

Effect of lyophilized water extracts of *Melissa officinalis* on the stability of algae and linseed oil-in-water emulsion to be used as a functional ingredient in meat products

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ABSTRACT

Previous work pointed out the possibility to enhance the nutritional value of meat products using long chain ω -3 PUFA enriched emulsions. Oil in water emulsions elaborated with a mixture of algae and linseed oils (15:10) in order to be used as functional ingredient were stabilized with BHA (butylhydroxyanisol) or with a lyophilized water extract of *Melissa officinalis* L. (Lemon balm). The lipid profile of the oil mixture showed a high amount of DHA (31.7 %), oleic (25.4 %) and α -linolenic acid (12.7 %) resulting in a very low ω -6/ ω -3 ratio (0.12). The lyophilized extract of *Melissa officinalis* showed a high antioxidant activity (being 62 ppm of the lyophilized water extract of *melissa* equivalent to 200 ppm of BHA, using the DPPH assay as reference), and high total phenolic content. Studying the oxidation process in the emulsions during 15 days at room temperature, it could be concluded that this extract was as efficient as BHA in order to control the Thiobarbituric Acid Reactive Substances (TBARS) formation.

Keywords: Functional meat products, PUFA, lipid oxidation, lemon balm, phenolic compounds.

INTRODUCTION

Dietary ω -3 PUFAs have been demonstrated to exert beneficial health effects decreasing the risk of many diseases, mainly coronary heart disease and other cardiovascular diseases (Simopoulos, 1997; Connor, 2000). However, the Western diet is deficient in ω -3 fatty acids and the ω -6/ ω -3 ratio is higher than the recommendations (Okuyama, Fujii & Ikemoto, 2000; Okuyama, 2001; Simopoulos, 2004). In consequence, with the aim of increasing the ω -3 intake, different ω -3 PUFA enriched functional foods are commercially available and research on new formulations is currently being done.

However, the higher unsaturation degree of lipids in food involves faster oxidation reactions (Gurr, Harwood & Frayn, 2002). This great susceptibility of PUFA to oxidative and rancidity processes limits the use of these fatty acids in foods and makes necessary the development of different strategies and techniques to protect ω -3 PUFA from oxidation.

The use of antioxidants can prevent the oxidative deterioration of foods enriched with ω -3 PUFA, however, similar antioxidants exert different effects on food emulsions (Jacobsen, Let, Nielsen & Meyer, 2008). Previous papers have demonstrated that it is possible to develop new meat products rich in long chain ω -3 PUFA elaborated with pre-emulsified oils and adding artificial antioxidants for improving the nutritional properties of these products (Muguerza, Gimeno, Ansorena & Astiasaran, 2004; Valencia, Ansorena & Astiasaran, 2006; Lee, Faustman, Djordjevic, Faraji & Decker, 2006; Valencia, Ansorena & Astiasaran 2007). Extracts from vegetal by-products, herbs and spices rich in polyphenols have been studied as potential antioxidants in different types of foods, such as edible oils, fish and meat products, showing in some cases comparable results to those obtained when using synthetic antioxidants (Lai, Gray, Smith, Booren, Crackel & Buckley, 1991; Balasundram, 2006). However, few studies have been carried out in simple o/w emulsions using vegetable sources of antioxidants (Abdalla & Roozen 1999; Duh, 1999; Viljanen, Halmos, Sinclair & Heinonen, 2005).

Melissa officinalis (Lemon balm) is an edible plant commonly used as an herbal infusion drink due its sedative, hypotensive and spasmolytic and digestive effects (Bisset, 1994; Ivanova, Gerova, Chervenkov & Yankonva, 2005). This plant belongs to the Lamiaceae family, which is considered an interesting source of antioxidant components (Lamaison, Petitjean-Freytet, Duke & Walker, 1993). In fact, the antioxidant properties of methanolic and ethanolic extracts of *Melissa officinalis* have been already pointed out (Zandi & Arnadi, 2000; Ferreira, Proença, Serralheiro & Araújo, 2006, López, Akerreta, Casanova, García-Mina, Cavero & Calvo, 2007). Aqueous ethanol extracts of these plants contained flavonoids and hydroxycinnamic acid derivates, known by their antioxidant capacity (Dastmalchi, Damien Dorman, Oinonen, Darwis, Laakso & Hiltunen, 2008). Dried parsley, oregano and olive mill wastewaters showed antioxidant activity in o/w emulsions enriched with 5 % tune oil (Jimenez-Alvarez, Giuffrida, Golay, Cotting, Lardeau & Keely, 2008).

The aim of this work was to evaluate the antioxidant capacity of a freeze-dried water extract of *Melissa officinalis* for stabilizing an emulsion rich in long chain ω -3 polyunsaturated fatty acids from algae and linseed oil in order to be used as a food ingredient in the elaboration of meat products.

MATERIAL AND METHODS

Materials

Algae oil DHASCO[®] oil is a commercially available oil obtained from *Cryptocodinium cohnii* and it was purchased from Martek Biosciences Corporation, (Columbia., USA). Linseed oil (Biolasi Productos Naturales, Guipúzcoa, Spain) was obtained in a local market and *melissa* dried leaves were purchased from Plantarom SL (Barcelona, Spain). Soya protein and BHA were donated by Dr. Mohino from ANVISA (Madrid). All the chemical reagents were obtained from Sigma-Aldrich Chemical Co. (MO., USA).

Lyophilized water extract preparation

Aqueous extracts of *Melissa officinalis* were prepared as follows: 50 g of dried leaves were weighted and added to 500 ml of distilled water, preheated at 100 °C. The mixture was subjected to reflux during 30 minutes at the temperature above. Extraction process was repeated with another 500 ml of distilled water, and both extracts were joined and completed with distilled water to a final volume of 1 L. Extracts were filtered using filter to remove insoluble particles. The extract was lyophilized with a freeze-dryer-cryodo (Telstar, Barcelona, Spain).

Determination of antioxidant capacity

Determination of Total phenolic content (TPC)

TPC was determined spectrophotometrically following the Folin-Ciocalteu colorimetric method (Singleton & Rossi, 1965). Dilutions of the lyophilized water extract of *melissa* ranging from 0.2 to 0.02 mg/ml were chosen in order to obtain absorbance readings within the standard calibration curve made from dilutions between 0.005 and 2 mg/ml of Gallic acid (GA). The reaction mixture was composed of 0.1 ml of suitable diluted sample, 7.9 ml of distilled water, 0.5 ml of Folin-Ciocalteu's reagent, and 1.5 ml of 20 % sodium carbonate anhydrous solution (added 2 minutes after the Folin-Ciocalteu's reagent). After the

initial mixing, the tubes were allowed to stand at room temperature for 2 hours in the dark. The optical density of the blue-colored resulting solution was measured at 765 nm using a Lambda 5-UV-VIS spectrophotometer (Perkin Elmer, Paris, France). The total phenolic content was expressed as mg GA/g lyophilized extract, using the corresponding calibration curve and taking into account the concentration of the diluted extracts. Absorbance measurements were made in duplicate for each diluted solution.

Determination of rosmarinic acid content

The determination of the rosmarinic content in the extract was done by HPLC-UV analysis. UV spectra were recorded on a Perkin Elmer UV/VIS Lambda 200 Series equipped with a photodiode array detector Series 200 PDA, using a C18 column (250mm×4.6mm, 5µm particle size; Perkin Elmer Brownlee columns, Massachusetts, USA), protected with a HPLC guard cartridge system (C18, 4mmx3mm; Phenomenex, Macclesfield, UK). Briefly, a 20mg/ml water solution of melissa dried extract was filtered through a 0.45µm membrane filter (Millipore, USA) and analysed using the chromatographic conditions described in Fecka and Turek (2008). The profile was recorded at 264nm. Identification of rosmarinic was done using the retention time of the pure standard compound and its characteristic UV spectra, and its quantification was performed using a calibration curve previously plotted.

DPPH method

The DPPH assay was performed according to the method of Blois (1958) with some modifications. Briefly, a DPPH solution of approximately 20 mg/ml was prepared in methanol and diluted to obtain an absorbance of 0.8 at 516 nm (working solution). 2 ml of diluted lyophilized extract of *melissa* of different concentrations (2.2×10^{-2} – 2.2×10^{-4} mg/ml) were allowed to react with 2 ml of DPPH working solution during 30 minutes in the dark, at room temperature. A control sample was prepared with 2 ml of methanol. The final

absorbance of the reaction mixture was measured at 516 nm (Lambda 5 UV-VIS Spectrophotometer, Perkin Elmer, Paris, France). The radical scavenging capacity of each dilution was calculated as percent of inhibition (% I), calculated according to the formula:

$$\% I = (Abs_{\text{control}} - Abs_{\text{sample}}) / Abs_{\text{control}} * 100$$

Where Abs_{control} was the absorbance of the control after 30 minutes of reaction and Abs_{sample} was the absorbance of the sample after 30 minutes of reaction. The percent of inhibition was plotted versus the concentration of the extracts. A calibration curve with Trolox (0.1 - 2000 $\mu\text{g/ml}$) was used for calculating the antioxidant capacity. Results were finally expressed as mg Trolox/g lyophilized extract of *melissa*. Absorbance measurements were done in duplicate for each dilution of lyophilized extract.

ABTS method

For ABTS assay, the procedure described by Re, Pellegrini, Proteggente, Pannala, Yang and Rice-Evans (1999) with some modifications was used. Briefly, the $ABTS^{++}$ chromogenic radical was generated by a chemical reaction mixing an aqueous solution of ABTS with $K_2S_2O_8$ (140 mM) to reach a 7 mM final concentration of ABTS. The mixture was kept in the dark for 12 - 16 hours at room temperature (stock solution). Before use, 1 ml of $ABTS^{++}$ stock solution was diluted with ethanol 50 % to an absorbance of 0.70 (\pm 0.02) at 741 nm (working solution). 3 ml of $ABTS^{++}$ working solution was allowed to react with 300 μl of suitably diluted water lyophilized water extract of *melissa* (0.125 mg/ml – 8×10^{-3} mg/ml) or control (ethanol - 50 %) during 6 minutes, and absorbance was measured at 741 nm (Lambda 5 UV-VIS Spectrophotometer, Perkin Elmer, Paris, France). The decrease in absorbance was recorded as percent of inhibition (% I) and was calculated according to the formula:

$$\% I = (Abs_{\text{control}} - Abs_{\text{sample}}) / Abs_{\text{control}} * 100$$

Where $Abs_{control}$ was the absorbance of the control after 6 minutes of reaction and Abs_{sample} was the absorbance of the sample after 6 minutes of reaction. The percent of inhibition was plotted versus the concentration of the extracts. A calibration curve with Trolox (0.1 - 60 $\mu\text{g/ml}$) was used for calculating the antioxidant capacity. Results were finally expressed as mg Trolox/g lyophilized extract of *melissa*. Absorbance measurements were made in duplicate for each diluted solution.

Emulsion formulation and processing

The emulsion was prepared mixing for two minutes eight parts of hot water with one part of isolated soya protein and then with ten parts of a mixture of oils (40% linseed oil and 60% algae oil) for other three minutes (Hoogenkamp, 1989a,b). Three batches were manufactured: A *melissa*, with 620.6 ppm of lyophilized water extract of *melissa* (ten fold the equivalent antioxidant activity of BHA measured by the DPPH method); *BHA*, with 200 ppm of the synthetic antioxidant and *Control*, without any type of antioxidant. The emulsions were placed in different flasks, one for each day of analysis. Analysis were done after 0, 3, 7 and 15 days of storage at room temperature. The experiment was performed in duplicate.

Chemical analysis

Fatty acids were determined in the oils mixture by gas chromatography. Boron trifluoride/methanol was used for the preparation of fatty acid methyl esters (AOAC, 2002). A Perkin-Elmer Autosystem XL gas chromatograph fitted with a capillary column SPTM-2560 (100 m x 0.25 mm x 0.2 μm) and flame ionization detection was used. The temperature of the injection port was 250 °C and of the detector was 260 °C. The oven temperature was programmed at 175 °C during 10 min and increased to 200 °C at a rate of 10 °C/min, then increased to 220 °C at a rate of 4 °C/min, which was kept for 15 min. The carrier gas was hydrogen, and the pressure was 20.5 psi. Split flow was 120 cm/s. The identification of the fatty acid methyl esters was done by comparison of the retention times of the peaks in the sample with those of standard pure compounds (Sigma, St. Louis, MO, USA) and by spiking

the sample with each standard individually. The quantification of individual fatty acids was based on the internal standard method, using heptadecanoic acid methyl ester (Sigma, St. Louis, MO, USA).

The progress of oxidation was determined by the TBARS value that was determined according to Tarladgis, Watts, Younathan, and Dugan (1960) with modifications by Tarladgis, Pearson, and Dugan (1964). Results are shown in mg malonaldehyde/kg sample (ppm). Analysis was done in triplicate.

Data Analysis

Anova test was used to determine significant differences ($p < 0.05$) among the different days and types of emulsions. The statistical package chosen for analysis was SPSS version 15.0 (SPSS inc. Chicago, Illinois, USA).

RESULTS AND DISCUSSION

The model system used in this paper to evaluate the effectiveness of a lyophilized water extract of *Melissa officinalis* as antioxidant was an emulsion rich in ω -3 PUFA. The composition of the mixture of algae and linseed oil used in the oil-in-water emulsion is shown in table 1. DHA was the main fatty acid present in the mixture (31.73 %), whose origin was basically the algae oil. This long chain ω -3 PUFA is known by its healthy role in cardiovascular diseases, immune disorders and others (Simopoulos, 1990; Nair Leitch, Falconer & Grag, 1997). Linseed oil contributed mainly with the supply of oleic acid, that accounted for a 25.42% in the mixture and α -linolenic acid, that characterizes this oil, was present in a 12.75%. Both oils supplied interesting fatty acids to the mixture, leading to excellent ratios from the nutritional standpoint. High PUFA/SFA and PUFA + MUFA/SFA ratios (2.33 and 3.63, respectively) and a very low ω -6/ ω -3 ratio (0.12) were obtained. Incorporated as an ingredient to other foods, this oils mixture would improve the health properties of its lipid fraction.

In order to stabilize these emulsions a lyophilized of water extract of *Melissa officinalis* was used. The antioxidant activity of this lyophilized water extract was determined, obtaining values of 424.43 (\pm 5.62) mg Trolox/g lyophilized extract of *melissa* for DPPH and 436.42 (\pm 7.69) mg Trolox/g lyophilized extract of *melissa* for ABTS. Comparing with the antioxidant activity of BHA, it can be concluded that 200 ppm of BHA is equivalent to 62 ppm of the lyophilized extract of *Melissa officinalis* using the DPPH assay. Regarding the phenolic content determined spectrophotometrically, the lyophilized extract contained 310 mg gallic acid/g dried extract. The analysis of the melissa extract by HPLC revealed that the main phenolic compound present in the lyophilized extract was rosmarinic acid, accounting for 67mg/g dried extract (fig 2). This compound is characterized by its high radical scavenging activity of free radicals due to its large number of electron donating hydroxyl groups (Dziedzic and Hudson, 1984). Chen, Lin, and Hsieh (2007) found that freeze dried water extracts of different nutraceutical extracts showed total phenolic compounds about 64 - 185 mg gallic acid/g, corresponding the highest amount to *Rosmarinus officinalis* (*rosemary*), which is rich in this phenolic acid. Hernández-Hernández, Ponce-Alquicira, Jaramillo-Flores and Guerrero-Legarreta found 39mg rosmarinic acid/g ethanolic extract of fresh rosemary.

In this work the efficacy of the natural extract for stabilizing the emulsion was analyzed through the measure of TBARS during 15 days at 20 °C. Figure 1 showed the TBARS evolution in the emulsions without any antioxidant (Control), with BHA (200 ppm) and with the lyophilized extract obtained from *melissa* (620.6 ppm). Except at time = 0, every analytical point showed higher TBARS values for the emulsions without antioxidants compared to those with antioxidants ($p < 0.05$). The need for an antioxidant effect to ensure the stability of this type of preparation is then demonstrated. Furthermore, no significant differences were found for TBARS during the 15 days between the emulsions with BHA and with melissa antioxidants ($p < 0.05$). Nevertheless, the behavior of this emulsions in a more complex matrix as a meat product should be evaluated. Besides this, if the evolution of

TBARS is followed during the experimental period, it can be observed that control emulsions showed significant increases of TBARS ($p < 0.05$), duplicating initial values. However, with the use of antioxidants TBARS values did not show significant increases in the case of the use of BHA and only a slight increase, not quantitatively relevant, in the case of melissa. Viljanen, Halmos, Sinclair, and Heinonen (2005) analyzing the effect of different vegetable juices on oil-in-water emulsion oxidative stability also observed a significant decrease on TBARS formation after 8 days at 40 °C, depending on the concentration of the juices in the emulsion, and consequently the concentration of anthocyanins and other phenolic compounds.

These results pointed out that the o/w emulsions elaborated with long chain ω -3 PUFA oils could be stabilized, in relation to the oxidation process, with a lyophilized water extract of *Melissa officinalis*, giving rise to an ingredient susceptible to be used as a functional ingredient in new formulations of meat products.

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FIGURE CAPTIONS

Figure 1. TBARS mean concentration (mg MDA/kg product) and standard deviation on linseed and algae oil in water emulsion stored at room temperature and measured at 0, 3, 7 and 15 days. Three batches were plotted: control (no antioxidant), BHA (200 ppm) and Melissa (620.6 ppm).

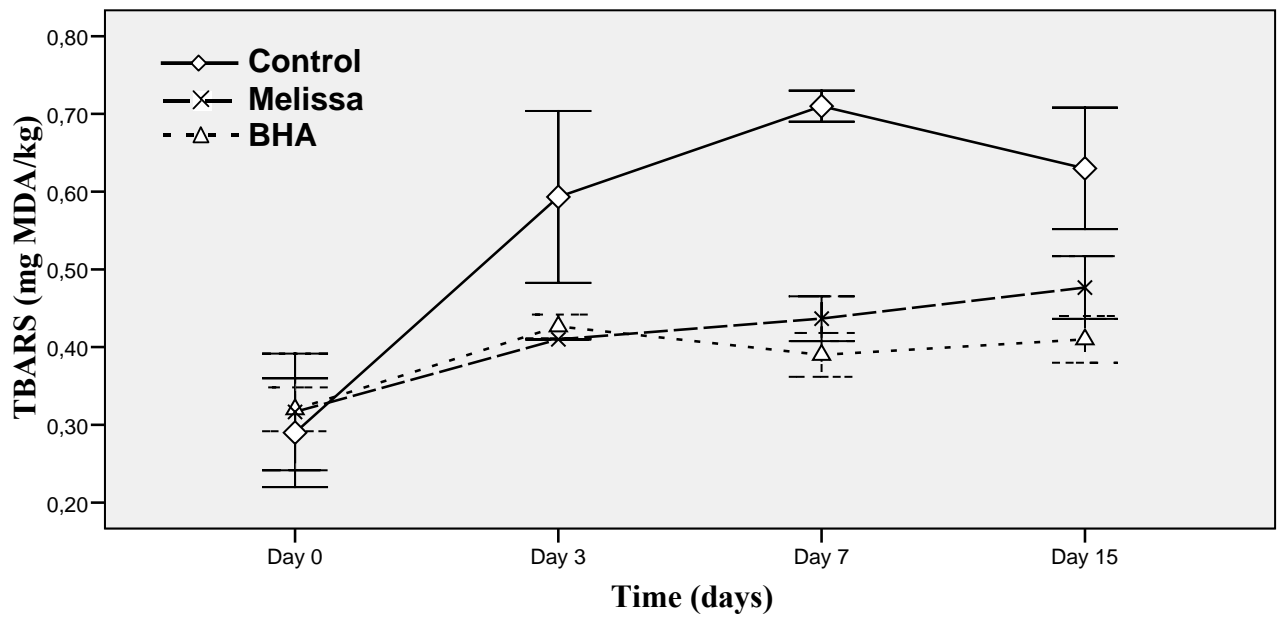


Figure 2. Chromatogram of the HPLC-UV analysis of phenolic compounds of the melissa extract. Acquisition is made at 264nm.

Figure 2.

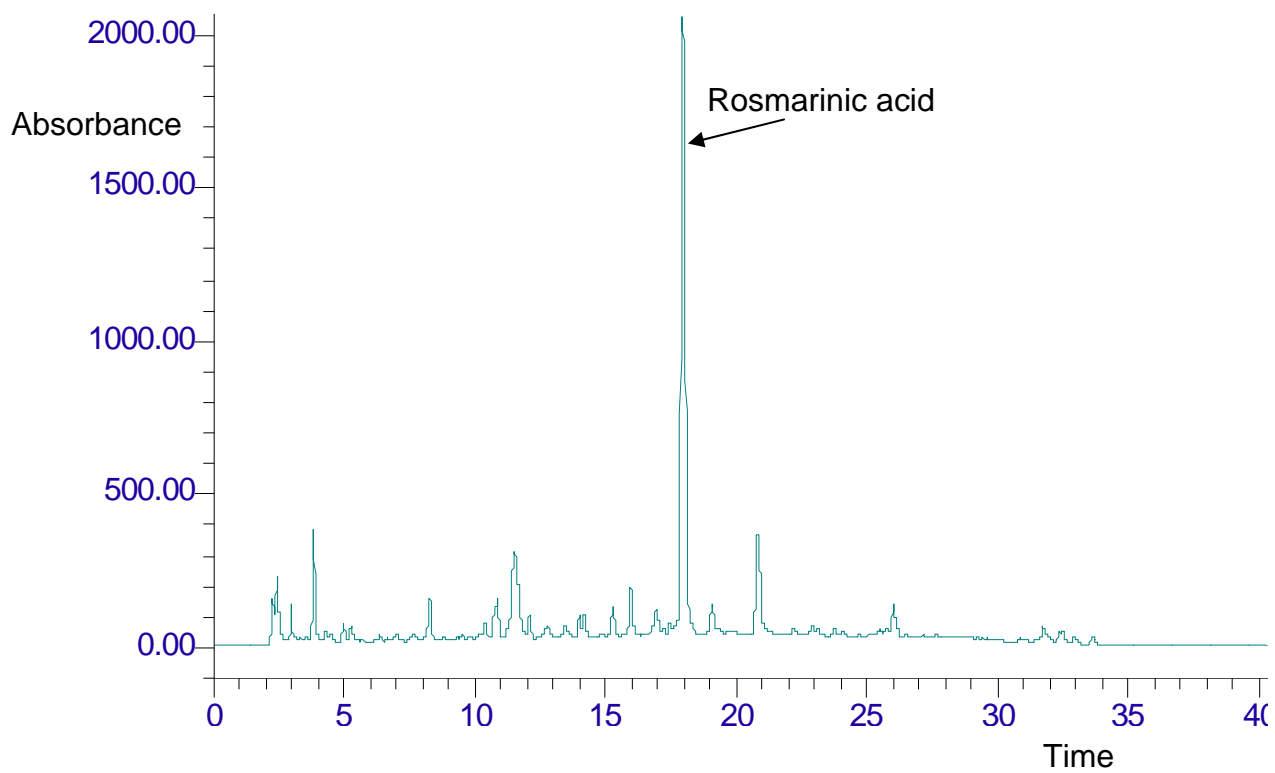


Table 1. Fatty acid profile of the algae and linseed oils mixture incorporated into the emulsion (g/100 g fatty acids).

Fatty acid	g /100 g fatty acid
Caprylic C8:0	0.30 ± 0.01
Capric C10:0	0.92 ± 0.02
Lauric C12:0	3.32 ± 0.05
Myristic C14:0	7.83 ± 0.06
Palmitic C16:0	7.01 ± 0.03
t-Palmitoleic C16:1t	0.04 ± 0.00
Palmitoleic C16:1	2.09 ± 0.01
Stearic C18:0	1.88 ± 0.03
Elaidic C18:1t	0.07 ± 0.01
Oleic C18:1	25.42 ± 0.06
Vaccenic C18:1	0.40 ± 0.01
t-Linoleic C18:2t	0.03 ± 0.00
c-t linoleic C18:2	0.05 ± 0.00
t-c linoleic C18:2	0.07 ± 0.00
Linoleic C18:2(ω-6)	5.18 ± 0.03
Arachidic C20:0	nd
γ-linolenic C18:3 (ω-6)	0.06 ± 0.00
Eicosenoic C20:1	0.06 ± 0.01
α-linolenic C18:3(ω-3)	12.75 ± 0.09
Eicosadienoic C20:2 (ω-6)	nd
Behenic C22:0	0.18 ± 0.02
Brasidic C20:1t	0.08 ± 0.00
Erucic C22:1	nd
Eicosatrienoic C20:3(ω-3)	nd
Arachidonic C20:4(ω-6)	0.04 ± 0.00
Eicosapentaenoic C22:5(ω-6)	0.09 ± 0.00
Nervonic C24:1	0.03 ± 0.00
Lignoceric C22:0	0.41 ± 0.01
Docosahexaenoic C22:6(ω-3)	31.73 ± 0.15
SFA	21.43 ± 0.11
MUFA	28.00 ± 0.06
PUFA	49.84 ± 0.05
ω-3	44.57 ± 0.07
ω-6	5.27 ± 0.03
ω-6/ω-3	0.12 ± 0.00
PUFA/SFA	2.33 ± 0.01
PUFA + MUFA/SFA	3.63 ± 0.02
Trans	0.33 ± 0.01