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(54) Titre : METHODE DE FRACTIONNEMENT DES ACIDES GRAS

(54) Title: PROCESS FOR THE FRACTIONATION OF FATTY ACIDS

(57) **Abrégé/Abstract:**

To prepare a fraction enriched in polyunsaturated fatty acids with a degree of unsaturation of 3 and more from a mixture of polyunsaturated fatty acids, an oil rich in polyunsaturated fatty acids is hydrolysed by a non-stereospecific lipase of Candida cylindracea, the mixture is esterified by enzymatic reaction in the presence of methanol and the reaction mixture is saponified under controlled conditions, which results in separation into an aqueous phase and organic phase, and the non-esterified fatty acids are then extracted from the aqueous phase by a non-polar solvent and the solvent is removed.

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Abstract

Process for the fractionation of fatty acids

To prepare a fraction enriched in polyunsaturated fatty acids with a degree of unsaturation of 3 and more from a mixture of polyunsaturated fatty acids, an oil rich in polyunsaturated fatty acids is hydrolysed by a non-stereospecific lipase of Candida cylindracea, the mixture is esterified by enzymatic reaction in the presence of 10 methanol and the reaction mixture is saponified under controlled conditions, which results in separation into an aqueous phase and organic phase, and the non-esterified fatty acids are then extracted from the aqueous phase by a non-polar solvent and the solvent is removed.

Process for the fractionation of fatty acids

The invention concerns an essentially enzymatic process for the fractionation of polyunsaturated fatty acids.

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Fatty acids of the n-6 and n-3 series have a nutritional value, in particular as precursors in the biosynthesis of prostaglandins. It may be advantageous to have available fractions enriched in these fatty acids for various
10 nutritional and cosmetic applications. These fatty acids are found in nature principally in the form of triglycerides. Free fatty acids are obtained industrially from triglycerides by hydrolysis at a high temperature and under a high pressure. In order then to fractionate these
15 fatty acids, several methods have been developed, for example crystallization, distillation, the formation of inclusion complexes or chromatographic techniques. Application of these methods may bring about degradation in the case of polyunsaturated fatty acids or prove to be too
20 costly to be applied industrially.

Enzymatic methods represent an alternative to the preceding methods since they enable reactions to be carried out under mild conditions using little energy and equipment which is
25 less stressed.

Enzymatic processes are known, for example from Matthew J. Hills et al in JAOCS, Vol.67, no.9, p.561-563, for fractionating fish oil and evening primrose oil fatty acids
30 based on the fact that the kinetics of the esterification by butanol catalysed by rape lipases and mucor miehei would be a function of the degree of saturation of the acid to be esterified. After esterification of fatty acids with a higher degree of saturation, the esters are separated from
35 the fatty acids which have not reacted, by thin layer chromatography.

The object of the invention is to provide a process for the fractionation of fatty acids from oils rich in

polyunsaturated fatty acids by enzymatic esterification which is applicable industrially, avoiding chromatographic separation.

5 The invention thus concerns an essentially enzymatic process for the fractionation of polyunsaturated fatty acids, in which esterification is carried out of a mixture of fatty acids by enzymatic catalysis, the esters formed are then separated from the fatty acids which have not
10 reacted and a fraction is obtained enriched in the desired polyunsaturated fatty acids, characterized in that the esters of the fatty acids are separated by controlled saponification of the fatty acids, extraction of the soaps formed with water, acidification of the aqueous phase and
15 extraction of the acids formed with a non-polar solvent.

In order to put the process according to the invention into practice, a mixture of starting fatty acids is used derived, preferably, from the total enzymatic hydrolysis of
20 the triglycerides of an oil rich in polyunsaturated fatty acids. This hydrolysis preferably takes place in a medium emulsified by a non regio-specific lipase of Candida cylindracea, preferably at room temperature, at neutral pH or close to neutrality and at atmospheric pressure.

25

As the starting oil, any natural oil may be cited of animal or vegetable or synthetic origin containing polyunsaturated fatty acids, in particular with a degree of unsaturation of three and more, for example blackcurrant seed oil, borage
30 oil, evening primrose oil, rich in gamma-linolenic acid (GLA) or "TGA" oil rich in arachidonic acid.

Esterification takes place in the presence of methanol and a solvent, for example hexane or a small quantity of water.
35 The reaction lasts 5 to 70 h and preferably 10 to 40 h, preferably at room temperature. The enzyme used may be immobilized or not. It is preferably immobilized so that it can be reused. It may be regio-specific or not.

After reaction, a mixture is obtained of free fatty acids and the methyl esters of fatty acids. The esterified fraction is impoverished in fatty acids with a high degree of unsaturation, for example in the case of blackcurrant seed oil particularly in GLA and in stearidonic acid (SA), which indicates that these acids are hardly esterified at all and consequently enriched in the free fatty acids fraction.

10

According to the invention, separation of free fatty acids from the esters takes place by saponification of fatty acids under mild conditions. These conditions are characterized by a reaction with a weak base in an aqueous medium, for example carbonates, phosphates, citrates of sodium, potassium or ammonium or their mixtures, preferably sodium carbonate, at a temperature starting from room temperature, for example 20°C up to about 80°C, with stirring and preferably increasing stirring and progressively the temperature from 45°C up to about 75°C. Separation is then observed between an aqueous phase and an organic phase and this separation can be accentuated, for example by addition of a saturated solution of sodium chloride.

25

It is possible by this process to react only the fatty acids which, in the form of soaps, become water soluble. The aqueous phase obtained can be then separated easily from the organic phase containing the liposoluble esters after decantation, for example by centrifuging. The free fatty acids can be recovered by acidification of the aqueous phase by an acid, for example concentrated hydrochloric acid, extracted by a solvent, for example hexane, and the solvent can then be removed, for example by evaporation.

The following examples illustrate the invention. In these, the percentages and parts are by weight, unless indicated

to the contrary. As regards the quantitative analysis of free fatty acids, this was carried out, following methylation by acetyl chloride, by gaseous phase chromatography (GPC), and thus on the basis of the methyl 5 esters.

Example 1

1.1. Preparation of the starting mixture of fatty acids. 10

A mixture of fatty acids was used as starting material derived from the enzymatic hydrolysis of a blackcurrant seed oil by means of a nonspecific lipase of Candida cylindracea. The mixture of fatty acids was obtained in 15 the following manner :

An oil in water emulsion was prepared containing 20% of blackcurrant seed oil and 1.2% of soya lecithin (Asol 100 (R) Lucas Meyer) dissolved in 78.8% of an aqueous solution 20 of 0.025 M phosphate buffer of pH 6.88 and carrying out 5 passes through a microfluidizer (110T, Microfluidics Corporation, Newton), which led to a mean diameter of the oil droplets of about 450 nm.

25 Lipase of Candida cylindracea type B, Biocatalysts Ltd., Cardiff, England, was solubilized in the phosphate buffer, and then centrifuged at 4000 g for 20 min. to remove the insoluble residues. The supernatant was used for the experiments. 10 ml of the preceding emulsion (containing 30 2 g of blackcurrant seed oil) were placed in a 25 ml stoppered Erlenmeyer flask, in a bath thermostatically controlled to a temperature of 37°C with magnetic stirring at 250 rpm to which the enzyme solution was added corresponding to 0.2 g of lipase.

35

After reacting for 4 h, the medium was centrifuged at 4000 g to break the emulsion and the lipid phase was recovered by extraction with ether. The extract was washed.

with water, dried over sodium sulphate, and the solvent then eliminated by evaporation. The fatty acids obtained were stored at -25°C protected from the light and under nitrogen.

5

The lipase in solution in the aqueous phase was recovered as well as the glycerol formed by ultrafiltration (module YM 10, cut off threshold 10,000, Amicon, Denver, USA), which gave a concentrated solution of lipase which could be 10 reused.

The degree of hydrolysis, corresponding to the percentage of fatty acids liberated during the reaction corresponded to 99.9%, determined by acid-base titration with the aid of 15 a Metrohm titroprocessor 692. The sample to be analysed, dissolved in 25 ml of an equivolume mixture of ethanol and ethyl ether was titrated with an alcoholic solution of KOH with a concentration of 0.1 N.

20 The mixture of fatty acids had the following composition determined by GPC in the form of methyl esters :

Fatty Acids		%
	C 16:0	7.1
25	C 18:0	1.7
	C 18:1	13.3
	C 18:2	45.6
	C 18:3 gamma	15.5
	C 18:3 alpha	12.2
30	C 18:4	2.9
	C 20:1	0.8
	C 20:2	0.2
	Others	0.8

35 1.2 Esterification

The fatty acids were esterified by methanol using 900 mg of the mixture of fatty acids in a mixture of 11 ml of hexane and 1 ml of methanol and 1200 mg of immobilised enzyme, 40 Lipozyme TM 20 from Mucor Miehei, Novo Nordisk, A/S Denmark. This enzyme has an increased specificity for

positions 1 and 3 of the glycerol skeleton of the triglycerides compared with position 2. The reaction was carried out at room temperature in a glass flask provided with a magnetic bar and placed on a mechanical stirrer for 5 20 h. After reaction, the enzyme was filtered off which, after rinsing and rehydration with 10% water by volume, could be reused. A mixture of free fatty acids and methyl esters of fatty acids was obtained.

10 1.3. Separation of fatty acids and methyl esters.

20 g of the preceding mixture of fatty acids and fatty acid methyl esters were heated at 40°C, and then 1.1 g of sodium carbonate was added dissolved in water with stirring. The 15 rate of stirring was then increased and the temperature raised to 75°C. When this latter temperature was reached, heating was discontinued and 20 ml of saturated salt solution was added. The formation of an organic phase and an aqueous phase was then noted. Everything was then 20 centrifuged for 10 min at 3000 rpm, two phases were separated and the aqueous phase which contained soaps was then solidified with a solution of hydrochloric acid. The fatty acids formed were then extracted with hexane and the hexane was then evaporated off. 1.5 g of fatty acids were 25 thus recovered enriched in GLA and in SA having the following composition (GPC of methyl esters):

Fatty Acids	%
C 16:0	1.3
30 C 18:1	2
C 18:2	6.5
C 18:3 gamma	73.4
C 18:3 alpha	2.4
C 18:4	13.6
35 Others	0.8

Example 2

The procedure was as in example 1, except that in the esterification stage (corresponding to 1.2 of example 1), a mixture of solvents was used consisting of 9 ml of methanol and 1 ml of water.

64.6% of GLA and 11.8% of SA were thus obtained in the enriched phase (consisting of the non esterified part obtained from the soaps).

10

Examples 3-7

The procedure was as in example 1, except for the fact that, in the esterification stage (corresponding to 1.2 in example 1), the enzyme reaction time was varied as indicated, and with the results of the % GLA and SA in the enriched phase indicated below (determined by GPC of the methyl esters) :

Example	3	4	5	6	7
Reaction time, h	5	10	30	40	70
% GLA	70	73.5	77.6	75.6	74.8
% SA	12	13	12.6	13.3	12.8

20

Examples 8-15

The esterification of a mixture of blackcurrant seed oil fatty acids was carried out as in example 1 (paragraph 1.2) with enzymes of various origins and regio-specificities by reaction at room temperature for 20 h. The results obtained were evaluated by determining the composition in fatty acids of the esterified fraction (by GPC in the form of methyl esters) :

30

Example	8	9	10	11	12	13	14	15
Enzyme of	<u>Rhizopus javanicus</u> Bio-catalysts	<u>Penicillium cyclopium</u>	<u>Penicillium roqueforti</u>	<u>Geotrichum candidum</u>	Lipo-mod, mixture of lipases	<u>Candida cylindracea</u> Type OF	<u>Rhizopus javanicus</u> , Type F-AP 15	<u>Aspergillus niger</u> type AP 6
C 13:0	-	-	-	0.5	-	-	-	-
C 16:0	7.6	5.8	8.8	11.2	8.4	7.9	8.1	7.6
C 16:1	-	0.1	-	-	-	-	-	-
C 18:0	1.2	1.1	1.8	1.8	1.5	1.4	1.3	1.4
C 18:1	15.8	15.7	16.3	12.8	16.9	15.9	15.4	17.4
C 18:2	57	59	54.9	48.7	56.8	56.2	56.1	57.7
C 18:3 gamma	2.1	1.6	2.2	1.2	1.8	2	5.1	2
C 18:3 alpha	14.5	15.3	13.5	7.2	12.3	14.4	12.9	13
C 18:4	-	-	-	1.8	1	0.3	-	-
C 20:1	0.6	0.6	0.8	0.6	-	0.8	0.6	0.8
C 20:2	-	0.2	-	-	-	0.2	-	-
Others	1.3	0.6	1.8	14.2	1.4	0.8	0.5	0.1

- : not quantifiable

5 The preceding results show that in all cases the esterified fraction was impoverished in GLA and in SA, which indicates that these two acids were hardly esterified at all and hence that the free fatty acids fraction was enriched in these acids.

10

Example 16

As a raw material, use was made of a total hydrolysate of a synthetic TGA oil from Suntory Ltd. Tokyo, Japan, extracted from Mortierella fungus, very rich in arachidonic acid.

15 After hydrolysis of the triglycerides, the mixture of free fatty acids was esterified as in example 1 (paragraph 1.2) with Lipozyme TM 20 for 20 h at room temperature. The methyl esters of the free fatty acids were then separated

by thin layer chromatography, and the composition of the mixture of free fatty acids was then analysed. The composition of the starting mixture of fatty acids and that of the mixture of free fatty acids obtained after 5 esterification (determined by GPC of the methyl esters) are indicated below :

	Composition of fatty acids %	Of the TGA oil	Of the esterified fraction
10	C 14:0	0.7	-
	C 16:0	17.2	5.6
	C 16:1	0.2	-
	C 17:0	0.3	0.2
15	C 18:0	9.2	5.5
	C 18:1	22.3	7.7
	C 18:2	8.3	2.4
	C 18:3 gamma	1.7	6.5
	C 18:3 alpha	1	-
20	C 20:0	0.9	0.9
	C 20:1	1	0.7
	C 20:2	0.7	0.5
	C 20:3	3.9	13.1
	C 20:4	20.2	40.2
25	C 22:0	3.5	4.2
	C 22:6	8.2	11.2
	Others	0.7	1.5

- : not quantifiable

30

The preceding results show that, as for blackcurrant seed oil, it was especially the fatty acids that were tri-unsaturated which were more enriched in the free fatty acid fraction. It will also be noted that, in spite of its low 35 percentage of TGA oil, GLA was selectively enriched relative to alpha linolenic acid (ALA).

- 10 -

CLAIMS:

1. Essentially enzymatic process for fractionating polyunsaturated fatty acids, comprising the steps of enzymatic catalysis with methanol for esterification of a mixture of fatty acids, separating formed methyl esters from unreacted fatty acids by saponification of the fatty acids with a weak base in an aqueous medium, extracting formed soaps with water, acidifying an aqueous phase, extracting the formed fatty acids with hexane and obtaining a fraction enriched in the polyunsaturated fatty acids.
2. Process according to claim 1, wherein the mixture of fatty acids is derived from the total enzymatic hydrolysis of the triglycerides of an oil rich in the polyunsaturated fatty acids.
3. Process according to claim 2, wherein the oil is an oil rich in fatty acids of the n-3 and n-6 series.
4. Process according to claim 3, wherein the oil is an oil rich in fatty acids which are gammalinolenic acid.
5. Process according to claim 3 or 4, wherein the oil is blackcurrant seed oil, borage oil or evening primrose oil.
6. Process according to any one of claims 1 to 5, wherein the saponification takes place by reaction with, as a weak base, a carbonate, phosphate or citrate of sodium, potassium or ammonium or mixtures thereof and separation of the reaction medium is obtained between an aqueous phase and an organic phase.
7. Process according to claim 6, wherein the saponification takes place by reaction with an aqueous solution of sodium carbonate.
8. Process according to claim 6 or 7, wherein the reaction takes place at a temperature of between 20°C and 80°C with stirring.

- 11 -

9. Process according to claim 6 or 7, wherein the reaction is carried out while increasing stirring and raising the temperature progressively from approximately about 45°C to approximately 75°C.
10. Process according to claim 6, wherein the separation of the phases is accentuated by adding a saturated solution of sodium chloride.
11. Process according to claim 6, wherein the aqueous phase containing water-soluble fatty acids is separated from the organic phase containing the liposoluble esters by centrifuging.
12. Process according to claim 6, wherein the free fatty acids are collected by acidifying the aqueous phase with an acid, followed by extraction with hexane and elimination of the hexane.
13. Process according to claim 12, wherein the acid is concentrated hydrochloric acid.
14. Process according to claim 12 or 13, wherein the extraction is by evaporation.