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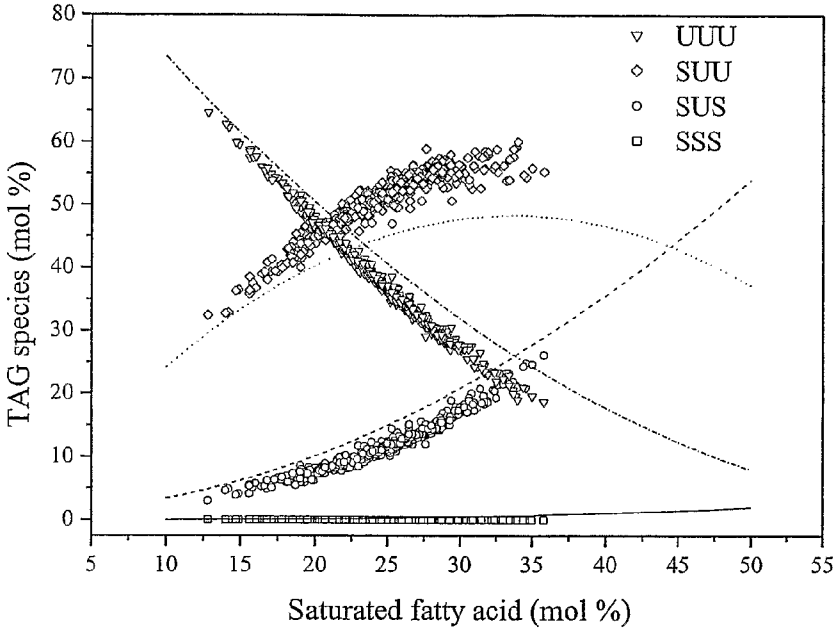
(54) Title: SUNFLOWER OIL, SEEDS AND PLANTS WITH MODIFIED FATTY ACID DISTRIBUTION IN THE TRIACYLGLYCEROL MOLECULE



(57) Abstract: The invention relates to a sunflower oil directly obtained from sunflower seeds with at least 12 % of stearic acid referred to the total fatty acid content, characterized in that the distribution coefficient of saturated fatty acids α between positions *sn*-1 and *sn*-3 is at least 0.38 and to a sunflower oil directly obtained from sunflower seeds with at least 12 % of stearic acid referred to the total fatty acid content, characterized in that the distribution coefficient of saturated fatty acids α between positions *sn*-1 and *sn*-3 is at least 0.28 when the oleic acid content is higher than the linoleic acid content in the oil. The invention also relates to the plants and seeds for producing the oil and to its use.

WO 2005/046315 A1

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SUNFLOWER OIL, SEEDS AND PLANTS WITH MODIFIED FATTY ACID DISTRIBUTION IN THE TRIACYLGLYCEROL MOLECULE

OBJECT OF THE INVENTION

The object of the present invention is a sunflower oil directly obtained from sunflower seeds with at least 12% of stearic acid referred to the total fatty acid content and with a modified fatty acid distribution between positions *sn*-1 and *sn*-3 of the triacylglycerol (TAG) molecule as compared to oil obtained from wild type sunflower seeds. The invention also relates to a sunflower plant and seeds containing an endogenous oil with the characteristics mentioned. It constitutes a further object of the present invention to provide a method for producing said sunflower plant and uses of said sunflower oil.

BACKGROUND ART

Oil and fats are made mainly from triglycerides, these are molecules formed by a glycerol backbone and three fatty acids esterified to the three hydroxyls groups of the glycerol (Gunstone et al. 1994). The chemical and physical, and also, the nutritional properties of oils are determined by the fatty acid composition of oils and the distribution of these fatty acids in the different triglyceride species. The three stereochemical positions of the fatty acids are denominated *sn*-1, *sn*-2 and *sn*-3. The fact that an oil is solid at a specific temperature or has a good stability is correlated with a reduced level of double bonds in the fatty acids. The main fatty acids found in oilseeds are the linoleic acid (18:2) with 18 carbon atoms and two double bonds and the oleic acid (18:1) with only one double bond, making these oils liquid at room temperature. Some oils, like the one from soybean and canola, have also linolenic

acid (18:3) with 18 carbons and 3 double bonds. Those fatty acids are unsaturated because they have one or more double bonds. The vegetable oils also have minor amounts of saturated fatty acids, without any double bond, like palmitic acid that has 16 carbons (16:0), stearic acid with 18 carbons (18:0), arachidic acid with 20 carbons (20:0) and behenic acid (22:0) with 22 carbons.

The unsaturated fatty acids are beneficial to health and the saturated fatty acids neutral or unhealthy, depending on the fatty acid and the position in the triglyceride molecule. On the other hand some tropical vegetable oils and animal fats have short and medium chain saturated fatty acids like lauric acid with 12 carbons (12:0) and myristic acid, a saturated fatty acid with 14 carbons (14:0), being the last one the worst for health. Palmitic acid and stearic acid are the usual saturated fatty acids found in temperate vegetable oils (**Table 1**). Palmitic acid is considered a little unhealthy and stearic acid is considered neutral.

However, it is very important to consider a second property that depends on the position of the fatty acids in the triglyceride molecule. A saturated long chain fatty acid is less harmful if it is not bound to the middle position (sn-2) of the glycerol. During fat digestion the pancreatic lipase hydrolyzes the fatty acids found in the *sn*-1 and *sn*-3 positions of the glycerol. While the fatty acid in the middle position is kept bound to the glycerol forming a monoglyceride that has detergent properties and is assimilated perfectly, the fatty acids liberated from the positions *sn*-1 and *sn*-3 react with calcium or magnesium producing insoluble salt with these metals, making the intestinal adsorption very difficult. As a result thereof

they are excreted. As shown in **table 1**, all saturated fatty acids of vegetable oils, with the exception of palm oil, are not located in position *sn*-2, for this reason they do not negatively affect the cholesterol level, although they have a high palmitic content like cocoa butter or medium palmitic like olive oil.

Table 1

Fatty acid composition of edible fat and oils (Álvarez-Ortega et al. 1997; Chow 1992; Gunstone et al. 1994).

Fat or oil	Fatty acid composition (%)								
	≤12:0	14:0	16:0	16:1	18:0	18:1	18:2	*Trans*	Saturated in *sn*-2
Lard		2	25	3	12	45	10	1	79
Butter	12	10	26	2	12	28	3	3	84
Palm		1	45		5	39	9		18
Olive			14	1	3	71	10		2
Cocoa			26		35	35	3		4
Sunflower			7		5	30	57		1
Sunflower High Oleic (HO)			5		4	88	2		1

For many food preparations the food industry needs plastic or solid fats (such as animal fats) with good stability. Bakery, pastry and, of course, margarine and spreads, require solid fat, while deep frying industry wants liquid oil resistant to thermo-oxidation. In the eighties, the food industry following nutrition expert recommendations and consumer demands switched from animal fats to vegetable oils. These oils do not have the appropriate properties to be used in these food preparations; they must be chemically modified through partial hydrogenation and/or trans-esterification. Hydrogenation reduces the doubles bounds of the unsaturated fatty acids with hydrogen and a heavy metal as a catalyst. During this process the saturated fatty acids increase, but at the same time the number of artificial

fatty acid isomers *cis* and *trans* increases. The *trans* isomers, although they are unsaturated fatty acids, have physical properties similar to saturated fatty acids. The main problem with these *trans* fatty acids is that they are even worse than the animal saturated fatty acids with respect to cholesterol levels, and they are involved in some essential fatty acid deficiencies or in certain cancers, like women's breast cancer.

Chemical trans-esterification leads to a redistribution of all fatty acids within the triglyceride molecules; later by fractionation a portion enriched in saturated triglycerides can be obtained. Through this process a healthy vegetable oil is converted into an unhealthy fat, like lard, with a lot of saturated fatty acids in position *sn*-2. This oil will increase the low density (bad) cholesterol. In conclusion, the processes used to chemically modify the vegetable oils are not particularly healthy, changing the properties of these oils in such a way that the new ones are less healthy. Taking into account the technological and nutritional data, the best oil should be a natural vegetable oil with an increased content of stearic acid as saturated fatty acid, preferably bound to the glycerol backbone through the *sn*-1 and *sn*-3 positions, and oleic or linoleic acids as unsaturated fatty acids which are bound to the three *sn* positions.

Several fatty acid sunflower mutant lines were selected and fixed after a mutagenesis program (Osorio et al. 1995). Some of these mutants have a high content of saturated fatty acids in the seed oil: CAS-3 with a least 26% of stearic acid; CAS-4 and CAS-8 with at least medium levels of stearic acid (12-16%). This material and other like CAS-29, 30 and 31, selected after biochemical studies

and further recombination, make a wide germplasm collection (**Table 2**).

Table 2

Fatty acid composition of selected sunflower material from the sunflower collection of Instituto de la Grasa, CSIC, Seville, Spain

Oil type	Line	Fatty acid composition (mol %)					
		16:0	18:0	18:1	18:2	20:0	22:0
Medium 18:0	CAS-4	5.9	11.9	27.8	53	0.6	0.7
High 18:0	CAS-3	5.4	26.1	14.2	51.3	1.4	1.3
High 18:0	CAS-14	8.4	37.3	12.4	37.9	2.2	1.8
High 18:0	CAS-30	5.8	32.1	9.4	49.3	1.9	1.5
High 18:0	CAS-29	6.7	31.9	21.2	36.2	1.8	2.2
High 18:0	CAS-31	7.2	31.7	14.7	43.3	1.4	1.7
High 18:0	CAS-15	5.2	26.3	57.8	7.2	1.2	1.9
High 18:0	CAS-26	10.1	23.3	59.1	4.5	1.2	1.8
High 18:0	CAS-24	7.2	24.5	60.6	4.4	1.4	1.9

The genetic characterization of the mutants has shown that the inheritance of the altered fatty acid levels is gametophytic and controlled by alleles at a reduced number of loci, making feasible their transference to target inbred lines in few backcross cycles. The study of the temporal and spatial expression of these mutant characters showed that the mutant characters are expressed only during seed formation, are little influenced by growth temperature and are not expressed in the vegetative tissues. These sunflower mutant lines do not have any collateral negative effect like the ones found in arabidopsis and canola high stearic mutants.

Plant triglycerides are produced by the glycerol-3-P pathway (Kennedy pathway). Initially (**Figure 1**), two

acylations of glycerol 3-phosphate in *sn*-1 and *sn*-2 positions with acyl-CoA esters occurs, producing phosphatidate by the enzymes, glycerol 3-phosphate acyltransferase (GPAT) and lysophosphatidate acyltransferase (LPAAT), respectively. The phosphatidate is then hydrolysed to diglyceride by the phosphatidate phosphohydrolase and, subsequently, the diglyceride can be further acylated by acyl-CoA to yield triglyceride (a reaction catalysed by the diglyceride acyltransferase, DAGAT). The last enzyme is exclusive to the triglyceride biosynthesis. Those acyltransferases regulate fatty acid stereochemical distribution.

During the analysis of the sunflower mutant triglyceride composition, 38 different molecular species were found (Fernández-Moya et al. 2000). But unexpectedly, the triglycerides synthesized by the high stearic lines do not have a random distribution in the positions *sn*-1 and *sn*-3 as expected by the Vander Wal (1960) theory that supposedly was settled for all oils. The enzymes responsible for this unusual distribution are the acyltransferases that synthesized the triglycerides from the acyl-CoA pool and glycerol-3-P. Taking into account that in vegetable systems the triglyceride synthesis implies that no saturated fatty acid will be bound to the *sn*-2 position of the glycerol (Álvarez-Ortega et al;. 1997), the specific enzymes responsible for this effect should be the glycerol-3P acyltransferase and/or the diglyceride acyltransferase.

In the research that led to the invention a mathematical coefficient, alpha S (αS), has been developed which calculates the relative distribution of saturated fatty acid in TAG *sn*-1 and *sn*-3 positions. The value of αS goes from 0, meaning one of the positions without any saturated fatty acid, to 0.5, both positions having the same

saturated fatty acid content. If a triglyceride distribution is made according to the Vander Wal theory, then $\alpha = 0.5$ and the different fatty acids are distributed evenly in the triglyceride. This is very important in the case of the saturated fatty acids distribution between positions *sn*-1 and *sn*-3, because if there is more saturated fatty acids in one of these positions α is smaller than 0.5 and the amount of disaturated triglycerides is smaller than theoretically expected. That is exactly what the inventors found in sunflower oils, mainly in the ones with stearic acid higher than 12%. The maximum amount of disaturated triglycerides, which are advantageous for making plastic fats for spreads, margarines, shortening, bakery, pastry, etc., is obtained when $\alpha = 0.5$, the smaller this value of α is in the saturated fatty acid distribution in positions *sn*-1 and *sn*-3 of the triglyceride molecule, the worst this oil is for this specific food purposes. Thus, for particular uses mutant sunflowers that have an advantageous relative distribution of saturated fatty acid in TAG *sn*-1 and *sn*-3 positions can be selected based on calculation of the value α.

This coefficient was calculated knowing the total saturated fatty acid composition of the triglyceride (S), the saturated fatty acid composition in *sn*-2 (S_2), which two can be calculated according to Álvarez-Ortega et al. (1997), and the triglyceride molecular species composition, which can be calculated according to Fernández-Moya et al. (2000).

Table 3 Saturated and unsaturated fatty acid percentage in each TAG position (S_1, S_2 and S_3) in function of S, S_2 and αS: Total saturated fatty acid content; S_2: *sn*-2 saturated fatty acid content; α: distribution coefficient of saturated fatty acid between *sn*-1 and *sn*-3 positions.

	TAG positions		
	1	2	3
Saturated(S)	$(3S-S_2)\alpha$ (S_1)	S_2	$(3S-S_2)(1-\alpha)$ (S_3)
Unsaturated (U)	$100-[(3S-S_2)\alpha]$ (U_1)	$100-S_2$ (U_2)	$100-[(3S-S_2)(1-\alpha)]$ (U_3)

The percentages of the different TAG subclasses (Trisaturated, SSS; Disaturated, SUS; Monosaturated , SUU; and Triunsaturated, UUU) are usually calculated using the following formulas:

$$SSS\ (\%) = S_1S_2S_3/10000 \quad (i)$$

$$SUS\ (\%) = (U_1S_2S_3 + S_1U_2S_3 + S_1S_2U_3)/10000 \quad (ii)$$

$$SUU\ (\%) = (S_1U_2U_3 + U_1S_2U_3 + U_1U_2S_3)/1000 \quad (iii)$$

$$UUU\ (\%) = U_1U_2U_3/10000 \quad (iv)$$

Using the values given for S_1, S_2, S_3, U_1, U_2 and U_3 in Table 3 we can calculate the value for distribution coefficient α by the following reasoning in the different TAG:

a) From trisaturated TAG species (SSS):

$$SSS\ (\%) = S_1S_2S_3/10000 \quad (i)$$

Substituting the values for S_1, S_2 and S_3 from **Table 3**, we obtain:

$$\mathbf{S_1S_2S_3} = [(2S-S_2)\alpha] \times S_2 \times [(3S - S_2)(1 - \alpha)] =$$

$$(3S\alpha - S_2\alpha) \times S_2 \times (3S - 3S\alpha - S_2 - S_2\alpha) =$$

$$3SS_2\alpha - S_2^2\alpha) \times (3S - 3S\alpha - S_2 - S_2\alpha) =$$

$$9S^2S_2\alpha - 9S^2S_2\alpha^2 - 3SS_2^2\alpha + 3SS_2^2\alpha^2 - 3SS_2^2\alpha + 3SS_2^2\alpha^2 + S_2^3\alpha - S_2^3\alpha^2 =$$

$$\mathbf{(-9S^2S_2 + 6SS_2^2 - S_2^3)\alpha^2 + (9S^2S_2 - 6SS_2^2 + S_2^3)\alpha} \quad (v)$$

Rearranging in (i),

$$S_1S_2S_3 - 10000SSS(\%) = 0$$

Substituting for $S_1S_2S_3$ value from equation (v),

$$(-9S^2S_2 + 6SS_2^2 - S_2^3)\alpha^2 + (9S^2S_2 - 6SS_2^2 + S_2^3)\alpha - 10000SSS\ (\%) = 0$$

α value is calculated as a quadratic equation ($ax^2 + bx + c = 0$) that can be simplified because a = -b

$$\alpha = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a} = \frac{-b}{2a} \pm \frac{\sqrt{b^2 - 4ac}}{2a} = 0.5 \pm \frac{\sqrt{a^2 - 4ac}}{2a}$$

where $a = -9S^2S_2 + 6SS_2^2 - S_2^3$, $b = 9S^2S_2 - 6SS_2^2 + S_2^3$ and $c = 10000SSS$ (%), SSS(%) being the total amount of trisaturated TAGs in the seed/oil.

b) From disaturated TAG species (SUS):

$$SUS\ (\%) = U_1S_2S_3 + S_1U_2S_3 + S_1S_2U_3)/10000 \quad (ii)$$

Subsituting the values for S_1, S_2, S_3, U_1, U_2 and U_3 from **Table 3**, we obtain:

$$U_1S_2S_3 = \{100 - [3S - S_2)\alpha]\} \times S_2 \times [(3S - S_2)(1 - \alpha)] =$$
$$300SS_2 - 300SS_2\alpha - 100S_2^2 + 100S_2^2\alpha - 9S^2S_2\alpha + 9S^2S_2\alpha^2 + 3SS_2^2\alpha -$$
$$3SS_2^2\alpha^2 + 3SS_2^2\alpha - 3SS_2^2\alpha^2 - S_2^3\alpha + S_2^3\alpha^2 \quad (vi)$$

$$S_1U_2S_3 = (3S - S_2)\alpha \times (100 - S_2) \times [(3S - S_2)(1 - \alpha)] =$$
$$900S^2\alpha - 300SS_2\alpha - 9S^2S_2\alpha + 3SS_2^2\alpha - 900S^2\alpha^2 + 300SS_2\alpha^2 + 9S^2S_2\alpha^2 -$$
$$3SS_2^2\alpha^2 - 300SS_2\alpha + 100S_2^2\alpha + 3SS_2^2\alpha - S_2^3\alpha + 300SS_2\alpha^2 - 100S_2^2\alpha^2 -$$
$$3SS_2^2\alpha^2 + S_2^3\alpha^2 \quad (vii)$$

$$S_1S_2U_3 = [(3S - S_2)\alpha] \times S_2 \times \{100 - [(3S - S_2)(1 - \alpha)]\} =$$
$$300SS_2\alpha - 9S^2S_2\alpha + 9S^2S_2\alpha^2 + 3SS_2^2\alpha - 3SS_2^2\alpha^2 - 100S_2^2\alpha +$$
$$3SS_2^2\alpha - 3SS_2^2\alpha^2 - S_2^3\alpha + S_2^3\alpha^2 \quad (viii)$$

Rearranging in (ii),

$$(U_1S_2S_3 + S_1U_2S_3 + S_1S_2U_3) - 10000SUS\ (\%) = 0$$

Substituting for $U_1S_2S_3$, $S_1U_2S_3$ and $S_1S_2U_3$ values from equations (vi), (vii) and (viii) respectively, and grouping in function of α:

$$(600SS_2 - 18SS_2^2 + 27S^2S_2 - 900S^2 - 100S_2^2 + 3S_2^3)\alpha^2 + (-600SS_2 + 18SS_2^2 - 27S^2S_2 + 900S^2 + 100S_2^2 - 3S_2^3)\alpha + 300SS_2 - 100S_2^2 - 10000SUS(\%) = 0$$

α value is calculated as a quadratic equation that can be simplified because a = -b

$$\alpha = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a} = \frac{-b}{2a} \pm \frac{\sqrt{b^2 - 4ac}}{2a} = 0.5 \pm \frac{\sqrt{a^2 - 4ac}}{2a}$$

where a = $600SS_2 - 18SS_2^2 + 27S^2S_2 - 900S^2 - 100S_2^2 + 3S_2^3$ and c = $300SS_2 - 100S_2^2 - 10000SUS(\%)$, SUS(%) being the total amount of disaturated TAGs in the seed/oil.

c) From monounsaturated TAG species (SUU):

$$SUU\ (\%) = (S_1U_2U_3 + U_1S_2U_3 + U_1U_2S_3)/10000 \quad (iii)$$

Substituting the values for S_1, S_2, S_3, U_1, U_2 and U_3 from **Table 3**, we obtain:

$$S_1U_2U_3 = 30000S\alpha - 900S^2\alpha + 900S^2\alpha^2 + 300SS_2\alpha - 300SS_2\alpha^2 - 10000S_2\alpha + 300SS_2\alpha - 300SS_2\alpha^2 - 100S_2^2\alpha + 100S_2^2\alpha^2 - 300SS_2\alpha + 9S^2S_2\alpha - 9S^2S_2\alpha^2 - 3SS_2^2\alpha + 3SS_2^2\alpha^2 + 100S_2^2\alpha - 3SS_2^2\alpha + 3SS_2^2\alpha^2 + S_2^3\alpha - S_2^3\alpha^2 \quad (ix)$$

$$U_1S_2U_3 = 10000S_2 - 300SS_2 + 300SS_2\alpha + 100S_2^2 - 100S_2^2\alpha - 300SS_2\alpha + 9S^2S_2\alpha - 9S^2S_2\alpha^2 - 3SS_2^2\alpha + 3SS_2^2\alpha^2 + 100S_2^2\alpha - 3SS_2^2\alpha + 3SS_2^2\alpha^2 + S_2^3\alpha - S_2^3\alpha^2 \quad (x)$$

$$U_1U_2S_3 = 30000S - 10000S_2 - 30000S\alpha + 10000S_2\alpha - 300SS_2 + 100S_2^2 + 300SS_2\alpha - 100S_2^2\alpha - 900S^2\alpha + 300SS_2\alpha + 900S^2\alpha^2 - 300SS_2\alpha^2 + 9S^2S_2\alpha - 3SS_2^2\alpha - 9S^2S_2\alpha^2 + 3SS_2^2\alpha^2 + 300SS_2\alpha -$$

$$100S_2^2\alpha - 300SS_2\alpha^2 + 100S_2^2\alpha^2 - 3SS_2^2\alpha + S_2^3\alpha + 3SS_2^2\alpha^2 - S_2^3\alpha^2$$

(xi)

Rearranging in (iii):

$$(S_1U_2U_3 + U_1S_2U_3 + U_1U_2S_3) - 10000SUU\ (\%) = 0$$

Substituting for $S_1U_2U_3$, $U_1S_2U_3$ and $U_1U_2S_3$ values from equations (ix), (x) and (xi) respectively, and grouping in function of α:

$$(-1200SS_2 + 18SS_2^2 - 27S^2S_2 + 1800S^2 + 200S_2^2 - 3S_2^3)\alpha^2 +$$
$$(1200SS_2 - 18SS_2^2 + 27S^2S_2 - 1800S^2 - 200S_2^2 + 3S_2^3)\alpha -$$
$$600SS_2 + 200S_2^2 + 30000S - 10000SUU(\%) = 0$$

α value is calculated as a quadratic equation that can be simplified because a = -b

$$\alpha = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a} = \frac{-b}{2a} \pm \frac{\sqrt{b^2 - 4ac}}{2a} = 0.5 \pm \frac{\sqrt{a^2 - 4ac}}{2a}$$

where a = $-1200SS_2 + 18SS_2^2 - 27S^2S_2 + 1800S^2 + 200S_2^2 - 3S_2^3$ and c = $-600SS_2 + 200S_2^2 + 30000S - 10000SUU(\%)$, SUU(%) being the total amount of monosaturated TAGs in the seed/oil.

d) From triunsaturated TAG species:

$$UUU(\%) = U_1U_2U_3/10000 \quad (iv)$$

Substituting the values for U_1, U_2 and U_3 from **Table 3**, we obtain:

$$U_1U_2U_3 = (600SS_2 - 6SS_2^2 + 9S^2S_2 - 900S^2 - 100S_2^2 + S_2^3)\alpha^2 +$$
$$(-600SS_2 + 6SS_2^2 - 9S^2S_2 + 900S^2 + 100S_2^2 - S_2^3)\alpha + 300SS_2 -$$
$$100S_2^2 - 30000S + 1000000 \quad (xii)$$

Rearranging in (iv):

$$U_1U_2U_3 - 10000UUU(\%) = 0$$

Substituting for $U_1U_2U_3$ value from equation (xii), and grouping in function of α:

$$(600SS_2 - 6SS_2^2 + 9S^2S_2 - 900S^2 - 100S_2^2 + S_2^3)\alpha^2 + (-600SS_2 + 6SS_2^2 - 9S^2S_2 + 900S^2 + 100S_2^2 - S_2^3)\alpha + 300SS_2 - 100S_2^2 - 30000S + 1000000 - 10000UUU(\%) = 0$$

α value is calculated as a quadratic equation that can be simplified because a = -b

$$\alpha = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a} = \frac{-b}{2a} \pm \frac{\sqrt{b^2 - 4ac}}{2a} = 0.5 \pm \frac{\sqrt{a^2 - 4ac}}{2a}$$

where $a = 600SS_2 - 6SS_2^2 + 9S^2S_2 - 900S^2 - 100S_2^2 + S_2^3$, and $c = 300SS_2 - 100S_2^2 - 30000S + 1000000 - 10000UUU(\%)$, UUU(%) being the total amount of triunsaturated TAGs in the seed/oil.

To avoid deviations by experimental errors in the determination by GLC of TAG species we define αS as the weighted average of α values calculated from SSS (αSSS), from SUS (αSUS), from SUU (αSUU) and from UUU (αUUU).

$$\alpha S = \frac{(\alpha SSS \times SSS(\%) + (\alpha SUS \times SUS(\%)) + (\alpha SUU \times SUU(\%)) + (\alpha UUU \times UUU(\%))}{(SSS(\%) + SUS(\%) + SUU(\%) + UUU(\%))}$$

In a random distribution of the saturated fatty acid between positions *sn*-1 and *sn*-3 of the triglyceride molecule 50% of each saturated fatty acid should be in each position, being the optimum to have the maximum SUS triglyceride molecules, S being a saturated and U an unsaturated fatty acid, respectively. **Figure 2** shows the proportion of the different TAG species in sunflower oil with increasing saturated fatty acid content if a random distribution between *sn*-1 and *sn*-3 occurs. Those curves have been generated substituting α for 0.5, using the content of sunflower saturated fatty acids in *sn*-2 position (Álvarez-

Ortega et al. 1997) and increasing values for total saturated fatty acid content in the formulas (i), (ii), (iii) and (iv).

The α coefficient of an oil could also be calculated by chemically analyzing the fatty acid composition of the three *sn* positions of the TAG molecule. This analysis could be made following the methods proposed by Laakso and Christie (1990) or Takagi and Ando (1991). These methods make it possible to know the fatty acid content of the three *sn* positions, but need a large size sample and could not be applied to a small sample, like a half-seed as in our method. In this case the formula is as follows, αS being the smallest of these two values, except when both are 0.5. In this case α = 0.5.

$$\alpha S = Min\left(\frac{S_1}{S_1 + S_3}; \frac{S_3}{S_1 + S_3}\right)$$

The inventors have studied this distribution in TAG in actual sunflower oils. The data show, as expected, that saturated fatty acids (S) are located mainly in positions *sn*-1 and *sn*-3 of the glycerol molecule in normal and high saturated sunflower oils and in very low amount in *sn*-2. The main fatty acids in this position are oleic and linoleic acids as expected and in accordance with the data of Álvarez-Ortega et al. (1997). However, the acyl groups were not distributed according to the 1,3-random, 2-random theory (Vander Wal, 1960). The saturated fatty acids, palmitic and stearic, were not evenly distributed. These results are in agreement with previous data (Reske et al., 1997) that show a preference for position *sn*-3 over the position *sn*-1 of saturated fatty acids, mainly when stearic content was increased (11%) with respect to commodity sunflower that had

4.8%. The TAG distribution of sunflower oils differing in the stearic acid content and in the oleic/linoleic ratio, from high oleic to high linoleic is shown in **Figure 3**. It was found that the theoretical values of sunflower TAG species groups (SSS, SUS, SUU and UUU) expected for different saturated fatty acid content, based on the observed composition in position *sn*-2 and total fatty acid content applying the 1,3-random 2-random theory, are different from the TAG compositions found in the analyzed seeds. The TAG composition has been determined by GLC and the data of these TAG species grouped by level of unsaturation (Fernández-Moya et al., 2000).

As **Figure 3** shows, sunflower saturated fatty acids follow an asymmetric distribution in TAG, the obtained values for SUU always being higher than the expected values and the values for SUS and UUU lower than the anticipated values by a non-specific distribution in positions *sn*-1 and *sn*-3.

These results are also in agreement with previous results with high stearic sunflower mutant TAG species containing two molecules of linoleic acid and one saturated fatty acid that were more abundant than expected by the 1,3-random 2-random theory (Fernández-Moya et al., 2000). The increment of SUU and the reduction of UUU TAG species were directly correlated with the total stearic acid content in the oil.

The distribution coefficient of the saturated fatty acids (α) between positions *sn*-1 and *sn*-3 in control and high stearic mutant lines has been calculated (**Table 4**). This coefficient is always between 0.19 and 0.37 when the linoleic acid content is higher than the oleic acid content and between 0.15 and 0.27 when the oleic acid content is higher than the linoleic acid content.

Table 4

Stearic content (18:0), total saturated fatty acid content (S), the different TAG groups (SUS, SUU and UUU), and the value of the distribution coefficient α in several normal and mutant lines of sunflower are shown. RHA-274 is from USDA-ARS, Northern Crop Science Lab, Fargo, ND. Other lines are from Sunflower Collection of Instituto de la Grasa, CSIC, Seville, Spain. The content of the different fatty acids in the lines are represented as: HS, high stearic; MS, medium stearic; HL, high linoleic and HO, high oleic.

Line	Type	18:0	S	SUS	SUU	UUU	α
RHA-274	Normal	5	11.7	2.9	29.5	67.7	0.31
CAS-3	HSHL	25.6	37	28.6	48.9	21.6	0.33
CAS-29	HSHL	33.2	42	28.9	55.8	14.7	0.37
CAS-4	MSHL	12.9	20.3	8.3	44.5	47.3	0.29
G-8	HO	4.9	9.4	1.1	22.7	76.2	0.19
CAS-15	HSHO	23.4	33.5	19.3	61.9	18.8	0.23
DG-9	MSHO	19.1	29.8	16.8	55.6	27.5	0.27

Therefore there is still a need for a sunflower oil that has a distribution coefficient above 0.38.

SUMMARY OF THE INVENTION

The object of the present invention is provision of a sunflower oil directly obtained from sunflower seeds with at least 12%, preferably at least 20% of stearic acid referred to the total fatty acid content, and in said oil the distribution coefficient of saturated fatty acids α between positions *sn*-1 and *sn*-3 of the TAG molecule is at least 0.38, preferably at least 0.42 and most preferably 0.46.

When the oleic acid content is higher than the linoleic acid content in the oil and the stearic acid

content is at least 12%, preferably at least 20% referred to the total fatty acid content, the distribution coefficient of saturated fatty acids α between positions *sn*-1 and *sn*-3 in the TAG molecule is at least 0.28, preferably 0.32, most preferably 0.36.

The present invention further relates to sunflower plants that form seeds containing an endogenous oil, obtainable from said sunflower seeds, with the characteristics indicated above and to sunflower seeds produced by said sunflower plant.

It constitutes a further object of the present invention to provide a method for producing a plant which forms seeds containing an endogenous oil with at least 12% of stearic acid referred to the total fatty acid content and where the distribution coefficient of saturated fatty acids α between positions *sn*-1 and *sn*-3 is at least 0.38 or 0.28 when the oleic acid content is higher than the linoleic acid content.

It constitutes another object of the present invention to provide hybrid plants and their progeny that have the above saturated fatty acid distribution between positions *sn*-1 and *sn*-3 and other desirable characteristics.

DETAILED DESCRIPTION OF THE INVENTION

The invention thus relates to a sunflower oil directly obtained from sunflower seeds with at least 12% of stearic acid referred to the total fatty acid content, characterized in that the distribution coefficient of saturated fatty acids α between positions *sn*-1 and *sn*-3 is at least 0.38, which seeds are obtainable by a method comprising the steps of:

a) providing seeds which contain an oil having a stearic acid content of at least 12% referred to the total fatty acid content in the oil;

b) providing seeds which contain an oil having a distribution coefficient α higher than 0.38;

c) crossing plants from the seeds provided in step a) and b);

d) harvesting the F1 seed progeny;

e) planting the F1 progeny seeds to grow plants;

f) self-pollinating the plants thus grown to produce F2 seed;

g) testing the seed for the presence of a stearic acid content of at least 12% and a distribution coefficient α of at least 0.38;

h) planting seeds having the desired levels of stearic acid content and distribution coefficient α to grow plants;

i) self-pollinating the plants thus grown to produce F3 seed; and

j) optionally repeating steps g), h) and i) until the desired levels of stearic acid content and distribution coefficient α are fixed.

The distribution coefficient of saturated fatty acids α between positions *sn*-1 and *sn*-3 is preferably at least 0.42, more preferably at least 0.46.

The invention further relates to a sunflower oil directly obtained from sunflower seeds with at least 12% of stearic acid referred to the total fatty acid content, characterized in that the distribution coefficient of saturated fatty acids α between positions *sn*-1 and *sn*-3 is at least 0.28 when the oleic acid content is higher than the linoleic acid content in the oil, which seeds are obtainable by a method comprising the steps of:

a) providing seeds which contain an oil having a stearic acid content of at least 12% referred to the total fatty acid content in the oil and where the oleic acid content is higher than the linoleic acid content;

b) providing seeds which contain an oil having a distribution coefficient α higher than 0.38 in the oil;

c) growing plants from the seeds provided in step a) and b) and crossing them;

d) harvesting the F1 seed progeny;

e) planting the F1 progeny seeds to grow plants;

f) self-pollinating the plants thus grown to produce F2 seed;

g) testing the seeds for the presence of a stearic acid content of at least 12%, a higher oleic acid content than the linoleic acid content and a distribution coefficient α of at least 0.28;

h) planting seeds having the desired levels of stearic, oleic and linoleic acids content and distribution coefficient α to grow plants;

i) self-pollinating the plants thus grown to produce F3 seed; and

j) optionally repeating steps g), h) and i) until the desired levels of stearic, oleic and linoleic acids content and distribution coefficient α are fixed.

Preferably the distribution coefficient of saturated fatty acids α between positions *sn*-1 and *sn*-3 is at least 0.32, more preferably at least 0.36.

The invention also relates to sunflower plants which form seeds containing an endogenous oil as defined above and to the seeds produced by these plants.

In addition, the invention relates to a method for producing a plant which forms seeds containing an endogenous oil with at least 12% of stearic acid referred to the total

fatty acid content and wherein the distribution coefficient of saturated fatty acids α between positions *sn*-1 and *sn*-3 is at least 0.38, which method comprises:

a) providing seeds which contain an oil having a stearic acid content of at least 12% referred to the total fatty acid content in the oil;

b) providing seeds which contain an oil having a distribution coefficient α higher than 0.38;

c) growing plants from the seeds provided in step a) and b) and crossing them;

d) harvesting the F1 seed progeny;

e) planting the F1 progeny seeds to grow plants;

f) self-pollinating the plants thus grown to produce F2 seed;

g) testing the seeds for the presence of a stearic acid content of at least 12% and a distribution coefficient α of at least 0.38;

h) planting seeds having the desired levels of stearic acid content and distribution coefficient α to grow plants;

i) self-pollinating the plants thus grown to produce F3 seed; and

j) optionally repeating steps g), h) and i) until the desired levels of stearic acid content and distribution coefficient α are fixed.

The seeds which contain an oil with at least 12% of stearic acid are provided by:

a) treatment of sunflower seeds having a stearic acid content of less than 12% with a mutagenic agent, in particular sodium azide or an alkylating agent, more particularly ethyl methane sulfonate;

b) producing plants there from which are pollinated to produce seeds;

c) testing the seeds for the desired stearic acid content;

d) optionally repeating steps b) and c).

The seeds which contain an oil where the distribution coefficient of saturated fatty acids α between positions *sn*-1 and *sn*-3 is at least 0.38 are provided by:

a) treatment of sunflower seeds having a distribution coefficient α value smaller than 0.38 with a mutagenic agent, in particular sodium azide or an alkylating agent, more particularly ethyl methane sulfonate;

b) producing plants there from which are pollinated to produce seeds;

c)testing the seeds for the desired distribution coefficient α value;

d) optionally repeating steps b) and c).

In an alternative embodiment the invention relates to a method for producing a plant which form seeds containing an endogenous oil with at least 12% of stearic acid referred to the total fatty acid content, where the oleic acid content is higher than the linoleic acid content and where the distribution coefficient of saturated fatty acids α between positions *sn*-1 and *sn*-3 is at least 0.28, which method comprises:

a) providing seeds which contain an oil having a stearic acid content of at least 12% referred to the total fatty acid content in the oil and where the oleic acid content is higher than the linoleic acid content;

b) providing seeds which contain an oil having a distribution coefficient α higher than 0.28 in the oil;

c) crossing plants from the seeds provided in step a) and b);

d) harvesting the F1 seed progeny;

e) planting the F1 progeny seeds to grow plants;

f) self-pollinating the plants thus grown to produce F2 seed;

g) testing the seeds for the presence of a stearic acid content of at least 12%, a higher oleic acid content than the linoleic acid content and a distribution coefficient α of at least 0.28;

h) planting seeds having the desired levels of stearic, oleic and linoleic acids content and distribution coefficient α to grow plants;

i) self-pollinating the plants thus grown to produce F3 seed; and

j) optionally repeating steps g), h) and i) until the desired levels of stearic, oleic and linoleic acids content and distribution coefficient α are fixed.

The seeds which contain an oil with at least 12% of stearic acid are provided by:

a) treatment of sunflower seeds having a stearic acid content of less than 12% with a mutagenic agent, in particular sodium azide or an alkylating agent, more particularly ethyl methane sulfonate;

b) producing plants therefrom which are pollinated to produce seeds;

c) testing the seeds for the desired stearic acid content;

d) providing seeds which contain an oil where the oleic acid content is higher than the linoleic acid content;

e) crossing plants from the seeds tested in step c) and from the seeds provided in step d);

f) harvesting the F1 seed progeny;

g) self-pollinating the plants thus grown to produce F2 seed;

h) testing the seeds for the presence of a stearic acid content of at least 12% and a higher oleic acid content than the linoleic acid content;

i) planting seeds having the desired levels of stearic, oleic and linoleic acid content;

j) self-pollinating the plants thus grown to produce F3 seed; and

k) optionally repeating steps h), i) and j) until the desired levels of stearic, oleic and linoleic acid content are fixed.

The seeds which contain an oil where the distribution coefficient of saturated fatty acids α between positions *sn*-1 and *sn*-3 is at least 0.38 are provided by:

a) treatment of sunflower seeds having a distribution coefficient α value smaller than 0.38 with a mutagenic agent, in particular sodium azide or an alkylating agent, more particularly ethyl methane sulfonate;

b) producing plants therefrom which are pollinated to produce seeds;

c) testing the seeds for the desired distribution coefficient α value;

d) optionally repeating steps b) and c).

The invention further relates to hybrid plants obtainable by crossing a first parent plant resulting from the methods above and a second parent plant having desirable characteristics, and to progeny of the hybrid plant. The second parent plant may also be a plant resulting from the method above.

The invention also relates to the use of the oil in the production of food products and to food products prepared with or containing this oil.

More in particular, the invention relates to a new type of sunflower mutant, that has a better α distribution

than previous sunflower lines. Thus, this mutant has better properties for the production of margarine, spreads, etc. than the presently available lines (**Figure 3**). The best triglycerides for margarine are the saturated-unsaturated-saturated types (SUS), preferably a triglyceride of the type saturated-oleic-saturated (SOS).

This new mutant line, called CAS-36, obtained by technological methods has been deposited in the ATCC and has been given the accession number PTA-5041. This mutant has the best TAG distribution according to the random theory. Data from oil from a sample of seeds of some CAS-36 plants are shown in **Table 5**.

Table 5

Stearic, total saturated fatty acid content (S), oleic/linolenic acid ratio (O/L), the different TAG groups, and the value of the distribution coefficient α in some CAS-36 mutant plants are shown.

Plant	18:0	S	O/L	TAG groups			α
				SUS	SUU	UUU	
CAS-36-A	28.5	37.7	0.95	32.4	41.8	23.7	0.5
CAS-36-B	28.4	38.8	0.15	35.1	46.2	18.7	0.5
CAS-36-D	31.2	39.7	0.93	35.8	44.8	18.4	0.5
CAS-36-E	38.2	47.7	0.06	49.3	44.7	6.1	0.45

In U.S. patent 6,475,548 there is a comparison to a reference spread product made with the untreated oil of WO 95/20313, and a margarine made with the stearin fraction of the oil of WO 95/20313. The spread made with the stearin fraction apparently gave good spreadability at temperatures close to refrigerator temperature, appropriate melting in the mouth and stability. The performance of such a fat blend apparently gave a similar performance of known high quality

fat compositions, without non-natural components like hydrogenated fat.

It is well known that stearin fractions of fats can be used in a fat phase of spreads to resolve problems in making spreads. For example in U.S. Pat. No. 4,438,149 spreads were prepared with a fat phase containing less than 70% butter fat. This product was too soft in consistency. But when the stearin fraction of the fat was used, a less expensive and more spreadable product was made. U.S. Patent No. 6,475,548 teaches a method of the preparation of a triglyceride fat suited for structuring a liquid vegetable oil or spreads. The method of the preparation of a triglyceride fat was with a high stearic high oleic sunflower oil (HSHOSF), with at least 12 wt. % of stearic acid residues and at least 40 wt. % of oleic acid residues that is subjected to wet fractionation or a dry fractionation and a stearin fraction was collected. Further the above patent teaches that the stearin fraction of the fat blend was gotten by exposing the starting HSHOSF oil to standard conditions for fractionation, either wet or dry fractionation. The fraction containing >30 wt.% of SUS and <40 wt. % of SUU triglycerides should be collected and fractionation would be stopped when the first 25 wt. % of solid fat has crystallised.

The present invention could likewise be employed in a process of the preparation of a triglyceride fat with a high stearic high oleic sunflower oil (HSHOSF) by a wet fractionation or a dry fractionation and then collecting a stearin fraction. More particularly, preparation of a triglyceride fat with a high stearic high oleic sunflower oil (HSHOSF)with at least 12% of stearic acid referred to the total fatty acid content, characterized in that the distribution coefficient of saturated fatty acids α between

positions *sn*-1 and *sn*-3 is at least 0.28, is subjected to wet fractionation or a dry fractionation and a stearin fraction is collected.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the triglyceride biosynthetic pathway.

Figure 2 is the theoretical distribution of sunflower TAG species distribution with respect to increasing saturated fatty acid content when the α coefficient value is 0.5. See figure legend for TAG nomenclature.

Figure 3 shows the triglycerides (TAG) distribution in seeds segregating for high oleic and high stearic characters versus the saturated fatty acid content. Theoretical distribution, as control, is represented as lines and the different oil samples distribution are represented as symbols, see figure legend for TAG nomenclature.

The invention will be further illustrated in the Examples that follow and that are not intended to limit the invention.

EXAMPLES

Introduction

The invention relates to a method for preparing sunflower seeds having an better distribution of saturated fatty acids in the different triglycerides molecular species as compared to wild type seeds. This method includes the step of treating parent seeds with a mutagenic agent during a period of time and in a concentration sufficient to induce one or more mutations in the gene trait involved in triglycerides biosynthesis. This results in an increased production of triglycerides species of the type SUS and lower content of SUU. These mutagenic agents include agents such as sodium azide or an alkylating agent, like ethyl methane

sulfonate, but any other mutagenic agent having the same or similar effects may also be used. The treated seeds will contain inheritable genetic changes.

These mutated seeds are then germinated and progeny plants are developed therefrom. To increase the traits in the lines the progeny can be crossed or selfed. The progeny seeds are collected and analyzed. Seeds having the near random or random triglyceride trait can then be crossed to any other line and the trait transferred. Optionally, there can be additional cycles of germination, culturing, and selfing to fix the homozygosity of the traits in the lines and crossing and collection of seeds.

Ethyl methane sulfonate was used as mutagenic agent in Example 1. A sunflower line with a α value higher than 0.4 has been obtained. The original sunflower parent line mutagenised was CAS-10 (Sunflower Collection of Instituto de la Grasa, CSIC, Seville, Spain). The oil of that line has an α value smaller than 0.38. The high oleic material used herein is derived from the Russian researched oleic lines (Soldatov, 1976) having an α value between 0.15 and 0.27.

The high oleic high stearic material used is derived from crosses of the high oleic line with mutant CAS-3 deposited under ATCC accession number 75968, and selecting for high oleic high stearic seeds as described in WO-0074470 "High oleic high stearic plants, seeds and oils". A method for the preparations of the high α value plant with more linoleic than oleic acid and vice versa have been described in the following examples.

EXAMPLE 1

Seeds were mutagenized with a solution of 70 mM of ethyl methane sulfonate (EMS) in water. The treatment was performed at room temperature during 2 hours while shaking

(60 rpm). After mutagenesis the EMS solution was discarded and seeds were washed during 16 hours under tap water.

Treated seeds were germinated in the field and plants were self-pollinated. The seeds collected from these plants were used to select new sunflower lines with modifications in the triglyceride distribution. By using the method of Garcés, R. and Mancha, M. (1993) the seed fatty acid composition and by using the method of Fernández-Moya et al. (2000) the triglyceride composition were determined by gas liquid chromatography.

A first plant with a α value of 0.42 was selected. The progeny was cultivated for five generations wherein the α value increased and the new genetic trait became stably fixed in the genetic material of the seed. This line is called CAS-36 and has a linoleic content that is higher than the oleic acid content. The minimum and the maximum α value of the line were 0.38 and 0.5 respectively.

Table 6 shows some data of the analysis of seeds from several CAS-36 plants and the necessary data to calculate the α values according with the proposed formula.

Table 6

Fatty acid, total saturated (S) and saturated in *sn*-2 position (S2) composition of TAG and TAG composition and α Sat calculated following the formula for some CAS-36 oils.

CAS-36 plant	Fatty acid (mol %)								TAG (mol %)				
lines	16:0	18:0	18:1	18:2	20:0	22:0	S	S_2	SSS	SUS	SUU	UUU	αSat
BU-59	8.32	28.49	30.42	31.9	0.88	0	37.69	4.16	2.17	32.38	41.79	23.66	0.5
CB-71	6.09	28.94	28.14	34.57	1.3	0.96	37.29	4.12	0.85	32.61	44.1	22.44	0.5
CB-88	6.3	25.03	41.5	25.73	0.9	0.54	32.77	3.65	0	24.41	49.47	26.12	0.45
CB-79	7.63	23.88	32.15	34.02	1.3	1.01	33.83	3.76	0	26.1	49.28	24.62	0.46

EXAMPLE 2

Sunflower plants were grown from the sunflower seeds of the high oleic high stearic line like the one shown in **Table 2**. Sunflower plants were also grown from the sunflower seeds of CAS-36. The lines were crossed. The plants were assisted by artificial pollination in order to ensure adequate seed production occurred. The F1 seed was produced on the high oleic high stearic line, or vice versa, and harvested. The F2 seeds with more than 0.28 α value, high stearic and more oleic than linoleic acids were selected. Although this produces the oil of the present invention the level of production is limited. Therefore fixed inbred lines evidencing seeds with these α values are desirable.

These homozygous fixed inbred high oleic high stearic lines can then be crossed to form hybrid seed, which will produce F2 seed evidencing the desired oil traits of the present invention. Toward this end the F1 seeds were planted and produced plants were selfed in isolated conditions and F2 seed was produced. The F2 seed was tested for the α value. The remaining portion of the seeds evidencing the trait was employed to grow plants to form F3 seed. The selfing and screening and selection process is repeated to develop the fixed homozygous line with an α value higher than 0.28, having all of them an α value of 0.5 or near 0.5.

Once the trait is fixed similar high oleic lines can be crossed to form hybrid seed having the trait as shown in **Table 7**. According to the invention sunflower plants and seeds from which said oil can be extracted have been obtained by means of a biotechnological process. The α value content is an inheritable trait and is fairly independent from the growing conditions.

Table 7

Stearic (18:0), total saturated fatty acid content (S), oleic/linolenic acid ratio (O/L), the different TAG groups, and the value of the distribution coefficient α in lines derived from crosses of CAS-36 mutant line and CAS-15 or CAS-24 (**Table 2**) high stearic lines are shown. The Oleic versus linoleic content ratios are represented as O/L, being >1 because the oleic content is higher than the linoleic content in the selected lines.

Sunflower lines	18:0	S	O/L	SUS	SUU	UUU	α Sat
HO1	33.5	45.8	2.48	47.5	42.3	10.2	0.49
HO2	25.4	35	1.45	28.5	47.8	23.6	0.5
HO3	22.8	33.8	1.05	26.7	47.8	25.5	0.5
HO4	20.2	28.2	10.91	18.8	47	34.2	0.5
HO5	29.8	38.9	1.48	36.4	44	19.6	0.5
HO6	40.8	51	1.7	54.3	38.6	5.1	0.47
HO7	31	42.3	6.19	37.8	51.3	10.9	0.41
HO8	28.6	36.6	1.8	27.8	54.2	18	0.35

EXAMPLE 3

Sunflower plants were grown from the sunflower seeds of the high linoleic high stearic line like CAS-3, CAS-30, or any other of the high linoleic high stearic lines shown in **Table 2**. Sunflower plants were also grown from the sunflower seeds of CAS-36. The lines were crossed. The plants were assisted by artificial pollination in order to ensure that adequate seed production occurred. The F1 seed was produced on the high linoleic high stearic line, or vice versa, and harvested. The F2 seeds with an α value higher than 0.38, high stearic acid content and more linoleic than oleic acids were selected.

Although this produces the oil of the present invention the level of production is limited. Therefore fixed inbred lines evidencing seeds with these α values are desirable. These homozygous fixed inbred high oleic high

stearic lines can then be crossed to form hybrid seed, which will produce F2 seed evidencing the desired oil traits of the present invention. Toward this end the F1 seeds were planted and plants produced were selfed in isolated conditions and F2 seed was produced. The F2 seed was tested for the α value.

The remaining portion of the seeds evidencing the trait was employed to grow plants to form F3 seed. The selfing, screening and selection process are repeated to develop the fixed homozygous line with high linoleic high stearic acids content and an α value higher than 0.38, having all of them an α value of 0.5 or near 0.5. Once the trait is fixed similar high linoleic high stearic lines can cross to form hybrid seed having the trait as shown in Table 8. According to the invention sunflower plants and seeds from which said oil can be extracted have been obtained by means of a biotechnological process. The α value content is an inheritable trait and is fairly independent from the growing conditions.

Table 8

Stearic (18:0), total saturated fatty acid content (S), oleic/linolenic acid ratio (O/L), the different TAG groups, and the value of the distribution coefficient α in lines derived from crosses of CAS-36 mutant line and any of the high linoleic high stearic lines shown in **Table 2**, like CAS-3 or CAS-30 (Table 2) are shown. The oleic versus linoleic content ratios are represented as O/L, being <1 because the oleic content is lower than the linoleic content in the selected lines.

Sunflower lines	18:0	S	O/L	SUS	SUU	UUU	α Sat
HL1	31.1	36.7	0.75	31.7	46.6	21.7	0.5
HL2	31.1	39	0.42	34.8	47.5	17.8	0.49

HL3	21.1	25	0.35	15.5	43.9	40.5	0.5
HL4	14.3	23.6	0.15	13.3	44.1	42.6	0.5
HL5	30.3	43.5	0.05	41.4	45.7	12.2	0.47
HL6	32.4	44.5	0.09	41.7	47.9	9.7	0.44
HL7	36.9	47.9	0.28	47.8	43.4	7.2	0.46
HL8	31.2	40.9	0.12	36.4	49.7	13.8	0.43
HL9	30.4	42	0.29	38.2	49.6	12.2	0.43

REFERENCES

1. Álvarez-Ortega. R., Cantisán, S., Martínez-Force, E. and Garcés, R. (1997) Characterization of polar and non-polar seed lipid classes from highly saturated fatty acid sunflower mutants. Lipids 32, 833-837.
2. Chow, C. K. (1992) Fatty acids in foods and their health implications. Marcel Dekker, New York.
3. Fernández-Moya, V., Martínez-Force, E. and Garcés, R. (2000) Identification of triacylglycerol species from high-saturated sunflower (*Helianthus annuus*) mutants. Agric. Food Chem. 48, 764-769.
4. Garcés, R. and Mancha, M. (1993) One-step lipid extraction and fatty acid methyl esters preparation from fresh plant tissues. Anal. Biochem. 211, 139-143.
5. Gunstone, F.D. Harwood, J.L. and Padley, F.B. (1994) The Lipid Handbook. Chapman and Hall . London.
6. Laakso, P. and Christie, W.W. (1990) Chromatographic resolution of chiral diacylglycerol derivatives: potential in the stereo-specific analysis of triacyl-sn-glycerols. Lipids 25, 349-353.
7. Osorio, J., Fernández-Martínez, J., Mancha, M. and Garcés, R. (1995) Mutant sunflowers with high concentration of saturated fatty acids in the oil. Crop Sci. 35, 739-742.
8. Reske, J., Siebrecht, J. and Hazebroed, J. (1997) Triacylglycerol composition and structure in genetically modified sunflower and soybean oils. J. Am. Oil Chem. Soc. 74, 989-998.

9. Takagi, T. and Ando, Y. (1991) Stereospecific analysis of triacyl-sn-glycerols by chiral HPLC. Lipids 26, 542-547.

10. Vander Wal, R.J. (1960) Calculation of the distribution of the saturated and unsaturated acyl groups in fats, from pancreatic lipase hydrolysis data. J. Am. Oil Chem. Soc. 37, 18-20.

11. Soldatov, K.I. (1976) Chemical mutagenesis in sunflower breeding. pp. 352-357. In: Proc. 7th Int. Sunflower Association. Vlaardingen, The Netherlands

CLAIMS

1. Sunflower oil directly obtained from sunflower seeds with at least 12% of stearic acid referred to the total fatty acid content, characterized in that the distribution coefficient of saturated fatty acids α between positions *sn*-1 and *sn*-3 is at least 0.38, which seeds are obtainable by a method comprising the steps of:

a) providing seeds which contain an oil having a stearic acid content of at least 12% referred to the total fatty acid content in the oil;

b) providing seeds which contain an oil having a distribution coefficient α higher than 0.38;

c) crossing plants from the seeds provided in step a) and b);

d) harvesting the F1 seed progeny;

e) planting the F1 progeny seeds to grow plants;

f) self-pollinating the plants thus grown to produce F2 seed;

g) testing the seed for the presence of a stearic acid content of at least 12% and a distribution coefficient α of at least 0.38;

h) planting seeds having the desired levels of stearic acid content and distribution coefficient α to grow plants;

i) self-pollinating the plants thus grown to produce F3 seed; and

j) optionally repeating steps g), h) and i) until the desired levels of stearic acid content and distribution coefficient α are fixed.

2. Sunflower oil according to claim 1, characterized in that the distribution coefficient of saturated fatty acids α between positions *sn*-1 and *sn*-3 is at least 0.42.

3. Sunflower oil according to claim 1, characterized in that the distribution coefficient of saturated fatty acids α between positions *sn*-1 and *sn*-3 is at least 0.46.

4. Sunflower oil according to any one of the claims 1-3, characterized in that the stearic acid content referred to the total fatty acid content is at least 20%.

5. Sunflower oil directly obtained from sunflower seeds with at least 12% of stearic acid referred to the total fatty acid content and where the oleic acid content is higher than the linoleic acid content in the oil, characterized in that the distribution coefficient of saturated fatty acids α between positions *sn*-1 and *sn*-3 is at least 0.28, which seeds are obtainable by a method comprising the steps of:

a) providing seeds which contain an oil having a stearic acid content of at least 12% referred to the total fatty acid content in the oil and where the oleic acid content is higher than the linoleic acid content;

b) providing seeds which contain an oil having a distribution coefficient α higher than 0.38 in the oil;

c) growing plants from the seeds provided in step a) and b) and crossing them;

d) harvesting the F1 seed progeny;

e) planting the F1 progeny seeds to grow plants;

f) self-pollinating the plants thus grown to produce F2 seed;

g) testing the seeds for the presence of a stearic acid content of at least 12%, a higher oleic acid content than the linoleic acid content and a distribution coefficient α of at least 0.28;

h) planting seeds having the desired levels of stearic, oleic and linoleic acids content and distribution coefficient α to grow plants;

i) self-pollinating the plants thus grown to produce F3 seed; and

j) optionally repeating steps g), h) and i) until the desired levels of stearic, oleic and linoleic acids content and distribution coefficient α are fixed.

6. Sunflower oil according to claim 5, characterized in that the distribution coefficient of saturated fatty acids α between positions *sn*-1 and *sn*-3 is at least 0.32.

7.Sunflower oil according to claim 5, characterized in that the distribution coefficient of saturated fatty acids α between positions *sn*-1 and *sn*-3 is at least 0.36.

8.Sunflower oil according to any one of the claims 5-7, characterized in that the stearic acid content referred to the total fatty acid content is at least 20%.

9. Sunflower plant which form seeds containing an endogenous oil according to claims 1-4.

10. Sunflower seeds produced by sunflower plants according to claim 9.

11. Sunflower oil obtainable from sunflower seeds produced by sunflower plants according to claim 9.

12. Sunflower plant which forms seeds containing an endogenous oil according to claims 5-8.

13. Sunflower seeds produced by sunflower plants according to claim 12.

14. Sunflower oil obtainable from sunflower seeds produced by sunflower plants according to claim 12.

15. Method for producing a plant which forms seeds containing an endogenous oil with at least 12% of stearic acid referred to the total fatty acid content and wherein the distribution coefficient of saturated fatty acids α between positions *sn*-1 and *sn*-3 is at least 0.38, which method comprises:

a) providing seeds which contain an oil having a stearic acid content of at least 12% referred to the total fatty acid content in the oil;

b) providing seeds which contain an oil having a distribution coefficient α higher than 0.38;

c) growing plants from the seeds provided in step a) and b) and crossing them;

d) harvesting the F1 seed progeny;

e) planting the F1 progeny seeds to grow plants;

f) self-pollinating the plants thus grown to produce F2 seed;

g) testing the seeds for the presence of a stearic acid content of at least 12% and a distribution coefficient α of at least 0.38;

h) planting seeds having the desired levels of stearic acid content and distribution coefficient α to grow plants;

i) self-pollinating the plants thus grown to produce F3 seed; and

j) optionally repeating steps g), h) and i) until the desired levels of stearic acid content and distribution coefficient α are fixed.

16. Method as claimed in claim 15, wherein the seeds which contain an oil with at least 12% of stearic acid are provided by:

a) treatment of sunflower seeds having a stearic acid content of less than 12% with a mutagenic agent, in particular sodium azide or an alkylating agent, more particularly ethyl methane sulfonate;

b) producing plants there from which are pollinated to produce seeds;

c) testing the seeds for the desired stearic acid content;

d) optionally repeating steps b) and c).

17. Method as claimed in claim 15, wherein the seeds which contain an oil where the distribution coefficient of saturated fatty acids α between positions *sn*-1 and *sn*-3 is at least 0.38 are provided by:

a) treatment of sunflower seeds having a distribution coefficient α value smaller than 0.38 with a mutagenic agent, in particular sodium azide or an alkylating agent, more particularly ethyl methane sulfonate;

b) producing plants there from which are pollinated to produce seeds;

c)testing the seeds for the desired distribution coefficient α value;

d) optionally repeating steps b) and c).

18. Method for producing a plant which form seeds containing an endogenous oil with at least 12% of stearic acid referred to the total fatty acid content, where the oleic acid content is higher than the linoleic acid content and where the distribution coefficient of saturated fatty acids α between positions *sn*-1 and *sn*-3 is at least 0.28, which method comprises:

a) providing seeds which contain an oil having a stearic acid content of at least 12% referred to the total fatty acid content in the oil and where the oleic acid content is higher than the linoleic acid content;

b) providing seeds which contain an oil having a distribution coefficient α higher than 0.38 in the oil;

c) crossing plants from the seeds provided in step a) and b);

d) harvesting the F1 seed progeny;

e) planting the F1 progeny seeds to grow plants;

f) self-pollinating the plants thus grown to produce F2 seed;

g) testing the seeds for the presence of a stearic acid content of at least 12%, a higher oleic acid content than the linoleic acid content and a distribution coefficient α of at least 0.28;

h) planting seeds having the desired levels of stearic, oleic and linoleic acids content and distribution coefficient α to grow plants;

i) self-pollinating the plants thus grown to produce F3 seed; and

j) optionally repeating steps g), h) and i) until the desired levels of stearic, oleic and linoleic acids content and distribution coefficient α are fixed.

19. Method as claimed in claim 18, wherein the seeds which contain an oil with at least 12% of stearic acid are provided by:

a) treatment of sunflower seeds having a stearic acid content of less than 12% with a mutagenic agent, in particular sodium azide or an alkylating agent, more particularly ethyl methane sulfonate;

b) producing plants therefrom which are pollinated to produce seeds;

c) testing the seeds for the desired stearic acid content;

d) providing seeds which contain an oil where the oleic acid content is higher than the linoleic acid content;

e) crossing plants from the seeds tested in step c) and from the seeds provided in step d);

f) harvesting the F1 seed progeny;

g) self-pollinating the plants thus grown to produce F2 seed;

h) testing the seeds for the presence of a stearic acid content of at least 12% and a higher oleic acid content than the linoleic acid content;

i) planting seeds having the desired levels of stearic, oleic and linoleic acid content;

j) self-pollinating the plants thus grown to produce F3 seed; and

k) optionally repeating steps h), i) and j) until the desired levels of stearic, oleic and linoleic acid content are fixed.

20. Method as claimed in claim 18, wherein the seeds which contain an oil where the distribution coefficient of saturated fatty acids α between positions *sn*-1 and *sn*-3 is at least 0.38 are provided by:

a) treatment of sunflower seeds having a distribution coefficient α value smaller than 0.38 with a mutagenic agent, in particular sodium azide or an alkylating agent, more particularly ethyl methane sulfonate;

b) producing plants therefrom which are pollinated to produce seeds;

c) testing the seeds for the desired distribution coefficient α value;

d) optionally repeating steps b) and c).

21. A hybrid plant obtainable by crossing a first parent plant resulting from the method as claimed in any one of the claims 15-17 and a second parent plant having desirable characteristics.

22. A hybrid plant as claimed in claim 21, wherein the first parent is a plant as claimed in claim 9.

23. A hybrid plant obtainable by crossing a first parent plant which is a plant as claimed in claim 9 and a second parent plant which is a plant as claimed in claim 9 with other desirable characteristics.

24. A hybrid plant as claimed in claim 23, wherein said hybrid plant is a plant as claimed in claim 9 with other desirable characteristics.

25. A hybrid plant obtainable by crossing a first parent plant resulting from the method as claimed in any one of the claims 18-20 and a second parent plant having desirable characteristics.

26. A hybrid plant as claimed in claim 25, wherein the first parent is a plant as claimed in claim 12.

27. A hybrid plant obtainable by crossing a first parent plant which is a plant as claimed in claim 12 and a second parent plant which is a plant as claimed in claim 12 with other desirable characteristics.

28. A hybrid plant as claimed in claim 27, wherein said hybrid plant is a plant as claimed in claim 12 with other desirable characteristics.

29. Progeny of the hybrid plant claimed in any one of the claims 21-28.

30. Oil as claimed in any one of the claims 1-8, 11 and 14 for the production of a food product.

31. Food product comprising an oil as claimed in any one of the claims 1-8, 11 and 14.

Fig. 1

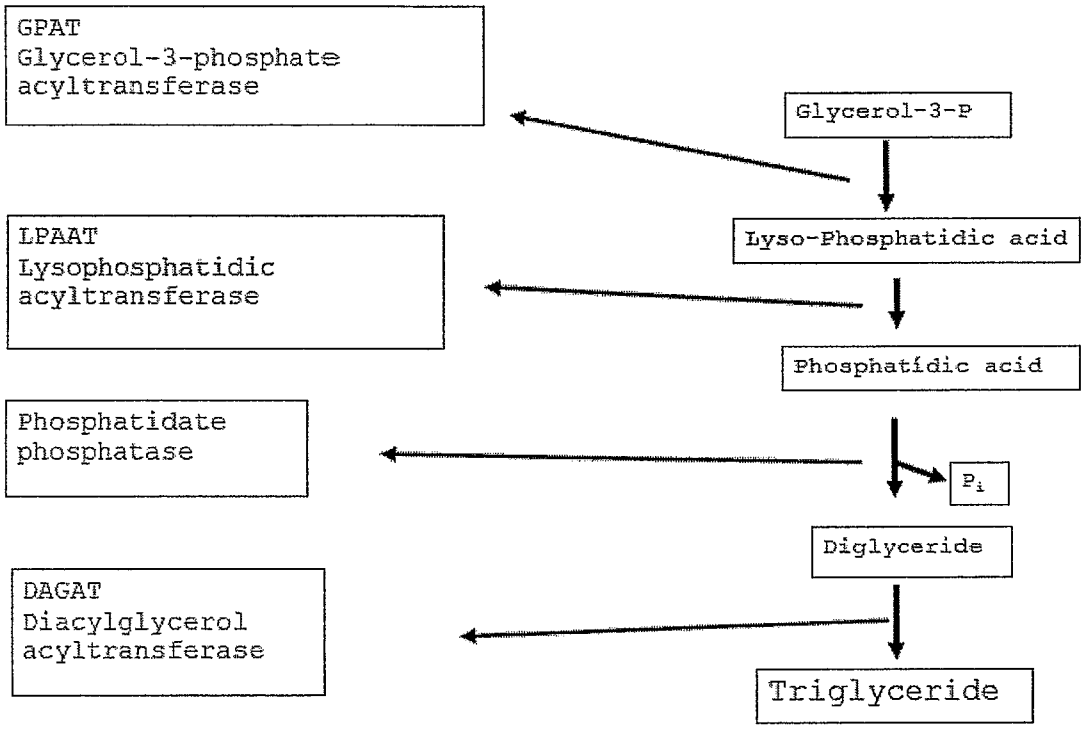

GPAT
Glycerol-3-phosphate acyltransferase
Glycerol-3-P
Lyso-Phosphatidic acid
LPAAT
Lysophosphatidic acyltransferase
Phosphatídic acid
Phosphatidate phosphatase
Pi
Diglyceride
DAGAT
Diacylglycerol acyltransferase
Triglyceride

Fig. 2

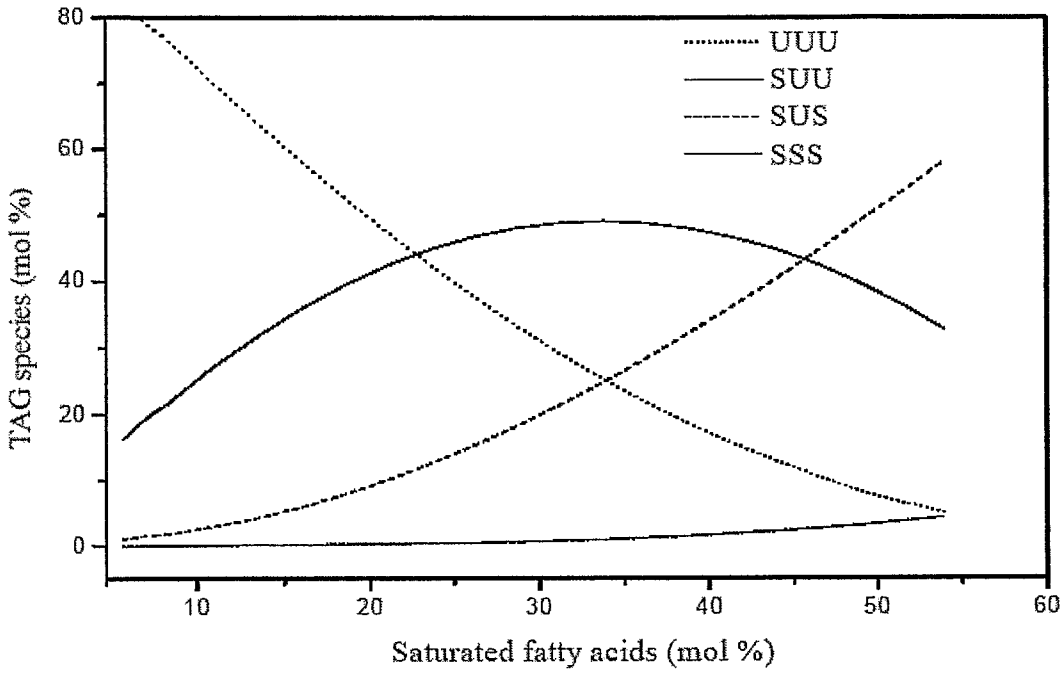
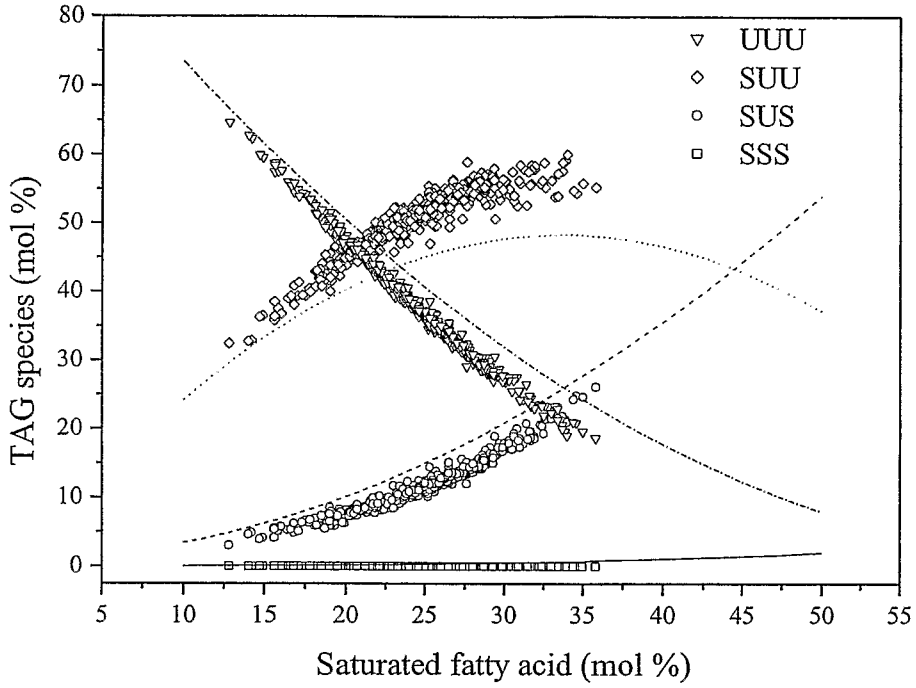

UUU
SUU
SUS
SSS
TAG species (mol %)
Saturated fatty acids (mol %)
0
20
40
60
80
10
20
30
40
50
60

Fig. 3


UUU
SUU
SUS
SSS
TAG species (mol %)
Saturated fatty acid (mol %)
0
10
20
30
40
50
60
70
80
5
10
15
20
25
30
35
40
45
50
55