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Effect of β-sitosterol in the antioxidative activity of oxidized lipid–amine reaction products

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ABSTRACT

Phosphatidylethanolamine (PE), phosphatidylcholine (PC), lysine (Lys), and mixtures of them were tested for activity in a polar compound-stripped olive oil (SOO) and in the same oil after addition of 1500 ppm of β-sitosterol (phytosterol-added olive oil, PAO) to evaluate the role of phytosterols in the antioxidant activity of oxidized lipid–amine products. None of the added compounds protected either SOO or PAO, when tested alone at 0–400 ppm. However, mixtures of PE/Lys and PC/Lys (100/300, 200/200, and 300/100 ppm) significantly increased the induction periods of both oils. Furthermore, there was a synergism between the phospholipids and Lys, which was a consequence of the reaction between the carbonyl compounds produced in the oxidation of the phospholipid fatty acid chains and the amino group of Lys. Some of these carbonyl–amine reaction products were determined by gas chromatography–mass spectrometry after converting them into volatile derivatives. In addition, stepwise multiple regression analysis demonstrated the relationship between the induction periods and the formed products. However, the contribution of carbonyl–amine reaction products to oil stability also depended on the type of oil, therefore suggesting a role of β-sitosterol in the antioxidative activity of the compounds produced by carbonyl–amine reactions. This contribution was also confirmed by the higher synergism observed for PE/Lys and PC/Lys mixtures in PAO than in SOO.

1. Introduction

The antioxidative properties of carbonyl–amine reaction products were firstly observed in the early 1950s in an oil system (Franzke & Ivanisky, 1954) and they have been later shown in many other food systems (Hwang, Shue, & Chang, 2001; Mesa, Silván, Olza, Gil, & del Castillo, 2008; Rufián-Henares & Delgado-Andrade, 2009). However, neither the exact nature of the antioxidants formed (Manzoco, Calligaris, Mastrocola, Nicoli, & Lerici, 2001) nor the influence of other food components in the observed antioxidative activities are well-known at present. Thus, for example, a recent study from this laboratory has shown that antioxidative activities of oxidized lipid–amine reaction products are increased considerably in the presence of tocopherols (Hidalgo, León, & Zamora, 2007). However, to our knowledge the influence of other food components has not been yet investigated.

This study was undertaken in an attempt to determine the role of β-sitosterol, as a representative phytosterol, in the antioxidative activity of the oxidized lipid–amine reaction products formed during the oxidation of aminophospholipids and phospholipid/amino acid mixtures. Phytosterols are a group of steroid alcohols naturally occurring in plants. They are minor components of fats and oils, being generally β-sitosterol the major phytosterol (50–80% of total sterols). In addition, they are now added to spreads and other foods to reduce cholesterol absorption (Gunstone, 2008).

Oxidized lipid–amine reaction products are formed as a last step in the lipid oxidation process when it takes place in the presence of amino compounds (Hidalgo & Zamora, 2002). These compounds contribute to the changes produced in both the sensorial properties of foods and their antioxidant properties (Zamora & Hidalgo, 2005a). In addition, a recent study has shown that these compounds can be produced in situ and their antioxidative activities evaluated at the same time that they are produced, when they are formed in the vessel of a Rancimat apparatus (Hidalgo, León, & Zamora, 2006). Thus, in the Rancimat vessel, the oil is exposed to a stream of atmospheric oxygen at elevated temperature, which produces its oxidation. This oxidation is slowed down when the oil contains substances with antioxidative properties (Mastelic et al., 2008; Ramalho & Jorge, 2008; Velasco, Dobarganes, Holgado, & Márquez-Ruiz, 2009). However, the produced lipid oxidation products formed can also react with surrounding amino compounds, if they are present, and produce in situ oxidized lipid–amine reaction

Abbreviations: IP, induction period; Lys, lysine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PAO, phytosterol-added olive oil; S_F, synergism factor; SOO, polar compound-stripped olive oil.

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products with antioxidative activities that can modify the induction period of the oil to an extent that depends on both the type of compounds formed and the presence of minor components in the oils.

This study describes the stability of a polar compound-stripped olive oil (SOO), and the same oil after addition of 1500 μg of β-sitosterol/g of oil (phytosterol-added olive oil, PAO), in the presence of phosphatidylethanolamine (PE), phosphatidylcholine (PC), lysine (Lys), and mixtures of them.

2. Materials and methods

2.1. Materials

Phosphatidylethanolamine (PE) was isolated from soybean lecithin by column chromatography on silicic acid/silica gel (1:1) using chloroform/methanol (9:1). Phosphatidylcholine (PC) was isolated from soybean lecithin according to Singleton, Gray, Brown, and White (1965). The identity and purity of the obtained phospholipids were confirmed by HPLC and ^{1}H and ^{13}C NMR spectroscopy. Each phospholipid exhibited a single peak in HPLC (Zamora & Hidalgo, 2003) and the characteristic ^{1}H and ^{13}C signals of their polar heads (Gunstone, 1993). The fatty acid composition of PE was as follows: palmitic acid (19.8%), stearic acid (3.2%), oleic acid (7.6%), linoleic acid (61.6%), and linolenic acid (7.8%). The fatty acid composition of PC was as follows: palmitic acid (3.6%), stearic acid (2.3%), oleic acid (8.3%), linoleic acid (75.9%), and linolenic acid (9.9%).

Virgin olive oil was obtained from SOS Cuétara S. A. (Andujar, Jaén, Spain). Natural tocopherols and other polar components in the oils were removed according to the procedure of Frankel, Cooney, Moser, Cowan, and Evans (1959), which was modified. Briefly, 500 g of oil diluted in 500 mL of hexane were mixed with 50 g of activated charcoal and shaken mechanically for 30 min. The suspension was filtered and the absorbent washed thoroughly with hexane. The solvent was removed and the procedure was repeated twice to yield a polar compound-stripped olive oil (SOO). This oil was free of tocopherols [as determined directly in the oil by HPLC using a fluorescence detector (American Oil Chemists' Society, 1999)] and this procedure was also valid for eliminating free sterols. Thus, the total sterol content of SOO was reduced to about one half, which is the usual amount of esterified sterols in olive oil (Moreda, Pérez-Camino, & Cert, 2004). In addition, the absence of free sterols was confirmed by thin layer chromatography on silica gel plates, eluting with hexane-diethyl ether (60:40), and using β-sitosterol as standard. This obtained stripped oil was used directly (SOO) or after addition of 1500 μg of β-sitosterol per g of oil (PAO). One thousand and five hundred micrograms of β-sitosterol per g of oil were added because this is a common sterol content in olive oils (Moreda et al., 2004).

6-Amino-2-(1*H*-pyrrol-1-yl)hexanoic acid was obtained by reaction of Lys with 4,5-epoxy-2-heptenal, as described previously (Zamora & Hidalgo, 2005b). Activated charcoal was purchased from Merck KGaA (Darmstadt, Germany), and β-sitosterol and Lys were obtained from Sigma Chemical Co. (St. Louis, MO). Other reagents and solvents were purchased from reliable commercial sources.

2.2. Measurement of antioxidative activity

Oxidative stabilities of SOO and PAO were compared with SOO and PAO samples containing PE, PC, Lys, or their mixtures added at concentrations of 100–400 μg per g of oil. Tested compounds (5 mg) were dissolved in 2 mL of water containing 1% of tween 20 and added to the oil at the concentration indicated. The total amount of solvent added to the oil was always 400 μL, included control oils.

Oil samples (2.5 g) were heated at 90 °C in a Metrohm Rancimat (Metrohm AG, Herisau, Switzerland). A continuous airstream (10 L/h) was passed through the heated sample, and the volatiles were absorbed in a conductivity cell. Conductivities were continuously monitorized until a sudden rise signified the end of the induction period (IP).

When mixtures of different compounds were added, the existence of a synergism between the components of the mixture was analyzed by determining the synergism factor (S_F). S_F is defined as the ratio between the determined IP and the theoretical IP calculated by adding the protection offered by the components of the mixture when tested alone.

2.3. Determination of carbonyl–amine reaction products

Some of the pyrrolic carbonyl–amine reaction products formed in the Rancimat vessel during the oxidation process were determined after converting these nonvolatile derivatives into volatile pyrroles by using the procedure described by Zamora and Hidalgo (2006). Briefly, 500 mg of oil were treated successively with 2 mL of 0.3 M sodium citrate, pH 3; 1 mL of *p*-anisidine solution (1 mg/mL in 0.3 M sodium citrate, pH 3); and 50 μL of BHT solution (0.8% in acetic acid). The resulting mixture was stirred, bubbled with nitrogen, and heated at 110 °C for 20 h under an inert atmosphere. After that time, the resulting mixture was extracted three times with 2 mL of chloroform–methanol (3:2), and the organic layer was recovered and taken to dryness under nitrogen. The residue was dissolved in 500 μL of chloroform. Two hundred microliters of this solution were mixed with 20 μL of 2-pentylpyridine solution (6.01 μmol/mL in methanol), which was used as internal standard, and analyzed by GC–MS.

GC–MS analyses were conducted with a Hewlett–Packard 6890 GC Plus coupled with an Agilent 5973 MSD (Mass Selective Detector-Quadrupole type). A fused-silica HP5-MS capillary column (30 × 0.25 mm i. d.; coating thickness 0.25 μm) was used. Working conditions were: carrier gas helium (1 mL/min at constant flow); injector: 250 °C; oven temperature: from 70 (1 min) to 240 °C at 5 °C/min and then to 300 °C at 10 °C/min; transfer line to MSD: 280 °C; ionization EI 70 eV. Identification of 1-(4-methoxyphenyl)-1*H*-pyrrole (**1**), 1-(4-methoxyphenyl)-2-ethyl-1*H*-pyrrole (**2**), and 1-(4-methoxyphenyl)-2-pentyl-1*H*-pyrrole (**3**) was carried out by comparison of their retention indexes and mass spectra with those of authentic compounds. Retention index and mass spectra of compounds **1–3** were described previously (Zamora & Hidalgo, 2005b). Chemical structures for these compounds are given in Fig. 1. For the determination of compounds **1**–**3**, the following ions were employed: *m/z* 173 for compound **1**, *m/z* 201 for compound **2**, *m/z* 243 for compound **3**, and *m/z* 120 for the internal standard.

Quantification of compounds **1**–**3** was carried out by preparing standard curves of 6-amino-2-(1*H*-pyrrol-1-yl)hexanoic acid in the 500 mg of oil. For each curve, five different concentration levels of pyrroles were used (0–1 nmol/mg of oil). The amount of the pyrrole added was directly proportional to the formed pyrrole/internal standard area ratio found ($r > 0.997$, $p < 0.0001$). Although 2-alkylpyrrole derivatives could not be employed in the calibration curves, they are quite similar to *N*-substituted pyrroles. Therefore, they are expected to have similar responses to that of 6-amino-2-(1*H*-pyrrol-1-yl)hexanoic acid, and the same calibration curve was employed for the quantification of the three pyrroles. The coefficients of variation obtained were always lower than 10%.

2.4. Statistical analysis

IPs are expressed as mean values ± standard deviations (SD) of, at least, two independent experiments. Statistical comparisons among different groups were made using analysis of variance.