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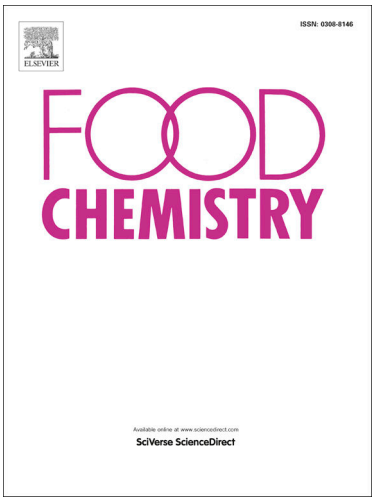

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Production of feruloylated lysophospholipids via a one-step enzymatic interesterification

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ABSTRACT

Incorporation of ferulic acid (FA) into egg-yolk phosphatidylcholine (PC) in a lipase-catalyzed acidolysis and interesterification process was studied using four commercially available immobilized lipases as catalysts and two acyl donors: ferulic acid (FA) and ethyl ferulate (EF). Novozym 435 and a binary solvent system of toluene/chloroform 9:1 (v/v) were found to be the most suitable biocatalyst and medium, respectively, and significantly increased the incorporation of FA into the phospholipid fraction. Subsequently response surface methodology (RSM) and Box-Behnken design were employed to evaluate the effects of substrate molar ratio, enzyme loading and time of the reaction on the process of interesterification. The selected optimized parameters were established as PC/EF molar ratio 1/15, enzyme load 30% (w/w) and incubation time 6 days. The process of interesterification at the optimized parameters carried out on a large scale afforded feruloylated lysophosphatidylcholine (FLPC) in high isolated yield of 62% (w/w).

Keywords: ferulic acid; ethyl ferulate; acidolysis; interesterification; immobilized lipases; egg-yolk phosphatidylcholine

1. Introduction

The importance of phenolic acids in human health and disease prevention is well recognized. One of the most abundant phenolic acids, with a wide array of putative health benefits, is ferulic acid (FA)

(4-hydroxy-3-methoxycinnamic acid). This compound was isolated for the first time from *Ferula foetida* (*Apiaceae*), around the mid 19th century (Von Hlasiwetz, & Barth, 1866). FA is found in cereal raw materials (maize, barley, wheat and rice) and whole-grain bread, in concentrations up to 3300 mg/100 g of edible product and 90% of the total polyphenol fraction (Zhao, & Moghadasian, 2008). Rich sources of FA include vegetables, fruits and coffee (Sakakibara, Honda, Nakagawa, Ashida, & Kanazawa, 2003), and it is estimated that daily intakes of FA may reach 150-250 mg/day depending on the eating habits (Zhao, & Moghadasian, 2008).

Since the bioactivities of FA were confirmed, and this phenolic acid was approved in Japan as a food additive (Itagaki et al., 2009), extensive studies on its application in foods and pharmaceuticals have begun. The number of reports on anti-inflammatory (Sakai, Ochiai, Nakajima, & Terasawa, 1997), anti-diabetic (Balasubashini, Rukkumani, & Menon, 2003), anti-carcinogenic (Chang et al., 2006) and antimicrobial (Saavedra et al., 2010) characteristics as well as hepato- (Shanmugarajan et al., 2008), cardio- (Suzuki et al., 2007) and neuroprotective properties (Jin et al., 2005) has increased significantly. However, they all demand that in order to achieve a therapeutic effect *in vivo*, the biologically active compound must reach the target sites in a sufficient concentration, which depends on bioavailability, i.e. the rate and extent of absorption from the digestive system.

Kern et al. (2003) evaluated the pharmacokinetic parameters of ferulic acid (FA) in the human body after a meal composed of cereal bran, which contained phenolic acid at 22.5 μmol/kg body weight. The maximum FA concentration (expressed as the sum of its metabolites) at 0.2 μmol/L was determined in the blood of volunteers after consumption (180 min), and bioavailability was determined to be only around 3%. The proposed reason for such low absorption was the chemical form in which FA occurs in natural sources, i.e. in combinations with mono-, di- and polysaccharides, sterols, polyamines, glycoproteins and lignins (Herrmann, & Nagel, 1989). Although dependent upon the source, individual FA fractions vary in foods, with the majority associated with dietary fiber (Adom & Liu, 2002). An additional factor limiting the promotion of health benefits associated with FA is its ready transformation into secondary metabolites.

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"In the text refer to the author's name (without initials) and year of publication (e.g. "Steventon, Donald and Gladden (1994) studied the effects..." or "...similar to values reported by others (Anderson, Douglas, Morrison & Weiping, 1990)..."). For 2-6 authors all authors are to be listed at first citation. At subsequent citations use first author et al."

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In studies on the rat model, it has been shown that orally administered free FA is absorbed from the stomach (74%), from where it is transferred to the liver via the portal vein, and there converted into sulfonic and glucuronide derivatives, which are subsequently excreted mainly via urine (Zhao, Egashira, & Sanada, 2004). The result of such rapid transformations is low concentrations of free, unmetabolized FA in the general circulation. These data were also confirmed in a study carried out on people. Yang, Tian, Zhang, Xu, & Chen (2007) analyzed FA pharmacokinetic parameters following administration of oral FA (50 mg), as a sodium salt (FA-Na). The maximum concentration of free FA in the blood was only 2.5 μmol/L after 24 minutes and its half-life was only 42 minutes.

Due to its low bioavailability, rapid metabolism and poor solubility in both hydrophilic and hydrophobic media, many attempts have been made to modify the structure of FA and to improve its uptake models *in vivo*. Among the many derivatives of phenolic acids obtained so far are conjugates with carbohydrates, amides, ethers, fatty alcohols and acylglycerols (Pei, Ou, Huang, 2015). The most attention was devoted to the last group of derivatives. Due to the antioxidant capacity and biological properties of FA its enzymatic incorporation into triacylglycerols (TAGs) was extensively studied as a method for production of novel structured phenolic lipids having the benefits of both functional and antioxidative properties. Laszlo and Compton focused on using lipase-catalyzed reactions of vegetable oils such as soybean oil to synthesize structured lipids with properties suitable for use as possible sunscreen reagents (Laszlo, Compton, Eller, Taylor & Isbell, 2003; Compton, Kenar, Laszlo & Felker, 2007). Many research groups have also investigated lipase-catalyzed reactions using fish oil or flaxseed oil with FA and other phenolic acids to synthesize nutraceutical phenolic lipids and food additives (Sabally, Karboune, St-Luiz & Kermasha, 2006; Sabally, Karboune, St-Luiz & Kermasha, 2007).

Currently, phospholipids (PLs) are of particular interest, as carriers of biologically active molecules (Gliszczyńska, Niezgoda, Gładkowski, Świtalska, & Wietrzyk, 2017). PLs, as hydrophobic compounds, pass through human intestine walls, penetrate the lymphatic system and from there enter the circulatory system via the thoracic vein. Giving a more lipophilic character to

phenolic acids, by linking them with PLs, means it is possible to increase their concentrations by avoiding "first-pass metabolism" (Zhao & Moghadasian, 2008). Therefore, the latest strategy for the modification of phenolic acids, for use by the food industry, is production of their conjugates with phospholipids, such as phosphatidylcholine (PC) (Yang, Mu, Chen, Xiu, & Chen, 2013; Balakrishna et al., 2017; Czarnecka, Świtalska, Wietrzyk, Maciejewska, & Gliszczyńska, 2018a, 2018b). The distinguishing feature of PC is its amphiphilic character, which is responsible for its unique physicochemical properties, specifically compatibility with biological membranes. In addition, in research on humans it has been shown that over 90% of PC is absorbed from the small intestine, and its peak concentration in the blood (at 20-30% of the given dose) can be observed approximately 6 hours after ingestion (Zierenberg, & Grundy, 1982). Based on these data, it can be concluded that PLs are characterized by high absorption from the digestive system and intense circulation in the bloodstream.

To our best knowledge there is only one report on the enzymatic synthesis of feruloylated lysophospholipids (FLPs) in a two-step lipase-catalyzed reaction. Yang et al. (2013) hydrolyzed soybean PC enzymatically and then carried out interesterification of obtained intermediate products (LPC and GPC) with ethyl ferulate (EF) using lipases. The aim of our study was to develop a biotechnological method for the preparation of feruloylated phospholipids via a one-step enzymatic reaction. Enzyme screening, organic solvent and effects of feruloyl donors (FA and EF) were first evaluated. Then the response surface methodology with 3 factors at 3 levels – enzyme loading, reaction time and substrate molar ratio – was used for optimization of reaction of interesterification of PC with EF and investigation of the affinities between the reaction variables and response (incorporation mol%).

2. Materials and methods

2.1. Materials and chemicals

The phosphatidylcholine (PC) was obtained from egg yolk of Lohman Brown hens according to a procedure described previously (Rychlicka, Niezgoda, & Gliszczyńska, 2018) and its purity was analyzed by TLC on silica gel-coated plates (65:25:4, v/v/v) and confirmed by HPLC (Gliszczyńska, Niezgoda, Gładkowski, Czarnecka, Świtalska, & Wietrzyk, 2016). Ethyl ferulate (EF) was synthesized according to the method described earlier (Li, Shi, Tang, Li, & Duan, 2009). Ferulic acid (FA, *trans*-4-hydroxy-3-methoxycinnamic acid, purity: 99%), Novozym 435 (lipase B from *Candida antarctica*, immobilized in a macroporous acrylic resin, >5000 U/g) and CALB (lipase B from *Candida antarctica*, immobilized on resin Immobead 150 >1800 U/g) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lipozyme RM IM (immobilized lipase from *Rhizomucor miehei*, >30 U/g) was provided by Fluka (Buchs, Switzerland) and Lipozyme TL IM (immobilized lipase from *Thermomyces lanuginosus*, 250 U/g) was supplied by Novozymes A/S (Bagsvaerd, Denmark). A boron trifluoride methanol complex solution (13-15% BF_3 × MeOH) and sodium methylate were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents used in chromatography and HPLC grade solvents (Merck LiChrosolv Reag.) were purchased from Merck (Darmstadt, Germany). Silica gel-coated aluminium plates (Kieselgel 60 F254, 0.2 mm) used in thin layer chromatography (TLC) and the silica gel (Kieselgel 60, 230-400 mesh) used in the column chromatography were also purchased from Merck.

2.2. Lipase-catalyzed acidolysis/interesterification of PC

The egg-yolk phosphatidylcholine (PC) (20 mg, 0.026 mmol) and ferulic acid (at molar ratio of substrates 1/5, PC/FA) in 2 mL of organic solvent were mixed at 55 °C and then 30% lipase (by weight of substrates) was added. The reactions were carried out using four different lipases. The different solvents, effect of molar ratio of substrates and time of the reaction were tested in another set of experiments for high-activity enzyme Novozym 435. Each experiment was carried out in triplicate. The reaction mixtures were agitated on a heating plate with a magnetic stirrer in 5 mL

screw-capped vials at 300 rpm in N_2 atmosphere and stopped at the selected time intervals by enzyme filtration (G4 Shott funnel with Celite layer). The same procedure was used for lipase-catalyzed interesterification reaction of egg-yolk phosphatidylcholine with ethyl ferulate (EF).

After evaporation of solvent *in vacuo*, the modified phospholipid fraction was separated from the free fatty acids and unreacted ferulic acid using solid-phase extraction (SPE). A silica gel column (Discovery DSC-Si SPE, 52654 – U 500 mg) was conditioned by successive washing with 10 mL of methanol, 10 mL of chloroform and 6 mL of chloroform/acetic acid (95:5, v/v). Evaporated reaction mixture (150 µL) was applied to an SPE cartridge. The FA and fatty acids were eluted with 15 mL of chloroform/acetic acid (95:5, v/v). Then the phospholipids were eluted with solvent mixtures of increasing polarity (10 mL of chloroform/propan-2-ol (1:1, v/v) and then 35 mL of methanol/water (25:4, v/v)). The phospholipid fraction was evaporated using a rotatory vacuum evaporator at 45 °C. The phospholipid fraction was analyzed by thin-layer chromatography (TLC) and the profile of acids in the modified phospholipid fraction was analyzed by gas chromatography (GC).

2.3. Preparative scale of lipase-catalyzed interesterification of PC with ethyl ferulate (EF)

The egg-yolk phosphatidylcholine (PC) (200 mg, 0.26 mmol) was mixed with ethyl ferulate (at a molar ratio of substrates 1/15, PC/FA) in 20 mL of chloroform/toluene mixture (9:1, v/v) and after dissolving substrates, Novozym 435 (30% by weight of substrates) was added. The reaction mixtures were agitated on a heating plate with a magnetic stirrer at 300 rpm in N_2 atmosphere at 55 °C and stopped after 4 days by enzyme filtration (G4 Shott funnel with Celite layer). After evaporation of solvent *in vacuo* reaction products were separated by column chromatography using silica gel as a stationary phase. The phospholipids were eluted with solvent mixtures of chloroform/methanol/water of increasing polarity (first 65:25:2, v/v/v, then 65:25:4). The procedure was as follows: silica gel was well solvated with chloroform and then the prepared slurry was gently poured into the glass column. The samples were dissolved in a minimal (3 mL) amount of chloroform and applied to the top of the column and then eluted with the above solvents. The eluted fractions were collected in test

Commented [U2]: EF?

tubes and identified by TLC on silica gel-coated aluminum plates according to the procedure described in Section 2.5.1. Separate and identified fractions of egg PC and modified LPC (FLPC) were combined and dried with magnesium sulfate ($MgSO_4$). After filtration, the solvent was evaporated *in vacuo*. The structure of FLPC was confirmed by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy, HPLC and high-resolution mass spectra (HRMS).

2.4. Experimental factorial design

A 3-level,-3-factor Box-Behnken design with three replicates at the center was employed in this study, requiring 15 experiments (Box & Behnken, 1960). The crucial factors involved in the study are substrate molar ratio PC/EF (1:5–1:25), enzyme loading (20–40% w/w) and time of the reaction 4–8 days). The design of experiments employed is presented in Table 1.

A factorial design approach was applied to maximize the experimental efficiency requiring a minimum of experiments to optimize the synthesis of feruloylated lysophospholipid. The data were analyzed using the STATISTICA 13.3 (StatSoft, Inc.) software.

2.5. Analysis of substrates and products

2.5.1. Thin-layer chromatography (TLC)

Progress of the reaction of enzymatic acidolysis/transesterification and qualitative analysis of reaction mixtures were controlled using the mixture of chloroform/methanol/water (65:25:4, v/v/v) as a developing system. The products were identified by spraying the TLC plates with 0.05% primuline solution (acetone:water, 8:2, v/v) and then exposing the plates to UV light (λ=365 nm).

2.5.2. Gas chromatography (GC)

Analysis of fatty acid profile of native PC and the modified phospholipid (PC/LPC) fraction was performed in two replications. The samples of native PC and phospholipid fraction obtained in enzymatic modification after their purification by SPE were transesterified to the fatty acid methyl

esters (FAME). Transesterification was conducted on the magnetic stirrer (300 rpm) by mixing 10 mg of native PC or modified PL fraction with 2 mL of 0.75 M sodium methoxide methanol solution for 20 minutes at 40 °C. Next 200 μL of acetic acid glacial was added and products were extracted with 2 mL of hexane and the organic layer was washed with a saturated NaCl solution and dried over anhydrous magnesium sulfate ($MgSO_4$). Samples were analyzed by GC.

The methyl ester of ferulic acid was obtained according to the following procedure: FA (10 mg) was heated under reflux (3 min) with 3 mL of BF_3 × MeOH complex solution. After cooling, the mixtures were extracted with 2 mL of hexane and the organic layer was washed with a saturated NaCl solution. Hexane extract was dried over anhydrous $MgSO_4$. Samples performed in two replications were next analyzed by GC.

The FAME were identified by comparing their retention times with those of a standard FAME mixture (Supelco 37 FAME Mix) purchased from Sigma Aldrich and a prepared methyl ester of FA. The quantitative analysis of incorporation of FA into the PL fraction (PC/LPC) expressed as FA mol% was carried out based on their peak areas and were calculated using GC ChemStation Version A.10.02.

FAME were analyzed via gas chromatography (GC) on an Agilent 6890N apparatus with a flame ionization detector (FID) (Agilent, Santa Clara, CA) fitted with a DB-WAX capillary column (30 m × 0.32 mm × 0.25 μm film thickness) manufactured by Agilent Technologies (Santa Clara, CA, USA). The oven temperature was first set at 90 °C and then raised to 200°C (rate of 5 °C/min) and next to 250 °C at 2 °C /min and held there for 5 min. The total analysis time was 49 min. The injector temperature and the flame ionization detector temperatures were 250 °C. Hydrogen was used as the carrier gas with a constant flow of 1.5 mL/min.

2.5.3. Spectroscopic spectra (NMR)

The NMR experiments were conducted on a Bruker Advance II 600 MHz spectrometer (Bruker, Billerica, MA, USA) operating at 600 MHz for ^{1}H, 150 MHz for ^{13}C and 243 MHz for ^{31}P, at 25 °C

using temperature stabilization. The sample of the product FLPC obtained from column chromatography was dissolved in 0.6 mL of $CDCl_3$/MeOH (2:1, v/v). The product was added directly into the NMR tube and used to obtain the NMR spectra. The chemical shifts are given in ppm downfield from tetramethylsilane (TMS) as the internal standard. In ^{31}P NMR spectra, chemical shifts were referenced to 85% H_3PO_4 as a standard.

1-(4-hydroxy-3-methoxy)cinnamoyl-2-hydroxy-sn-glycero-3-phosphocholine (FLPC)

Colorless greasy solid (62% yield, R_f 0.06); ^1H NMR (600 MHz, $CDCl_3/CD_3OD$ 2:1 (v/v)), δ: 2.80 (s, 9H, $-N(CH_3)_3$), 3.20 (m, 2H, CH_2-β), 3.49 (s, 4H, $-OCH_3$), 3.52-3.60 (m, 2H, CH_2-3′), 3.65 (m, 1H, H-2′), 3.80-3.89 (three m, 5H, CH_2-1′, CH_2-α, -OH), 5.93 (d, 1H, J = 15.9 Hz, H-2), 6.42 (m, 1H, H-5″), 6.65, 6.71 (two m, 2H, H-2″, H-6″), 7.23 (d, 1H, J = 15.9 Hz, H-3); ^{13}C NMR (150 MHz, $CDCl_3/CD_3OD$ 2:1 (v/v)) δ: 53.61 ($(-N(\underline{C}H_3)_3$), 55.38 ($-OCH_3$), 58.99 (C-α), 64.90 (C-1′), 66.23 (C-β), 66.61 (C-3′), 68.63 (C-2′), 110.33 (Ar), 113.92 (Ar), 115.26 (C-2), 122.90 (Ar), 126.25 (Ar), 145.90 (Ar), 147.96 (C-3), 149.24 (Ar), 167.77 (C-1); ^{31}P NMR (243 MHz, $CDCl_3/CD_3OD$ 2:1 (v/v)) δ: -0.42; HRMS (ESI): *m/z* calculated for $C_{16}H_{26}NO_8P$ $[M + H]^+$ 434.1580; found 434.1579.

2.5.4. High pressure chromatography (HPLC)

The purity of the native phosphatidylcholine was determined by HPLC on a DIONEX UltiMate 3000 chromatograph from Thermo Fisher Scientific (Olten, Switzerland) equipped with a Corona charged aerosol detector (CAD) from ESA Biosciences (Chelmsford, MA). A BetaSil DIOL column (Thermo Scientific, 150 × 4.6 mm, 5 μm) was used for analysis. The injection volume was 15 μL for all analyzed samples. The temperature for the autosampler compartment was 20 °C and the column temperature was maintained at 30 °C. The analysis was performed in a gradient mode with a constant flow of 1.5 mL/min. Solvents used were as follows: solvent A (1% HCOOH, 0.1% TEA in water), solvent B (hexane) and solvent C (2-propanol). The elution program started with 3/40/57

(%A/%B/%C (v/v/v)), at 5 min = 10/40/50, at 9 min = 10/40/50, at 9.1 min = 3/40/57 and at 19 min = 43/40/57. Total time of analysis was 19 min.

The FA, EF, and FLPs were analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) at 30 °C. The chromatographic separation was carried out with a Zorbax XDB-C18 column (250 × 4.6 mm, 5 μm) with a UV/DAD detector (at 320 nm) according to the procedure described by Yang, Mu, Chen Xiu & Yang (2013). The mobile phase was a binary solvent of A (water containing 3% acetic acid) and B (acetonitrile) at a flow rate of 1 mL/min. The gradient was operated as follows: 0 min: 90% A and 10% B; 0-10 min: changed to 30% A and 70% B; 10-20 min: maintained 30% A and 70% B; 20-20.5 min: changed to 90% A and 10% B. Samples (1 mg) were blown by nitrogen and diluted in a mixture of 0.7 mL of acetonitrile and 0.3 mL of water. The injection volume was 10 μL.

2.5.5. High-resolution mass spectra (HRMS)

High-resolution mass spectra (HRMS) were obtained using an electron spray ionization (ESI) technique on a Waters ESI-Q-TOF Premier XE spectrometer.

3. Results and discussion

3.1. Screening of lipases in the acidolysis reaction

For the enzymatic synthesis of feruloylated phospholipids (FPLs) we started with the reaction of acidolysis of PC isolated from egg yolk (purity >99% according to HPLC) with ferulic acid as an acyl donor. For the experiments we involved four commercially available lipases in immobilized form, which make it possible to carry out the reactions at higher temperatures in the presence of organic solvent. The selection of the type of biocatalysts was carried out mainly in terms of their selectivity. Two of the selected biocatalysts, Lipozyme and Lipozyme TL IM, are classified as 1,3-regioselective lipases, whereas the next two, CALB and Novozym 435, are considered as non-specific

enzymes, but in most of the modifications reported in the literature they showed high selectivity towards the *sn*-1 position of TAGs and PC.

The initial reaction parameters used for acidolysis were selected on the basis of literature examples of TAG modification with phenolic acids (Sun et al., 2009). At the screening stage, reactions were conducted using a molar ratio of substrates PC:FA 1:5, with toluene as the reaction medium and 30% (w/w) enzyme dosage. In most studies on the modification of TAG with FA via lipase-catalyzed esterification the optimal temperature was 50 °C, which we also applied in our study. Although the used biocatalysts exhibited different activities (according to suppliers) we decided to apply them at the same weight ratios, because it is important to reduce the total costs of the process when choosing an enzyme for industrial applications. The progress of the acidolysis reaction was monitored by collecting samples of the product mixtures after 1, 2, 3 and 4 days. Then we separated the phospholipid fraction PC/LPC from the acids by solid phase extraction (SPE) and analyzed products after their derivatization by gas chromatography (GC). We were focused on obtaining a phospholipid fraction containing the highest degree of incorporation of ferulic acid, so in this preliminary research we did not fractionate PLs into individual fractions of phosphatidylcholine (PC) and lysophosphatidylcholine (LPC).

The degree of incorporation of FA into phosphatidylcholine increased in the following order: *TL IM* < *RM IM* < *CALB* < *Novozym 435*. The time course of incorporation of FA into the phospholipid fraction (PC/LPC) by the studied lipases is shown in Fig.1 (A).

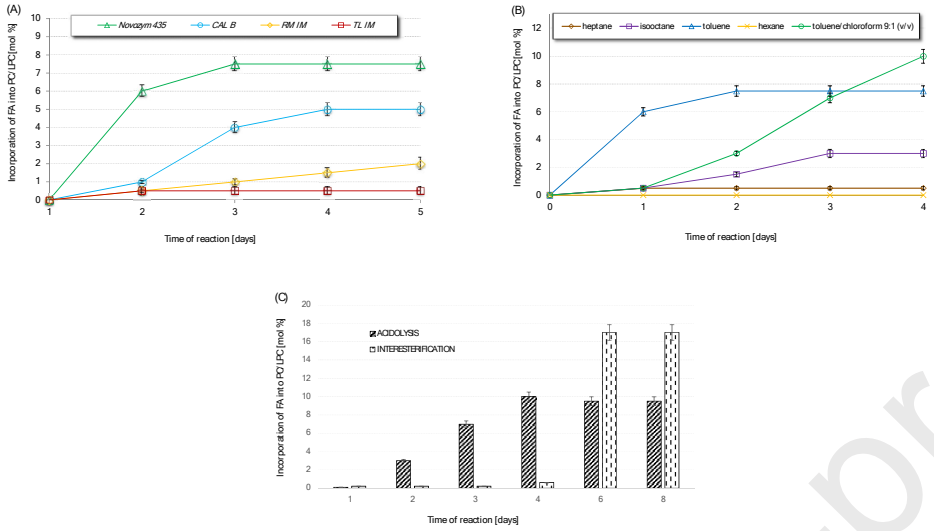

Fig. 1. (A) Time course of different lipase-catalyzed acidolysis between egg-yolk phosphatidylcholine (PC) and ferulic acid (FA) (reaction conditions: 1:5 PC/FA molar ratio, enzyme load 30% (w/w), toluene 2 mL, temperature 50 °C), **(B)** Effect of different organic solvents on the incorporation of FA into egg-yolk PC in acidolysis reaction (reaction conditions: 1:5 PC/FA molar ratio, enzyme load 30% (w/w) Novozym 435, solvent: heptane/ isooctane/toluene/hexane/toluene:chloroform 9:1 (v/v) 2 mL, temperature 50 °C), **(C)** Effect of feruloyl donors on the incorporation of FA into egg-yolk PC in acidolysis/transesterification reactions (reaction conditions: 1:5 PC:FA/EF molar ratio, toluene/chloroform 9:1 (v/v) 2mL, temperature 50 °C, enzyme load 30% (w/w) Novozym 435).

Only two biocatalysts exhibited significant activities. In the reaction catalyzed by Novozym 435 the incorporation degree reached a maximum 7.5 mol% after 3 days, whereas CALB-catalyzed acidolysis afforded a lower incorporation degree, 5 mol% after 4 days. The other two enzymes, RM IM and TL IM, were not able to catalyze this type of reaction (incorporation of FA was around or lower than 2 mol%); therefore Novozym 435 was selected as the most effective biocatalyst for the

synthesis of FPLs for the following experiments. The obtained results are in accordance with previously reported studies in which Novozym 435 was found to be a good biocatalyst for esterification of phenolic acids with medium or high chain lengths of fatty alcohols and TAGs (Stamatis, Sereti, & Kolisis, 1999; Karboune, St-Louis, & Kermashasha, 2008; Sun et al., 2009). During the enzymatic synthesis of ferulate esters Compton et al. found that among tested lipases only Novozym 435 was the only enzyme able to catalyze the esterification of FA and transesterification of EF (Compton, Laszlo & Berhow, 2000).

3.2. Effect of solvent on Novozym 435-catalyzed reaction of acidolysis

An appropriately selected medium of enzymatic reactions should be non-toxic in relation to the enzyme and not cause its premature inactivation. Generally, the enzymes have better performance in lipophilic environments (Buisman et al., 1998). Unfortunately, phenolic acids have low solubility in lipophilic solvents. It is a major hindrance of interaction between them and biocatalysts. For this type of substrate, the literature specifies that the proper solvents for lipase-catalyzed reactions are those characterized by the logarithm of the partition coefficient (log P) in the range of 2-4 (Villeneuve, 2007). For this reason, *n*-hexane (log P=3.5), *n*-heptane (log P=4), isooctane (log P=2.9) and toluene (log P=2.5) were selected as the reaction medium in the present experiments. To overcome the limited solubility of phenolic acid in the organic solvents we also tested a binary organic solvent system which Sabally et al. developed for the first time during the esterification of phenolic acids with linolenyl alcohol (Sabally, Karboune, Yeboah, & Kermasha, 2005). The mixtures of different organic solvents in two-step lipase-catalyzed interesterification of soybean PC with ethyl ferulate (EF) were next studied by Yang, Mu, Chen, Xiu, & Yang (2013). Their results showed that a binary solvent system of toluene/chloroform (log P=2.5/2.01) mixed in the volume ratio of 9:1 significantly increased the conversion of substrate. Therefore, we also decided to apply it in our study in order to improve the solubility of FA and its conversion, and to maintain the activity of Novozym 435.

Analyzing the results presented in Fig. 1 (B), it can be concluded that highly hydrophobic solvents such as *n*-heptane, *n*-hexane and isooctane significantly limit the process of acidolysis. Using them we determined the incorporation of FA into PC only at the level below 4 mol%. In the variant with toluene as a solvent we observed that the incorporation of FA into the PL fraction increased to 7.5 mol%. The highest incorporation (10 mol%) was achieved after 4 days for acidolysis carried out in a binary solvent system in the mixture of toluene/chloroform (9:1, v/v). It was the result of better solubility of PLs in the chloroform than in other solvents as well as better solubility of ferulic acid. This mixture of solvents was chosen for further research.

3.3. Effect of feruloyl donors

As observed in the above results, the highest incorporation of FA (10 mol%) into the PC structure was achieved when the medium of the reaction was the mixture of organic solvents toluene/chloroform (9:1, v/v). However, the obtained level of incorporation of ferulic acid into PC was still low; therefore in the next step of the study we used the ethyl ester of ferulic acid (EF) as an acyl donor. EF was synthesized according to a procedure described previously (Li et al., 2009). The results, presented in Fig. 1 (C), showed that by using EF as the feruloyl donor it is possible to obtain much higher incorporation of FA (17 mol% at 6 day) into the phosphatidylcholine. These results can be explained by the lower melting point (64 °C) of EF than that (174 °C) of FA and good solubility of EF with phosphatidylcholine. Thus, EF is a better feruloyl donor than FA and is preferred for the transesterification.

3.4. Statistical analysis of enzymatic interesterification of PC with EF

Response surface methodology (RSM) is an empirical statistical technique used to obtain statistically acceptable results from a lower number of experimental runs. It is a good technique for the optimization of the process, appraising the effect of multiple parameters around reaction variables (Melo, Pastore & Macedo, 2005).

To analyze the dependent variables for the development of optimal parameters of the interesterification reaction of PC with EF, a factorial design was developed to provide information about the effects of the selected variables on the synthesis of feruloylated phospholipids (FPLs). The choice of substrate molar ratio PC/EF, enzyme loading and time of reaction were examined in a Box-Behnken design (Table 1). The optimal conditions of synthesis can be ascertained from the experimental data.

Table 1 Response values (incorporation of FA into PC/LPC fraction) of the three factors for the experiment with 15 interesterifications.

Run	Substrate molar ratio PC:FE	Enzyme load [%]	Reaction time [days]	Incorporation of FA into PC/LPC [%] (Experimental)	Incorporation of FA into PC/LPC [%] (Predicted)
1	5	20	6	3 ± 0.3	4.6
2	5	40	6	4 ± 0.6	4.8
3	25	20	6	14 ± 1.1	13.1
4	25	40	6	14 ± 0.8	12.6
5	15	20	4	13 ± 0.7	12
6	15	40	4	12 ± 0.6	11.6
7	15	20	8	12 ± 0.9	12.4
8	15	40	8	12 ± 0.6	12.8
9	5	30	4	4 ± 0.1	3.5
10	25	30	4	13 ± 0.1	14.8
11	5	30	8	9 ± 0.4	7.2
12	25	30	8	12 ± 0.3	12.5
13	15	30	6	18 ± 0.5	18
14	15	30	6	18 ± 0.7	18
15	15	30	6	18 ± 0.2	18

[a] Data are presented as mean ± SD of two independent analyses.

For each of the 3 variables, analysis of variance (ANOVA) was performed. From Table 2 and Fig. 2A the factors that have a significant effect (p-value < 0.05) on incorporation are the substrate molar ratio (L and Q), enzyme load (Q) and time of the reaction (Q). Other evaluated factors, i.e. enzyme load (L) and time of the reaction (L), were not statistically significant (p-value > 0.05); nor were the interactions between them (1 by 2, 1 by 3 and 2 by 3).

Table 2 Analysis of variance (ANOVA) for interesterification variables pertaining to the response of percent incorporation of FA into PC/LPC.

Evaluated factors	Sum of squares	Degrees of freedom	Medium square	F-value	p-value
(1) Substrate molar ratio (L)	136.1250	1	136.1250	49.50000	0.000896
substrate molar ratio (Q)	132.9231	1	132.9231	48.33566	0.000946
(2) Enzyme load (L)	0.0000	1	0.0000	0.000000	1.000000
Enzyme load (Q)	39.0000	1	39.0000	14.18182	0.013078
(3) Time of reaction (L)	1.1250	1	1.1250	0.40909	0.550582
Time of reaction (Q)	23.0769	1	23.0769	8.39161	0.033916
1 by 2	0.2500	1	0.2500	0.09091	0.775162
1 by 3	9.0000	1	9.0000	3.27273	0.130229
2 by 3	0.2500	1	0.2500	0.09091	0.775162
Error	13.7500	5	2.7500		
Total error	334.9333	14			

The response surface plots provide the variation of incorporation of FA into PC with independent variables and are presented in Fig. 2. Each 3D surface signifies a limitless combination of two variables by keeping one of the variables at a constant level. Figure 2B shows the 3D surface for incorporation of FA into phospholipids versus molar ratio PC:EF and enzyme loading when the time of reaction was kept at 6 days. The incorporation was increased up to 18 mol% with an increase in molar ratio from 1:4 to 1:20 and 18% to 30% (w/w) respectively. These results are in accordance with the literature data indicating that a high substrate molar ratio shifts the reaction equilibrium to the product side and improves the incorporation (Haresh & Pandit, 2010).

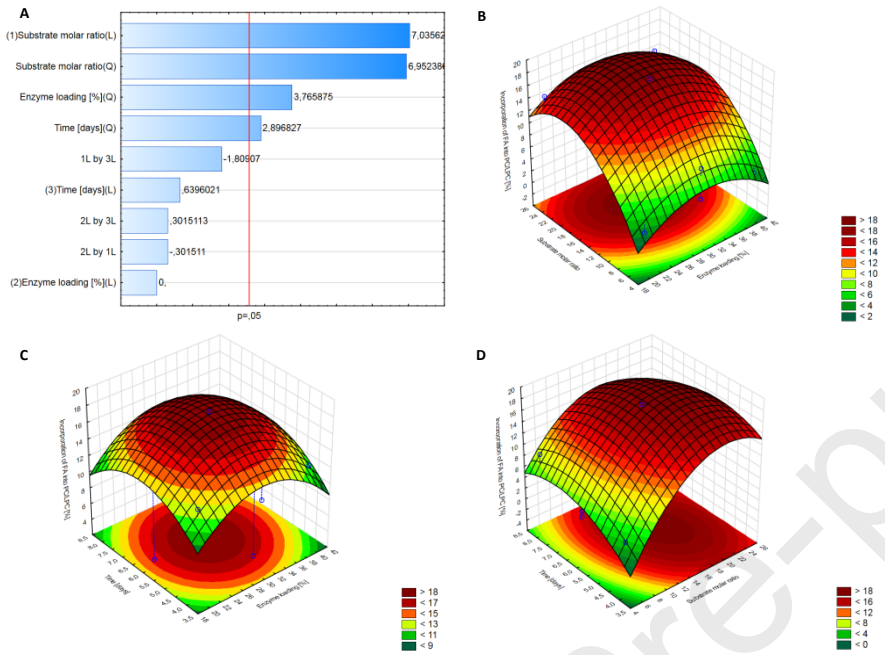

Fig. 2. A Pareto chart of the analyzed effect for incorporation of FA into PC, **B** Surface response chart of the effect of substrate molar ratio and enzyme loading on the incorporation of FA into PC/LPC, **C** Surface response chart of the effect of reaction time and enzyme loading on the incorporation of FA into PC/LPC, **D** Surface response chart of the effect of reaction time and substrate molar ratio on the incorporation of FA into PC/LPC.

Figure 2C represents the response surface plot for incorporation as a function of the reaction time and the enzyme loading at a fixed substrate PC:EF molar ratio of 1:15. It is observed that an increase in time of the reaction from 4 days to 6 days and enzyme loading from 18% to 30% may enhance the incorporation up to 18 mol%. It can be observed that there is a decrease in the incorporation by changing the enzyme loading from 30% to 40%. These results are in agreement with previous papers reporting that productivity of oleyl ferulate or feruloylated lysophospholipids did not increase when the enzyme concentration attained a certain value (Chen et al., 2011; Yang et al., 2013).

Figure 2D shows the interaction between time of the reaction and substrate molar ratio when the enzyme loading was held at 30%. The minimum time of the reaction of 4 days and substrate molar ratio of 1:6 give a minimum incorporation of around 4 mol%, while the increase in time to 6 days with a ratio of 1:15 produces maximum incorporation of 18 mol%. Thus, from the obtained results optimal parameters were found and applied for the synthesis of FLPs on a large scale.

3.5. Identification of reaction products

Under the optimized parameters – reaction medium (toluene/chloroform 9:1(v/v), lipase (Novozym 43, enzyme load 30%), substrate molar ratio (PC:EF, 1:15), temperature (50 °C) and reaction time (6 days) – we performed one-step interesterification of PC with EF on the larger scale. After 6 days the extracted mixture of products was analyzed by TLC. Based on the standards PC-egg yolk, LPC-egg yolk, FA, EF, as references, in the mixture of products spotted on the TLC we observed a new spot with the R_f value 0.06. We found that the new spot migrated more slowly than PC-egg yolk (R_f=0.45) and LPC-egg yolk (R_f=0.1) and possesses higher polarity. The obtained phospholipid fraction contained modified LPC (FLPC) and PC-egg yolk. The possible changes occurring during the enzymatic interesterification of egg-yolk phosphatidylcholine with ethyl ferulate are presented in Figure 3. In the next step the product FLPC was separated from the unreacted EF, fatty acids and PC-egg yolk by the chromatography column and subsequently analyzed to confirm its structure.

FLPC was analyzed by RP-HPLC with a UV/DAD detector using the program previously reported for this compound by Yang et al. (2013), where presence of a phenolic ring with an alkoxy group in ferulic acid produced fluorescent absorption under UV light (320 nm). The RP-HPLC elution profile at 320 nm showed that according to the results obtained by Yang et al. there was a peak with retention time of 5.253 min (Supplementary Materials).

To determine the structure of FLPC, NMR experiments were next performed. In the NMR spectra all characteristic signals from the glycerol, choline and ferulic acid were identified (Supplementary

Materials). In the ^{1}H NMR spectrum signals from the protons of the benzene ring (6.42–6.71 ppm) and olefinic protons (δ = 5.93 and 7.23 ppm) were visible. The multiplet of proton H-2′ in the range 3.80–3.89 proved that the *sn*-2 position was non-esterified. The structure of FLPC was fully confirmed by the ^{13}C, ^{31}P and ESI-MS spectra as well. On the spectrum from electrospray ionization-mass spectroscopy (ESI-MS) in the positive ion mode by low energy bombardment we observed for FLPC a molecular ion $[M]^{+}$ at m/z 434.15, which is characteristic for this compound.

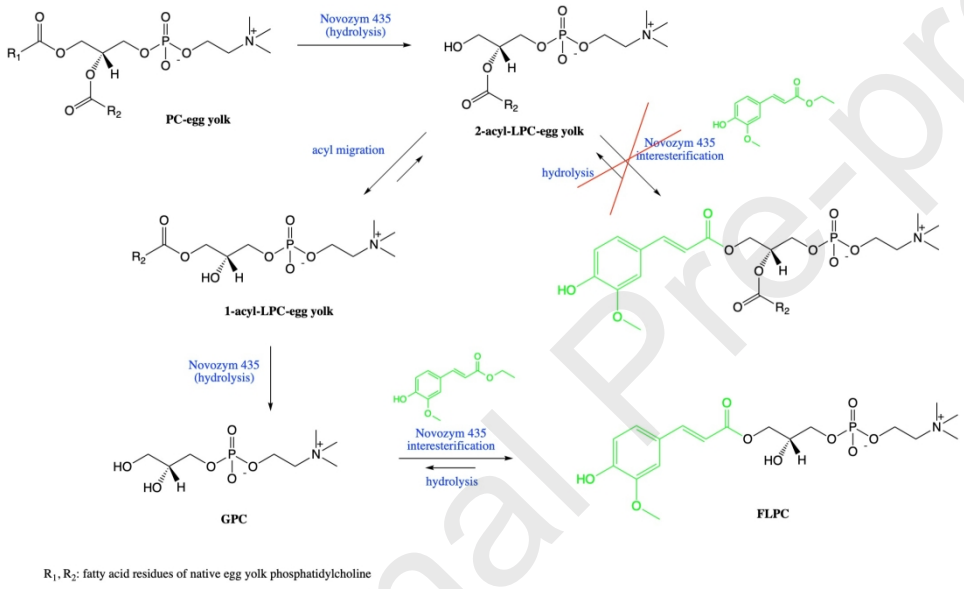

Fig. 3. Possible changes occurring during the enzymatic interesterification of egg-yolk phosphatidylcholine with ethyl ferulate.

FLPC was obtained in a high 62% (w/w) isolated yield and the regiospecificity of Novozym 435 in the performed reaction of interesterification was proved by the analysis of total fatty acid composition of the native PC and modified PL fraction, where we could observe that the decrease of palmitic acid (16:0) and stearic acid (18:0) accompanied the increased level of FA (Table 3).

Table 3 Fatty acid composition (% according to GC) of native egg-yolk PC and FA-enriched PL fraction obtained after Novozym 435-catalyzed interesterification of egg-yolk PC with EF.

Fatty and FA acids	Native PC	Modified PL fraction
C16:0 (PA)	32 ± 1.2	9 ± 0.9
C16:1 (OPA)	1 ± 0.3	0 ± 0
C18:0 (SA)	14 ± 1	3 ± 1.2
C18:1 (OA)	27 ± 1.1	27 ± 0.8
C18:2 (LA)	21 ± 0.6	30 ± 0.5
C20:4 (AA)	5 ± 0.4	13 ± 0.2
FA	-	18 ± 0.4

[a] Data are presented as mean ± SD of two independent analyses.

4. Conclusions

A novel biotechnological route for the production of feruloylated lysophosphatidylcholine was successfully developed. First, we examined four commercially available regioselective lipases, type of acyl donor and organic solvent. Optimization of the process for interesterification of egg-yolk phosphatidylcholine with EF was performed by RSM using Novozym 435 as a biocatalyst in a binary solvent system, toluene:chloroform 9:1 (v/v). Three variables – substrate molar ratio, enzyme loading, and reaction time – were assessed. Feruloylated lysophosphatidylcholine (FLPC) was obtained in a high isolated yield of 62% (w/w). The presented method is thus promising in the area of enzymatic production of phospholipids containing biologically active phenolic acids with potential application in the food industry as food additives, natural emulsifiers or nutraceuticals with pro-health activity.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could potentially have influenced the work reported in this paper.

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Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- Production of feruloylated lysophosphatidylcholine (FLPC) via a one-step interesterification was successfully developed.
- Synthesis of FLPC was carried out using Novozym 435 as biocatalyst.
- Feruloylated lysophosphatidylcholine was obtained in high 62% (w/w) isolated yield.