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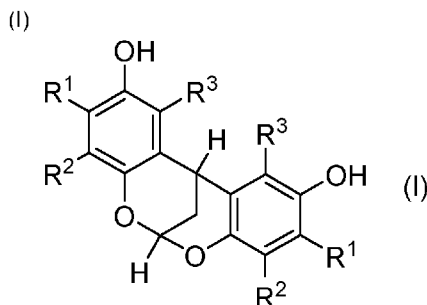
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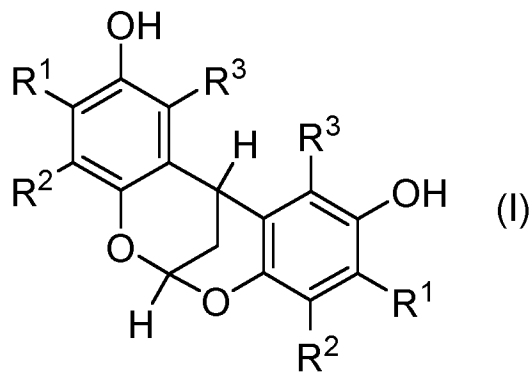
(57) Abstract: The present invention is directed towards the use of at least one compound of formula (I) wherein R^1 , R^2 and R^3 are independently from each other H or linear C_{1-6} -alkyl or branched C_{3-8} -alkyl, as antioxidant in PUFA-containing edible oils such as marine oil, microbial oil, fungal oil, algal oil and PUFA-containing plant oil for human consumption. The present invention is further directed towards these PUFA-containing edible oils comprising at least one compound of formula (I). The present invention is further directed to a method of preserving the shelf life of PUFAs and/or their esters in an edible oil comprising the step of adding at least one compound of formula (I) to said edible oil, as well as to a method of limiting the amount of oxidation of PUFAs and/or their esters in an edible oil which is exposed to air, comprising adding at least one compound of formula (I) to said edible oil, preferably in an amount of said compound of formula (I) ranging from 10 to 500 ppm, preferably ranging from 30 to 300 ppm, more preferably ranging from 100 to 250 ppm, based on the total amount of said edible oil.

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USE OF TWIN-CHROMANOLS AS ANTIOXIDANTS IN OIL

The present invention is directed towards the use of at least one compound of formula (I) as antioxidant in oil,

5



wherein the oil contains polyunsaturated fatty acids and/or their esters, and wherein the oil is for human consumption, and

10 wherein R^1 , R^2 and R^3 are independently from each other H or linear C_{1-6} -alkyl or branched C_{3-8} -alkyl.

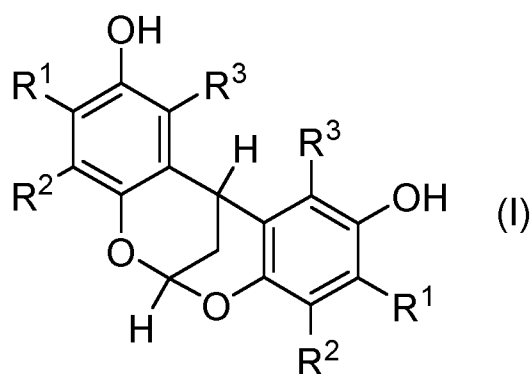
Oils containing polyunsaturated fatty acids and/or their esters are gaining more and more attention, because of their beneficial health effects in
15 humans.

Since these oils are only of limited stability, because they are oxidized very easily, there is a need to provide efficient antioxidants for their
20 stabilization.

20

Detailed description of the invention

This need is fulfilled by the present invention, which is directed to the use of at least one compound of formula (I) as antioxidant in oil,



wherein the oil contains polyunsaturated fatty acids and/or their esters, and wherein the oil is for human consumption, and

- 5 wherein R^1 , R^2 and R^3 are independently from each other H or linear C_{1-6} -alkyl or branched C_{3-8} -alkyl;
and with the preferences for the substituents R^1 to R^3 as given below.

Compounds of formula (I)

- 10 Preferred are compounds of formula (I), wherein R^1 , R^2 and R^3 are independently from each other H or linear C_{1-4} -alkyl or branched C_{3-4} -alkyl.

More preferred are compounds of formula (I), wherein R^1 is H or methyl or ethyl or n-propyl or iso-propyl or *tert*-butyl; and/or R^2 and R^3 are

- 15 independently from each other H or methyl or ethyl.

Even more preferred are compounds of formula (I), wherein R^1 is methyl or *tert*-butyl and/or R^2 and R^3 are independently from each other H or methyl.

- 20 Especially preferred compounds of the present invention include the following compounds:

Compound of formula (I) with $R^1 = R^2 = R^3 =$ methyl;

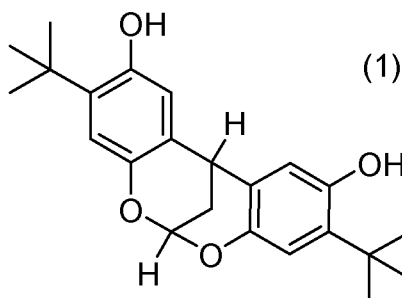
Compound of formula (I) with $R^1 =$ *tert*-butyl and $R^2 = R^3 =$ H,

- 25 Compound of formula (I) with $R^1 =$ methyl and $R^2 = R^3 =$ H,

Compound of formula (I) with $R^1 = R^3 =$ methyl and $R^2 =$ H,

Compound of formula (I) with $R^1 = R^2 = R^3 =$ H.

Most preferred is the following compound of formula (1) (3,9-di-*tert*-butyl-12*H*-6,12-methanodibenzo[*d,g*][1,3]dioxocine-2,10-diol):



5

Use as antioxidants

The compounds of the present invention are efficient as antioxidants in
10 PUFA-containing oils for human consumption.

“PUFA(s)” means polyunsaturated fatty acid(s) such as docosahexaenoic acid (“DHA”) and/or eicosapentaenoic acid (“EPA”) and/or docosapentaenoic acid (“DPA”) and/or oleic acid and/or stearidonic acid and/or linoleic acid
15 and/or alpha-linolenic acid (“ALA”) and/or gamma-linolenic acid and/or arachidonic acid (“ARA”) and/or the esters of all of them, whereby the term “esters” encompasses monoglycerides, diglycerides and triglycerides as well as C₁₋₆-alkyl esters such as especially the methyl esters and the ethyl esters, whereby the triglycerides are often dominant.

20

DHA, EPA, ALA and stearidonic acid are omega-3 fatty acids, whereas linoleic acid, gamma-linolenic acid and ARA are omega-6 fatty acids.

The term “DPA” encompasses two isomers, the omega-3 fatty acid clupanodonic acid (7*Z*,10*Z*,13*Z*,16*Z*,19*Z*-docosapentaenoic acid) and the
25 omega-6 fatty acid osbond acid (4*Z*,7*Z*,10*Z*,13*Z*,16*Z*-docosapentaenoic acid).

In accordance with the invention, the polyunsaturated fatty acid (PUFA) is preferably DHA and/or EPA and/or DPA and/or any ester thereof, more preferably the polyunsaturated fatty acid (PUFA) is preferably DHA and/or EPA and/or any ester thereof.

5

Examples of PUFA-containing oils for human consumption are

- marine oil, such as preferably fish oil,
- microbial biomass containing polyunsaturated fatty acids and/or their esters (“**microbial oil**”), preferably containing high amounts of docosahexaenoic acid (“DHA”) and/or eicosapentaenoic acid (“EPA”) and/or docosapentaenoic acid (“DPA”) and/or their esters, and
- 10 - oil containing high amounts of PUFAs and/or their esters, preferably containing high amounts of docosahexaenoic acid (“DHA”) and/or eicosapentaenoic acid (“EPA”) and/or docosapentaenoic acid (“DPA”) and/or their esters, extracted from microbial biomass, such as fungus (“**fungus oil**”) or algae (“**algal oil**”), and
- 15 - plant oil with relatively high amounts of PUFAs and/or their esters, (“**PUFA-containing plant oil**”), such as e.g. canola seed oil, linseed/flaxseed oil, hempseed oil, pumpkin seed oil, evening primrose oil, borage seed oil, blackcurrent seed oil, sallow thorn/sea buckthorn oil, chia seed oil, argan oil and walnut oil.
- 20

Marine oils, microbial oils and algal oils are especially preferred.

25 **Further objects of the present invention**

Thus, in addition, the present invention is

- (1) directed to the use of the compounds of formula (I) with the preferences as given above as antioxidants in marine oils, microbial oils, oils containing
- 30 high amounts of PUFAs and/or their esters extracted from microbial biomass and plant oils with relatively high amounts of PUFAs and/or their esters for human consumption; as well as

(2) directed to these PUFA-containing oils for human consumption comprising such compounds of formula (I) with the preferences as given above.

5 Furthermore, the present invention is directed to

(3) an edible oil comprising a compound of formula (I) and PUFAs and/or their esters;

(4) a method of preserving the shelf life of PUFAs and/or their esters in an edible oil comprising the step of adding at least one compound of formula

10 (I) to said edible oil, preferably in an amount of said compound of formula (I) ranging from 10 to 500 ppm, preferably ranging from 30 to 300 ppm, more preferably ranging from 100 to 250 ppm, based on the total amount of the edible oil;

(5) a method of limiting the amount of oxidation of PUFAs and/or their
15 esters in an edible oil which is exposed to air, comprising adding at least one compound of formula (I) to said edible oil, preferably in an amount of said compound of formula (I) ranging from 10 to 500 ppm, preferably ranging from 30 to 300 ppm, more preferably ranging from 100 to 250 ppm, based on the total amount of the edible oil.

20

For all these objects (1) to (5) of the present invention the preferences with respect to the compound of formula (I) and the PUFA-containing oil for human consumption, i.e. the PUFA-containing edible oil, as given above and below apply.

25

Further antioxidants

The compounds of formula (I) can be used in combination with one or more other antioxidants as described below.

30 In an embodiment of the present invention the PUFA-containing oils of the present invention comprising a compound of formula (I) additionally comprise ascorbyl palmitate and/or carnosic acid.

Instead of ascorbyl palmitate other esters of ascorbic acid such as the esters of ascorbic acid with linear C₁₂₋₂₀ alkanols, preferably the esters of ascorbic acid with linear C₁₄₋₁₈ alkanols, may also be used, so that further embodiments of the present invention are directed to PUFA-containing oils of the present invention comprising a compound of formula (I) that additionally comprise esters of ascorbic acid with linear C₁₂₋₂₀ alkanols, preferably esters of ascorbic acid with linear C₁₄₋₁₈ alkanols, more preferably ascorbyl palmitate.

10 The PUFA-containing oils of the present invention comprising a compound of formula (I) may also comprise additionally alpha-tocopherol and/or gamma-tocopherol, whereby either an ester of ascorbic acid with a linear C₁₂₋₂₀ alkanol with the preferences as given above may additionally be present.

15 The PUFA-containing oils themselves are described in more detail below.

PUFA-containing oils

In the context of the present invention the term “PUFA-containing oil” encompasses

- 20 - marine oil, such as especially fish oil,
- microbial biomass containing polyunsaturated fatty acids (“PUFAs”), especially docosahexaenoic acid (“DHA”) and/or eicosapentaenoic acid (“EPA”) and/or docosapentaenoic acid (“DPA”) and/or their esters (“**microbial oil**”);
- 25 - oil containing high amounts of PUFAs, especially containing high amounts of DHA and/or EPA and/or DPA and/or their esters extracted from microbial biomass as e.g., fungi (“fungal oil”) or algae (“algal oil”);
- Plant oil with high amounts of PUFAs and/or their esters (“**PUFA-containing plant oil**”), such as e.g. canola seed oil, linseed/flaxseed oil, hempseed oil, pumpkin seed oil, evening primrose oil, borage seed oil, blackcurrent seed oil, sallow thorn/sea buckthorn oil, chia seed oil, argan oil and walnut oil.
- 30

The term “DHA” does not only encompass the acid but also derivatives thereof such as monoglycerides, diglycerides and triglycerides as well as C₁₋₆-alkyl esters such as the methyl and ethyl esters. The same applies for
5 “EPA” and “DPA” and all the other PUFAs.

Fish oil and algal oil are commonly used for human consumption. Instead of fish oil and algal oil also other PUFA-containing oils may be used for human consumption, i.e.:

- 10 - microbial biomass containing PUFAs (“microbial oil”)
- oil containing high amounts of PUFAs extracted from microbial biomass, such as especially fungal oil, and
- plant oil with high amounts of PUFAs.

15 The above-mentioned PUFA-containing oils may not only be used as alternative of fish oil and algal oil, but also in addition.

Details of these PUFA-containing oils for human consumption are given below.

20

Marine oil

Examples of suitable marine oils include, but are not limited to, Atlantic fish oil, Pacific fish oil, or Mediterranean fish oil, or any mixture or combination thereof.

25

In more specific examples, a suitable fish oil can be, but is not limited to, pollack oil, bonito oil, pilchard oil, tilapia oil, tuna oil, sea bass oil, halibut oil, spearfish oil, barracuda oil, cod oil, menhaden oil, sardine oil, anchovy oil, capelin oil, herring oil, mackerel oil, salmonid oil, tuna oil, and shark
30 oil, including any mixture or combination thereof.

Other marine oils suitable for use herein include, but are not limited to, squid oil, cuttle fish oil, octopus oil, krill oil, seal oil, whale oil, and the like, including any mixture or combination thereof.

- 5 For stabilizing marine oil an amount of at least one compound of formula (I) ranging from 10 to 500 ppm, preferably ranging from 30 to 300 ppm, more preferably ranging from 100 to 250 ppm, based on the total amount of the marine oil, is usually sufficient. The same applies for the other PUFA-containing oils such as microbial oil, algal oil, fungal oil and PUFA-
10 containing plant oil.

A commercially available example of marine oil is the fish oil “MEG-3” (Bleached 30S TG Fish oil) from DSM Nutritional Products, LLC (US) whose specification and composition is shown in **Tables 1 and 2** below:

15

Table 1

ANALYSIS	SPECIFICATIONS
Colour	Max. 6 Gardner Colour
Free Fatty Acid (as % Oleic)	Max. 0.4%
<i>p</i> -Anisidine Value	Max. 12 (at time of release)
Peroxide Value	Max. 3 milli equivalents/kg (at time of release)
% Moisture	Max. 0.05%
Cold Test	Remains clear at 0 °C for 3 hours
Cholesterol	Report Actual
TOTOX ((2 x Peroxide Value) + (<i>p</i> -Anisidine Value))	Max. 20

The peroxide value is defined as the amount of peroxide oxygen per 1
20 kilogram of oil. Traditionally this is expressed in units of milliequivalents or meq/kg.

Winterization is part of the processing of fish oil, and it is performed to remove solid fat in the oil. The “cold test” is performed to check if any solid
25 fat is present and precipitated in the oil when cooled to 0°C within a

specific period of time. In this fish oil (Product Code: FG30TG), any such precipitation is checked for 3 hours at 0°C.

Table 2

5

Fatty Acid Profile	
EPA (A%)	Min. 18
EPA mg/g (as TG)	Min. 170
DHA (A%)	Min. 12
DHA mg/g (as TG)	Min. 110
EPA + DHA (A%)	Min. 30
Total Omega 3 (A%)	Min. 34

“TG” = triglyceride;

“A%” = “area %” = area percentage by GC based on 24 peak analysis (meaning the 24 highest peaks have been analyzed)

10

Oil containing high amounts of PUFAs, especially containing high amounts of DHA and/or EPA and/or DPA and/or their esters, extracted from microbial biomass as e.g., fungi (“fungal oil”) or algae (“algal oil”)

15 **Algal oil**

“Algal oil” is an oil containing high amounts of DHA and/or EPA and/or DPA and/or their esters extracted from algae as microbial source/biomass.

20 An example of algal oil is the commercially available “Algal oil containing EPA+DPA” from DSM Nutritional Products, LLC (US) whose composition is shown in the **Table 3** below:

Table 3

Fatty Acid Profile	
DHA + EPA content, mg/g oil	587 mg/g
DHA content, mg/g oil	401 mg/g
EPA content, mg/g oil	186 mg/g
TOTOX ((2 x Peroxide Value) + (p-Anisidine Value))	5

Free Fatty Acid	0.6%
Moisture	< 0.05%

- A further example of a crude oil containing high amounts of DHA and/or EPA extracted from microbial sources as e.g., algae, is the oil extracted from
- 5 Algae *Schizochytrium* Biomass, whose specification is given in the following Table 4.

Table 4

Specification	Aqua (Base Product)
DHA + EPA, mg/g oil	minimal 500 mg/g
DHA content, mg/g oil	minimal 250 mg/g (at least 25% -> 40%)
EPA content, mg/g oil	minimal 100 mg/g (at least 10% -> 25%)
Minimal ratio EPA:DHA	1:4
Maximal ratio EPA:DHA	1:1
TOTOX ((2 x Peroxide Value) + (p-Anisidine Value))	maximum 35
Free fatty acid	maximal 5%
Moisture	maximal 0.75%
DPA n-3 (omega-3 docosapentaenoic acid), %	< 6
Arachidonic Acid, %	< 2
Stearic, %	< 2.5
Palmitic, %	< 30
Shelf life	6 months at 25° C
Total Fat	Record
Crude Fat	> 92%

10

Microbial biomass containing polyunsaturated fatty acids (“PUFAs”), especially docosahexaenoic acid and/or eicosapentaenoic acid and/or docosapentaenoic acid (“DPA”) and/or their esters

- 15 The biomass preferably comprises cells which produce PUFAs heterotrophically. According to the invention, the cells are preferably selected

from algae, fungi, particularly yeasts, bacteria, or protists. The cells are more preferably microbial algae or fungi.

Suitable cells of oil-producing yeasts are, in particular, strains of *Yarrowia*,
5 *Candida*, *Rhodotorula*, *Rhodospiridium*, *Cryptococcus*, *Trichosporon* and *Lipomyces*.

Oil produced by a microorganism or obtained from a microbial cell is referred to as "microbial oil". Oil produced by algae and/or fungi is
10 referred to as an algal and/or a fungal oil, respectively.

As used herein, a "microorganism" refers to organisms such as algae, bacteria, fungi, protist, yeast, and combinations thereof, e.g., unicellular organisms. A microorganism includes but is not limited to, golden algae
15 (e.g., microorganisms of the kingdom *Stramenopiles*); green algae; diatoms; dinoflagellates (e.g., microorganisms of the order *Dinophyceae* including members of the genus *Crypthecodinium* such as, for example, *Crypthecodinium cohnii* or *C. cohnii*); microalgae of the order *Thraustochytriales*; yeast (*Ascomycetes* or *Basidiomycetes*); and fungi of the
20 genera *Mucor*, *Mortierella*, including but not limited to *Mortierella alpina* and *Mortierella sect. schmuckeri*, and *Pythium*, including but not limited to *Pythium insidiosum*.

In one embodiment, the microorganisms of the kingdom *Stramenopiles* may
25 in particular be selected from the following groups of microorganisms:
Hamatores, *Proteromonads*, *Opalines*, *Developayella*, *Diplophrys*,
Labrinthulids, *Thraustochytrids*, *Biosecids*, *Oomycetes*,
Hypochytridiomycetes, *Commation*, *Reticulosphaera*, *Pelagomonas*,
Pelagococcus, *Ollicola*, *Aureococcus*, *Parmales*, *Diatoms*, *Xanthophytes*,
30 *Phaeophytes* (brown algae), *Eustigmatophytes*, *Raphidophytes*, *Synurids*,
Axodines (including *Rhizochromulinales*, *Pedinellales*, *Dictyochales*),
Chrysomeridales, *Sarcinochrysidales*, *Hydrurales*, *Hibberdiales*, and *Chromulinales*.

- In one embodiment, the microorganisms are from the genus *Mortierella*, genus *Crypthecodinium*, genus *Thraustochytrium*, and mixtures thereof. In a further embodiment, the microorganisms are from *Crypthecodinium*
- 5 *Cohnii*. In a further embodiment, the microorganisms are from *Mortierella alpina*. In a still further embodiment, the microorganisms are from *Schizochytrium sp.* In yet an even further embodiment, the microorganisms are selected from *Crypthecodinium Cohnii*, *Mortierella alpina*, *Schizochytrium sp.*, and mixtures thereof.
- 10 In a still further embodiment, the microorganisms include, but are not limited to, microorganisms belonging to the genus *Mortierella*, genus *Conidiobolus*, genus *Pythium*, genus *Phytophthora*, genus *Penicillium*, genus *Cladosporium*, genus *Mucor*, genus *Fusarium*, genus *Aspergillus*, genus *Rhodotorula*, genus *Entomophthora*, genus *Echinosporangium*, and genus
- 15 *Saprolegnia*.

- In an even further embodiment, the microorganisms are from microalgae of the order *Thraustochytriales*, which includes, but is not limited to, the genera *Thraustochytrium* (species include *arudimentale*, *aureum*,
- 20 *benthicola*, *globosum*, *kinnei*, *motivum*, *multirudimentale*, *pachydermum*, *proliferum*, *roseum*, *striatum*); the genera *Schizochytrium* (species include *aggregatum*, *limnaceum*, *mangrovei*, *minutum*, *octosporum*); the genera *Ulkenia* (species include *amoeboidea*, *kerguelensis*, *minuta*, *profunda*, *radiate*, *sailens*, *sarkariana*, *schizochytrops*, *visurgensis*, *yorkensis*); the
- 25 genera *Aurantiacochytrium*; the genera *Oblongichytrium*; the genera *Sicyoidochytrium*; the genera *Parientichytrium*; the genera *Botryochytrium*; and combinations thereof. Species described within *Ulkenia* will be considered to be members of the genus *Schizochytrium*. In another embodiment, the microorganisms are from the order *Thraustochytriales*. In
- 30 yet another embodiment, the microorganisms are from *Thraustochytrium*. In still a further embodiment, the microorganisms are from *Schizochytrium sp.*

In certain embodiments, the oil can comprise a marine oil. Examples of suitable marine oils are the ones as given above.

5 The biomass according to the invention preferably comprises cells, and preferably consists essentially of such cells, of the taxon *Labyrinthulomyces* (*Labyrinthulea*, net slime fungi, slime nets), in particular, those from the family of *Thraustochytriaceae*. The family of the *Thraustochytriaceae* (*Thraustochytrids*) includes the genera *Althomia*, *Aplanochytrium*, *Aurantiochytrium*, *Botryochytrium*, *Elnia*, *Japonochytrium*,
10 *Oblongichytrium*, *Parietichytrium*, *Schizochytrium*, *Sicyoidochytrium*, *Thraustochytrium*, and *Ulkenia*. The biomass particularly preferably comprises cells from the genera *Aurantiochytrium*, *Oblongichytrium*, *Schizochytrium*, or *Thraustochytrium*, more preferably from the genus *Schizochytrium*.

15

In accordance with the invention, the polyunsaturated fatty acid (PUFA) is preferably DHA and/or EPA and/or their esters as defined above.

20 The cells present in the biomass are preferably distinguished by the fact that they contain at least 20 weight-%, preferably at least 30 weight-%, in particular at least 35 weight-%, of PUFAs, in each case based on cell dry matter.

25 In a very preferred embodiment of the current invention, cells, in particular a *Schizochytrium* strain, is employed which produces a significant amount of EPA and DHA, simultaneously, wherein DHA is preferably produced in an amount of at least 20 weight-%, preferably in an amount of at least 30 weight-%, in particular in an amount of 30 to 50 weight-%, and EPA is produced in an amount of at least 5 weight-%, preferably in an amount of at
30 least 10 weight-%, in particular in an amount of 10 to 20 weight-% (in relation to the total amount of lipid as contained in the cells, respectively).

Preferred species of microorganisms of the genus *Schizochytrium*, which produce EPA and DHA simultaneously in significant amounts, as mentioned before, are deposited under ATCC Accession No. PTA-10208, PTA-10209, PTA-10210, or PTA-10211, PTA-10212, PTA-10213, PTA-10214, PTA-10215.

5

DHA and EPA producing *Schizochytrium* strains can be obtained by consecutive mutagenesis followed by suitable selection of mutant strains which demonstrate superior EPA and DHA production and a specific EPA:DHA ratio. Any chemical or nonchemical (e.g. ultraviolet (UV) radiation) agent capable of inducing genetic change to the yeast cell can be used as the mutagen. These agents can be used alone or in combination with one another, and the chemical agents can be used neat or with a solvent.

10

Methods for producing the biomass, in particular, a biomass which comprises cells containing lipids, in particular PUFAs, particularly of the order *Thraustochytriales*, are described in detail in the prior art (see e.g. WO 91/07498, WO 94/08467, WO 97/37032, WO 97/36996, WO 01/54510). As a rule, the production takes place by cells being cultured in a fermenter in the presence of a carbon source and a nitrogen source, along with a number of additional substances like minerals that allow growth of the microorganisms and production of the PUFAs. In this context, biomass densities of more than 100 grams per litre and production rates of more than 0.5 gram of lipid per litre per hour may be attained. The process is preferably carried out in what is known as a fed-batch process, i.e. the carbon and nitrogen sources are fed in incrementally during the fermentation. When the desired biomass has been obtained, lipid production may be induced by various measures, for example by limiting the nitrogen source, the carbon source or the oxygen content or combinations of these.

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In a preferred embodiment of the current invention, the cells are grown until they reach a biomass density of at least 80 or 100 g/l, more preferably at least 120 or 140 g/l, in particular at least 160 or 180 g/l (calculated as

dry-matter content). Such processes are for example disclosed in US 7,732,170.

5 Preferably, the cells are fermented in a medium with low salinity, in particular, so as to avoid corrosion. This can be achieved by using chlorine-free sodium salts as the sodium source instead of sodium chloride, such as, for example, sodium sulphate, sodium carbonate, sodium hydrogen carbonate or soda ash. Preferably, chloride is used in the fermentation in amounts of less than 3 g/l, in particular, less than 500 mg/l, especially
10 preferably less than 100 mg/l.

PUFA-containing plant oils: Plant oils with relatively high amounts of PUFAs, especially with high amounts of DHA and/or EPA such as e.g.,

15 **canola seed oil**

The plant cells may, in particular, be selected from cells of the families *Brassicaceae*, *Elaeagnaceae* and *Fabaceae*. The cells of the family *Brassicaceae* may be selected from the genus *Brassica*, in particular, from oilseed rape, turnip rape and Indian mustard; the cells of the family
20 *Elaeagnaceae* may be selected from the genus *Elaeagnus*, in particular, from the species *Olea europaea*; the cells of the family *Fabaceae* may be selected from the genus *Glycine*, in particular, from the species *Glycine max*.

25 **Examples:**

- Canola seed oil with a content of DHA of at least 9% by weight, of at least 12% by weight, of at least 15% by weight, or of at least 20% by weight, based on the total weight of the canola seed oil;
- Canola seed oil with a content of EPA of at least 9% by weight, of at
30 least 12% by weight, of at least 15% by weight, or of at least 20% by weight, based on the total weight of the canola seed oil.

Examples of PUFA-containing plant oils containing high amounts of other PUFAs than EPA and/or DHA and/or DPA and/or their esters are linseed/flaxseed oil, hempseed oil, pumpkin seed oil, evening primrose oil, borage seed oil, blackcurrent seed oil, sallow thorn/sea buckthorn oil, chia seed oil, argan oil and walnut oil.

The invention is now further illustrated in the following non-limiting examples.

10

Examples

Example 1: Synthesis of compound of formula (1)

3,9-Di-*tert*-butyl-12*H*-6,12-methanodibenzo[*d,g*][1,3]dioxocine-2,10-diol was prepared following a procedure by N. Seto and M. Morigaki (N. Seto, M. Morigaki, *Silver Halide Color Photographic Sensitive Material*, 1992, JPH04330440).

Example 2: Antioxidant activities of compound of formula (1) in fish oil and algal oil

The antioxidant activity of compound of formula (1) was evaluated in both fish and algal oil in comparison with mixed natural tocopherols (MNT). Fish oil did not contain any antioxidants (= blank oil) whereas algal oil contained about 1.5 mg/g of MNT. Thus, the blank oil, i.e. oil without any antioxidant, (= fish oil) and oil containing "MNT" (= algal oil) have been used as benchmark. Any compound better in antioxidant activity than the blank oil indicates that it has antioxidant activity. The comparison with MNT gives an indication about the amount of the antioxidant effect, relative to the activity of MNT.

30

"MNT" are mixed natural tocopherols commercially available as e.g., "Tocomix 70 IP" from AOM (Buenos Aires, Argentina). Tocomix 70 IP comprises d-alpha-tocopherol, d-beta-tocopherol, d-gamma-tocopherol and

d-delta-tocopherol, whereby the total amount of tocopherols is at least 70.0 weight-% and the amount of non-alpha tocopherols is at least 56.0 weight-%.

Materials and methods

- 5 The compound of formula (1) was used in both fish and algal oils to see its antioxidant effect in these oils. Antioxidant effect was determined using mainly the Oil Stability Index (OSI). A storage stability study was performed to compare the variation of primary oxidation products, the hydroperoxides, generated during oxidation, measured in terms of peroxide value (PV) and
- 10 the secondary oxidation products which were measured and determined as anisidine reactive substances or *p*-anisidine value (*p*-AV) of oil samples containing this compound.

Oxidative stability

- 15 Two concentration levels were used. Compound of formula (1) was added in the concentrations of 0.5 mg/g (low level) and 2 mg/g (high level) to 5 g of oil and used in the Oxidative Stability Instrument operated at 80°C with the continuous air flow rate at 5.5 psi. All samples were run in duplicate. The Protection Factors (PF) for compound of formula (1) in oil were calculated
- 20 in percentage as:

$$\text{PF (\%)} = \frac{100\% \times (\text{OSI of the sample with antioxidant} - \text{OSI of the sample without antioxidant})}{\text{OSI of the sample without antioxidant}}$$

Results

- 25 OSI values of the fish oil samples containing compound of formula (1), in comparison to the same levels of MNT, are shown in **Tables 5 and 6**. The compound of formula (1) provides slightly lower protection in fish oil than MNT.
- 30 Table 5: Oxidative stability of FG30TG fish oil with compound of formula (1) (SD = standard deviation)

	OSI (h)	SD
Blank (FG30TG)	4.3	0.4
0.5 mg/g of compound of formula (1)	6.4	0.5
2 mg/g of compound of formula (1)	6.2	0.5
0.5 mg/g of MNT	7.0	0.5
2 mg/g of MNT	7.8	0.1

Table 6: Protection Factor of compound of formula (1) in fish oil

	Protection Factor (%)
0.5 mg/g of MNT	58.6
0.5 mg/g of compound of formula (1)	75.5
2 mg/g of MNT	77.2
2 mg/g of compound of formula (1)	43.9

- 5 The antioxidant effect of the compound of formula (1) in algal oil is different from the effect in fish oil. As shown in Table 7 the compound of formula (1) showed higher OSI values than the crude algal oil indicating a considerable antioxidant activity of said compound in algal oil. The compound of formula (1) clearly showed much better Protection Factor than
- 10 MNT at both concentration levels (Table 8).

Table 7: Oxidative stability of crude algal oil with compound of formula (1) (SD = standard deviation)

	OSI (h)	SD
Crude algal oil (containing ~1.5 mg/g of MNT)	14.9	0.5
0.5 mg/g of compound of formula (1)	17.7	0.6
2 mg/g of compound of formula (1)	20.0	0.4
0.5 mg/g of MNT	15.5	1.2
2 mg/g of MNT	16.9	0.1

15

Table 8: Protection Factor of compound of formula (1) in crude algal oil

	Protection Factor (%)
0.5 mg/g of compound of formula (1)	18.8
2 mg/g of compound of formula (1)	33.7

0.5 mg/g of MNT	3.4
2 mg/g of MNT	13.0

Also, the synergistic effect of the compound of formula (1) with ascorbyl palmitate (AP) and/or carnosic acid (CA) was determined using the OSI values (for the results see Table 9) and the Protection Factor (for the results see Table 10).

Table 9: Improvement of the effect of the compound of formula (1) in FG30TG fish oil using AP and/or CA (SD = standard deviation)

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	OSI (h)	SD
Blank (FG30TG)	4.43	0.1
0.5 mg/g of CA	20.9	2.1
0.5 mg/g of AP + 0.5 mg/g of CA	35.0	5.0
2 mg/g of compound of formula (1)	5.6	0.3
2 mg/g of compound of formula (1) + 0.5 mg/g of AP	7.5	0.1
2 mg/g of compound of formula (1) + 0.5 mg/g of CA	29.2	3.1
2 mg/g of compound of formula (1) + 0.5 mg/g of AP + 0.5 mg/g of CA	47.3	1.8

Table 10: Improvement of the Protection Factor of the compound of formula (1) in FG30TG fish oil using AP and/or CA (SD = standard deviation)

	Protection Factor (%)
Blank (FG30TG)	47.4
0.5 mg/g of CA	338.9
0.5 mg/g of AP + 0.5 mg/g of CA	636.9
2 mg/g of compound of formula (1)	17.9
2 mg/g of compound of formula (1) + 0.5 mg/g of AP	58.4
2 mg/g of compound of formula (1) + 0.5 mg/g of CA	514.2
2 mg/g of compound of formula (1) + 0.5 mg/g of AP + 0.5 mg/g of CA	896.3

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Storage stability

A storage stability study for fish oil (XBUFG30TG) containing 2 different levels of compound of formula (1) (0.5 and 2 mg/g) was performed to compare the variation of primary and secondary oxidation products generated during storage at ambient temperature as a result of oxidation of the oil in the presence of these compounds.

Hereby, accurately weighed amounts of compound of formula (1) (0.5 mg/g and 2 mg/g) were added, each individually, to 60 g of fish oil samples in glass amber bottles, thoroughly mixed under nitrogen and stored at ambient temperature storage for 2 weeks. All sample bottles were left open to air, away from light. 0.5 mg/g of the compound of formula (1) were soluble in fish oil upon thorough mixing, whereas in an amount of 2 mg/g it was only partially soluble in fish oil.

The primary oxidation products, hydroperoxides, were determined in terms of peroxide value (PV), and the secondary oxidation products were measured and determined as anisidine reactive substances or *p*-anisidine value (*p*-AV). Conjugated dienoic acid (CD) levels which are also indicative of the primary oxidation were also determined. Peroxide values (PV), *p*-anisidine values (*p*-AV) and conjugated dienes as percentage dienoic acid were determined at different times for 2 weeks.

Tables 11, 12 and 13 show the PV (peroxide value), *p*-AV (*p*-anisidine value) and CD (Conjugated dienoic acid %) of the fish oil samples stabilized with the compound of formula (1) at low (0.5 mg/g) and high levels (2 mg/g).

Table 11: Variation of PV [meq/kg] with compound of formula (1) in fish oil FG30TG

	Initial	1 day	3 days	6 days	10 days	15 days
Blank (FG30TG)	1	1	0	3	7	13
0.5 mg/g of MNT	1	1	0	2	2	2
0.5 mg/g of compound of	1	1	1	2	5	0

formula (1)						
2 mg/g of MNT	1	1	1	3	8	14
2 mg/g of compound of formula (1)	1	1	2	3	7	0

Table 12: Variation of *p*-AV (*p*-anisidine value) with compound of formula (1) in fish oil FG30TG

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	Initial	1 day	3 days	6 days	10 days	15 days
Blank (FG30TG)	1	8	7	11	11	13
0.5 mg/g of MNT	1	8	11	10	10	10
0.5 mg/g of compound of formula (1)	1	10	10	11	11	14
2 mg/g of MNT	1	10	10	11	11	14
2 mg/g of compound of formula (1)	1	10	10	11	11	11

Table 13: Variation of CD (conjugated dienoic acid in %) with compound of formula (1) in fish oil FG30TG

	Initial	1 day	3 days	6 days	10 days
Blank(FG30TG)	1	1	1	1	1
0.5 mg/g of MNT	1	1	1	1	1
0.5 mg/g of compound of formula (1)	1	1	1	1	1
2 mg/g of MNT	1	1	1	1	1
2 mg/g of compound of formula (1)	1	1	1	1	1

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Results and discussion

All fish oil samples containing the compound of formula (1) had lower levels of PV than those of the samples which did not contain said compound or any other antioxidants indicating their antioxidant effect against the oxidation of PUFA in fish oil.

5

Although there was no considerable difference among the samples in the p-AV during storage, the samples containing high levels of MNT (2 mg/g) and the sample containing 0.5mg/g of compound 1 showed the highest level of p-AV after 15 days of storage (Table 12). All other samples containing the compound of formula (1) did not show clear difference in p-AV among them. There was no change in the values of conjugated dienes at all during the study period (Table 13).

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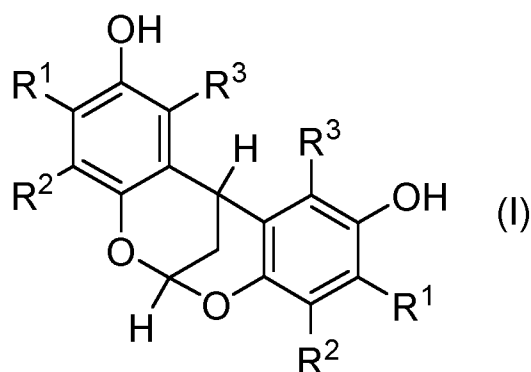
Conclusion

The compound of formula (1) showed antioxidant activity in both fish and algal oil.

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Claims

1. Use of at least one compound of formula (I) as antioxidant in oil,



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wherein the oil contains polyunsaturated fatty acids and/or their esters, and wherein the oil is for human consumption, and whereby R¹, R² and R³ are independently from each other H or linear C₁₋₆-alkyl or branched C₃₋₈-alkyl.

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2. The use according to claim 1, whereby in compound of formula (I) R¹, R² and R³ are independently from each other H or linear C₁₋₆-alkyl or branched C₃₋₈-alkyl.

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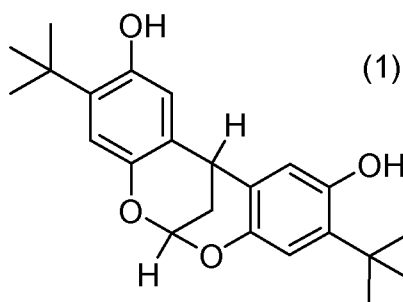
3. The use according to claim 1 and/or 2, whereby in compound of formula (I) R¹, R² and R³ are independently from each other H or linear C₁₋₄-alkyl or branched C₃₋₄-alkyl.

4. The use according to claim 1 and/or 2, whereby in compound of formula (I) R¹ is H or methyl or ethyl or n-propyl or *iso*-propyl or *tert*-butyl; and/or R² and R³ are independently from each other H or methyl or ethyl.

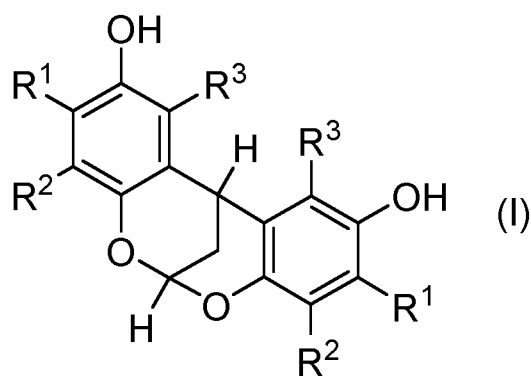
5. The use according to any one or more of claims 1 to 4, whereby in compound of formula (I) R¹ is methyl or *tert*-butyl and/or R² and R³ are independently from each other H or methyl.

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6. The use according to any one or more of claims 1 to 5, whereby the compound of formula (I) is a compound of formula (I) with $R^1 = R^2 = R^3 =$ methyl or a compound of formula (I) with $R^1 = \textit{tert}$ -butyl and $R^2 = R^3 =$ H or a compound of formula (I) with $R^1 =$ methyl and $R^2 = R^3 =$ H or a compound of formula (I) with $R^1 = R^3 =$ methyl and $R^2 =$ H, or a compound of formula (I) with $R^1 = R^2 = R^3 =$ H or any mixture thereof.
7. The use according to any one or more of claims 1 to 5, whereby the compound of formula (I) is the compound of formula (1) (3,9-di-*tert*-butyl-12*H*-6,12-methanodibenzo[*d,g*][1,3]dioxocine-2,10-diol):



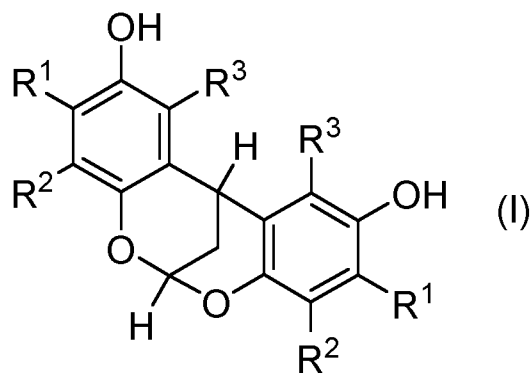
8. Oil containing polyunsaturated fatty acids (PUFAs) and/or their esters for human consumption comprising at least one compound of formula (I),



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wherein R^1 , R^2 and R^3 are independently from each other H or linear C_{1-6} -alkyl or branched C_{3-8} -alkyl.

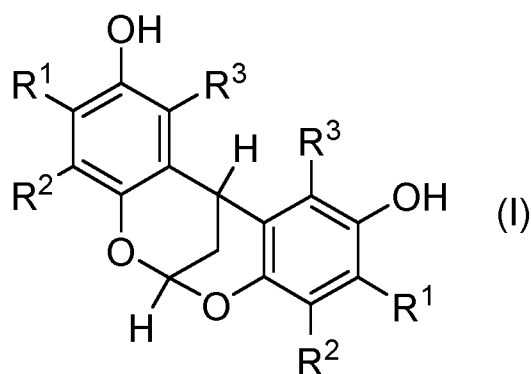
9. The oil containing polyunsaturated fatty acids (PUFAs) and/or their esters for human consumption according to claim 8, whereby the PUFA-containing oil is marine oil or microbial oil or fungal oil or algal oil or PUFA-containing plant oil, preferably whereby the PUFA-containing oil is marine oil or algal oil, more preferably whereby the PUFA-containing oil is algal oil.
10. The oil containing polyunsaturated fatty acids (PUFAs) and/or their esters for human consumption according to claim 8 and/or claim 9 additionally comprising esters of ascorbic acid with linear C₁₂₋₂₀ alkanols, preferably esters of ascorbic acid with linear C₁₄₋₁₈ alkanols, more preferably ascorbyl palmitate.
11. The oil containing polyunsaturated fatty acids (PUFAs) and/or their esters for human consumption according to any one or more of claims 8 to 10 additionally comprising alpha-tocopherol and/or gamma-tocopherol.
12. Marine oil for human consumption comprising at least one compound of formula (I),



- wherein R¹, R² and R³ are independently from each other H or linear C₁₋₆-alkyl or branched C₃₋₈-alkyl.

13. The marine oil according to claim 12 additionally comprising esters of ascorbic acid with linear C₁₂₋₂₀ alkanols, preferably esters of ascorbic acid with linear C₁₄₋₁₈ alkanols, more preferably ascorbyl palmitate.
- 5 14. The marine oil according to claim 12 and/or claim 13 additionally comprising alpha-tocopherol and/or gamma-tocopherol.
15. Microbial oil for human consumption comprising at least one compound of formula (I),

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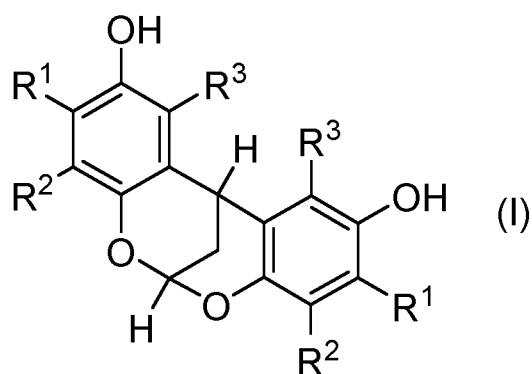


wherein R¹, R² and R³ are independently from each other H or linear C₁₋₆-alkyl or branched C₃₋₈-alkyl.

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16. The microbial oil according to claim 15 additionally comprising esters of ascorbic acid with linear C₁₂₋₂₀ alkanols, preferably esters of ascorbic acid with linear C₁₄₋₁₈ alkanols, more preferably ascorbyl palmitate.
- 20 17. The microbial oil according to claim 15 and/or claim 16 additionally comprising alpha-tocopherol and/or gamma-tocopherol.
18. Algal oil for human consumption comprising at least one compound of formula (I),

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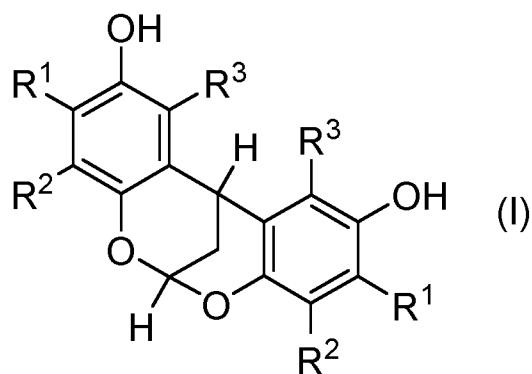


wherein R^1 , R^2 and R^3 are independently from each other H or linear C_{1-6} -alkyl or branched C_{3-8} -alkyl.

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19. The algal oil according to claim 18 additionally comprising esters of ascorbic acid with linear C_{12-20} alkanols, preferably esters of ascorbic acid with linear C_{14-18} alkanols, more preferably ascorbyl palmitate.
20. The algal oil according to claim 18 and/or claim 19 additionally comprising alpha-tocopherol and/or gamma-tocopherol.
21. An edible oil comprising a compound of formula (I) and PUFAs and/or their esters,

15



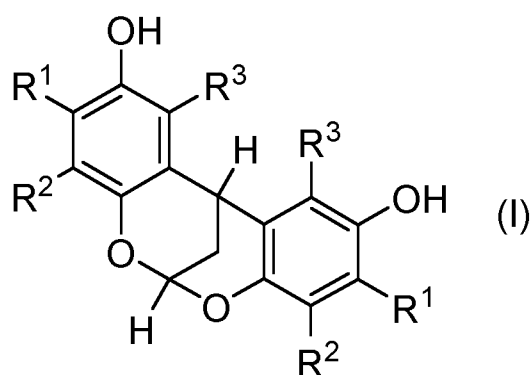
wherein R^1 , R^2 and R^3 are independently from each other H or linear C_{1-6} -alkyl or branched C_{3-8} -alkyl.

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22. The edible oil according to claim 21, whereby the edible oil is marine oil or microbial oil or fungal oil or algal oil or PUFA-containing plant oil, preferably whereby the edible oil is marine oil or algal oil, more preferably whereby the edible oil is algal oil.

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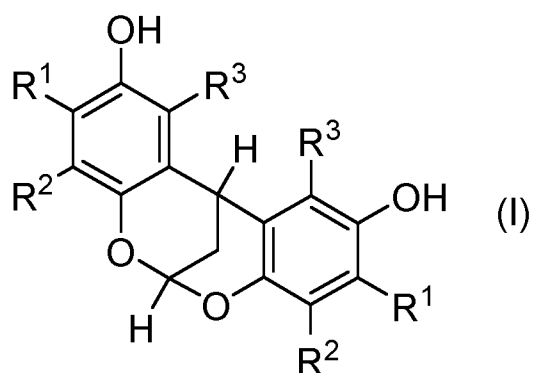
23. A method of preserving the shelf life of PUFAs and/or their esters in an edible oil comprising the step of adding at least one compound of formula (I) to said edible oil,



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wherein R^1 , R^2 and R^3 are independently from each other H or linear C_{1-6} -alkyl or branched C_{3-8} -alkyl.

- 15 24. The method according to claim 23, whereby the compound of formula (I) is added to said edible oil in an amount ranging from 10 to 500 ppm, preferably ranging from 30 to 300 ppm, more preferably ranging from 100 to 250 ppm, based on the total amount of the edible oil.
- 20 25. A method of limiting the amount of oxidation of PUFAs and/or their esters in an edible oil which is exposed to air, comprising adding at least one compound of formula (I) to said edible oil,



wherein R¹, R² and R³ are independently from each other H or linear C₁₋₆-alkyl or branched C₃₋₈-alkyl.

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26. The method according to claim 25, whereby the compound of formula (I) is added to said edible oil in an amount ranging from 10 to 500 ppm, preferably ranging from 30 to 300 ppm, more preferably ranging from 100 to 250 ppm, based on the total amount of the edible oil.

10

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/058118

A. CLASSIFICATION OF SUBJECT MATTER
INV. A23D9/00 A23L3/3499 A61K31/355 A61P39/06
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
A23D A23L A61K A61P C11C
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/121177 A2 (COLBY PHARMACEUTICAL COMPANY [US]; BASU HIRAK S [US] ET AL.) 21 October 2010 (2010-10-21)	1-26
Y	example 7 paragraphs [0238] - [0240] claims 1, 6-7, 20, 25-26 ----- -/--	1-26

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents :

<p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier application or patent but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>
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Date of the actual completion of the international search 11 June 2019	Date of mailing of the international search report 27/06/2019
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Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Renard, Delphine
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/058118

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>STANIEK K ET AL: "The protection of bioenergetic functions in mitochondria by new synthetic chromanols", BIOCHEMICAL PHARMACOLOGY, ELSEVIER, US, vol. 70, no. 9, 1 November 2005 (2005-11-01), pages 1361-1370, XP027715843, ISSN: 0006-2952 [retrieved on 2005-11-01] abstract figure 1; compounds Twin-chromanol 5. Conclusions; page 1369</p> <p style="text-align: center;">-----</p>	1-26
Y	<p>WOLFGANG GREGOR ET AL: "Antioxidant Properties of Chromanols Derived from Vitamin E and Ubiquinone", ANNALS OF THE NEW YORK ACADEMY OF SCIENCES, vol. 1031, no. 1, 1 December 2004 (2004-12-01), pages 344-347, XP055591622, US ISSN: 0077-8923, DOI: 10.1196/annals.1331.040 abstract figure 1; compounds Twin-chromanol page 346, last paragraph</p> <p style="text-align: center;">-----</p>	1-26
Y	<p>WOLFGANG GREGOR ET AL: "Antioxidant Properties of Natural and Synthetic Chromanol Derivatives: Study by Fast Kinetics and Electron Spin Resonance Spectroscopy", JOURNAL OF ORGANIC CHEMISTRY, vol. 70, no. 9, 1 April 2005 (2005-04-01), pages 3472-3483, XP055591630, US ISSN: 0022-3263, DOI: 10.1021/jo047927s abstract compounds Twin-chromanol last two sentences before "experimental section"; page 3481, left-hand column</p> <p style="text-align: center;">-----</p>	1-26
Y	<p>US 2010/178369 A1 (ARLEDGE NICOLE LEE [US] ET AL) 15 July 2010 (2010-07-15) claims 1-20 paragraphs [0008], [0029], [0035], [0040] - [0058]</p> <p style="text-align: center;">-----</p>	1-26

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2019/058118

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