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ORIGINAL PAPER

Acylglycerol synthesis including EPA and DHA from rainbow trout (*Oncorhynchus mykiss*) belly flap oil and caprylic acid catalyzed by *Thermomyces lanuginosus* lipase under supercritical carbon dioxide

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Abstract

Supercritical carbon dioxide ($SCCO_2$) was studied as a medium for the esterification of eicosapentaenoic acid (*n*-3 C20:5, EPA) or docosahexaenoic acid (*n*-3 C22:6, DHA) and caprylic acid (C8:0, CA) in structured triacylglycerols (sTAG) using *Thermomyces lanuginosus* lipase as biocatalyst. Process variables (*n*-3 long-chain polyunsaturated fatty acid: CA, *n*-3 LCPUFA:CA content ratio), glycerol content (wt. %), and supercritical time, temperature and pressure were optimized by the Response Surface Methodology through a central composite design of 2^{5-1} + star. Synthesis of sTAG with EPA, DHA and CA under $SCCO_2$ was significantly affected by the *n*-3 LCPUFA:CA content ratio and supercritical time. MALDI-TOF mass spectrometry revealed that acylglycerols with the highest levels of EPA or DHA content in the *sn*-2 position were obtained when the following variables conditions were applied: 50% (*n*-3 LCPUFA:CA content ratio), 40 °C (supercritical temperature), 20 MPa (supercritical pressure), 4 h (supercritical time) and 20.0 wt. % (glycerol concentration). For such experimental conditions**,** esterification catalyzed by *Thermomyces lanuginosus* lipase under supercritical carbon dioxide allowed obtaining sTAG synthesized with 54.95% of CA, 11.64% of EPA and 13.77% of DHA.

Keywords EPA–DHA · Supercritical carbon dioxide · Acylglycerol enzymatic synthesis · Rainbow trout · MALDI-TOF mass spectrometry · Response surface methodology

Introduction

EPA and DHA have important physiological functions in different organs. One of their main characteristics is to be incorporated into the cell membrane phospholipids, modifying the fluidity and thickness of the membrane, as well as altering the specific interactions with membrane proteins and being substrates for the formation of a series of lipid derivatives called eicosanoids (EPA derivatives) and docosanoids (DHA derivatives), which exert important functions in cell metabolism and anti-inflammatory effects [1]. Both fatty acids have also multiple beneficial effects in various clinical situations, such as cardiovascular diseases. These effects vary according to the type of *n*-3 LCPUFA ingested, the dietary source, the daily dose and inherent factors of each individual [2, 3].

These benefits for health have raised a great interest in obtaining concentrates of EPA and DHA from marine organisms due to the fact that they are recognized as the most important natural sources of such *n*-3 LCPUFA, this arising from the marine phytoplankton as primary producer, and then following the trophic chain up to marine invertebrates and fish [4].

Rainbow trout (*Oncorhynchus mykiss*) has received a great attention because of a wide farming production in many countries. Most previous research has shown a high

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yield of *n*-3 LCPUFA content of this species [5, 6]. Rainbow trout belly flap is a by-product resulting from the trimming process, which is obtained from the central part of the stomach after a longitudinal cut of the fish, without removing skin, bones and stapes [7, 8].

Authors have reported that the bioavailability of EPA + DHA from re-esterified triglycerides was superior (124%) compared with natural fish oil, whereas the bioavailability from ethyl esters was inferior (73%) [2].

Methods for concentration of *n*-3 LCPUFA are abundant, but only few of them are suitable for large-scale production [9]. The available methods include adsorption chromatography, fractional or molecular distillation, enzymatic splitting, low-temperature crystallization, supercritical fluid extraction and urea complexation. Each technique has its own advantages and drawbacks. Considerable effort has been applied to replace chemical concentration methods with milder and greener enzymatic techniques involving lipases [10]. The lipase from TL IM (*Thermomyces lanuginosus*) and immobilized lipase is a specific lipase for hydrolysis in the *sn*-1,3 positions. The TL IM lipase selects the acyl group of the *sn*-1,3 position of the glycerol skeleton, also having the ability to select certain substrates and their stereoisomers being a regioselective enzyme [11–15]. $SCCO_2$ is a markedly attractive process solvent for enzymatic catalysis as it offers even more advantages over organic solvents, e.g., it is nonflammable, nontoxic, and inexpensive. It is considered a Generally Recognized as Safe (GRAS) compound and has revealed a variable density, great solvent power [16]. Combining the advantages of supercritical fluid (SCF) and CO_2, several studies have employed $SCCO_2$ as a medium for enzymatic catalyzed reactions on oxidation, hydrolysis, transesterification, esterification and enantioselective synthesis [17].

Response surface methodology (RSM) enables the evaluation of the effects of multiple parameters, alone or in combination, on response variables and also predicts their behavior under a given set of conditions [18]. The aim of this study was to optimize operational variables by RSM for the synthesis of triacylglycerols that include EPA or DHA from *n*-3 LCPUFA concentrates obtained from rainbow trout (*Oncorhynchus mykiss)* belly flap (RTBF) oil, CA and glycerol, this reaction being catalyzed by the *sn*-1,3 from TL IM lipase and using as reaction solvent $SCCO_2$.

Materials and methods

Chemicals and materials

Rainbow trout belly flap (*Oncorhynchus mykiss*) oil (RTBF) was obtained from Salmones Antárctica S. A. (Aysén, Chile) and processed in the pilot plant (Island of Chiloé, Chile); it was stored at − 80 ºC until used. Lipozyme® TL IM, a 1,3-specific lipase originating from *Thermomyces lanuginosus*, was used for acylglycerol esterification; for it, the enzyme was immobilized on a non-compressible silica gel carrier (Novozymes A/S, Denmark) gift provided by Blumos S. A. (Santiago, Chile). CA was purchased from Sigma Chemical Co. Internal standard, for gas chromatography (GC) analysis, tricosanoic acid methyl ester (C23:0) was obtained from Nu-Chek-Prep, Inc. Hexane and toluene used for GC analysis were HPLC grade and obtained from Merck S.A. (Santiago, Chile). All other chemicals and solvents were of analytical grade. CO_2 with a purity of more than 99.95% was purchased from Linde (Santiago, Chile).

n-3 LCPUFA process

The preparation of free *n*-3 LCPUFA from RTBF was carried out by hexane/isopropanol oil extraction followed by saponification and urea inclusion method [8, 19–23]. Values of the process variables for urea inclusion were: 4.2:1 (urea:FA ratio), − 15 °C (crystallization temperature), 24 h (crystallization time), and 1000 rpm (stirring speed) [8]. Total *n*-3 LCPUFA content was 80.3 (g/100 g total FA), which contained 74.0 (g/100 g total FA) of EPA + DHA.

Enzymatic esterification by $SCCO_2$

A high-pressure batch reactor, which can stand up to 400 bar (1 bar ≈ 0.1 MPa) with an inside volume of 200 mL, was used to carry out the esterification reactions. The reactor column was placed in an isothermal oven to control the reaction temperature. Liquid CO_2 contained in a cooling unit and a layer of molecular sieve to remove the extra water were pressurized with a high-pressure pump and stored in a buffer container. To prevent a CO_2 content decrease during processing, the buffer container was used as a pressure source to supply the higher pressure liquid CO_2. Reactions were carried out under varied conditions of supercritical pressure, supercritical temperature, and supercritical time. *n*-3 LCPUFA concentrates, CA and glycerol content were previously mixed and incubated with TL IM, 1,3-specific lipase and then added at various weight ratios into the reactor. Immobilized lipase was added in a 10% of total weight of the substrates according to Lin et al. [17] (Table 1). Once the reaction was carried out for the desired time, depressurization and elution in a collector glass tube was performed.

Purification of sTAG by neutralization with NaOH

The sTAG obtained by enzymatic esterification under $SCCO_2$ was purified by fatty acid neutralization with NaOH to remove the remnant free fatty acids of the reaction according to Hita et al. [24] and Jiménez et al. [25] and then collected in hexane for GC analysis. The purification state of

Table 1 Central composite rotatable design 2^{5-1} + star and values obtained for the different experimental response variables of acylglycerol synthesis with EPA, DHA and CA catalyzed by *Thermomyces lanuginosus* lipase under supercritical carbon dioxide

Trials	Process variables[a]					Response variables[b]		
	A	B	C	D	E	Y1	Y2	Y3
1	25	50	150	2	30	4.68	4.17	86.97
2	75	50	150	2	10	24.36	20.64	35.86
3	25	70	150	2	10	6.06	7.54	78.52
4	75	70	150	2	30	18.15	17.49	55.43
5	25	50	250	2	10	15.17	14.79	52.59
6	75	50	250	2	30	28.58	20.05	20.74
7	25	70	250	2	30	13.96	14.07	60.49
8	75	70	250	2	10	31.55	17.95	18.62
9	25	50	150	6	10	2.73	1.95	92.23
10	75	50	150	6	30	27.62	15.11	37.28
11	25	70	150	6	30	3.48	3.10	89.2
12	75	70	150	6	10	15.95	9.51	62.08
13	25	50	250	6	30	2.39	3.98	88.34
14	75	50	250	6	10	15.88	8.71	59.05
15	25	70	250	6	10	3.47	3.88	89.61
16	75	70	250	6	30	18.63	16.86	20.05
17	0	60	200	4	20	0.00	0.00	100.00
18	100	60	200	4	20	28.71	23.96	0.00
19	50	40	200	4	20	13.77	11.64	54.95
20	50	80	200	4	20	8.79	12.21	74.54
21	50	60	100	4	20	14.53	8.38	57.6
22	50	60	300	4	20	17.10	9.26	53.55
23	50	60	200	0	20	0.20	0.51	0.00
24	50	60	200	8	20	10.25	5.57	71.11
25	50	60	200	4	0	0.00	0.00	0.00
26	50	60	200	4	40	10.8	16.19	61.07
27	50	60	200	4	20	13.65	11.53	54.46
28	50	60	200	4	20	16.41	12.66	46.16
29	50	60	200	4	20	21.65	6.68	48.72

[a]Process variable: A (*n*-3 LCPUFA:CA content ratio), B (supercritical temperature, °C), C supercritical pressure, bar), D (supercritical time, h), E (glycerol content, wt %)

[b]Response variables: Y1 (EPA content, g/100 g total FA), Y2 (DHA content, g/100 g of total FA), and Y3 (CA content, g/100 g total FA in concentrate)

each sample was followed by thin layer chromatography (TLC).

FA analysis by gas chromatography

Previously purified sTAG from the enzymatic esterification were converted into fatty acids methyl esters (FAME) with sodium methylate 0.2 N. GC analysis was performed in a GC HP 5890 series II, injection system, flame ionization detector, split and capillary column SPTM-2560 of 100 m × 0.25 mm × 0.2 µm (Supelco, Bellefonte, PA, USA). The program used was according to Berríos et al. [22]. Identification of FAME profiles were performed using as reference the standard Nu-Chek GCL463 with the program DataApex (Ltd., Prague Czech Republic) Clarity™. The concentration of the different FAME was determined from the calibration curves by assessment of the peak/area ratio. Quantification of all kinds of FA (g/100 g total FA) was achieved by employing C23:0 methyl ester as internal standard [23].

Experimental design of enzymatic esterification of sTAG in $SCCO_2$ and optimization of response variables

On the basis of the RSM, a 2^{5-1} + star central composite design of 5 factors and 29 runs with three replicates of the central point was used to estimate the experimental error (Table 1). The range of the design variables was 0–100% (concentrated *n*-3 LCPUFA:CA content ratio,

variable A), 40–80 °C (supercritical temperature, variable B), 100–300 bar (supercritical pressure, variable C), 0–8 h (supercritical time, variable D) and 0–40 wt% (glycerol content, variable E). The lipase enzyme remained at 10% concentration of the total substrates (Table 1)**.** The following response variables (Y variables) of the experiment design were chosen: Y1 (EPA content, g/100 g total FA), Y2 (DHA content, g/100 g of total FA), and Y3 (caprylic acid, content, CA g/100 g total FA in concentrate). All experiments were carried out randomly to minimize the effect of unexplained variability in the observed responses due to extraneous factors.

A polynomial regression model was assumed for predicting individual Y variables. The model proposed for each response of Y value was expressed in the following equation:

$$Yi = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j + \varepsilon,$$

where Y is the dependent variable (EPA incorporation, DHA incorporation and CA incorporation); β_0, β_i, and β_{ii} are intercept, linear, and quadratic coefficients, respectively; β_{ij} denotes the interaction coefficient term for the interaction of variables i and j; X_i represents the process variables and ε corresponds to the random error [26].

Multiple regression equations were fitted to the responses by discarding no significant terms ($p > 0.05$) to obtain response surfaces.

Multiple-response optimization

A multiple-response optimization was performed to optimize several responses simultaneously. After the multifactor analysis of variance and the second-order model prediction determinations, the maximizing desirability function that ranged between 0 and 1 scores was obtained as a function of the optimization of multiple responses.

MALDI-TOF mass spectrometry of sTAG

The mass spectrometry analysis was performed in a Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight (MALDI-TOF) Microflex (Bruker Daltonics Inc., MA, USA). mMass program version 5.5.0 was used for the analysis [27–29].

Working solutions of the oils with a concentration of 1.0 mg/mL were prepared in chloroform/isopropanol 1:1. The 5-chloro-2-mercaptobenzothiazole (CMBT) matrix was prepared at a concentration of 10.0 mg/mL in methanol. The working solutions and the matrix were mixed in a 1:1 ratio and 0.7 µL was deposited on a micro-scout sample plate (Bruker Daltonics Inc., MA-USA). The acquisition of mass spectra was carried out in a MALDI-TOF Microflex (Bruker Daltonics Inc., MA-USA) in positive ion mode and detection by reflection. The equipment was calibrated with an external standard corresponding to a mixture of peptides. The final spectra correspond to the accumulation of 900 laser shots. mMass program version 5.5.0 was used for the spectra analysis [27–29]. The identification was performed with the monoisotopic *m/z* signals using Compounds Search option by comparison with the theoretical monoisotopic signals of different types of glycerolipids (GL) contained in the LIPID MAPS database version 16-11-2013. A mass tolerance of 0.10 Da was used and in agreement with the protonated forms, sodium or potassium adducts (species $[M+H]^+$, $[M+Na]^+$ or $[M+K]^+$) in positive polarity [30]. Subsequently, the annotated coincidences were examined manually and, in each case, the experimental isotope distribution was compared with the theoretical, through the Show Isotopic Pattern option. The same procedure was performed by mixing the samples with the α-cyano-4-hydroxycynamic acid (CHCA), 2,5-dihydroxybenzoic acid in methanol (DHB1) and 2,5-dihydroxybenzoic acid in methanol and trifluoroacetic acid 0.10% v/v (DHB2) matrices.

Statistical analysis

A statistical analytical system for multiple regression analysis, analysis of variance (ANOVA), canonical analysis and analysis of ridge maximum of data in the response surface regression (RSREG) procedure was used.

Estimated response surfaces and contours of estimated response surface using the fitted quadratic polynomial equations obtained from RSREG analysis and holding the process variables with the least effect on the response at a constant value and changing the levels of the other two variables were developed. The 95% confidence intervals of each quality parameter were calculated, taking into account the number of replicates and considering the standard deviation of each sample. The lack-of-fit test was performed by comparing the variability of the current model residuals with the variability between observations at replicate settings of the factors was performed. Statgraphics®Centurion XVI-2011 software (StatPoint Technologies, Inc., Rockville, USA) was used.

Results and discussion

Experimental values of acylglycerol synthesis including EPA, DHA and CA under $SCCO_2$

Experimental values of the responses variables (Y1, Y2 and Y3 for EPA, DHA and CA contents, respectively) obtained by the esterification process after purification are expressed in Table 1.

According to Cormier et al. [31], CO_2 compressibility values and small changes in thermal parameters can lead to large changes in the local density under supercritical conditions. Thus, the closer the system temperature and pressure to their critical values, the greater the effect of local density enhancement. In this study, these facts could be explained by the large difference between some of the experimental trials when taking into account EPA, DHA and CA content responses variables that were incorporated into the sTAG (Table 1). It should be noted that in the case of the non-incorporation of *n-3* LCPUFA, i.e., 0: 100% (*n-3* LCPUFA:CA content ratio), the process of esterification of EPA and DHA content incorporation does not occur and the acylglycerols alone contain CA and glycerol. Meanwhile, the absence of glycerol content is not possible for the reaction of the esterification process to occur, as well as for the incorporation of EPA, DHA and CA into acylglycerols. Thus, chromatograms obtained from the purified sTAG of the runs 17, 18 and 25 of the experimental design (Table 1), corresponding to 0: 100% (*n*-3 LCPUFA:CA content ratio) (variable A), 100:0% (*n*-3 LCPUFA:CA content ratio) (variable A) and 0 wt.% (glycerol concentration) (variable E), respectively, experimentally corroborates that when there is no presence of *n*-3 LCPUFA in the variable A, the sTAG only contains CA in the structure (Fig. 1a) and when the variable A is 100:0% (*n*-3 LCPUFA:CA content ratio), the sTAG contains only FA of the optimized *n*-3 LCPUFA concentrates (data from Pando et al. [8]) as shown in Fig. 1b. No presence of FA from *n*-3 LCPUFA concentrates or CA was detected in the chromatogram of Fig. 1c due to the fact that there is no glycerol present in the chromatogram of the purified sample, indicating the lack of esterification when there is no glycerol skeleton to form sTAG.

Predictive second-order polynomial model and effect of process esterification on individual response EPA, DHA and CA

Second-order polynomial model for each individual response (Y1, Y2 and Y3 for EPA, DHA and CA contents, respectively) resulted in the corresponding adjusted regression equations (Table 2). This table shows the regression coefficients of predictive second-order polynomial model for response variables. The results of fitting a multiple regression model describe the effect of the different process variables on the response variables considered for the synthesis. The regression coefficient indicated that for Y1, Y2 and Y3, linear term of variable A (*n*-3 LCPUFA:CA content ratio) was significant (p value < 0.05). The variable A affected positively the esterification of the EPA, DHA in the sTAG. In the case of the Y1, the significant terms were variable A (*n-3* LCPUFA:CA content ratio) and interaction of variable C (supercritical pressure, bar) and variable D (supercritical time, h) (p value < 0.05). In the case of the variable Y2, the significant terms were variable A (*n*-3 LCPUFA:CA content ratio) and variable D (supercritical time) (p value < 0.05).

In the case of variable Y3, the significant terms were variable A (*n-3* LCPUFA:CA content ratio), variable D (supercritical time) and interaction between variable B (supercritical temperature) and variable C (supercritical pressure) (p value < 0.05).The variable A affected negatively the esterification of the CA in the glycerol molecule due to the fact that there is less availability of CA. In this case, this negative effect was eliminated maintaining the variable Y3 at 50% in the optimization (Table 3). The Variable D (supercritical time) showed an effect that was positive in the CA incorporation into sTAG (p value < 0.05). This effect could be explained because CA would occupy the *sn*-1 and *sn*-3 positions, since such positions are occupied commonly by saturated fatty acids [32]. Furthermore, such two positions are in the same spatial orientation, leading to a steric hindrance for the enzyme to achieve the esterification in short time. The Durbin-Watson (DW) statistic tests in the three dependent variables (Y1, Y2 and Y3) presented p values greater than 0.05 (Table 2), this indicating that there is no serial autocorrelation in the residuals (at the 5% significance level).

Liu et al. [33] studied by RSM the effect of operational variables on the enzymatic esterification of *n-3* LCPUFA concentrate from tuna oil (*Thunnus albacares*) and glycerol with a non-specific lipase. These authors found that in the equation of the polynomial model, the linear and quadratic terms for temperature and glycerol were significant (p value < 0.01) to synthesize sTAG, structured diacylglycerols (sDAG) and monoacylglycerols (sMAG) with C16:1, C16:2, C20:4, *n-3* C20:5, C22:3 C22:4, *n-3* C22:5 and *n-3* C22:6, among other FA.

Effect of esterification process on the incorporation of EPA, DHA and CA into sTAG

Figure 2a–e shows the contours and estimated response surface as a function of *n-3* LCPUFA:CA content ratio and/or supercritical process variables of the incorporation of EPA, DHA and CA in the synthesis process of sTAG. The linear, quadratic and interaction terms in the second order polynomial were used to generate a three-dimensional response surface graph [8]. The synthesis process variables were subsequently placed in the different axes to analyze their influence on incorporation EPA, DHA and CA into structured triacylglycerol (Fig. 2). In this figure, Panel a shows the response surface for EPA incorporation as a function of the *n-3* LCPUFA concentration ratio and supercritical time. In Fig. 2a, b it can be observed that the highest values of EPA and DHA were reached by taking into account high values of *n-3* LCPUFA:CA content ratio for all range supercritical time, respectively (p value < 0.05). It can be observed that

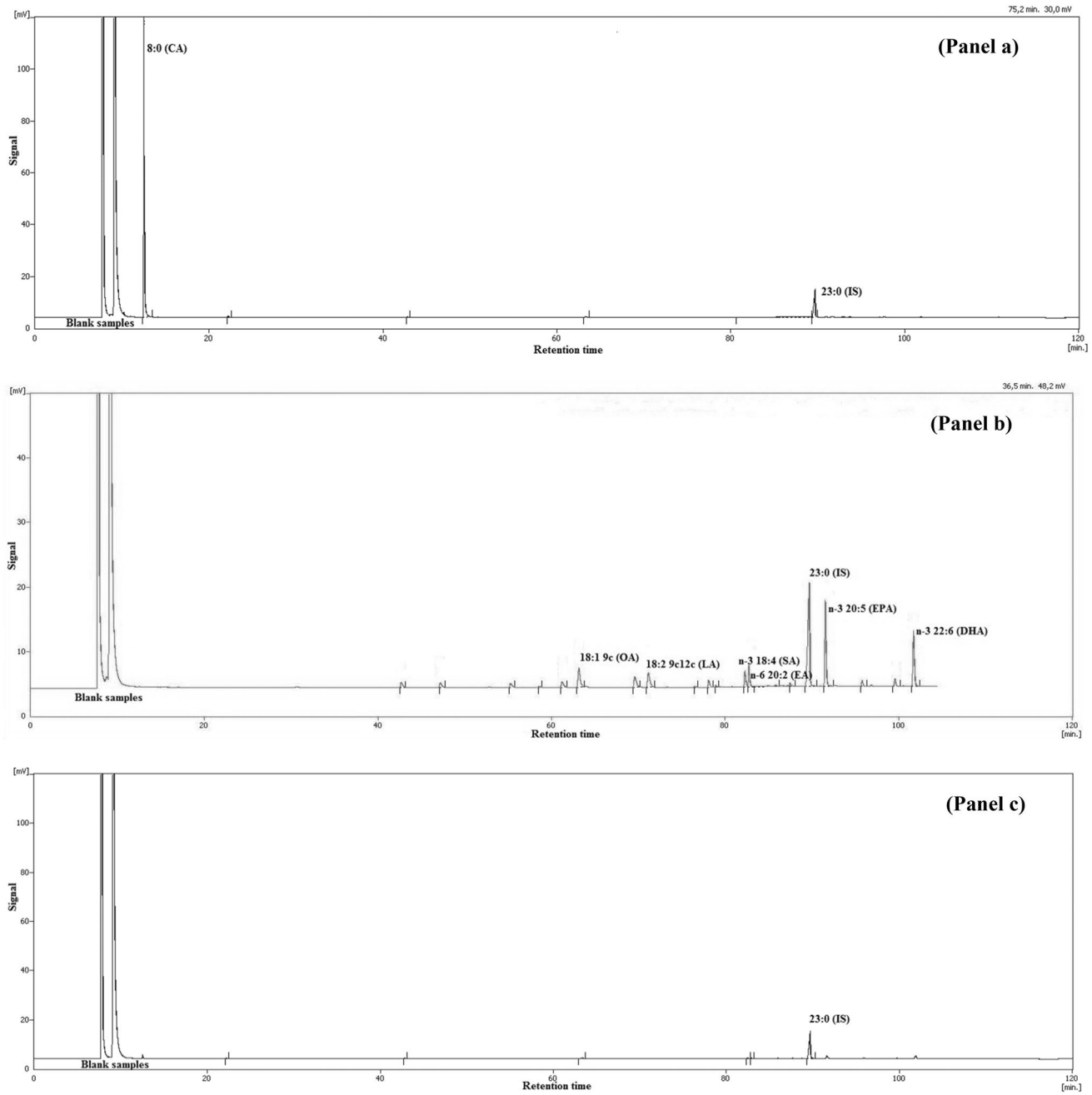

Fig. 1 Fatty acid chromatograms of the experimental design of sTAG synthesis in $SCCO_2$ corresponding to **a** 0: 100 (*n-3* LCPUFA:CA content ratio), **b** 100:0 (*n-3* LCPUFA:CA content ratio) and **c** 0 wt., % (glycerol content)**.** Blank samples: toluene and hexane. IS: internal standard 23:0 methyl tricosanoate

the EPA and DHA incorporation in sTAG increased with the *n-3* LCPUFA concentrations ratio. Thus, the EPA and DHA incorporation presented a maximum value in the response surface at high levels of *n-3* LCPUFA concentrations ratio and at intermediate levels of supercritical time.

In the current study, optimization of the process variables for the synthesis of EPA into sTAG showed an optimum value of 41.7 (g/100 g total FA). Optimization of the process variables for the synthesis of DHA content-enriched sTAG resulted in an optimum value of 27.5 (g/100 g total FA) (Table 3a). The analysis of the regression results (R^2 coefficient) provided a variability value of 77.93% ($p < 0.05$) in the experimental design for Y1 (EPA content) and 68.00% for Y2 (DHA content) (p value < 0.05) (Table 2).

Liu et al. [33] found contents of DHA and EPA of 73.4% and 13.5%, respectively, in enzymatic synthesis of

Table 2 Regression coefficients and *p* values of predictive second-order polynomial model for the different response variables of the incorporation of EPA, DHA or CA in the sTAG synthesis

Process variables[a]	Response variables[b]					
	Y1 (EPA content, g/100 g total FA)		Y2 (DHA content, g/100 g of total FA)		Y3 (CA content, g/100 g total FA)	
	Coefficient	*p* value	Coefficient	*p* value	Coefficient	*p* value
Constant	– 36.52		– 8.79		– 149.76	
Linear						
A	0.31	**0.00**	0.20	**0.00**	0.78	**0.00**
B	– 0.08	0.44	0.01	0,92	– 0.97	0.16
C	0.14	0.22	0.02	0,29	1.28	0.51
D	8.87	0.21	2.14	**0.04**	35.53	**0.00**
E	0.86	0.36	0.18	0,05	0.05	0.89
Quadratic						
A × A	–	–	–	–	–	–
B × B	–	–	–	–	0.05	0.11
C × C	–	–	–	–	–	–
D × D	– 0.48	0.06	– 0.38	0.25	–	–
E × E	– 0.02	0.07	–	–	–	–
Interaction						
A × B	–	–	–	–	–	–
A × C	–	–	–	–	– 0.01	0.17
A × D	–	–	–	–	– 0.14	0.11
A × E	–	–	–	–	–	–
B × C	–	–	–	–	– 0.02	**0.04**
B × D			–	–	– 0.35	0.11
B × E			–	–	–	–
C × D	– 0.03	**0.04**	–	–	10.08	0.35
C × E			–	–	–	–
D × E	0.08	0.43	–	0.25	– 13.27	0.23
R^2	77.93		68.00		77.35	
Adjusted R^2	69.11		59.28		64.23	
SE	5.15		4.26		18.95	
MAE	3.37		2.73		10.63	
DW value	1.80	0.21	2.01	0.53	1.80	0.17
Lag 1 residual autocorrelation	0.07		– 0.05		0.08	

Bold values indicate p value < 0.05

[a]Process variable: A (*n*-3 LCPUFA:CA content ratio), B (supercritical temperature, °C), C (supercritical pressure, bar), D (supercritical time, h), E (glycerol content, wt. %)

[b]Response variables: Y1 (EPA content, g/100 g total FA), Y2 (DHA content, g/100 g of total FA), and Y3 (CA content, g/100 g total FA). Other abbreviations: R^2 (determination coefficient), SE (standard error), MAE (mean absolute error) and DW (Durbin–Watson)

acylglycerols from glycerol and *n-3* polyunsaturated fatty acids concentrates, prepared from tuna oil using Lipozyme Novo 435 under different reaction. The results indicated the following conditions: glycerol (2.5 g), hexane (5 ml), the initial water content (0.60%), temperature (40 °C), and molecular sieves (1 g).

The $SCCO_2$ as a medium for esterification of EPA, DHA and CA in the sTAG resulted in a favoring process solvent for enzymatic catalysis by *Thermomyces lanuginosus* lipase above critical temperature of 31.1 °C and critical pressure of 72.9 atm [16]. Interestingly, CO_2 above its critical point, does not liquefy, but reaches a dense gaseous state and behaves like a solvent which allow accelerating the mass transfer in enzymatic reaction. This was observed in the significant effect of supercritical time on EPA, DHA and CA incorporation into sTAG (Table 3a, Fig. 2).

The incorporation of CA decreased when *n-3* LCPUFA/ CA ratio increased at both low and high values of pressurization times Fig. 2c. The incorporation of CA decreased when *n-3* LCPUFA/ CA ratio increased at both low and

Table 3 Process variables optimization and multiple response optimization of the response variables for the synthesis of sTAG with *n*-3 LCPUFA:CA content ratio

Response variables[b]	Process variables[a]					
Y	A *n*-3 LCPUFA:CA content ratio	B Supercritical temperature (°C)	C Supercritical pressure (bar)	D Supercritical time (h)	E Glycerol content (wt. (%))	Y Content (g/100 g total FA)
(a) Optimization of the process variables for the synthesis of sTAG						
EPA	96.8	40.0	300	0.9	23.4	41.7
DHA	99.9	69.3	294	2.8	40.0	27.5
CA	59.3	60.4	208	3.0	20.0	33.0
(b) Multiple response optimization of the process and response variables for the synthesis of sTAG with EPA, DHA and CA						
sTAG	75.5	40.0	300	3.1	24.0	
EPA						31.5
DHA						19.6
CA						49.6

Optimum desirability: optimal value: 0.93. Conditions: DHA—maximize; EPA—maximize; CA—hold at 50.0

[a]Process variable: A (*n*-3 LCPUFA:CA content ratio), B (supercritical temperature, °C), C (supercritical pressure, bar), D (supercritical time, h), E (glycerol content, wt. %)

[b]Response variables: EPA, DHA and CA content: g/100 g total FA. sTAG: structured triacylglyceride

high values of pressurization times (Fig. 2c). The CA incorporation presented a maximum value in the response surface at low levels of *n-3* LCPUFA concentrations ratio and at high levels of supercritical time. However, optimized conditions found for the synthesis of sTAG holding a 50% of CA were 33.0 (g/100 g total FA) with 59.3% *n-3* LCPUFA:CA content ratio under CO_2 supercritical conditions of 60.4 °C and 208 bar and 20.0 wt. % glycerol content during 3 h (Table 3a). In the actual study, the R^2 coefficient indicated that the fitted model explained 77.35% of the variability of CA content ($p \leq 0.05$) (Table 2).

Figure 2d shows the response surface for incorporation EPA content into sTAG as a function of the interaction between supercritical pressure (variable C) and supercritical time (variable D). The EPA incorporation increased with the supercritical pressure and by reducing the supercritical time (p value < 0.05). This result agrees with the inverse relationship found between variables C and D, i.e., negative interactions of regression coefficient predictive of second-order polynomial model (Table 2). Figure 2e shows the contours and estimated response surface as function of supercritical temperature and supercritical pressure variables for the incorporation of CA, in the synthesis process of sTAG. It is observed that the highest values of CA were reached by taking into account high values of supercritical temperature and low values supercritical pressure (p value < 0.05). Also, the incorporation of CA increased at low values supercritical temperature and high values of pressurization pressure.

Optimization of the process variables for the synthesis of sTAG

Table 3a shows the model proposed for each response of individual Y value and the process variables optimization for the synthesis of sTAG with EPA, DHA and CA content, respectively. The goal was to maximize the EPA and DHA content and maintain the percentage of CA at 50% in the sTAG, respectively. The optimal conditions for EPA content were 96.8 (*n*-3 LCPUFA:CA content ratio), 40.0 °C (supercritical temperature), 300 bar (supercritical pressure), 0.9 h (supercritical time) and 23.4 wt. % (glycerol content). Concerning the DHA content, the optimal conditions were 99.9 (*n*-3 LCPUFA:CA content ratio), 69.3 °C (supercritical temperature), 294 bar (supercritical pressure), 2.8 h (supercritical time) and 40 wt. % (glycerol content). Finally, optimal condition for CA content were 59.3 (*n*-3 LCPUFA:CA content ratio), 60.4 °C (supercritical temperature), 208 bar (supercritical pressure), 3.0 h (supercritical time) and 20 wt. % (glycerol content).

Robles et al. [34] reported the synthesis of TAG with PUFA from microalgae *Phaeodactylum tricornutum* and *Porphyridium cruentum* and glycerol by esterification with the non-specific lipase of *Candida antarctica*. In this case, the optimal conditions were established as: 100 mg (enzyme concentration), 900 mL (hexane volume), 50 °C (temperature), 1.20:3.00 (glycerol/PUFA molar ratio), 0% (water content), 1 g (sieve molecules content) and 200 rpm (stirring speed). With the aim of synthesizing

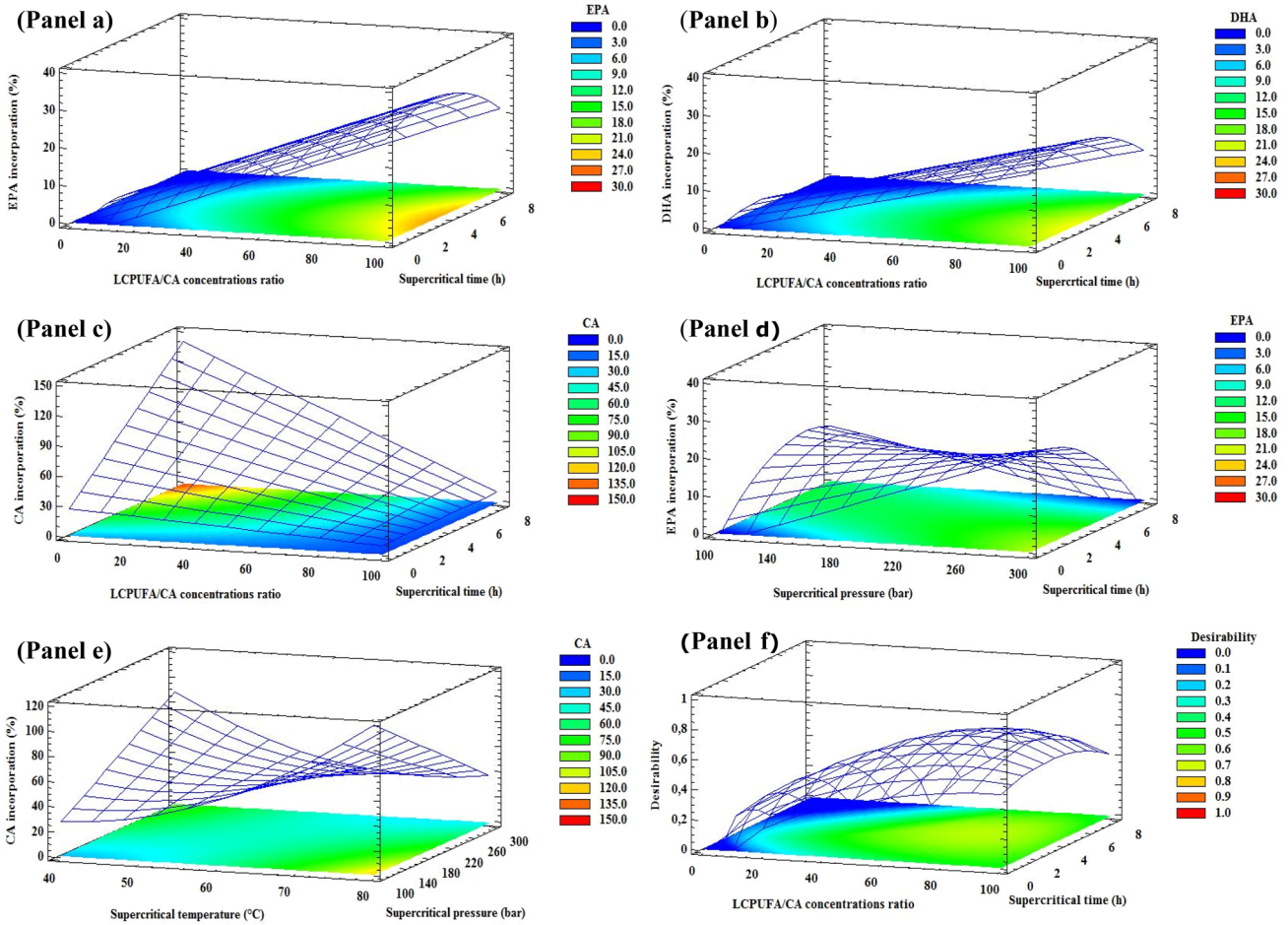

Fig. 2 Estimated response surface and contour response surface for incorporation of EPA, DHA and CA into sTAG by synthesis process under different conditions. **a** EPA incorporation into sTAG as function of *n-3* LCPUFA:CA content ratio and supercritical time process variables, **b** DHA incorporation into sTAG as function of *n-3* LCPUFA:CA content ratio and supercritical time process variables, **c** CA incorporation into sTAG as function of *n-3* LCPUFA:CA content ratio and supercritical time process variables, **d** EPA incorporation into sTAG as function supercritical pressure and supercritical time process variables, **e** CA incorporation into sTAG as function of supercritical temperature and supercritical pressure process variables, **f** Combination of factors that maximizes the desirability function *n-3* LCPUFA:CA content ratio and supercritical time

sTAG, Noriega-Rodríguez et al. [35] synthesized previously *n-3* LCPUFA by starting from sardine (*Sardinops sagax caeruleus*) oil and glycerol using the non-specific lipase of *C. antarctica* and developing RSM procedure. To obtain a 95% of production of sTAG, the optimal conditions of its variables were 4.20 (FFA/glycerol ratio) with a time greater than 12 h and at 72 °C. Imanparast et al. [36] optimized the medium conditions for the lipase production from *Actinomadura sediminis* UTMC 2870 to esterify PUFA concentrates (obtained from flaxseed oil) with glycerol. The product contained 50% (w/w) of PUFAs, including 42% (w/w) of α-linolenic acid (*n*-3 C18:3, ALA,) 9.7% (w/w) of linoleic acid (*n*-6 C18:2, LA,), 35% (w/w) of monounsaturated fatty acids (MUFAs) including 35% of oleic acid (*n*-9 C18:1, OA), and 14% (w/w) of saturated fatty acids (SFA).

Multiple response optimization variables for the synthesis of sTAG

Desirability

Table 3b shows the multiple response optimization variables for synthesizing a sTAG with EPA, DHA and CA. The goal was to simultaneously maximize the EPA and DHA contents and maintain the percentage of CA at 50% in the sTAG. The optimal process variables combination for acylglycerol synthesis from rainbow trout belly oil and CA catalyzed by *T. lanuginosus* lipase under $SCCO_2$ resulted: 75.5 (*n*-3 LCPUFA:CA content ratio), 40.0 °C (supercritical temperature), 300 bar (supercritical pressure), 3.1 h (supercritical time) and 24.0 wt. % (glycerol content). As result, the optimum value of multiple response optimization of the

response variables for the synthesis of sTAG was 31.5 EPA (g/100 g FA content), 19.6 DHA (g/100 g FA content) and 49.6 of CA (g/100 g FA content) (Table 3b).

To optimize the synthesis of a sTAG with EPA and DHA and CA, the desirability of Derringer was used. Optimum desirability indicated the combination of process variables levels which maximizes the desirability function over the indicated region, so that the optimum situation would be attained. A combined response surface of the optimized response variables was obtained on the basis of the responses obtained for EPA, DHA and CA contents Fig. 2f. The optimal process variables combination for acylglycerol synthesis from rainbow trout belly oil and CA catalyzed by *T. lanuginosus* lipase under $SCCO_2$ resulted: 75.5 (*n*-3 LCPUFA:CA content ratio), 40.0 °C (supercritical temperature), 300 bar (supercritical pressure), 3.1 h (supercritical time) and 24.0 wt. % (glycerol content). As result, the predicted values for the maximum stationary points of multiple response optimization for the synthesis of sTAG were 31.5 EPA (g/100 g FA content), 19.6 DHA (g/100 g FA content) and 49.6 of CA (g/100 g FA content) (Table 3b).

The maximum score of desirability function was 0.93 (range 0–1) for sTAG with EPA, DHA and CA when the percentage of incorporation into sTAG was simultaneously maximize the EPA and DHA contents and maintain the percentage of CA at 50% in the sTAG (Table 3b).

Figure 2f shows the multiple response surface and contour expressed as the desirability as function of *n-3* LCPUFA:CA ratio and supercritical time. It can be observed that desirability increased with *n-3* LCPUFA:CA ratio and supercritical time, reaching the maximum desirability with *n-3* LCPUFA:CA ratio of 75.5 and a supercritical time of 3.1 h. The same behavior was observed for CA and DHA desirability, this reaching the maximum desirability with an *n-3* LCPUFA:CA ratio of 60 and a supercritical time of 3.20 h.

Positional analysis of sTAG by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry

The analysis by mass spectrometry suggested the synthesis of acylglycerols with EPA or DHA at the *sn*-2 position of the structure when the reaction was catalyzed by the stereospecific enzyme *T. lanuginosus* TL IM in $SCCO_2$. Figure 3a shows the spectrum obtained between *m*/*z* 200 and 1100 of the sTAGs mixed with the CMBT matrix. The signal *m*/*z* 685.4177 (arrow) would correspond to a sDAG with FA in the following positions of its structure according to the mMass report: *n-3* C18:3 in *sn*-1 position and *n-3* C22:6 in *sn*-2 position; *n-6* C18:3 in *sn*-1 position and *n-3* C22:6 in *sn*-2 position; and *n-6* C20:4 in *sn*-1 position and *n-3* 20:5 in *sn*-2 position Fig. 3b. The signal *m*/*z* 691.3700 would correspond to sDAG with *n-3* C22:6 in the *sn*-2 position. MALDI-TOF is an important technique that has been used in the characterization of triacylglycerols in different types of oils [37–41], in the determination of oil adulterations [42–44] and even in the relative quantification with results comparable to those obtained by gas chromatography [45]. Schiller et al. [46], Calvano et al. [37] and Picariello et al. [42] found that an advantage of MALDI-TOF is the minimal sample preparation, the discrimination ability of the components in the absence of chromatographic separation and the high sensitivity, whereas Shinn et al. [40] and Yener and Van Valenberg [40] suggest that the lack of chromatographic separation has the disadvantage of an important suppression effect, for example, polar lipids interfere in the ionization of non-polar lipids, or the more abundant forms of saturated TAGs affect the ionization of less abundant unsaturated TAGs, which would explain the low intensity of the identified signals shown in the Fig. 3a at *m*/*z* 685.4177 and *m*/*z* 691.3700. Additionally, the ionization process, although of a soft type, can induce the fragmentation of the TAGs to originate DAGs or free fatty acids, artificially increasing the own content of the samples, this explaining the large amount of signals observed in the range *m*/*z* 580–650 of the spectrum [37–40, 48] and the DAG with EPA and DHA seen in the purified TAG sample Fig. 3b.

On the other hand, the visualization of the compounds requires the use of a matrix, which would be one of the factors that favor the fragmentation of the compounds, as well as providing signals to the spectrum up to *m*/*z* 500, thus making the identification more complex [37–49]. Interestingly, identification by MALDI-TOF can be considered as tentative where the high resolution provided ensures a high certainty in the identification; however, the structural confirmation of the position of the fatty acids in the structure of the TAGs would only be possible through fragmentation [37, 39, 43, 45]. Nevertheless, the availability of complementary information obtained from other analysis techniques or from the previous related literature has been used in the current study to propose the identifications without performing fragmentation [38, 39, 48]. As a result, the experimental data obtained would indicate the presence of fatty acids *n-3* C18:3, n-6 18:3, *n-6* C20:4, *n-3* C20:5 and *n-3* C22:6 consistent with those described in the literature for fish oils [50–53].

Conclusions

As a conclusion, the current study has shown that it is possible to synthesize acylglycerols including EPA and DHA from rainbow trout (*Oncorhynchus mykiss*) belly oil and caprylic acid catalyzed by *Thermomyces lanuginosus* lipase under $SCCO_2$ condition.

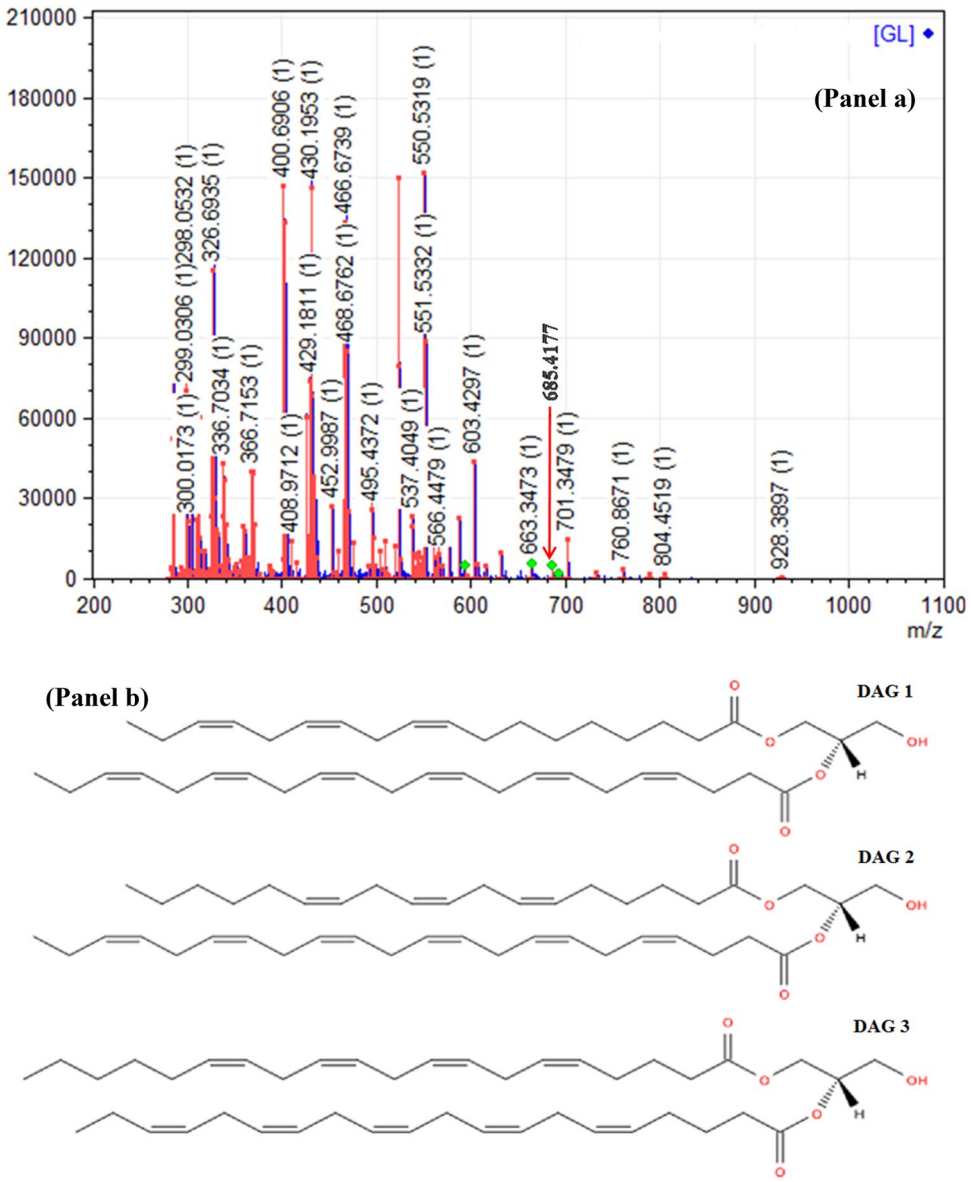

Fig. 3 MALDI-TOF mMass report spectrum of glycerolipid (GL) profile. **a** Spectra obtained between *m/z* 200 and 1100 from a purified sample of sTAGs mixed with the CMBT matrix. The signal *m/z* ratio of 685.41 (arrow) would correspond to a sDAG with FA in the following positions of its structure according to the mMass report: *n*-3 C18:3 in *sn*-1 position and *n*-3 C22:6 in *sn*-2 position; *n*-6 C18:3 in *sn*-1 position and *n*-3 C22:6 in *sn*-2 position; and *n*-6 C20:4 in *sn*-1 position and *n-3* C20:5 in *sn*-2 position, **b** Structures with *n-3* C20:5, EPA or *n-3* C22:6, DHA into the *sn*-2 position of DAG from the 685.41 signal, DAG 1: LMGL02010242DG(18:3(9Z,12Z,15Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0[iso2], DAG 2: LMGL02010497DG(18:3(6Z,9Z,12Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:9[iso2], DAG3: LMGL02010217DG(20:4(5Z,8Z,11Z,14Z)/20:5(5Z,8Z,11Z,14Z,17Z)/0:0[iso2]. Data base: LIPID MAPS for glycerolipids

$SCCO_2$ was found to be an effective system to replace the use of organic solvents in the enzymatic synthesis of sTAG. The RSM optimization allowed predicting the experimental conditions of the process variables to synthesize sTAG with the goal to simultaneously maximize the EPA and DHA contents and maintain the percentage of CA at 50% in the sTAG. Synthesis of sTAG with EPA, DHA and CA under $SCCO_2$ was significantly affected by the *n-3* LCPUFA:CA content ratio and supercritical time. The most convenient conditions to be employed to reach high EPA and DHA incorporation and DHA contents should include high scores of *n-3* LCPUFA:CA content ratio and intermediate value of supercritical time. Using this method, the analysis by GC–MS established that the optimal condition in the sTAG included the highest levels of EPA or DHA content in the *sn*-2 position of the identified sDAG. Optimal conditions were established to esterify *n-3* LCPUFA and CA with glycerol in a one-step greener enzymatic reaction to produce acylglycerols with a high biological value that can be employed in future applications for the prevention of cardiovascular diseases, correct neuronal functioning, reduction of inflammation in type 2 diabetes, or according to nutritional recommendations that require EPA and DHA administration combined with CA which is rapidly metabolized for the liver and does not accumulate in adipose tissue, adding even more value to synthesized acylglycerols.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Compliance with ethics requirements This article does not contain any studies with human participants or animals performed by any of the authors.

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