acids. Moreover, and irrespective of the saturated fatty acid content in the ranges studied, the amount of saturated acyl-CoA available for TAG biosynthesis does not modify the distribution of the saturated fatty acids between the *sn*-1 and *sn*-3 positions, as reflected by the α value, probably due to the different affinities of the GPAT and the DAGAT.

ABBREVIATIONS USED

A, arachidic acid; B, behenic acid; E, stearic acid; L, linoleic acid; O, oleic acid; P, palmitic acid; S, saturated fatty acids; TAG, triacylglyceride; U, unsaturated fatty acids.

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