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Process for the enrichment with $\Delta 6$ fatty acids of a mixture of fatty acids

1 This invention relates to the selective enrichment with polyunsaturated fatty acids, of which the first double bond is in the 6 position, more especially with γ -linolenic acid, of a mixture containing polyunsaturated fatty acids of which
5 the first double bond is in the 6 or 9 position.

 The biological importance of γ -linolenic acid (6,9,12-octadecatrienoic acid ($\Delta 6, \omega 6$)) is well known. It is an essential intermediate in the metabolic process which takes place in a living organism from linoleic acid (9,12-octadeca-
10 dienoic acid ($\Delta 9, \omega 6$)) and which leads by way of dihomogamma-linolenic acid (5,8,11-eicosatrienoic acid) to arachidonic acid (5,8,11,14-eicosatetraenoic acid). This property is utilized in its numerous dietetic, cosmetic and pharmaceutical applications. It is also known that α -linolenic acid (9,12,15-
15 octadecatrienoic acid ($\Delta 9, \omega 3$)) does not participate in this metabolic process in the same way.

 The majority of vegetable oils contain the $\Delta 9$ polyunsaturated acids, linoleic acid ($\omega 6$) and α -linolenic acid ($\omega 3$). A very interesting source of the $\Delta 6$ polyunsaturated
20 acid, γ -linolenic acid ($\omega 6$), is in the seeds of fruit of the genus Ribes, particularly black currant. The seed oil of this fruit contains appreciable quantities of $\Delta 9$ polyunsaturated acids in the form of linoleic acid ($\omega 6$) and α -linolenic acid ($\omega 3$). By way of illustration, the seed oil of this fruit
25 consists of triglycerides of the following fatty acids in percent by weight:

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1	Fatty acids	Black currant	Red currant	Gooseberry
	C16:0	6-7	4-5	7-8
5	C18:0	1-2	1-2	1
	C18:1 cis	9-10	14-15	15-16
	C18:1 trans	0.5	0.5-1	1-2
	C18:2,Δ9,12	47-49	41-42	39-41
	C18:3,Δ6,9,12	15-19	4-5	10-12
10	C18:3,Δ9,12,15	12-14	29-31	19-20
	C18:4,Δ6,9,12,15,	3-4	2.5-3.5	4-5

For certain applications, particularly pharmaceutical applications, it is desirable to enrich the mixture of fatty acids emanating from these oils selectively with Δ6 polyunsaturated acids, particularly with γ-linolenic acid. Now, the conventional method of fractionating fatty acids by high-performance liquid chromatography (HPLC) does not enable γ-linolenic acid to be separated from α-linolenic acid.

We have surprisingly found that these two isomers may be separated by a simple method hitherto used for separating fatty acids having different degrees of saturation. In particular, the separation of unsaturated fatty acids from saturated fatty acids is described, for example, in FR-PS 1 603 383, corresponding to GB-PS 1 240 513, which relates to the enrichment with γ-linolenic acid of a mixture of fatty acids emanating from the oil of the evening primrose (Oenothera) which moreover contains hardly any α-linolenic acid.

The present invention provides a process for the selective enrichment with polyunsaturated fatty acids, of which the first double bond is in the 6 position, of a mixture containing polyunsaturated fatty acids of which the first double bond is in the 6 or 9 position, characterized in that the mixture is treated with urea dissolved in an alkanol containing 1 to 4 carbon atoms in a ratio by weight of fatty acids other than the Δ6 polyunsaturated to urea of from 1:2.0 to 1:4.5, in that the insoluble inclusion complex formed is separated and in that a fraction enriched with Δ6 polyunsaturated fatty acids is collected in the liquid phase.

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1 The $\Delta 6$ polyunsaturated fatty acid is preferably γ -linolenic acid.

5 The process is preferably carried out using a mixture of fatty acids obtained by saponification of a starting material containing seed oil of fruit of the genus Ribes, preferably oil of black currant (Ribes nigrum). Thus, it is possible to saponify the crude or refined oil or, alternatively, directly to treat the seeds, preferably converted into flake form. Saponification is carried out conventionally by
10 treating the starting material with a concentrated strong base, for example sodium hydroxide, preferably in hot aqueous-alcoholic medium, advantageously containing a sequestering agent for metal ions, such as for example disodium ethylene diamine tetraacetate, separating the unsaponifiables with a
15 solvent, for example hexane, and neutralizing the aqueous phase, for example with hydrochloric acid in concentrated aqueous solution. After saponification, the mixture obtained is preferably protected against oxidation by addition of an antioxidant, for example 100 to 600 ppm (parts per
20 million) of propyl gallate or, preferably, 200 to 400 ppm of ascorbyl palmitate.

The soaps obtained during neutralization of the crude oil during refining may also be used as starting product.

The fractionation is carried out under conditions which
25 promote the selective formation of a complex of fatty acids other than $\Delta 6$ with urea in a medium in which the urea is soluble, but not the inclusion complexes formed. A suitable medium is an alkanol containing
from 1 to 4 carbon atoms, preferably ethanol or methanol,
30 methanol being particularly suitable by virtue of its high dissolving power on urea. The solution is preferably saturated and should contain from 45 to 50% by weight of urea. The saturated solution is advantageously prepared by dissolving the urea in the methanol, for example at around
35 60°C, while stirring until a transparent solution is obtained.

1 The quantity of urea is proportional to the total
quantity of fatty acids to be eliminated from the mixture.
When the mixture of fatty acids emanates from the saponification
of the starting material mentioned above, a ratio by weight
5 of starting material to urea of preferably about 1:3 is used.
The quantity of methanol used is advantageously from 2 to 6
times and preferably about 3 times the quantity by weight of
starting material used. After vigorous stirring of the
mixture, it is cooled to a temperature of from 0 to 12°C and
10 preferably to a temperature of from 4 to 6°C over a period of
from 10 to 20 h. After separation of the precipitate, for
example by centrifuging, the unreacted urea is neutralized
by treating the solution with an acid, for example with
preferably concentrated hydrochloric acid in aqueous solution,
15 and the fatty acids are extracted with a solvent, preferably
hexane, which is then eliminated, preferably by evaporation
in vacuo.

In one preferred embodiment, the fraction enriched
as described above is treated once more with urea in a ratio
20 by weight of total fatty acids to urea of from 1:1.4 to
1:1.6. This gives a fraction containing from 92 to 96% by
weight of Δ6 polyunsaturated fatty acids of which from 77
to 81% consist of γ-linolenic acid.

If desired, the substantially pure γ-linolenic acid may
25 be prepared from the fraction emanating from the second
fractionation with urea by reverse high-performance liquid
chromatography which enables the γ-linolenic acid to be
separated from stearidonic acid (C18:4, Δ6,9,12,15).

Alternatively, a mixture containing the fatty acids
30 emanating from saponification may be separated by chromato-
graphy and then fractionated with urea as described in the
foregoing.

The free fatty acid fractions obtained in accordance
with the invention may be used as such or in the form of an
35 oil obtained by recombination with glycerol for any of the

- 1 various applications of γ -linolenic acid, for example in the
nutritive compositions or the medicaments described in
published European Patent Applications 92 085 (corresponding
to GB-PA 2.118.567) and 92 076 (corresponding to US-PS
5 4.526.793) and administered orally, enterally, parenterally
or topically.

The fractions recombined with glycerol in the form of an
oil are particularly suitable for topical application in
dermatology and in cosmeto-dermatology. The fractions
10 containing the substantially pure γ -linolenic acid may also
be used as starting product in the synthesis of dihom- γ -
linolenic acid.

The invention is illustrated by the following Examples
in which the percentages and parts are by weight, unless
15 otherwise indicated.

EXAMPLE 1

To 30 kg of refined and deodorized black currant oil are
20 added 63.9 kg of a 14.2% aqueous-ethanolic solution of sodium
hydroxide containing 95 g of disodium ethylene diamine tetra-
acetate. The mixture is heated to 60°C and is then stirred
at that temperature for 30 minutes. 12 kg of water are then
added and the solution is cooled to 30°C.

25 After addition of 79 kg of hexane and stirring for 1 hour
at 30°C, the mixture is left for 15 minutes to separate and
the upper phase containing the unsaponifiables is eliminated.
30 kg of 32% aqueous hydrochloric acid are then added to the lower
phase with stirring (to pH 1), ensuring that the temperature
30 does not exceed 30°C. After decantation, the lower phase
is eliminated and the upper phase is concentrated in a water-
jet vacuum pump at 40°C.

The 28.5 kg of the fatty acid mixture obtained is added
with continuous stirring to a saturated, clear solution of
35 90 kg of urea in 190 kg of methanol at 60°C. The mixture

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1 is cooled to 5°C and kept at that temperature for 15 h. The solid phase formed is then separated by centrifuging whilst the liquid phase is left standing for 4 h at 5°C. The liquid phase is again separated from the solid phase by centrifuging.

5 180 kg of liquid phase are thus collected. 45.9 kg of hexane, 39.9 kg of a 32% aqueous hydrochloric acid solution and 106 kg of water are then added to the liquid phase and the mixture is heated to 30°C. After stirring for 1 h at 30°C, the mixture is left standing for 10 mins., after which

10 the upper phase is collected by decantation. The lower phase is then extracted with 12 kg of hexane while stirring for 15 minutes at 30°C. The mixture is left standing for 15 mins., after which the upper phase is collected by decantation and combined with the preceding upper phase.

15 50 kg of water are then vigorously mixed with the combined phases. The mixture is then left standing for 3 h at ambient temperature and the upper phase containing the fatty acids is separated and dried at 40°C by evaporation in a water-jet vacuum pump. 6.82 kg of fatty acids are thus

20 obtained (yield 22.7%, based on the oil used). The mixture has the following composition by weight, as determined by gas chromatography:

		%
25	C18:1	0.6
	C18:2,Δ9,12	22.1
	C18:3,Δ6,9,12	55.6
	C18:3,Δ9,12,15	10.7
	C18:4,Δ6,9,12,15	11.0

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COMPARISON EXAMPLE

The fatty acids from black currant oil used in the previous Example are fractionated by preparative high-performance liquid chromatography in the form of their methyl esters

35 using reverse-phase RP-18 columns.

The elution of a sample having a concentration of 20% in the following solvent mixture: 67.5% methanol/22.5% ethanol/10% water, at a rate of 150 ml/min. gives the following fractions:

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- 1) a first fraction representing 7.6% of the mixture and containing 52% of stearidonic acid, C18:4,Δ6,9,12,15;
- 2) a second fraction representing 25.4% of the mixture and containing 41% of α-linolenic acid, C18:3,Δ9,12,15 and 43% of γ-linolenic acid, C18:3,Δ6,9,12;
- 3) a third fraction representing 45.8% of the mixture and containing 87% of linoleic acid, C18:2,Δ9,12 and
- 4) a fourth fraction representing 21.2% of the mixture and containing 85% of saturated fatty acids.

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Comparison of the ratios between the quantities of γ-linolenic acid and α-linolenic acid calculated for the mixture obtained in accordance with Example 1, for fraction 2) of the Comparison Example and for black currant oil provides an indication of the selectivity of the enrichment process according to the invention:

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	Mixture of Exam- ple 1	Black currant oil	Fraction 2) above
25			
% γ-linolenic acid	5.2	1.3	1.1
% α-linolenic acid			

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It can be seen from the values indicated above that, contrary to the process according to the invention, the conventional method of chromatography does not enable the γ- and α-linolenic acids to be separated from a mixture containing them, so that the mixture cannot be enriched with γ-linolenic acid.

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EXAMPLE 2

A mixture of 10.2 kg of urea and 21.5 kg of methanol is heated to 60°C until a saturated, clear solution is obtained. The 6.82 kg of fatty acid mixture emanating from the fractionation according to Example 1 are then added with stirring to that solution. The mixture is then cooled to 5°C and left standing at that temperature for 15 h. The solid phase formed is then separated by centrifuging whilst the liquid phase is left standing for 4 h at 5°C. The liquid phase is then centrifuged again and the crystals formed are eliminated.

5.9 kg of hexane, 5.1 kg of a 32% aqueous hydrochloric acid solution and 13.5 kg of water are then added to 23 kg of the solution obtained. The mixture heated to 30°C is then vigorously stirred for 15 minutes. It is then left standing for 10 mins. to separate and the upper phase containing the fatty acids is separated off. 3 kg of hexane are then added to the lower phase and the mixture is vigorously stirred for 15 mins. The mixture is then left standing for 15 mins, after which the upper phase is collected by decantation and combined with the preceding upper phase.

The combined phases are then mixed with 30 kg of water while stirring for 15 mins. The mixture is left standing for 3 h, after which the lower phase is eliminated by decantation and the upper phase is collected and dried by evaporation at 40°C in a water-jet vacuum pump. 400 ppm (parts per million) of ascorbyl palmitate are then added to the 4.05 kg of fatty acid mixture obtained (yield 13.5%, based on the mixture of fatty acids used).

The fatty acid mixture obtained has the following composition by weight, as determined by gas chromatography:

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	<u>%</u>
C18:2, Δ9, 12	2.5
C18:3, Δ6, 9, 12	78.6
C18:3, Δ9, 12, 15	2.3
C18:4, Δ6, 9, 12, 15	16.6

EXAMPLE 3

Black currant seeds are converted into flakes. To
10 35 kg of these flakes are added 150 kg of a 14.2% aqueous-
ethanolic solution of sodium hydroxide containing 223 g of
disodium ethylene diamine tetraacetate, the suspension
heated to 60°C is stirred for 1 hour and the residue formed
is separated by filtration in vacuo. The filter is then
15 rinsed and washed with a quantity of ethanol
equal to the weight of the flakes used, after which the
majority of the ethanol is evaporated in the form of an
azeotropic mixture. A 32% aqueous hydrochloric acid solution
is then added to the residue until a pH of 1 is obtained.
20 After decantation, the majority of the fatty acids are
separated in the form of the upper organic phase formed.
35 kg of hexane are added to the lower aqueous phase which is
then decanted again. The lower phase is washed a second
time with 35 kg of hexane. The combined organic phases
25 (comprising the fatty acids and the two solutions in hexane)
are then washed twice with 70 kg of water and the solvent is
evaporated at 40°C in a water-jet vacuum pump. The residue
represents 6.72 kg of a mixture of fatty acids of which the
composition as determined by gas chromatography is identical
30 with that of the mixture obtained by saponification of the
oil used in Example 1. The mixture is then subjected to
fractionation under the conditions of Example 1.

EXAMPLE 4

35 A 20% solution of the mixture of fatty acids emanating



1 from the double fractionation of Example 2 is prepared in
the following solvent mixture: 67.5% methanol/22.5% ethanol/
10% water.

5 10 ml of this solution are then injected into a preparative
high-performance liquid chromatography apparatus provided with
reverse-phase RP-18 silica gel columns. The above solvent
mixture is used as the mobile phase at a rate of 100 ml/min.
and the quantitative identification is carried out with a
refractive index detector.

10 The two principal fatty acids (γ -linolenic and stearidonic)
of the mixture obtained in accordance with Example 2 (78.6%
 γ -linolenic acid, 16.6% stearidonic acid) are effectively
separable by the above method, the two eluted fractions
obtained having the following compositions:

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		<u>%</u>	
	- first fraction:	C18:3,Δ6,9,12	49
	(representing 20%		
20	of the mixture)	C18:4,Δ6,9,12,15	51
			<u>%</u>
	- second fraction:	C18:3,Δ6,9,12	96
	(representing 80%		
25	of the mixture)	C18:3,Δ9,12,15	2.5
		C18:4,Δ6,9,12,15	1.5

The second fraction may advantageously serve as starting
product in the synthesis of dihomog γ -linolenic acid.

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EXAMPLE 5

100 g of the fatty acid mixtures obtained in accordance
with Examples 1 to 4 are reacted with 15 g of glycerol for
35 5 h at 210°C under a pressure of 5 mm Hg. 96 g of triglycerides



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1 are thus obtained in a yield of 92%. The oils obtained are suitable for dermatological and cosmeto-dermatological applications.

CLAIMS

1. A process for the selective enrichment with polyunsaturated fatty acids, of which the first double bond is in the 6 position, of a mixture containing polyunsaturated fatty acids of which the first double bond is in the 6 or 9 position, characterized in that the mixture is treated with urea dissolved in an alkanol containing 1 to 4 carbon atoms in a ratio by weight of fatty acids other than the $\Delta 6$ polyunsaturated to urea of from 1:2.0 to 1:4.5, in that the insoluble inclusion complex formed is separated and in that a fraction enriched with $\Delta 6$ polyunsaturated fatty acids is collected in the liquid phase.
2. A process as claimed in Claim 1, characterized in that the starting mixture of fatty acids is obtained by saponification of seeds of fruit of the genus Ribes or of an oil extracted therefrom.
3. A process as claimed in Claim 2, characterized in that the seeds or oil are of blackcurrant, Ribes nigrum.
4. A process as claimed in any of Claims 1 to 3, characterized in that the ratio of total fatty acids to urea is 1:3.
5. A process as claimed in Claim 1, characterized in that the fraction enriched with $\Delta 6$ polyunsaturated fatty acids obtained is treated with urea in a ratio by weight of total fatty acids to urea of 1:1.5, in that the insoluble inclusion complex formed is separated and in that a



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fraction containing from 92 to 96% by weight of Δ^6 polyunsaturated fatty acids, of which 77 to 81% consist of γ -linolenic acid, is collected.

6. A process as claimed in any of Claims 1 to 5, characterized in that the mixture is treated with a saturated solution of urea in methanol.

7. A process as claimed in Claim 5, characterized in that the substantially pure γ -linolenic acid is separated from the mixture of Δ^6 polyunsaturated fatty acids by reverse-phase high performance liquid chromatography.

8. A process as claimed in any of Claims 1 to 7, characterized in that the mixture enriched with Δ^6 polyunsaturated fatty acids is esterified with glycerol to obtain an oil enriched with Δ^6 polyunsaturated fatty acids.

9. A process as claimed in any of Claims 1 to 8 characterized in that the Δ^6 polyunsaturated fatty acid is γ -linolenic acid.

10. A fraction containing the γ -linolenic acid obtained by the process claimed in any of Claims 1 to 7 or 9.

11. An oil obtained containing the γ -linolenic acid obtained by the process as claimed in claims 8 or 9.

12. A composition containing the oil claimed in Claim 11.

13. The use of a fraction obtained in accordance with Claim 7 as starting product in the synthesis of dihomogamma-linolenic acid.

