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Enzymatic synthesis of structured phenolic lipids by acidolysis of flaxseed oil with selected phenolic acids

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Abstract

Structured phenolic lipids were obtained by lipase-catalyzed acidolysis of flaxseed oil with selected phenolic acids, including hydroxylated and/or methoxylated derivatives of cinnamic, phenyl acetic and benzoic acids. Increasing the molar ratio of flaxseed oil to the selected phenolic acids from 4:1 to 8:1 resulted in an increase in the maximum bioconversion yield from the range of 5–60% to that of 7–74%, respectively. In addition, the bioconversion yield of phenolic lipids seemed to be dependent on the structure characteristics of phenolic acids and their electronic distribution. The highest bioconversion yield of 74% and 68% was obtained for the acidolysis of flaxseed oil with cinnamic and 3,4-dihydroxyphenyl acetic acids, respectively. Using *p*-coumaric acid as substrate, the bioconversion yield (45%) was higher compared to those obtained with ferulic and sinapic acids (19–29%). APCI-MS analyses confirmed the formation of seven cinnamoylated lipids, eight *p*-coumaroylated lipids and six 3,4-dihydroxyphenyl acetoylated lipids. The results also show that the acidolysis reaction resulted in an increase of $C_{18:3}$ *n*-3 proportion from 53% in the original flaxseed oil to 60–72% in the phenolic lipids. Although the radical scavenging activity of 3,4-dihydroxyphenyl acetoylated lipids was lower than that of their phenolic acid component, it was close to that of α-tocopherol. However, *p*-coumaroylated lipids showed radical scavenging activity similar to that of coumaric acid.

Keywords: Phenolic acid; Flaxseed oil; Lipase; Acidolysis; Organic solvent media

1. Introduction

The health and nutritional benefits of ω-3 polyunsaturated fatty acids (PUFAs) for the infant growth and the prevention of various human diseases are now well established [1–3]. Flaxseed oil is considered as one of the major important source of ω-3 linolenic acid (18:3 *n*-3) and its content ranges from approximately 40% to 60% of the total fatty acids [4]. Linolenic acid was reported to be the principal precursor for PUFAs, of which eicosapentaenoic (20:5 *n*-3) and docosahexaenoic acid (22:6 *n*-3) are the most prevalent, and by itself may have beneficial effects in health and in control of chronic diseases [5,6].

Because of the high demand for nutraceutical products beneficial to health, there has been an increasing interest in the modification and structuring lipids to produce functional fats and oils [7–9]. In addition, the use of structured triacylglycerols (TAGs) was found to be the most effective way of delivering desired fatty acids and increasing their absorption rates for nutritive and therapeutic purposes [10]. Most structured TAGs were designed with various enrichment levels of selected fatty acids to provide the beneficial effects of PUFAs [9,11,12]. The incorporation of phenolic acids into TAGs could potentially result in structured phenolic lipids; these novel molecules may offer numerous combined beneficial properties of both PUFAs and phenolic compounds. In addition to their many vital properties in biological systems as antimicrobial, anticarcinogenic and antimutagenic compounds, most phenolic acids are known to be potent antioxidants [13,14]. However, the use of the phenolic acids as antioxidants in fat and oil systems is limited by their hydrophilic nature [15,16]. Improvements or changes in the solubility and miscibility characteristics of the phenolic compounds can also be achieved upon their incorporation into TAGs.

The literatures [17–21] indicated few reports on the production of structured TAGs containing a phenolic acid moiety, where lipase plays a key role as biocatalyst [17–21]. In previous work [21], carried out in our laboratory, selected phenolic lipids were obtained by the incorporation of dihydrocaffeic acid into flaxseed oil. As part of an on-going research in our labora-

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tory [18,20–22], the presented work was aimed at the enzymatic synthesis of potential phenolic lipids by acidolysis of flaxseed oil with selected phenolic acids. The specific objectives were to investigate the effects of the structure of phenolic acid as well as the reaction time on the bioconversion yield of phenolic lipids. The molecular structures of phenolic lipids were characterized and their free radical scavenging activity was also determined.

2. Materials and methods

2.1. Chemicals

Commercially lipase from *Candida antarctica* immobilized on macroporous acrylic resin (Novozyme 435, with an activity of 10,000 propyl laurate units, PLU, per gram) was obtained from Novo Nordisk A/S (Bagsværd, Denmark). All phenolic acids, including cinnamic, caffeic, *p*-coumaric, 3,4-dihydroxyphenyl acetic, ferulic, sinapic, 3,4-dihydroxybenzoic and 3,4-dimethoxycinnamic acids and ethyl ferulate, as well as 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) were purchased from Sigma Chemical Co. (St-Louis, MO). Flaxseed oil was obtained as a gift from Arista Industries, Inc. (Wilton, CT). Acetic acid glacial certified ACS, sulfuric acid and all organic solvents of high-performance liquid chromatography (HPLC) grade were obtained from Fisher Scientific (Fair Lawn, NJ). Standards for thin-layer chromatography (TLC) and gas–liquid chromatography (GLC) were purchased from Nu-check Prep (Elysian, MN).

2.2. Acidolysis reaction

Lipase-catalyzed acidolysis of flaxseed oil with the selected phenolic acids, including cinnamic, caffeic, *p*-coumaric, 3,4-dihydroxyphenyl acetic, ferulic, sinapic, 3,4-dihydroxybenzoic and 3,4-dimethoxycinnamic acids as well as ethyl ferulate, was carried out at substrate molar ratios of 4:1 (20:5 mM) and 8:1 (40:5 mM) in 50 mL screwed Erlenmeyer flasks, according to the modified method of Sabally et al. [20]. Prior to the enzymatic reaction, a stock solution of each of the selected phenolic acids (26.7 mM), was freshly prepared in 2-butanone, while that of flaxseed oil (160 mM; 139.73 g/L) was prepared in hexane. Defined amounts of substrate stock solutions were diluted with sufficient amount of hexane to obtain a final substrate molar ratio of flaxseed oil to the elected phenolic acid of 4:1 and 8:1 in 10 mL hexane/2-butanone solvent mixture (85:15, v/v). The enzymatic reaction was initiated by the addition of 30 mg solid Novozym 435 lipase and was performed under vacuum at 55 °C with continuous agitation at 150 rpm. Control trials, without enzyme, were carried out in tandem with the enzymatic reactions. At defined time intervals over a 10-day period, 0.3 mL of the reaction mixture was withdrawn and dried down under vacuum, using an Automatic Environmental Speed Vac system (Savant Instruments Inc., Holbrook, NY). Samples were then flushed with a stream of nitrogen and stored at −80 °C for further analysis. All reactions were run in triplicate.

2.3. Analysis of the reaction components

The extent of the acidolysis reaction was monitored by HPLC analysis, according to the method developed previously in our laboratory [21], using a Beckman HPLC system (Model 126, Beckman Instruments Inc., San Ramon, CA) equipped with an ultraviolet diode-array (UV-DAD) detector (Model 168). The separation was performed on a Zorbax SB-C18 reversed-phase column (5 μm, 250 mm × 4.6 mm, Agilent Technologies, Wilmington, DE), using an isocratic elution with acetonitrile/methanol mixture (7:5, v/v), followed by two linear gradients with isopropanol. The reaction components were monitored, simultaneously, at 235 and 280 nm with a continous scanning in the region of 190–500 nm at 1-s intervals. The bioconversion yield, at a defined reaction time, was calculated as the total peak area of phenolic lipid products, monitored at 280 nm, divided by that of phenolic acid in the blank, multiplied by 100.

The molecular structures of phenolic lipids were characterized, using HPLC interfaced to atmospheric pressure chemical ionization-mass spectrometry (APCI-MS). The APCI-MS system (ThermoFinnigan, San Jose, CA) was equipped with Surveyor LC pump, auto-sampler coupled to a LCQ advantage mass spectrometer (ion trap) and with Xcalibur® software (Version 1.3) to control the system acquisition and data processing. The mass spectrometer was operated in positive-ion mode with full scan detection in the *m*/*z* range of 200–1500, where the source of fragmentation was turned on (collision energy of 15 V). The ion spray and capillary voltage were set at 4.0 kV and 15.6 V, respectively.

The reaction components of the acidolysis reaction were also separated by TLC on preparative Silica gel 60F plates, with fluorescent indicator (Whatman, Fisher Scientific). The developing solvent consisted of a mixture of chloroform/acetone/acetic acid (96:4:1, v/v/v). Upon visualization of the TLC plates under UV light (235 nm), the bands corresponding to phenolic lipids were scraped and recovered as a single fraction, by extraction with isopropanol, for the fatty acid analysis as well as for the scavenging activity determination [23]. The fatty acid methyl esters were analyzed by GLC as described previously [21].

2.4. Determination of free radical scavenging activity

The free radical scavenging activity of phenolic lipids, purified by TLC, was evaluated using DPPH$^{\bullet}$ as a stable free radical, according to the modified method of Chen and Ho [24]. In a 1-mL spectrophotometric cuvette, 40 μL of phenolic lipid and their corresponding phenolic acid solutions were added to 960 μL of a DPPH$^{\bullet}$ ethanolic solution (0.12 mM). The reduction of DPPH$^{\bullet}$ was monitored spectrophotometrically at 517 nm until the reaction reached a plateau against a blank assay containing only DPPH$^{\bullet}$, using a Beckman spectrophotometer (Model 650, Beckman Instruments, Inc., Fullerton, CA). The percentage of the scavenged DPPH$^{\bullet}$ was calculated as the absorbance of the DPPH$^{\bullet}$ control at steady state minus that of the sample divided by that of the control, multiplied by 100.

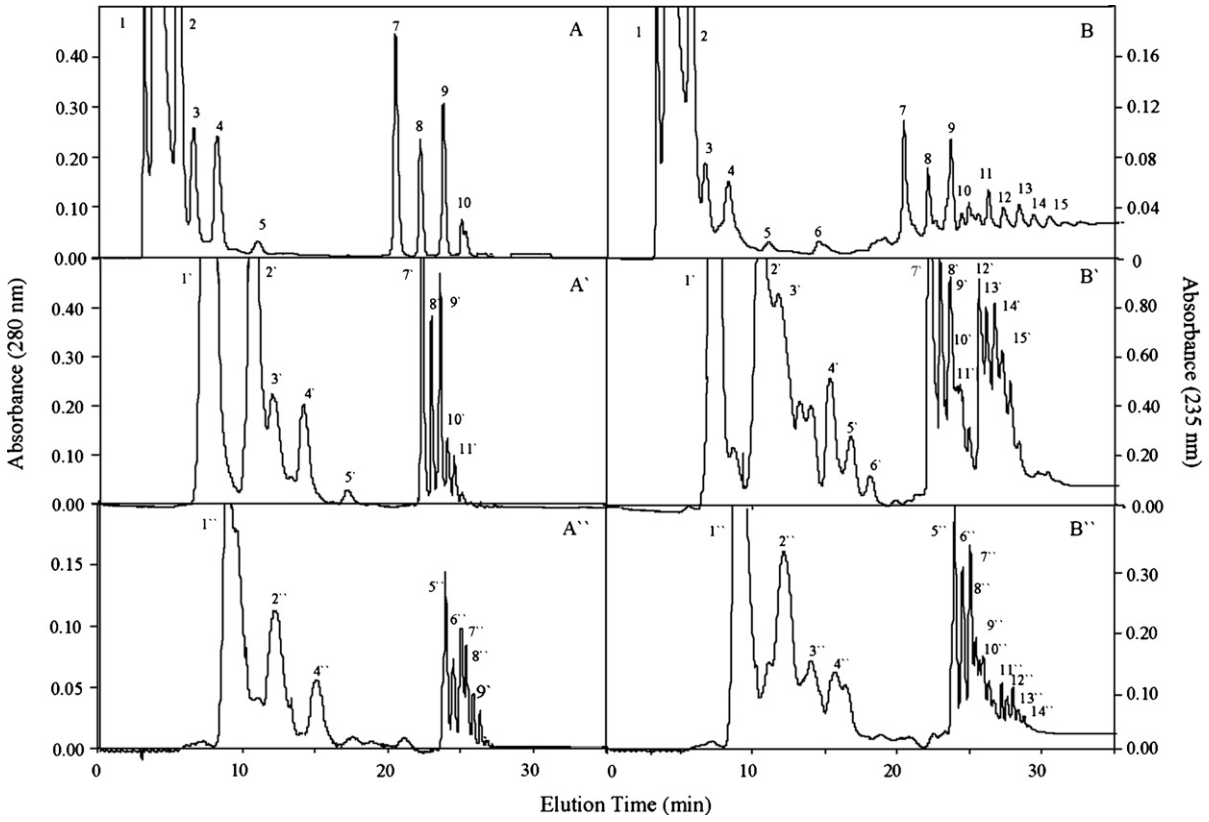


Fig. 1. HPLC chromatograms of the reaction components of lipase-catalyzed acidolysis of flaxseed oil with cinnamic (A and B), *p*-coumaric (A′and B′) and 3,4-dihydroxyphenyl acetic (A″ and B″) acids monitored at 280 and 235 nm.

3. Results and discussion

3.1. Effect of phenolic acid structure on the bioconversion yield of phenolic lipids

In order to determine the optimal phenolic acid structure, the enzymatic synthesis of phenolic lipids by acidolysis of flaxseed oil with the selected phenolic acids was investigated in a selected organic solvent medium using Novozym 435 as biocatalyst (Scheme 1). In order to maximize the bioconversion yield, two selected molar substrate ratios of flaxseed oil to phenolic acid of 4:1 and 8:1 were therefore investigated. Although the hydrophobic organic solvents are more appropriate for the enzymatic synthesis than the hydrophilic ones, their capacity of solubilization of hydrophilic substrates is limited [25,26]. To overcome the limited solubility of the hydrophilic phenolic acids in the hydrophobic organic solvents, a binary organic solvent mixture of hexane and 2-butanone, which is a non-reactive and non-toxic environment, has been used in our previous work [20,22]; however, the ratio of both solvents has been found to affect the enzymatic reaction rate, the bioconversion yield as well as the selectivity of the reaction [20,21]. The hexane/2-butanone organic solvent mixture at a ratio of 85:15 (v/v) was reported as the appropriate reaction medium for the biosynthesis of phenolic lipids, leading to a high bioconversion yield and favoring the production of phenolic diacylglycerols over that of monoacylglycerols [21].

Table 1 shows that the increase in the molar ratio of flaxseed oil to phenolic acid from 4:1 to 8:1 resulted by an increase of the bioconversion yield from the range of 5–60% to that of 7–74%, respectively; however, the extent of this increase seemed to be

Table 1
Lipase catalyzed acidolysis of flaxseed oil with selected phenolic acids in hexane/2-butanone solvent mixture (85:15, v/v)

Phenolic compounds	Bioconversion yield (%)[a]		
	4:1[b]	8:1[c]	8:1[d]
Cinnamic acid	60.4 (±3.0)[e]	73.9 (±6.3)	63.5 (±5.6)
Caffeic acid	8.4 (±0.1)	11.3 (± 0.1)	9.4 (± 0.1)
p-Coumaric acid	22.4 (±2.1)	44.6 (±1.9)	22.2 (±2.3)
3,4-Dihydroxyphenyl acetic acid	49.5 (±2.7)	55.9 (±3.1)	68.9 (±1.4)
Ethyl ferulate	8.2 (±0.8)	8.7 (±2.5)	5.6 (±2.5)
Ferulic acid	11.2 (±0.1)	28.9 (±2.5)	19.6 (±0.3)
Sinapic acid	10.2 (±0.1)	18.7 (±0.2)	9.3 (±0.1)
3,4-Dihydroxybenzoic acid	6.9 (±0.9)	5.8 (±0.7)	3.3 (±0.5)
3,4-Dimethoxycinnamic acid	5.3 (±2.1)	6.6 (±0.9)	9.9 (±1.2)

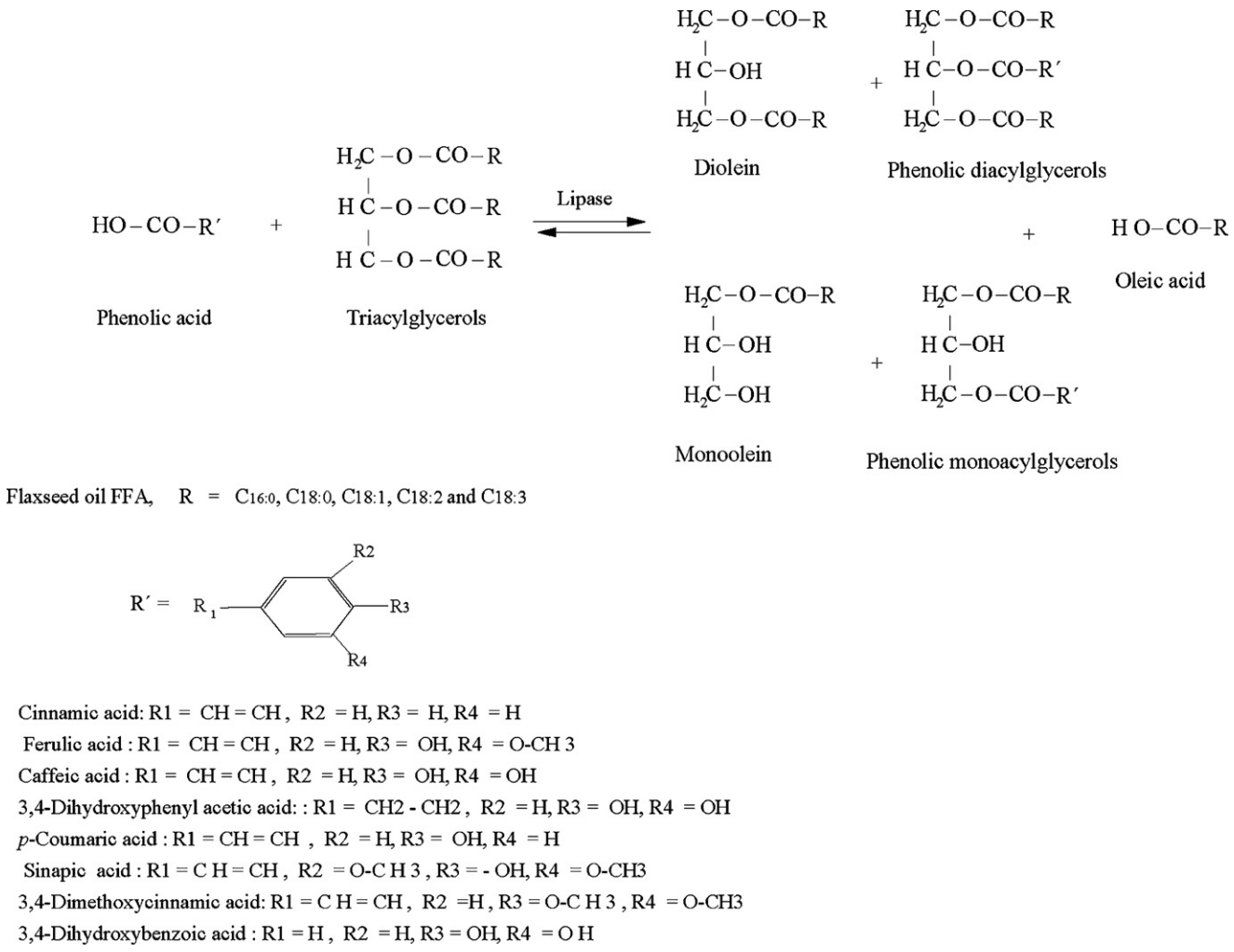
[a] Bioconversion yield was calculated as the peak area of phenolic lipid products after 8 days of reaction, monitored at 280 nm, divided by that of phenolic acid in the blank, multiplied by 100.

[b] Transesterification reaction was carried using a molar ratio of flaxseed oil to phenolic acid of 4:1.

[c] Transesterification reaction was carried using a molar ratio of flaxseed oil to phenolic acid of 8:1.

[d] Transesterification reaction was carried using a molar ratio of flaxseed oil to phenolic acid of 8:1 in the presence of Silica gel at a concentration of 2.2 mg/mL.

[e] Data are average of three determinations and standard deviations are given.



Scheme 1. Reaction scheme of lipase-catalyzed acidolysis reaction of flaxseed oil with selected hydroxylated and/or methoxylated derivatives of cinnamic, phenyl acetic and benzoic acids.

dependent on the nature of phenolic acid structure. The overall results suggest that the excess of flaxseed oil may have favored the enzymatic synthesis of phenolic lipids over the hydrolysis or the acyl interchange reactions between the TAGs. As compared to the substrate molar ratio of 1:4, the highest bioconversion yield obtained at 1:8 may be attributed to the effects of the mass action of excess flaxseed oil, used as a reactant, as well as of the increase in the interactions between the TAGs and the lipase [27]. The literatures [19,28] generally indicated that, with a high substrate molar ratio, there was an increase in the bioconversion yield as well as a decrease in the reaction time and the acyl migration of the structured lipids. Sabally et al. [21] reported that although a high molar ratio of oil did not decrease the reaction time, it did enhance the reaction rate and the total bioconversion yield of lipase-catalyzed transesterification of flaxseed oil with dihydrocaffeic acid. Nevertheless, other investigations [18,20,29] did not report any increase in the bioconversion yield of structured lipids with the use of a high substrate molar ratio.

The bioconversion yield of phenolic lipids (Table 1) was dependent on the structural characteristics of phenolic acids, which are hydroxylated and/or methoxylated derivatives of cinnamic, phenylacetic and benzoic acids. The highest bioconversion yield of 74% was obtained when cinnamic acid was used as substrate; however, the *p*-hydroxylation of its aromatic ring (*p*-coumaric acid) resulted in a decrease in the bioconversion yield to 45%. Moreover, the presence of additional two *p*-hydroxyl groups on the benzene cycle of caffeic acid decreased the bioconversion yield to 11%. The overall of the experimental findings (Table 1) suggests that the presence of *p*-hydroxyl groups on the benzene cycle of cinnamic acid derivatives may have an inhibitory effect on the lipase activity. However, the inhibitory effect of *p*-hydroxyl groups was much less significant, with a bioconversion yield of 56%, when there was no double bond on the side chain, conjugated with the aromatic ring of 3,4-dihydroxyphenyl acetic acid. These results may indicate that the inhibitory effect of *p*-hydroxyl substituents was most likely due to their electronic donating effect rather than to their steric hindrance in the enzyme active site. It has been reported [15] that the electronic donating effect of *p*-hydroxyl groups led to a deactivation of the reactivity of the electrophilic carbon center of carboxylic function. Similar findings have also been reported for the esterification of *p*-coumaric with 1-octanol in a solvent-free system [16] and for the transesterification of selected cinnamic

acid derivatives with triolein [31]; these authors have indicated that the inhibitory effect of *p*-hydroxyl group is higher when the side chain on the aromatic ring was unsaturated.

Table 1 also indicates that the presence of additional one or two methoxyl groups on the aromatic ring of ferulic and sinapic acids resulted by low bioconversion yield of 29% and 19%, respectively. The inhibitory effect of the methoxyl substituents of cinnamic acid derivatives on the enzyme activity has also been reported previously [15,16,30,31]. In our investigation, the effect of methoxyl group on the enzyme activity was somewhat different from that of *p*-hydroxyl one; the replacement of one hydroxyl group (caffeic acid) by one or two methoxyl groups (ferulic and sinapic acids) in the *p*-hydroxylated cinnamic acid led to 9–19% increases in the bioconversion yield. On the other hand, a low bioconversion yield of 7% was obtained when the two methoxyl groups were substituted on the aromatic ring of cinnamic acid (3,4-dimethoxycinnamic acid). The electronic donating and steric hindrance effects of the methoxyl groups seemed to be dependent on the other substituents on the aromatic ring of cinnamic acid derivatives. In addition, Guyot et al. [30] reported a decrease in the esterification yield of 3,4-dimethoxycinnamic acid from 60% to 12% by increasing the alcohol carbon chain length from 4 to 8, respectively.

The results (Table 1) also indicate that a lower bioconversion yield (9%) was obtained with ethyl ferulate as substrate than that with ferulic acid (29%); this low bioconversion yield may be due to the formation of ethanol as the hydrolysis by-product, which possibly slowed down the reaction by inhibiting the enzyme activity and by its competition with ferulic acid [19]. Compton et al. [19] have obtained a higher bioconversion yield of 77% for the lipase-catalyzed transesterification of ethyl ferulate with triolein by the removal of ethanol under vacuum (16 mmHg). The lowest bioconversion yield of 6% obtained with 3,4-dihydroxybenzoic acid, could be attributed to its rigid coplanar conformation as a result of the presence of aromatic substituent adjacent to the carboxyl group [15]. Similar low bioconversion ($\leq$3%) has been reported by Guyot et al. [30] for the esterification of 3,4-dihydroxybenzoic acid with 1-octanol in a solvent-free media as well as by Safari et al. [31] for the transesterification of the same phenolic acid with triolein in hexane/2-butanone solvent mixture (85:15, v/v).

In order to increase the bioconversion yield of phenolic lipids, Silica gel was added to the reaction mixture as an adsorbent support for glycerol. Indeed, the accumulation of the undesirable glycerol by-product could contribute to the formation of a hydrophilic hindrance layer in the enzyme micro-environment and the subsequent apparition of mass transfer limitations [18,27]. In addition, a high amount of glycerol could lead to the formation of the undesirable phenyl glycerol product, which lacks the fatty acid moiety [19]. Table 1 indicates that the addition of Silica gel in the reaction mixture provided certain enhancement of the bioconversion yield of 3,4-dimethoxy cinnamoylated and 3,4-dihydroxyphenyl acetoylated lipids by 4% and 13%, respectively. In contrast, slight decreases of 2% to 22% in the bioconversion yield of other phenolic lipids were obtained upon the addition of Silica gel; these findings could be attributed to the ability of Silica gel to strip off the water layer from the enzyme molecule, which is essential for its activity [25,26]. In addition, since the enzymatic synthesis of phenolic lipids may have been obtained through the intermediate hydrolysis and/or synthesis reactions [19], the effect of the removal of glycerol on these intermediate reactions could also account for the low bioconversion yields, obtained in the presence of Silica gel. Nevertheless, an increase in the maximum bioconversion yield of cinnamoylated lipids, from 42% to 55%, has been obtained upon the addition of Silica gel in the reaction mixture in our previous study [18]. On the basis of the experimental findings (Table 1), cinnamic, 3,4-dihydroxyphenyl acetic and *p*-coumaric acids were subjected to further investigation.

3.2. Structural characterization of phenolic lipids

Fig. 1 shows the HPLC elution profiles of the reaction components of lipase-catalyzed acidolysis reactions of flaxseed oil with the selected phenolic acids, including cinnamic, 3,4-dihydroxyphenyl acetic and *p*-coumaric acids, monitored at 235 and 280 nm. Peaks #1 (Fig. 1A and B), 1′ (Fig. 1A′and B′) and 1″ (Fig. 1A″ and B″), which absorb at both 235 and 280 nm, were characterized as cinnamic, *p*-coumaric and 3,4-dihydroxycinnamic acids, respectively. At 235 nm, the TAGs of flaxseed oil were identified as peaks # 11–15, 12′–15′ and 10″–14″ in the elution profiles of the reaction components obtained with cinnamic (Fig. 1B), *p*-coumaric (Fig. 1B′) and 3,4-dihydroxycinnamic (Fig. 1B″) acids as substrates, respectively. On the other hand, seven predominant peaks # 2–4, and 7–10, were characterized as the main phenolic lipid end products of the acidolysis reaction of flaxseed oil with cinnamic acid, since they have shown a UV-spectral scanning profile comparable to that of its phenolic acid component (peak # 1) (Fig. 1A and B). Similarly, the *p*-coumaroylated lipids were identified as peaks # 2′–4′, and 7′–11′ in the elution profile monitored at 235 and 280 nm (Fig. 1A′ and 1B′). Six peaks # 2″, 4″, and 5″–8″ were also found to display a characteristic UV-spectral scanning profile of 3,4-dihydroxycinnamic acid (Fig. 1 A″ and B″). Although the peaks # 5 (Fig. 1A and B) and 5′ (Fig. 1 A′and B′) absorbed at both 235 and 280 nm, they showed a UV-spectral scanning profile different from their phenolic acid component.

In order to characterize the molecular structure of phenolic lipids, further analyses of the eluting peaks by APCI-MS spectrometry in the positive-ion mode were conducted. The phenolic group substitution on the glycerol backbone of the phenolic lipids is shown for simplicity as the regioselectivity of the reaction is unknown (Figs. 2 and 3). The fragmentation patterns of the main phenolic lipid end products, monitored in the elution profiles at 235 and 280 nm, are shown in Figs. 2 and 3. The fragmentation pattern of peak # 2 (Fig. 2A) shows abundant molecular ions at *m/z* 465.3 $[M+H–H_2O]^+$ and 483.3 $[M+H]^+$ corresponding to monolinolenyl cinnamate and a fragment ion at *m/z* 335.3 $[M+H–H_2O]^+$ representing monolinolenin. Peak # 3 (Fig. 2A′) and 4 (Fig. 2A″) were characterized as monolinoleyl and oleyl cinnamates, respectively, with an abundant molecular ion $[M+H–H_2O]^+$ at *m/z* 467.4 and 469.4 and a fragment ion $[M+H–H_2O]^+$ at *m/z* 337.3 and 339.4, corresponding to monolinolein and monoolein, respectively. Similarly, peaks # 2′

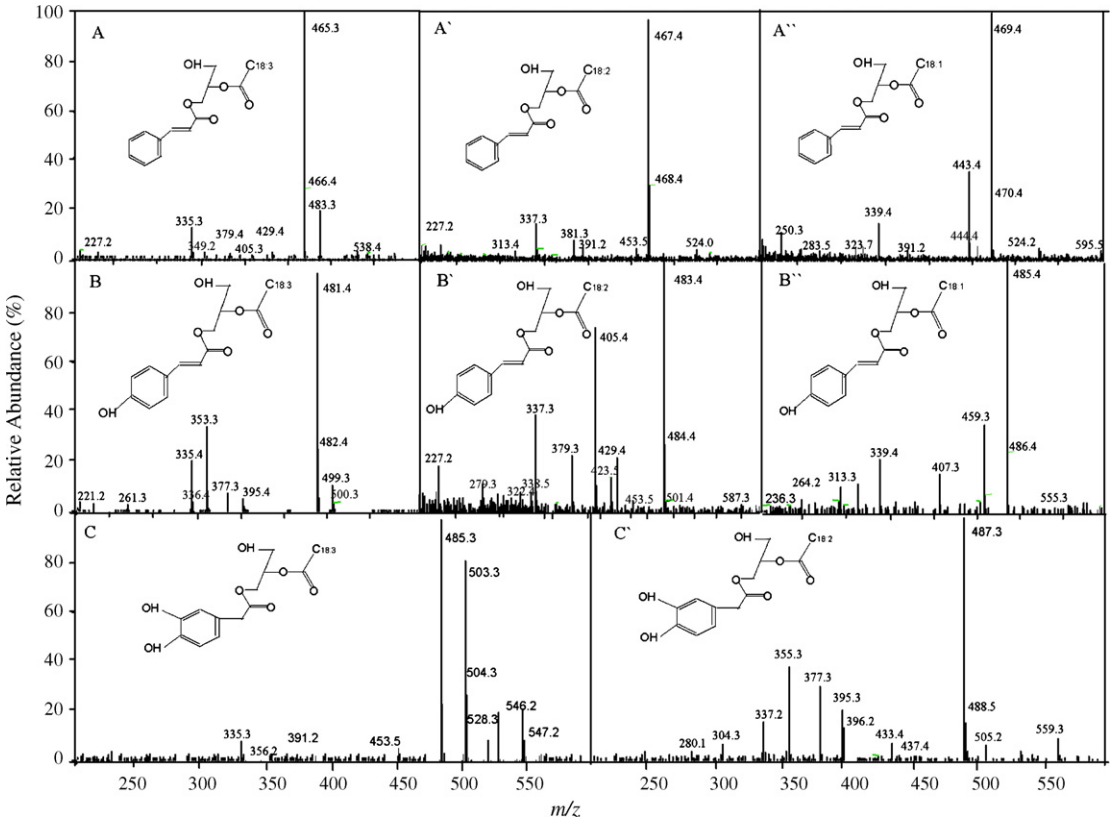


Fig. 2. APCI-MS Spectra of the fragmentation patterns of cinnamoylated monoacylglycerols (A, A′and A″, identified by peaks # 2–4 on A), *p*-coumaroylated monoacylglycerols (B, B′and B″, identified by peaks # 2′–4′ on A′) and 3,4-dihydroxyphenyl acetoylated monoacylglycerols (C and C′, identified by peaks # 2″ and 4″ on A″).

(Fig. 2B), 3′ (Fig. 2B′) and 4′ (Fig. 2B″), obtained in the elution profile of the reaction components with *p*-coumaric acid as substrate, were characterized as monolinolenyl, monolinoleyl and oleyl *p*-coumarates, respectively, with an abundant molecular ion $[M+H–H_2O]^+$ at *m/z* 481.4, 483.4 and 485.4 and a fragment ion $[M+–H_2O]^+$ at *m/z* 335.3, 337.3 and 339.4 characterized as their corresponding monoacylglycerols. The fragmentation of peaks # 2″ (Fig. 2C) produced a fragment ion at *m/z* 335.3 $[M+H–H_2O]^+$ as well as abundant molecular ions at m/z 503.3 $[M+H]^+$ and 485.3 $[M+H–H_2O]^+$, which are characteristics of monolinolenyl 3,4-dihydroxyphenyl acetate. On the other hand, the fragmentation pattern of peak # 3″ (Fig. 2C′) exhibited two molecular ions at *m/z* 505.2 $[M+H]^+$ and 487.3 $[M+H–H_2O]^+$ corresponding to monolinoleyl 3,4-dihydroxyphenyl acetate and fragment ions at *m/z* 337.2 $[M+H–H_2O]^+$ and 355.3 $[M+H]^+$ of monolinolein.

The APCI-MS analysis (Fig. 3) also confirms the enzymatic synthesis of four cinnamoylated diacylglycerols corresponding to the eluting peaks # 7–10 in the HPLC chromatogram (Fig. 1A and B). The fragmentation of peaks # 7 (Fig. 3A) and 9 (Fig. 3A″) resulted in a major molecular ion $[M]^+$ at *m/z* 743.6 and 747.7, respectively, characterized as dilinolenyl and dilinoleyl cinnamate as well as in a fragment ion $[M+H–H_2O]^+$ at *m/z* 595.7 and 599.7, corresponding to dilinolenin and dilinolein, respectively. While the fragmentation pattern of peaks # 8 (Fig. 3A′) and 10 (Fig. 3A‴) showed an abundant mixed phenolic diacylglycerol molecular ion $[M]^+$ at *m/z* 745.6 and 747.9, corresponding to linoleyl linolenyl and oleyl linolenyl cinnamates, respectively. The APCI-MS spectra (Fig. 3B–B‴) also reveal the enzymatic synthesis of five *p*-coumaroylated diacylglycerols (peaks # 7′–11′) by acidolysis of flaxseed oil with *p*-coumaric acid (Fig. 1A′). Peak # 7′ was characterized as dilinolenyl *p*-coumarate, with a major molecular ion $[M]^+$ at *m/z* 759.7, and fragment ions at *m/z* 613.8 and 335.5 (Fig. 3B), which correspond to dilinolenin $[M+H]^+$ and monolinolenin $[M+H–H_2O]^+$, respectively. Similarly, peaks # 9′ (Fig. 3B″) and 11′ (Fig. 3B⁗) were characterized as dilinoleyl and dioleyl *p*-coumarate, respectively, with abundant molecular ions $[M]^+$ at *m/z* 763.6 and 767.8, and two fragment ions $[M+H–H_2O]^+$ at *m/z* 599.7–603.6 and 337.7–339.3, corresponding to their mono- and diacylglycerols, respectively. On the other hand, the fragmentation pattern of peaks # 8′ (Fig. 3B′) and 10′ (Fig. 3B‴) was found to be corresponded to a mixed phenolic diacylglycerols, with an abundant molecular ion $[M]^+$ at *m/z* of 761.7 and 763.6, respectively, which were characterized as linoleyl linolenyl and oleyl linolenyl *p*-coumarates, respectively; these molecular ions produced fragment ions, characteristic of their mixed diacylglycerols $[M+H]^+$ at *m/z* 615.7 and 617.8 as well as of their monoacylglycerols $[M+H–H_2O]^+$ at *m/z* 335.6–337.5 and 335.6–339.6, respectively. The results also indicate that the fragmentation pattern of peaks # 5″ (Fig. 3C) and 7″ (Fig. 3C″), which possess a major molecular ion $[M]^+$ at *m/z* 763.5 and 767.5, respectively, corresponding to dilinolenyl and dilinoleyl 3,4-dihydroxyphenyl acetates, respectively. Peak # 6″ (Fig. 3C′)

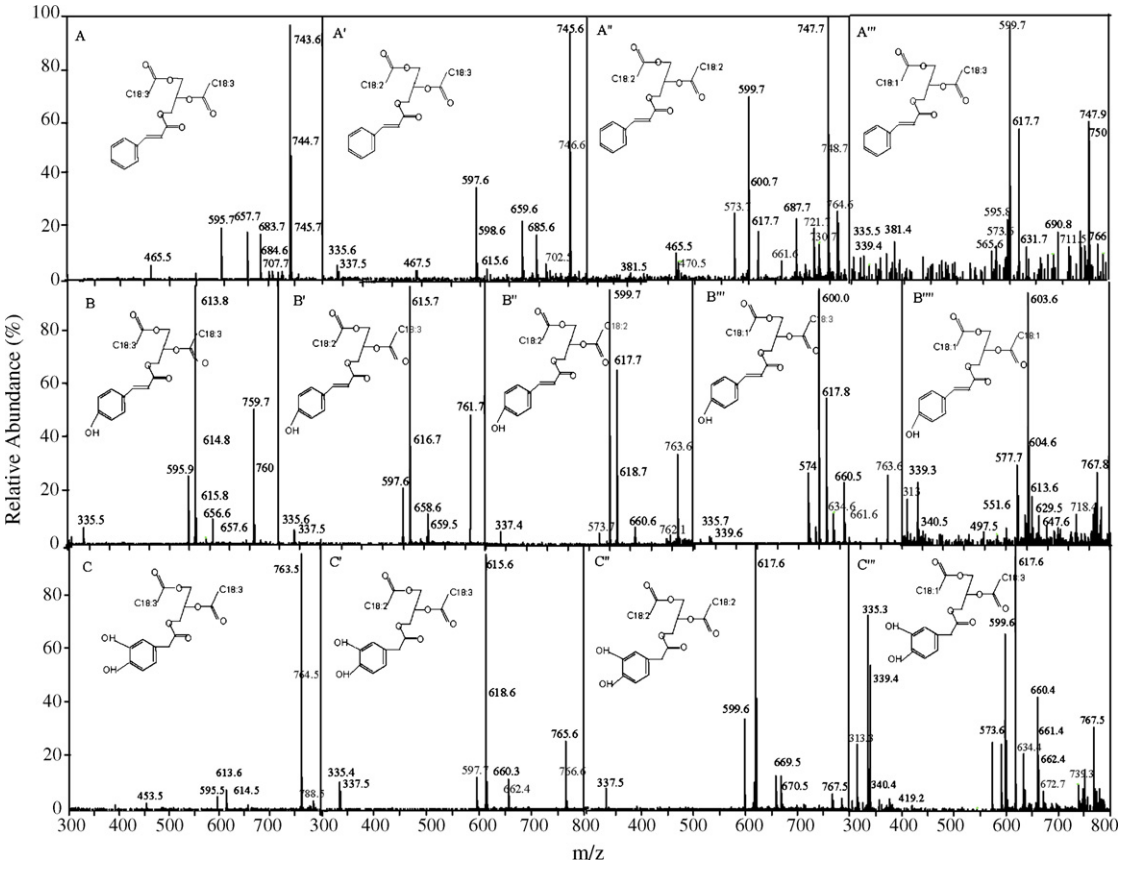


Fig. 3. APCI-MS Spectra of the fragmentation patterns of cinnamoylated diacylglycerols (A, A′, A″ and A‴, identified by peaks # 7–10 on Fig. 2A), *p*-coumaroylated diacylglycerols (B, B′, B″, B‴and B⁗, identified by peaks # 7′–11′ on Fig. 2A′) and 3,4-dihydroxyphenyl acetoylated diacylglycerols (C, C′, C″ and C‴, identified by peaks # 5″–8″ on Fig. 2A″).

was characterized as linoleyl linolenyl 3,4-dihydroxyphenyl acetate, with an abundant molecular ion $[M]^+$ at *m/z* 765.6 and different fragment ions at *m/z* 335.4, 337.5 and 615.6, corresponding to monolinolenin $[M + H–H_2O]^+$, monolinolein $[M + H–H_2O]^+$ and linoleyl linolenyl glycerol $[M + H]^+$, respectively. The fragmentation of peak # 8″ (Fig. 3C‴) resulted in a characteristic pattern of oleyl linolenyl 3,4-dihydroxyphenyl acetate, with an abundant molecular ion at *m/z* of 767.5 $[M]^+$ and fragment ions at 335.3, 339.4 and 617.6, corresponding to monolinolenin $[M + H–H_2O]^+$, monoolein $[M + H–H_2O]^+$ and oleyl linolenyl glycerol $[M + H]^+$, respectively. The loss of H_2O, from a monoacylglycerol during HPLC/APCI-MS analyses, has been previously reported by Compton et al. [19], Sabally et al. [21] and Safari et al. [31]. Overall, the HPLC/APCI-MS analyses confirmed the formation of various phenolic mono- and diacylglycerols by lipase-catalyzed acidolysis of flaxseed oil with the selected phenolic acids. Likewise, Sabally et al. [21] have characterized six dihydrocaffeoylate lipids as end products of lipase-catalyzed transesterification of flaxseed oil with dihydrocaffeic acid.

3.3. Time course of phenolic lipids synthesis

Fig. 4 illustrates the time course of the synthesis of selected phenolic lipids throughout a 10-day period of lipase-catalyzed acidolyis of flaxseed oil with one of the selected phenolic acids, cinnamic, *p*-coumaric and 3,4-dihydroxyphenyl acetic acids. The overall results indicate that the enzymatic synthesis of cinnamoylated and *p*-coumaroylated lipids were initiated after an elapsing period of 1 day of reaction and proceeded with different reaction rates over the time course. These results indicate the importance of the intermediate products for the enzymatic synthesis of phenolic lipids. Similar trends were previously reported [18,19,31] for the lipase-catalyzed transesterification of phenolic acids with triolein. On the other hand, the addition of Silica gel in the reaction mixture, containing 3,4-dihydroxyphenyl acetic acid as substrate, resulted by a less variation in the extent of the bioconversion yield of phenolic lipids over the reaction time course; these results confirm the effect of the glycerol, as hydrophilic hindrance component, on the enzyme activity [18,27].

The results (Fig. 4) also show that the bioconversion yield of cinnamoylated monoacylglycerols increased rapidly within the first 4 days of reaction to 34%, and thereafter with a relatively lower extent up to a maximum of 51% after 8 days of reaction; beyond this reaction time, the bioconversion yield was decreased to 36% after an additional 2 days. This decrease was most likely due to a shift in the thermodynamic equilibrium reaction toward hydrolysis or acyl migration, as a result of the increase in the concentration of free fatty acids [18]. The enzymatic synthesis of cinnamoylated diacylglycerols showed a different trend whereby a steady increase in the bioconver-

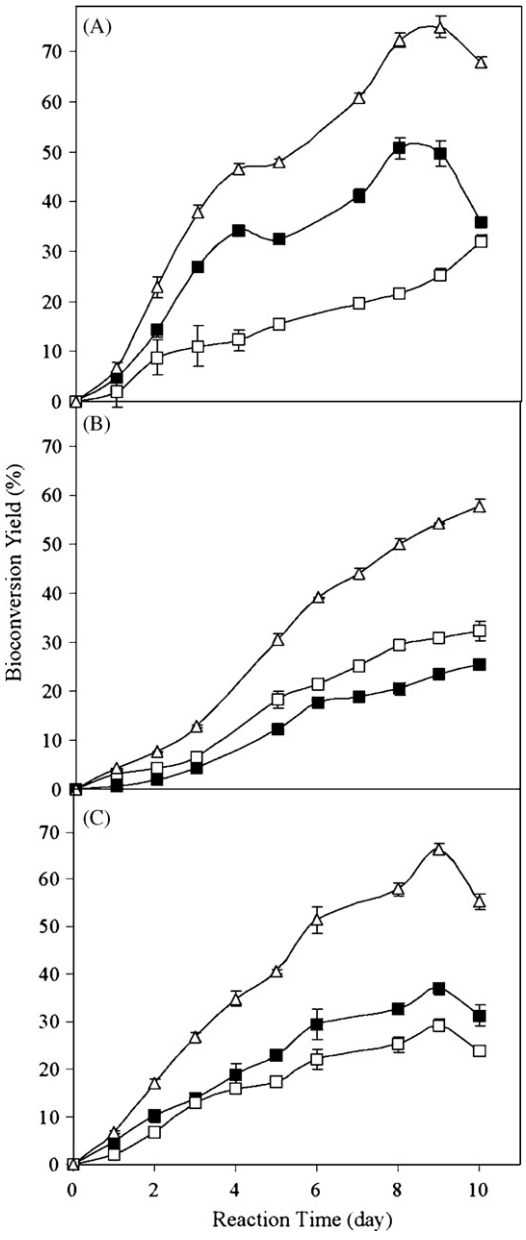


Fig. 4. Time course of lipase-catalyzed the production of phenolic monoacylglycerols (■), phenolic diacylglycerols (□) and total phenolic lipids (Δ), over a 10-day period of reaction, using cinnamic (A), *p*-coumaric acid (B) and 3,4-dihydroxyphenyl acetic (C) acids as selected phenolic acid substrate.

sion yield was obtained over the 10-day reaction period up to a maximum of 32%. On the other hand, the enzymatic synthesis of *p*-coumaroylated lipids proceeded with a low reaction rate within the first 3 days, and thereafter with a higher extent to reach a maximum bioconversion yield of 58% after 10 days of reaction period. Contrary to the cinnamoylated lipids, the *p*-coumaroylated mono- and diacylglycerols showed similar bioconversion yield trend over the reaction time course; however, the extent of bioconversion yield of *p*-coumaroylated monoacylglycerols was lower than that of their corresponding diacylglycerols with maximum ones of 25% and 35%, respectively. The results also show steady increase in the bioconversion yield of 3,5-dihydroxyphenyl acetoylated mono- and diacylglycerols up to maximum of 37% and 29%, respectively, after 9 days of reaction time; however, this increase was followed by a decrease in the tenth day of the enzymatic reaction to 31% and 24%, respectively. Although the trend of the enzymatic synthesis of 3,4-dihydroxyphenyl acetoylated mono- and diacylglycerols was similar throughout the 10-day reaction period, the extent of the bioconversion yield of dihydroxyphenyl acetoylated monoacylglycerols was higher.

Using cinnamic and 3,4-dihydroxyphenyl acetic acids as substrates, the lipase-catalyzed acidolysis of flaxseed oil was more in favor of the synthesis of phenolic monoacylglycerols, whereas with *p*-coumaric, it showed a higher selectivity towards the synthesis of phenolic diacylglycerols. This reaction selectivity was probably the consequence of substrate partition and lipase specificity. The polarity of the reaction medium and the substrate molar ratio have been reported to have a high effect on the selectivity of lipase-catalyzed esterification and transesterification reactions [18,20,21,27].

3.4. Determination of relative fatty acid composition

In order to evaluate the change in the relative fatty acids composition of the flaxseed oil TAGs, following its acidolysis with selected phenolic acids, the flaxseed oil and total phenolic lipid fractions were recovered by TLC and subjected to GLC analysis. The results (Table 2) show that the predominant fatty acids in flaxseed oil were oleic ($C_{18:1}$ *n*-9), linoleic ($C_{18:2}$ *n*-6) and linolenic ($C_{18:3}$ *n*-3) acids, with 21.3%, 13.8% and 52.4% of the total fatty acids, respectively. However, the relative proportion of these fatty acids in the selected produced phenolic lipids was different, indicating hence a modification in the profile of fatty acids of flaxseed oil upon its acidolysis with the selected phenolic acids. The proportion of $C_{18:3}$ *n*-3 was increased from 52.9% in the flaxseed oil to 60.1%, 68.3% and 72.2% in the cinnamoylated, *p*-coumaroylated and 3,4-dihydroxyphenyl acetoylated lipids, respectively, whereas that of $C_{18:1}$ *n*-9 was decreased from 21.3%, originally, to 19.3%, 11.2% and 9.3%, respectively. In contrast, there was no significant difference in the profile proportion of $C_{18:2}$ *n*-6 in the phenolic lipids as compared to that in the flaxseed oil. The results (Table 2) also show a decrease in the proportions of palmitic and stearic acids in the phenolic lipids as compared to that in the flaxseed oil. The overall results suggest a higher specificity of lipase for the exchange of the acyl group of $C_{18:1}$ *n*-9, palmitic and stearic acids, with that of phenolic acid as compared to those of $C_{18:3}$ *n*-3 and $C_{18:2}$ *n*-6. Similarly, Sabally et al. [21] have also reported an increase in the relative proportion of $C_{18:3}$ *n*-3 as well as a decrease in those of $C_{18:1}$ *n*-9 and $C_{18:2}$ *n*-6 upon incorporation of dihydrocaffeic acid in the flaxseed oil.

3.5. Determination of free radical scavenging activity

The free radical scavenging activity of selected phenolic lipids and that of their phenolic acid component was determined (Table 3), using DPPH• as the free radical. The scavenging activity assays were performed with different amounts of phenolic compounds and with an excess of DPPH• radical. 3,4-

Table 2
Relative fatty acid compositions of flaxseed oil and their phenolic lipids produced by enzymatic acidolysis with selected phenolic acids

Fatty acids	Relative fatty acid (%)[a]			
	Flaxseed oil	Cinnamoylated lipids	*p*-Coumaroylated lipids	3,4-Dihydroxyphenyl acetoylated lipids
$C_{16:0}$	4.5 (0.3)[b]	3.5 (0.8)	3.0 (0.3)	3.8 (0.3)
$C_{18:0}$	7.3 (0.4)	2.3 (0.7)	3.1 (0.5)	1.5 (0.2)
$C_{18:1}$ *n*-9	21.3 (1.3)	19.3 (1.3)	11.2 (1.1)	9.3 (0.4)
$C_{18:2}$ *n*-6	13.8 (0.9)	14.9 (0.8)	14.2 (0.8)	13.2 (1.3)
$C_{18:3}$ *n*-3	52.9 (3.6)	60.1 (4.5)	68.3 (4.7)	72.2 (6.2)

[a] Relative percentage composition was based on peak area of fatty acid over total peak area expressed as percentage.
[b] Data are average of three determinations and the standard deviations are given.

Table 3
Radical scavenging ability of selected phenolic lipids and their corresponding phenolic acids

Phenolic compounds	Maximum scavenged DPPH• (%)[a]		
	50 μM[b]	100 μM[b]	200 μM[b]
Cinnamic acid	N.D.[c]	N.D.	N.D.
Cinnamoylated lipids	0.8 (±0.1)[d]	1.1 (±0.1)	2.6 (±0.5)
p-Coumaric acid	2.9 (±1.0)	3.9 (±0.1)	5.3 (±0.9)
p-Coumaroylated lipids	2.7 (±1.2)	3.7 (±0.5)	4.5 (±2.2)
3,4-Dihydroxyphenyl acetic acid	58.1 (±8.0)	81.8(±2.5)	92.2 (±0.4)
3,4-Dihydroxyphenylacetoylated	13.0 (±1.5)	28.5 (±2.7)	53.3 (±2.1)
α-Tocopherol	22.2 (±1.6)	34.5 (±2.4)	58.2 (±1.6)

[a] Percentage of maximum scavenged DPPH• was calculated as the difference between the absorbance of the sample at 517 nm and that of DPPH•blank at the steady state divided by the absorbance of DPPH•blank, multiplied by 100.
[b] The percentage of maximum scavenged DPPH• was estimated using different concentrations of the phenolic compounds as scavengers
[c] Not detected.
[d] Data are average of three determinations and standard deviations are given.

Dihydroxyphenyl acetic acid was found to be the most potent scavenger among the investigated phenolic acids, with a radical scavenging activity higher than that of α-tocopherol, used as a control. On the other hand, cinnamic acid, which lacks the hydroxyl group, did not react against the DPPH• radical. In addition, the monophenol *p*-coumaric, with no substituent on the aromatic ring, showed lower radical activity than that of α-tocopherol. These results are in agreement with those previously reported [32], using the DPPH• as the free radical. Despite major advances in understanding the molecular mechanisms underlying the radical scavenging activity, there are still some controversies regarding the relationship between the molecular structure of phenolic compounds and their reactivity as radical scavengers. However, the main structural characteristics of phenolic acids, required for an efficient radical scavenging activity, were reported [14,24,33] to be the number of hydroxyl groups on the benzene ring being the most important as well as the *ortho* substitution with the electron donor methoxyl group. Hence, the highest scavenging activity of 3,4-dihydroxy acetic acid may be due to the presence of a second hydroxyl group, which is known to increase the resonance stabilization and the formation of quinone [14,24].

The results (Table 3) also show that the esterification of carboxyl group of the selected phenolic acids affected their radical scavenging activity by different ways. *p*-Coumaroylated lipids showed a similar scavenging activity than that of phenolic acid component. Although no radical scavenging activity was obtained with cinnamic acid, their phenolic lipids were able to react against the DPPH• radical. On the other hand, the 3,4-dihydroxyphenyl acetoylated lipids displayed a lower radical scavenging activity than that of their phenolic acid component. The effect of the modification of the carboxyl group of phenolic acid on its radical scavenging activity has been previously reported [21,24,31,33] and ascribed mainly to the conformational changes in the structure of phenolic acids upon their esterification.

4. Conclusion

Selected structured phenolic lipids were synthesized by lipase-catalyzed acidolysis of flaxseed oil with hydroxylated or/and methoxylated derivatives of cinnamic, phenyl acetic and benzoic acids. The bioconversion yield as well as the reaction selectivity was found to be dependent on the structural characteristics of phenolic acids and their electronic distribution. The acidolysis reaction have led to an increase in the relative proportion of linolenic acid ($C_{18:3}$ *n*-3) in the phenolic lipids as compared to that of flaxseed oil. In addition, the radical scavenging activity of the synthesized phenolic lipids indicated that the structural modification of the carboxyl group of phenolic acids has an effect on their radical scavenging activity.

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