In vitro bioaccessibility and physicochemical properties of phytosterol linoleic ester synthesized from soybean sterol and linoleic acid

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Abstract

Phytosterols are bioactive components capable of reducing cholesterol level in serum and reducing risk of arteriosclerosis. In this study, conditions for the synthesis of maximum yield of phytosterol linoleic ester (PLE) was optimized and the physicochemical properties and *in vitro* bioaccessibility of the PLE was assessed. Under the optimized condition of 1:1.1 mole ratio of phytosterol and linoleoyl chloride at 80^{o}C for 1.5h, the conversion rate of phytosterol reached 96.1%. Its solubility in oil increased 20 times, up to 33.8%. Also, peroxide value of PLE was much lower than linoleic acid (32.9 and 47.0 mmol/kg), which means better oxidative stability. Bioaccessibility of PLE was affected by time, concentration of bile extract, and dissolved medium. It was 4.93% alone, increased by 2.5times compare to phytosterol; or 53.46% in oil, under the condition of 40mg/mL bile extract for 120min. In conclusion, under the tested condition, phytosterol conversion rate, its solubility in oil and bioaccessibility were improved significantly. The method showed great potential in manufacture high quality and quantity of PLE.

Key words: Phytosterol, linoleic acid, synthesis method, phytosterol linoleic ester, bioaccessibility, *in vitro*

Abbreviations： PS, phytosterol; LA, linoleic acid; PLE, phytosterol linoleic ester; CR, conversion rate; LC, linoleyl chloride; SGF, simulated gastric fluid; SIF, simulated intestinal fluid;

1. Introduction

Phytosterols are bioactive compounds in plant, and also integral components of oil unsaponifiable matter. The composition and content of phytosterol (PS) differs in different vegetable oils, with the most important being β-sitosterol, stigmasterol, campesterol and brassicasterol (Moreau, Whitaker, & Hicks, 2002; Piironen, Lindsay, Miettinen, Toivo, & Lampi, 2000). Studies have shown the importance of PS in reducing cholesterol levels in the serum (Brufau, Canela, & Rafecas, 2008; Wolfs, de Jong, Ocké, Verhagen, & Monique Verschuren, 2006). Linoleic acid (LA) is a common polyunsaturated fatty acid with large amounts found in safflower seed oil, sunflower oil, walnut oil and soybean oil. LA is an essential fatty acid and can also reduce the risk of arteriosclerosis in animal model and humans. Both PS and LA are regarded essential molecules since they cannot be synthesized in the human body. These molecules must be obtained from food sources.

Phytosterol is insoluble in water and its solubility in oil is just about 1% (Yang, Oyeyinka, & Ma, 2016). This trait limits its wide application in food/pharmaceutical industry. In order to enlarge its application and improve the bioaccessibility of PS, researchers have utilized the esterification method to produce phytosterol esters from PS and fatty acids. Chemical synthesis and biological synthesis are the two main methods at present. Chemical synthesis shows advantageous, as it provides good conversion rate (CR), and high productivity, but it has several drawbacks too. For example, chemical esterification requires the use of catalysts including magnesium oxide, lanthanum oxide, zinc oxide, aluminum oxide, and aluminum triiodide (Hang & Dussault, 2010; Meng, Pan, & Yang, 2010; Robles-Manuel, Barrault, &

Valange, 2011; Valange et al., 2007). The major challenge is the difficulty in separating catalyst from the final product, also the high temperature may lead to the production of by-product. Biological synthesis uses a relatively low temperature, produces no or less by-product, but takes a long time with low CR products (Villeneuve et al., 2005; Vu, Shin, Lim, & Lee, 2004).

Recently we synthesized PS esters using PS from soybean and acetic anhydride (Yang et al., 2016). And the optimum condition for the production of high yield of PS ester (99.4%) was found to be a temperature of 135^{o}C for 1.5 h with a mole ratio 1:1 for phytosterol and acetic anhydride, respectively. Furthermore, Fourier transform infrared spectroscopic and gas chromatography-mass spectrometric studies revealed that no other harmful by-products were formed during the process (Yang et al., 2016). With the growing interest in the synthesis of high-quality PS ester products using new technology, it may be necessary to investigate promising alternatives to the traditional chemical methods. Hence, in this paper, PLE was first synthesized from soybean sterol and LA using acyl chloride method in order to optimize reaction conditions. The physicochemical properties and *in vitro* bioaccessibility of the PLE were thereafter assessed.

2 Materials and Methods

2.1 Materials

Linoleic acid (≥ 99%), trypsin, pepsin, sodium taurocholate, as well as lipase (Type II), colipase and cholesterol esterase from bovine pancreas were purchased from Sigma-Aldrich company (America). Acetone and acetonitrile used were of chromatography grade. Hexane, PCl_3, NaOH, $NaHCO_3$, NaCl, $CaCl_2$, HCl, KH_2PO_4 were analytical grade. Soybean sterol (≥95%, seperated and purified from soybean oil deodorized distillate), soybean oil, rapeseed oil, peanut oil, corn oil, sunflower oil were obtained from Jiusan Grains & Oils Industries Group Co., Ltd (China). Standards of campesterol (≥98%), stigmasterol (≥98%), β-sitosterol (≥98%) were purchased from Chengdu Purification Technology Development Co., Ltd

(China). Components of soybean sterol were analyzed by using a GC (7890A, Agilent, USA), the soybean sterol contents were β-sitosterol; 46.7%, stigmasterol; 27.4% and campesterol; 25.3%.

2.2 Preparation of linoleyl chloride

LA was reacted with phosphorus trichloride (PCl_3) as shown in following Equation1.

$$3C_{17}H_{31}COOH + PCl_3 \rightarrow 3C_{17}H_{31}COCl + H_3PO_3 \quad (1)$$

PCl_3 is a kind of colorless liquid with pungent smell, it has a melting point of -112^oC and boiling point of 76^oC. So, the reaction temperature should not be too high. The mole ratio of LA to PCl_3 used in the reaction was 3:1. LA was put into a reaction bottle which connected to a condenser device. PCl_3 was then transferred slowly into the bottle at room temperature (25^oC), and the solution was constantly stirred. The resulting mixture was kept at a constant temperature of 60^oC for 3h. Then, the lower layer of H_3PO_3 was separated, and crude linoleoyl chloride (LC) was obtained. The crude product was exposed to vacuum at 65^oC for 0.5h so that the residual PCl_3 could be removed by distillation.

2.3 Synthesis and purification of PLE

PS was esterified with LC as shown in Equation 2.

$$C_{17}H_{31}COCl + R-OH \rightarrow C_{17}H_{31}COOR + HCl \quad (2)$$

R—OH represents β-sitosterol, stigmasterol, and campesterol respectively in the equation. Because the reaction rate of acetylation is fast, the temperature does not need to be too high. PS was reacted with LC in different mole ratio of 1:1, 1:1.1, 1:1.2, 1:1.3, and 1:1.4 respectively. The mixture was reacted at different temperatures (90, 80, 70, 60, and 50^oC) and at different time (0.5, 1, 1.5, 2, and 2.5h). The generated HCl gas was absorbed by a diluted NaOH solution to promote the reaction. Crude product of PLE was dissolved in hexane and excessive saturated $NaHCO_3$ solution was added into the hexane and mixed thoroughly to remove the non-reacted LC. Finally, the upper layer of hexane was collected and distilled in vacuum to remove hexane, and product of PLE was obtained.

105 **2.4 Analysis of the conversion rate**

106 **2.4.1 Preparation of PLE standards**

107 There are no commercial standards of PLE. LC, campesterol (≥98%), stigmasterol (≥98%),
108 and β-sitosterol (≥98%) were used to synthesis standards of PLE at 80^{o}C for 1.5h. Mole ratio
109 of PS and LC was 1: 1.1. The crude standards of campesterol linoleic ester, stigmasterol
110 linoleic ester, β-sitosterol linoleic ester were dissolved in hexane respectively. Excessive
111 saturated $NaHCO_3$ solution was added into hexane and mixed thoroughly. Then the hexane
112 layer was taken out and dried with nitrogen gas flow. Aliquot of sample was analyzed by
113 HPLC (2695, Waters, USA) using area normalization method, and a HPLC condition was
114 used (see 2.4.2 section). A noted insignificant amount of PS in the product (PLE ≥98%),
115 connoted that the standard was qualified.

116 Standards of campesterol linoleic ester, stigmasterol linoleic ester, β-sitosterol linoleic ester
117 were weighed accurately and dissolved in acetone to make a standard solution. 10μL of five
118 different concentrations (0.01, 0.05, 0.25, 0.5, 1.0mg/mL） of the standard solution were used
119 to generate a standard curve.

120 **2.4.2 HPLC analysis**

121 Conversion rate (CR) analysis of PS ester was performed with HPLC (2695, Waters, USA)
122 equipped with an ultraviolet detector (UV, 2489, Waters, USA). Mobile phase was
123 acetonitrile and acetone (1:3, V/V), flow rate was 1.0mL/min. Chromatography column was
124 Symmetry-C18 (4.6mm×150mm, 5μm) and the detection wavelength was 210nm. CR was
125 calculated as Equation 3.

$$CR = \left(\frac{\text{Mass of PLE}}{\text{Mass of PS + Mass of PLE}}\right) \times 100\% \qquad (3)$$

126

127 **2.5 Structural and physicochemical properties**

128 **2.5.1 Fourier transform infrared spectrometer (FTIR) analysis**

129 Attenuated total reflectance (ATR) analysis was performed using a FTIR (Cary 630, Agilent,

USA) for spectra measurement in the frequency range of 4000-650cm^{-1}.

2.5.2 Thermodynamic analysis

Differential scanning calorimeter (DSC 1, Mettler-Toledo, Switzerland) was used to determine the thermodynamic properties of the PLE. Sample mass of PLE was 7.3mg (PS was 5.1mg). The PLE sample was heated from －20 to 90^{o}C (PS was from 0 to 180^{o}C) and then cooled from 90 to －20^{o}C (PS was from 180 to 0^{o}C) using a programmed temperature of 10^{o}C /min. Flow of nitrogen gas was 50mL/min.

2.5.3 Solubility in oil

The solubility of the synthesized PLE was assessed using previously described method except that mixture of PLE and oil was stirred evenly at 80^{o}C (Yang et al., 2016). Briefly, excessive PLE was taken and added into soybean oil, rapeseed oil, peanut oil, corn oil, sunflower oil respectively. The mixture of PLE and oil was then stored at －5, 5, 15, and 25^{o}C until the oil became clear and transparent. The upper layer of oil was taken out. PS content was analyzed by applying GC method as previously described (Naeemi, Ahmad, Alsharrah, & Behbahani, 1995).

2.5.4 pH stability

PH stability was studied at different pH values（2.0-12.0）for the PLE application in food. 300mL water was taken and divided into six portions (each 50mL), solution of HCl and NaOH were used to adjust the pH value to 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0. 200 mg PLE was added into each of the mixtures and stirred for 30 minutes at room temperature (25^{o}C). 20mL hexane was added into the mixture and the resulting mixture was fully stirred for 5 minutes, then the organic phase was decanted and dried with nitrogen gas flow. The residue was dissolved in acetone for HPLC analysis.

2.5.5 Oxidative stability

LA, PLE, soybean oil, soybean oil of 1% PS and soybean oil of 1% PLE were taken into an open container and placed into a 60^{o}C oven according to AOCS Recommended Practice Cg 5-

97 (2009). Peroxide value was determined at different time (24, 48, 72, 96, 120, 144 h) according to AOCS-method 8b-90 (2009). The peroxide value was used as the evaluation of oxidative stability of PLE.

2.6 *In vitro* gastrointestinal digestion

2.6.1 *In vitro* gastric digestion

Simulated gastric fluid (SGF) was prepared according to the methods described in a literature (Anwesha, Kelvinkt, Rpaul, & Harjinder, 2009). 2g of NaCl and 7mL of HCl (37%, w/v) were dissolved in 800mL water. PH was adjusted to 1.2 and pepsin (6.4mg/mL) was added into the SGF and stirred evenly before use.

80mL of SGF was introduced into two respective 250mL conical flasks and then agitated in a Water Bath Orbital Shaker (MaxQ7000, Thermo Fisher, USA) at 37^{o}C and 120rpm for 10min. PS and PLE (2g each) were taken and added into SGF respectively, the mixtures were homogenized. Then 10mL of SGF was taken out, 50mL solvent of methanol and chloroform (1:2 v/v) was added and vortexed for 5min. Organic phase was taken out after layering and dried with nitrogen gas flow. The dried residue was dissolved in acetone and examined by HPLC as described above. The results were recorded as *in vitro* gastric digestion of 0min. The other mixtures were taken out 10mL at 5, 10, 20, 30, 60, 90min for HPLC analysis.

2.6.2 *In vitro* intestinal digestion

Simulated intestinal fluid (SIF) was prepared according to method of previous researchers (Fu et al., 2015) with some modifications. SIF of fasted state; 23.4g NaCl, 6.8g KH_2PO_4 and 1.665g $CaCl_2$ were dissolved in 1000mL water and pH was adjusted to 7.2. Then the solution was divided into two 500mL portions. The final concentration (0mg/mL and 2.5mg/mL) of bile extract were made respectively. 6.0mg/mL lipase, 10μg/mL colipase, 5 units cholesterol esterase and 1% trypsin were added before use. SIF of fed state was prepared as the same method of fasted state with the only difference being the final concentration (10mg/mL and 40mg/mL) of bile extract. SGF and samples were prepared as previously described in section

2.6.1. 10mL of SGF was taken and the pH of SGF was adjusted to the same value as SIF by using 1.0N NaOH solution.10mL of SIF was incubated at 37^{o}C, then was added. The resulting mixture and sample preparation were done as described in Section 2.6.1.

The dried residue of nitrogen flow was examined by GC (7890A, Agilent, USA.) according to the method in literature (Toivo, Piironen, Kalo, & Varo, 1998) with little modification. The carrier gas was nitrogen with a flow rate of 2mL/min. The capillary GC column was HP-5 (30m×0.25mm, 0.25μm). Temperature of the column was from 180 to 280°C using a programmed temperature of 10°C/min, then 280°C was kept for 20minutes. Temperatures of injector and detector were 300 and 280°C respectively. The injection volume was 1μL. Bioaccessibility was calculated as presented in Equation 4. Mass of PLE was converted to the mass of PS.

$$\text{Bioaccessibility（%）} = \left(\frac{\text{Mass of PS dissolved in micelle}}{\text{Total mass of PS in different product}}\right) \times 100\% \qquad (4)$$

The bioaccessibility result was recorded as *in vitro* intestinal digestion of 0min. The resulting SIF mixtures were reacted for 10, 30, 60, 90, 120 and 180min using the same method, and their bioaccessibility results at different time were calculated.

Because PS and phytosterol ester are mostly used in oil, 2g soybean oil of each containing either 1% PS or 1% PLE were taken to examine the bioaccessibility of PS and PLE when oil was used as a carrier.

2.7 Statistical analysis

All the experiments were done in triplicate and the results were expressed as the means and standard deviations. Statistical analysis was performed by one-way analysis of variance using the Statistical Package for the Social Sciences v. 20.0 (SPSS, Chicago, IL, USA).

3 Results and discussions

3.1 Effects of reaction conditions on synthesis of PLE

Different mole ratios (1:1, 1:1.1, 1:1.2, 1:1.3, and 1:1.4) of PS and LC respectively, varying temperatures (50, 60, 70, 80 and 90^{o}C) and different time (0.5, 1, 1.5, 2, and 2.5h) were used to optimize the synthesis of PLE. CR of PS and LC to form PLE increased with increasing mole ratio, increasing reaction time and increasing temperature, reaching a maximum value of 97.5, 96.8 and 96.5% respectively (Table 1). It appears that the optimum conditions (temperature of 80^{o}C for 1.5h) to generate moderate amounts of PLE was a mole ratio of 1:1.1 of PS and LC respectively. The CR under these conditions was 96.1% (Table 1). Although from the chemical equation (2), the mole ratio of the reaction is 1:1 of PS and LC respectively. However, at this mole ratio the CR was low (90.1%) compared with other mole ratios. At the optimum mole ratio of 1:1.1 of PS and LC respectively with a CR of 96.1%, there was no need to increase the quantity of LC. The more LC that was used the more difficult the separation was, and this led to wastages. Beyond the optimum mole ratio of 1:1.1, optimum reaction time of 1.5h and optimum temperature of 80^{o}C, there was no significant changes in the CR. Thus, the reaction time of 1.5h is suggested as an optimal energy saving time period. Previous research on the synthesis of PE reported very long reaction times (approx. 8h) for chemical methods (Meng et al., 2010; Robles-Manuel et al., 2011) and enzymatic methods (24-168h) (Pan et al., 2012; Torres, Torrelo, Vazquez, Señorans, & Reglero, 2008). Therefore, the reaction time of PS and LC in the current study provides a remarkably shorter reaction time in comparison with those of chemical and enzyme methods. Furthermore, the CR in this study appear slightly higher than values (80-93.4%) reported for enzymatic methods (Pan et al., 2012; Torres et al., 2008), but comparable to those reported for chemical methods (89-98%) (Meng et al., 2010; Robles-Manuel et al., 2011). Another interesting finding in this study is the relatively low reaction temperature of 80^{o}C which is substantially lower than the reaction temperature (170-240^{o}C) reported for chemical methods (Meng et al., 2010; Robles-Manuel et al., 2011; Valange et al., 2007). High temperature treatment confers a darkened

product due to oxidation during heating. Consequently, it is suggested that reaction temperature should be 80^{o}C in order to prevent loss in product quality.

3.2 HPLC analysis

PLE product (Fig. 1A) and three components of PLE standard (Fig. 1B) were analyzed by HPLC. Retention time of campesterol linoleic ester, stigmasterol linoleic ester, and β-sitosterol linoleic ester were 12.3 min, 13.0 min, and 13.7 min respectively, which corresponds to the standards in Fig. 1A. Product purity (quantified by PLE standards) was 95.7% in mole ratio of 1:1.1 under the condition of 80^{o}C for 1.5 h. The product purity was sufficient to meet the requirement in food, medicine, nutraceuticals and cosmetic applications. Although the product may have residual PS and LA, the quantities of these two components are negligible amount and may not be detrimental to both product quality and human health.

3.3 FTIR

Structural analysis using FTIR was used to confirm the formation of PLE (Fig. 2). FTIR analysis of PS shows a strong absorption peak at around 3400 cm^{-1} (Fig. 2A), but the peak was absent in PLE (Fig. 2B). The peak at 3400 cm^{-1} corresponds to the absorption of -OH. There were two additional strong absorption peaks at 1734 cm^{-1} and 1172 cm^{-1} found in the FTIR spectra of PLE (Fig. 2B). These peaks corresponds to the stretching vibration of C=O and C－O－C respectively and confirms that the －OH group of PS had been esterified. Previous studies by Panpipat, Xu, and Guo (2013), reported absorption peaks at 1741cm^{-1} and 1172 cm^{-1} for β-sitosterol myristate. Hang and Dussault (2010) reported that absorption spectra at 1730cm^{-1} for campesterol acetic ester. Other researchers also reported absorption peaks at 1736cm^{-1} and 1171 cm^{-1} for steryl ester of polyunsaturated fatty acid (Shimada et al., 1999). The results in this paper are similar with previous studies, which confirm the formation of ester bonds.

3.4 Thermodynamic analysis

By the use of DSC, the melting and crystallizing points of PLE were 22.75^{o}C and －4.18^{o}C

respectively (Fig. 3A), which were substantially lower than those of PS (melting point = 139.34^{o}C; crystallizing point =119.6^{o}C) (Fig. 3B). The DSC result suggests that the esterification process significantly reduced the melting and crystallizing points of PS. Previous studies mentioned that the melting point of commercial phytosterol ester was 18.4^{o}C (Daels, Foubert, & Goderis, 2017). Crystallization onset temperature of β-Sitosteryl conjugated linoleic acid (CLA) was at −0.7^{o}C, and with maximum at −7^{o}C (Vu et al., 2004). Our result is a little different with the literatures. This may be attributed to the differences of phytosterol esters. Commercial phytosterol ester was a mixture of β-sitosterol (78.5%), Campesterol (10.0%), β-Sitostanol (9.7%), Brassicasterol (1.3%) and Stigmasterol (0.6%). β-Sitosteryl CLA contained CLA isomers (72%), palmitic (6%) and oleic acid (17%). Because the melting temperature of PLE is low, which makes it liquid in the body. It can also be inferred that bioaccessibility of PS may change after esterification.

3.5 Solubility of PLE in different edible oils

The solubility of PLE in soybean oil, corn oil, rapeseed oil, peanut oil and sunflower oil was assessed at temperatures varying between –5 and 25^{o}C, because edible oils are commonly stored at room or below room temperature (Yang et al., 2016). PLE was soluble in all the edible oils used in this study and the solubility increased with increasing temperature (Table 2). However, the solubility of PLE was not significantly affected by the type of oil used. Yang et al. (2016) reported that the solubility of phytosterol ester in soybean, rapeseed, and sunflower oil at –5 and 5^{o}C were not very different. According to their report variation in the solubility in the vegetable oils could probably be linked to the differences in fatty acid composition in the different oils at their respective ester links.

3.6 pH stability

The stability of PLE hydrolyzed for 30 min at room temperature (25^{o}C) under different pH (2.0-12.0) conditions showed that the purity of the products were significantly ($P > 0.05$) unchanged (Fig. 5). The stability result suggested that PLE was stable within the pH range of

284 food (2.0-12.0). Also, it was not easily decomposed, therefore it could be used in different
285 food applications with wide pH range.

286 **3.7 Oxidative stability**

287 The oxidative stabilities of LA, PLE, soybean oil, soybean oil of 1% PLE and soybean oil of
288 1% PS analyzed for seven days are shown in Fig. 6. With increasing storage period, the
289 peroxide value of LA increased faster than others at 60^{o}C (under the condition of accelerated
290 oxidation). Oxidative stability of PLE was better than LA, and it was less stable compared
291 with soybean oil. Changes in the peroxide value of soybean oil were not very obvious during
292 a short time storage after PLE and PS were added. But, the peroxide value of PLE-soybean oil
293 was higher during a long time storage compared the value in soybean oil alone. The peroxide
294 value of PS-soybean oil was lower than soybean oil. Studies had shown that, oxidative
295 stabilities decreased when the adding concentration of sterol ester were above 1 and 3% for
296 rapeseed oil and flaxseed oil (Qianchun et al., 2011). The sterol ester used in the literature
297 was also steryl ester of polyunsaturated fatty acid. The lower oxidative stability of LA
298 compared to other samples could be attributed to the presence of 2 unsaturated double bonds.
299 These bonds are prone to oxidation and are well-known to promote oxidative rancidity in oils.
300 PS has a stable structure of polycyclic hydrocarbons which are difficult to oxidize. The
301 oxidative stability of PLE is between LA and PS. PLE and PS were added into soybean oil.
302 On one hand, PLE accelerated the oxidation of soybean oil, and on the other hand PS played
303 the role of an antioxidant. If PLE is added into edible oil as a functional food, it may possibly
304 confer a shortened edible oil shelf life. Therefore, it is recommended to take some anti-
305 oxidative measures. In contrast to our results, Winkler and Warner (2008) found the effect of
306 added phytosterols (1.0-2.5% by weight) on the oxidative stability of soybean oil were not
307 significantly different. However, the soybean oil they used was a special kind (stripped
308 tocopherols and phytosterols).

309 **3.8 *In vitro* gastric digestion**

Bioaccessibility of PLE in SGF was analyzed by HPLC. The contents were almost unchanged after the PLE hydrolysis in SGF for 0, 5, 10, 20, 30, 60 and 90 min. This might be attributed to the limited hydrolysis of PLE under body temperature (37^{o}C) in SGF. In addition, PLE was stable in SGF. Extension of the time did not affect the content of PLE. Because there is no protein in PLE; the proteases in the stomach did not act on PLE. This enabled the PLE to avoid being broken down in stomach. If PLE is hydrolyzed in stomach, it will produce PS and LA. PS is crystalline in nature and present in the stomach acid. Hence, it is difficult to be absorbed by the intestinal cells. Also, it is very possible that the PLE enters into intestinal tract in liquid form which is more easily assimilated in the human body. Mayer, Weiss, and Mcclements (2013) did similar lipid digestion research of vitamin E acetate (a group of synthesized oil-soluble compounds like PLE). They found the vitamin E acetate was relatively stable to gastric conditions. Their reports are in agreement with our results. Other studies of PS bioaccessibility have shown that presence of milk increase bioaccessibility of total and individual PS, since milk lipids are an effective delivery system for highly lipophilic microconstituents (Alemany et al., 2013). PLE is also a kind of lipid, we may infer that PLE is more effective in delivery system than PS.

3.9 The effects of bile extract and time on bioaccessibility in SIF

Different concentrations (1-40mg/mL) of bile extract have been used in different simulated gastrointestinal experiments (Fu et al., 2015; Lesmes & Mcclements, 2012; Liang et al., 2012). PH values of SIF are also different in reported *in vitro* experiments. Simulated intestinal fluid (Fu et al., 2015), simulated small intestinal fluid (Dan & Socaciu, 2014; Mayer et al., 2013) and simulated duodenal fluid (Granado-Lorencio, Donoso-Navarro, Sánchez-Siles, Blanco-Navarro, & Pérez-Sacristán, 2011; Granado-Lorencio et al., 2007) are the most widely used SIF with pH values ranging from 6.8 to 8.0.

Bioaccessibilities in SIF of fasted state (0mg/mL, 2.5mg/mL) and fed state (10mg/mL, 40mg/mL) at pH 7.2 for 4h were reported in this study (Table 3). Bioaccessibility increased

with increasing bile extract concentration. This is consistent with the conclusion that concentration of bile extract has an effect on lipid absorption in other reports (Fu et al., 2015; Lesmes & Mcclements, 2012). Under fed state, bioaccessibilities of PS and PLE in the oil solution were 21.72%, 53.64%, respectively, the bioaccessibilities of PS and PLE were lower (1.59% and 4.93%). Under fasted state, the bioaccessibilities of the four substances were not satisfactory and were almost not absorbed when the concentration of the bile extract was 0mg/mL. In addition, they were very low (0.12% to 1.37%) when the concentration of the bile acid was 2.5mg/mL. This may be attributed to the weak emulsifying effect of low bile extract concentration, which affected the absorption of PS and PLE.

Food can be digested more completely in the intestine for more than 2h. Different digestion time (2-6h) in the intestine has been reported (Chen & Li, 2012; Mcdougall, Fyffe, Dobson, & Stewart, 2005; Silva, Bezerra, Santos, & Correia, 2015). In this study, bioaccessibilities of PS and PLE were investigated at different times (0-180min) and at pH 7.2 under the condition of 40mg/mL bile extract (Table 4). Bioaccessibilities of ester and oil changed more noticeably at different time in SIF. Values of PLE ranged from 0.61% to 5.28%, 1% PS oil; 1.19% to 28.16%, and 1% PLE oil; 2.97% to 59.78%. This may be attributed to the time of hydrolysis of lipase and the concentration of bile extract. The longer the reaction time, the higher the hydrolytic effect of lipase and the higher the bioaccessibilitiy. When the concentration of bile extract is high, the emulsification effect is stronger and the bioaccessibilitiy is better. As the PS is not a kind of ester, bioaccessibilitiy is not affected obviously by the time increased.

4. Conclusions

This study demonstrated that PLE can be efficiently synthesized by the chloride method from soybean sterol and LA. The CE of PS was above 96% at 80^{o}C for 1.5h with a 1:1.1 mole ratio of PS and LA, this condition was chosen as an optimum method to synthesize PLE. The physicochemical properties of the synthyzed PLE was analyzed by HPLC and FTIR. Melting

and boiling points of PLE were significantly lower than PS. Solubility of PLE in oil was higher than PS, and PLE had a good pH stability. Accelerated oxidation experiment showed that PLE was easily oxidized, but it was more stable than LA. Bioaccessibilitiy of PLE was better than PS using *in vitro* experiments, especially when oil was used as a vehicle. PLE synthesized using current method showed a strong potential application in food and nutraceuticals

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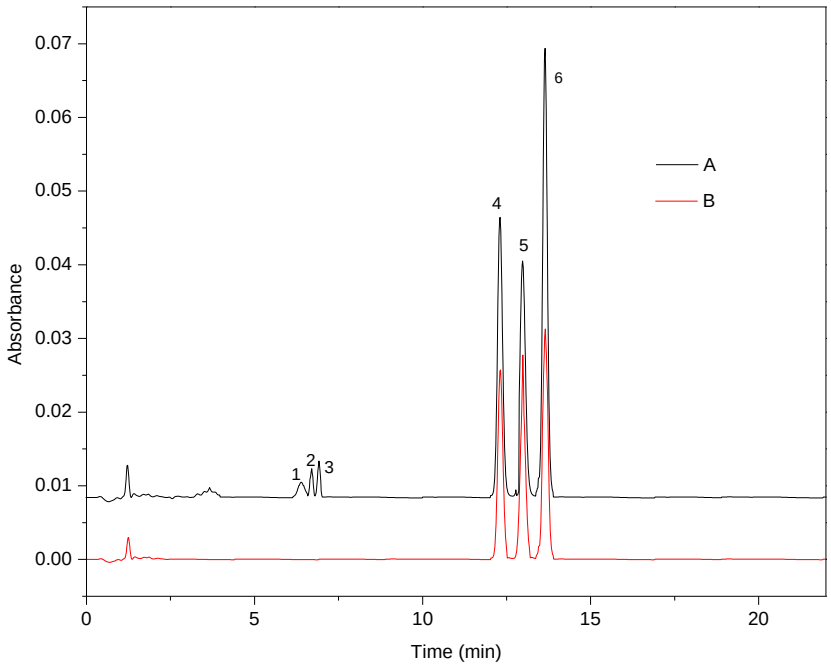

Fig. 1 HPLC analysis of phytosterol linoleic ester product and standards

(A) Phytosterol linoleic ester product, (B) Phytosterol linoleic ester standards

(1) campesterol (2) stigmasterol (3) β-sitosterol, (4) campesterol linoleic ester,

(5) stigmasterol linoleic ester (6) β-sitosterol linoleic ester

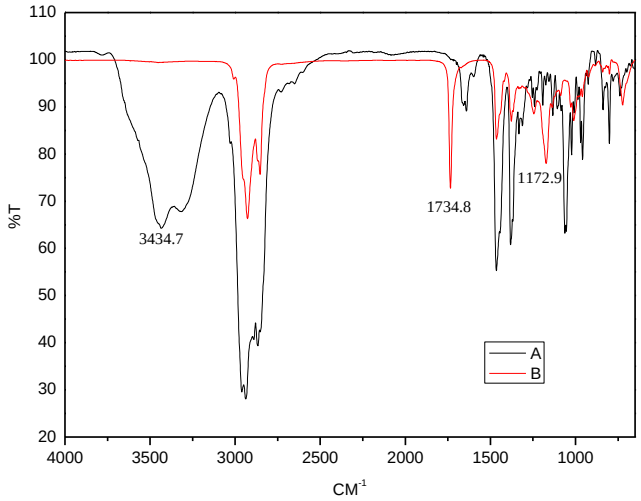

Fig. 2 FTIR spectra of phytosterol (A) and phytosterol linoleic ester (B)

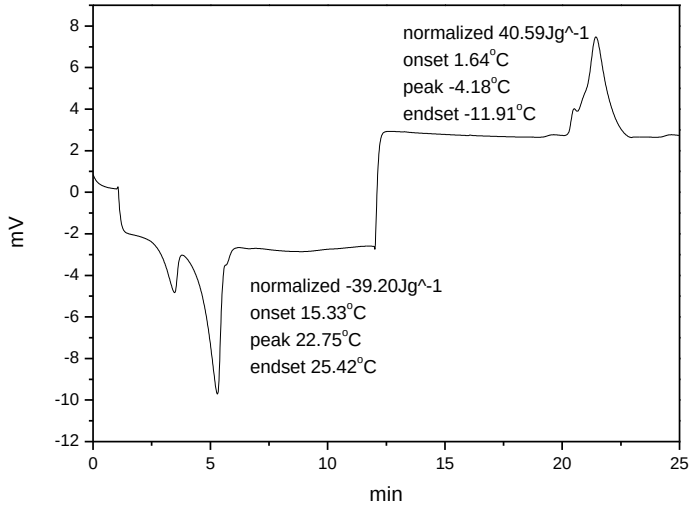

Fig. 3 Thermodynamic analysis of phytosterol linoleic ester

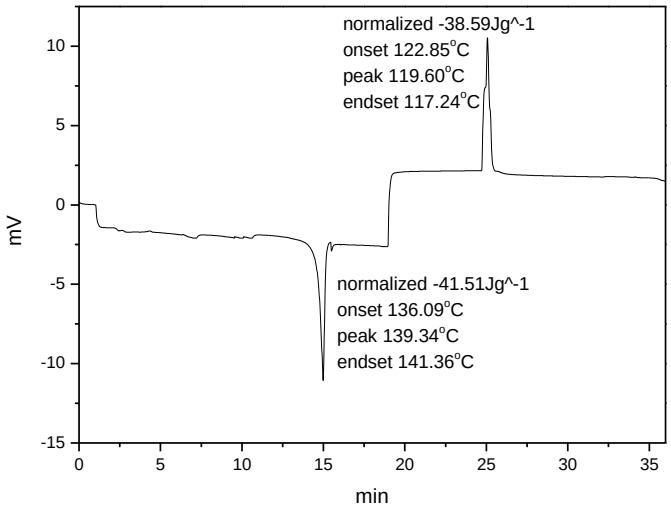

Fig. 4 Thermodynamic analysis of phytosterol

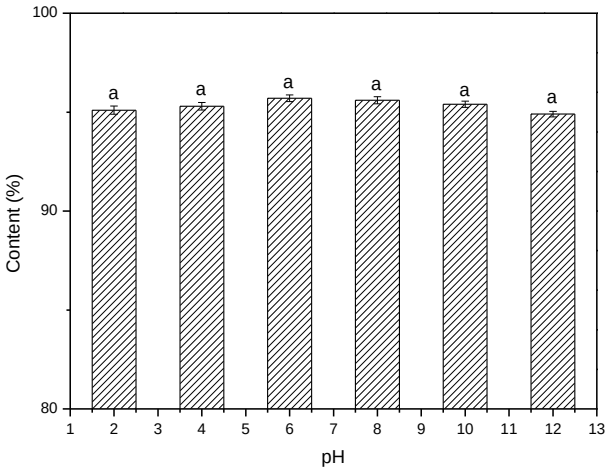

Fig. 5 Contents of phytosterol linoleic ester at different pH value for 30 minutes

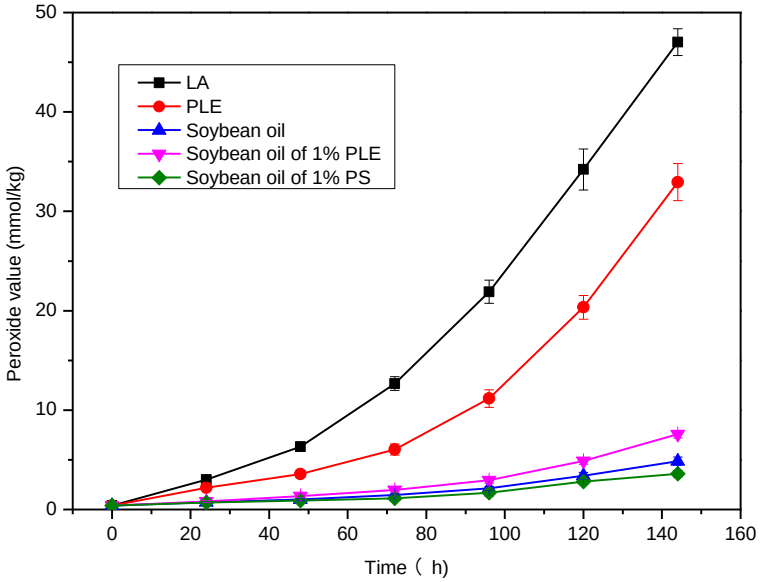

Fig. 6 Oxidative stability of phytosterol linoleic ester at 60^{o}C at different time

524 Table 1. Effect of mole ratio (PS ratio LC), temperature, and time on synthesis of PLE.

Effect of mole ratio (80°C,1.5 h)		Effect of time (1:1.1,1.5 h)		Effect of temperature (1:1.1,1.5 h)	
Mole ratio	CR (%)	Time (h)	CR (%)	Temperature (^{o}C)	CR (%)
1:1	90.1±1.04	0.5	85.6±1.03	50	60.1±1.87
1:1.1	96.1±1.17	1.0	91.7±1.12	60	76.2±1.18
1:1.2	96.3±1.33	1.5	96.1±1.17	70	85.4±1.27
1:1.3	96.7±1.46	2.0	96.4±1.35	80	96.1±1.17
1:1.4	97.2±1.21	2.5	96.8±1.41	90	96.5±1.69

525

526 Table 2. Solubility of PLE in different oils (g/100 g) at different temperature

Temperature	Soybean	Rapeseed	Peanut	Corn	Sunflower
–5℃	13.2^{a}±1.21	14.3 ^{a} ±0.67	---	13.3 ^{a} ±0.58	13.5 ^{a} ±0.86
5℃	17.3 ^{a} ±0.76	18.2 ^{a} ±0.89	---	17.5 ^{a} ±1.12	17.4 ^{a} ±0.67
15℃	23.9 ^{a} ±0.97	24.2 ^{a} ±1.02	23.1^{a}±1.33	23.4 ^{a}±0.95	23.3 ^{a} ±1.53
25℃	32.7 ^{a} ±1.17	33.8 ^{a} ±1.23	32.2 ^{a} ±0.92	32.3 ^{a} ±0.85	32.4 ^{a} ±1.19

527 Mean± SD. Mean with different superscript along the row are significantly different (p<0.05).
528

529

530 Table 3. Effects of bile extract on bioaccessibility at pH 7.2 for 120min

Products	Bioaccessiblities of different bile extract concentrations (%)			
	0mg/mL	2.5mg/mL	10mg/mL	40mg/mL
PS	0	0.12±0.02	0.36±0.04	1.59±0.24
PLE	0	0.32±0.03	1.21±0.12	4.93±0.31
1% PS oil	0	0.66±0.11	5.02±0.24	21.72±1.35
1% PLE oil	0	1.37±0.14	11.75±0.27	53.64±1.79

531

532

533 Table 4. Effects of time on bioaccessibility at pH 7.2 and bile extract concentration of
534 40mg/mL

Products	Bioaccessiblities at different time (%)						
	0min	10min	30min	60min	90min	120min	180min
PS	0	0.42±0.08	1.07±0.14	1.51±0.16	1.56±0.19	1.59±0.24	1.61±0.21
PLE	0	0.61±0.11	1.28±0.16	1.67±0.18	2.37±0.23	4.93±0.31	5.28±0.34
1% PS oil	0	1.19±0.16	2.21±0.29	6.13±0.64	11.79±1.02	21.72±1.35	28.16±1.47
1% PLE oil	0	2.97±0.32	7.79±0.51	19.77±0.87	35.48±1.18	53.64±1.79	59.78±1.63

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