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Oxidative stability of margarine enriched with different structures of β-sitosteryl esters during storage

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ABSTRACT

Oxidations of margarine incorporated with β-sitosteryl esters of hexanoic (Sito-C6), lauric (Sito-C12), palmitic (Sito-C16) and oleic (Sito-C18:1) acids were evaluated during storage at ambient temperature (25 °C) and at 55 °C for 20 days in comparison with native β-sitosterol. All oxidative indices, including hydroperoxide value (HPV), thiobarbituric acid reactive substances (TBARS), *p*-Anisidine value (AnV) and 7-keto derivatives, tended to increase with increasing storage time. Overall, losses of sterol esters in margarine during storage at both temperatures fitted a first order kinetic model. Margarine with unsaturated ester oxidized faster ($k = 0.0355\ day^{-1}$, $R^2 = 0.8126$) than those with saturated counterpart and native β-sitosterol, particularly at higher temperature (55 °C). As a consequence, sterol structure and storage temperature were the main factors influencing the oxidative stability of margarine enriched phytosterol esters during storage.

1. Introduction

Phytosterol esters have been synthesized for fortification in various food products as a functional ingredient and cholesterol lowering property was the main target for these esters (Abumweis et al., 2006; Clifton, Noakes, Sullivan, & Erichsen, 2004; Noakes, Clifton, Doornbos, & Trautwein, 2005). Phytosterol esters are able to solubilize in lipid and then they are commonly supplied into fat-based food products rather than its crystalline free form in order to achieve the effective dose. It has been reported that the enrichment of phytosterols into spreads, mayonnaise/salad dressing and milk/yoghurt was associated with a greater efficacy in LDL-cholesterol reduction than incorporation into other foods, suggesting the important role of the food matrix in the cholesterol-lowering effect (Abumweis et al., 2006). However, phytosterols and derivatives as lipid compounds are prone to oxidation, particularly when being subjected to heat treatments and long term storage. Oxidation of phytosterol fatty acid esters followed the same free radical mechanism as that of free phytosterols (Lercker & Rodrigues-Estrada, 2002). The mechanism of sterol oxidation was similar to free radical oxidation of fatty acids (Rudzińska, Przybylski, Zhao, & Curtis, 2010). Additionally, lipid matrix has been reported as one of the factors influencing the oxidation of phytosterols and derivatives (Soupas, Juntunen, Lampi et al., 2004). Soupas, Juntunen, Lampi et al. (2004) established that the phytosterol structure and lipid matrix are the main factors affecting the formation of oxidative derivatives. In the case of unsaturated lipid matrix, the oxidation of lipid matrix and sterol was coupled. The oxidative process has started with oxidation of the unsaturated lipids and continues to the sterol compounds. Furthermore, phytosterols would be particularly prone to oxidation when they are incorporated in emulsions (Rudzińska, Przybylski, & Wąsowicz, 2014). Since the oxidation can be enhanced at the interface when compared to oil or water phase (Cercaci, Rodriguez-Estrada, Lercker, & Decker, 2007). Margarine, one of the emulsion-based products, is commonly used for cooking which may enhance oxidation of phytosterol present therein, leading to the formation of several oxidation products. It has been reported that phytosterol oxidation and degradation occurred during storage or heating of enriched margarine (Rudzińska, 2014; Lin et al., 2017; Scholz, Menzel, Lander, & Engel, 2016). However, no systematic research has been carried out on oxidative stability of margarine incorporated with series of β-sitosteryl fatty acid esters during storage. As a consequence, this study aimed to evaluate the oxidative stability of margarine enriched with four enzymatically synthesized phytosterol fatty acid esters including β-sitosteryl hexanoate (Sito-C6), β-sitosteryl laurate (Sito-C12), β-sitosteryl palmitate (Sito-C16) and β-sitosteryl oleate (Sito-C18:1), in comparison with native phytosterol (β-sitosterol), during storage at ambient temperature (25 °C) and at elevated temperature (55 °C) for 20 days. The structure-stability relationship and the influence of storage temperature on the

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rate of oxidation were mainly focused in this study.

2. Materials and methods

2.1. Chemicals

β-Sitosterol was purchased from Sigma-Aldrich (St. Louis, MO, USA). Hexanoic acid, lauric acid, palmitic acid and oleic acid were from Nu-Chek Prep, Inc. (Elysian, MN, USA) with a minimum purity of 95%. Immobilized *Candida antarctica* lipase A, NZL-101 was purchased from Codexis, Inc. (Pasadena, CA, USA). All solvents such as methanol, diethyl esther, *n*-hexane and acetone were of HPLC grade and obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Synthesis of phytosteryl fatty acid esters

In this study, β-sitosterol was selected as a model substrate for synthesis of phytosteryl fatty acid esters because it is the most abundant phytosterol found naturally in plants and plant products. A series of aliphatic fatty acid esters of β-sitosterol were prepared according to the method of Panpipat, Dong, Xu, and Guo (2013) by mixing β-sitosterol with short chain- (hexanoic acid), medium chain- (lauric acid), long chain- (palmitic acid) and unsaturated- fatty acid (oleic acid) at a mole ratio of 1.0:1.0 (mol/mol). The concentrations of β-sitosterol and fatty acid were fixed at 0.2 M. The reaction was typically performed at 40 °C for 24 h with agitation at 500 rpm using hexane as a solvent in the presence of 5% *Candida antarctica* lipase A (CAL A) (wt% of β-sitosterol). The reaction was terminated by filtering out the enzyme. The resulting reaction mixtures were applied onto a thin layer chromatography (TLC) with silica gel to confirm the formation of products. The plates were developed with cyclohexane/ethyl acetate (4:1, v/v).

To remove unreacted fatty acids, the reaction mixture (1 g) was dissolved in 10 ml diethyl ether. The extracts were deacidified by three consecutive treatments with 6 ml, each, of 2% aqueous sodium carbonate solution, followed by repeated extractions with water, to remove the unesterified fatty acids as sodium salts. The diethyl ether phase was dried over anhydrous sodium sulfate to yield 530 mg of a product containing < 2% unesterified fatty acids. Future more, the deacidified reaction mixture (around 0.5 g), as obtained earlier, was dissolved in 5 ml hexane:diethyl ether (9:1, v/v) and applied to a column (20 cm × 1.5 cm i.d.) packed with Silica Gel 60 (Merck) as a slurry in hexane. Elution with 30 ml hexane:diethyl ether (95:5, v/v) yielded 168 mg crude reaction products containing 67% steryl esters. Subsequent elution with 30 ml hexane:diethyl ether (1:1, v/v) yielded unreacted fatty acids together with small proportions of unreacted sterols. Thereafter, 130 mg of the crude steryl esters from column chromatography, was dissolved in 2 ml acetone at 50 °C. Water, 0.2 ml, was added dropwise under shaking. After cooling to 7 °C, the acetone-water mixture was centrifuged and the supernatant removed. The pellet was redissolved in 2 ml acetone and the procedure repeated. After four cycles, the pellet (85 mg) consisted of 90% steryl esters. The resulting products were then dissolved in diethyl ester and subsequently applied to TLC in order to confirm the purity. The steryl esters prepared using this method had the purity over 99% identified by FTIR and ^{1}H NMR (Panpipat et al., 2013). The chemical structure of β-sitosterol and its fatty acid esters used in this study are presented in Fig. 1.

2.3. Margarine sample and storage procedure

Margarine was prepared by mixing 80% (w/w) lipid phase with 20% (w/w) aqueous phase. In the lipid phase, it composed of 79.3% (w/w) of mixed canola oil and hydrogenated sunflower oil and 0.7% (w/w) of mixed emulsifier containing glyceryl monostearate, glyceryl distearate and lecithin. For the aqueous phase, 1% (w/w) sodium chloride was incorporated. Margarines were enriched with β-sitosteryl esters or commercial β-sitosterol at a level of 1.7% (w/w). Enriched margarines were packed in plastic containers and subjected to storage at ambient temperature (25 °C) and accelerated temperature at 55 °C in a controlled temperature oven (Memmert, Schwabach, Germany) for 20 days. Samples were taken at day 0, 5, 10, 15 and 20 for analyses. Measurements were made for lipid hydroperoxide (HPV), thiobarbituric acid reactive substances (TBARS), *p*-Anisidine value (AnV), 7-keto derivatives and non-oxidized phytosterol esters. Remaining of phytosterol esters and their kinetic parameters were also calculated. The first order kinetic model corresponded to $ln\ (Sito_t/Sito_0) = -kt$ was used. The kinetic plot of $ln\ (Sito_t/Sito_0)$ against time (t) was made and the slope was defined as k value. $Sito_t$ and $Sito_0$ referred to phytosterol ester content at time t and the initial phytosterol ester content, respectively. The R^2 coefficient of determination was also reported.

2.4. Determination of lipid hydroperoxide value (HPV)

HPV was determined using a modified method of Shantha and Decker (1994). Samples were mixed with 2.8 ml of choloform/methanol (2:1, v/v) and 30 μl of thiocyanate/Fe^{2+} solution and then vortexed. The thiocyanate/Fe^{2+} solution was made by mixing one part of 3.94 M thiocyanate solution with one part of 0.072 M Fe^{2+} solution (obtained from the supernatant of a mixture of one part of 0.144 M $FeSO_4$ and one part of 0.132 M $BaCl_2$ in 0.4 M HCl). After 20 min, the absorbance was measured at 510 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). HPV was determined using a cumene hydroperoxide standard curve.

2.5. Determination of p-Anisidine value (AnV)

AnV was determined spectrophotometrically using the standard method 2504 of IUPAC (1987) using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan)

2.6. Determination of thiobarbituric acid reactive substances (TBARS)

TBARS was determined by using spectrophotometric method as described by Ke and Woyewoda (1979). Sample (50 mg) was mixed with 5 ml of TBA solution. The solution was incubated in boiling water for 45 min. Mixed solution was cooled down to room temperature by using ice bath. Thereafter, TCA solution (4 ml) was added. The resulting solution was centrifuged at 2500 × *g* in a RC-5B plus centrifuge (Sorvall, Norwalk, CT, USA) for 10 min in order to separate the pink water phase from the chloroform phase (bottom). The water phase was collected and subjected to measure the absorbance at 538 nm against blank that was prepared as above without oil sample. Standard curve was made by varying concentration of 1,1,3,3-tetraethoxypropane (TEP) from 0 to 1.4 mg/l.

2.7. Analysis of phytosterol oxide

Analysis of phytosterol oxide was carried out according to the method of Soupas, Juntunen, Säynäjoki et al. (2004) by using 19-hydroxycholesterol as an internal standard in which the main steps were cold saponification (overnight at 25 °C), extraction of unsaponifiable material with diethyl ether, purification of oxides by SiOH-SPE, derivatization of oxides to TMS ethers and analysis by gas chromatography-mass spectrometry (GC-MS). The oxidized sample was dissolved in 5 ml of hexane/diethyl ether (9:1, v/v) and purified by the SiOH-SPE. In brief, the SiOH-SPE cartridge was activated with 5 ml of hexane, after that 1 ml of oxidized sample was applied. The cartridge was washed with 5 ml of hexane/diethyl ether (9:1 v/v) and 5 ml of hexane/diethyl ether (1:1 v/v) to remove a polar compounds and non-oxidized phytosterols, respectively. Phytosterol oxides were eluted with 5 ml of acetone. 19-Hydroxycholesterol (10 μg) was added to these acetone extracts to act as references, and the extracts was then dried under nitrogen, dissolved in 100 μl of pyridine, and subjected to silylation by