ACTA SCIENTIARUM POLONORUM
Acta Sci. Pol. Technol. Aliment. 21(3) 2022, 305–310
www.food.actapol.net pISSN 1644-0730 eISSN 1898-9594 http://dx.doi.org/10.17306/J.AFS.2022.1071

ORIGINAL PAPER

Received: 29.06.2022
Accepted: 22.07.2022

CHARACTERIZATION OF DAIDZEIN ASSOCIATES IN SOYBEAN OIL AND THEIR INTERACTIONS WITH PHOSPHATIDYLCHOLINE REVERSED MICELLES

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ABSTRACT

Background. Soybean oil is one of the most commonly consumed vegetable oils in the world. It is rich in unsaturated fatty acids and contains surface-active compounds which, due to their amphiphilic nature, are capable of forming association colloids. These structures are considered to be autoxidation centers. Interactions of amphiphilic antioxidants present in soybean oil and association colloids may influence their solubility and antioxidant activity, as well as changing the organoleptic properties of the oil (such as opacity). The aim of this study was to characterize the interactions of isoflavone daidzein (DAI) with phosphatidylcholine association colloids (reverse micelles) and get insights into the aggregates formed from daidzein itself in soybean oil.
Materials and methods. In this investigation, static and dynamic light scattering techniques (SLS, DLS) were applied to take measurements. Association colloids (reverse micelles) containing daidzein were formed from DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) in soybean oil from which the amphiphilic minor components were removed.
Results. Daidzein was found to be able to self-assemble and form associates in soybean oil, as well as incorporating itself into DOPC reverse micelles. This antioxidant increases the hydrodynamic diameter of DOPC structures only above a specified critical concentration (600 μmol/kg according to DLS measurements), while structures composed of DAI are formed at its lower concentrations (above 50 μmol/kg as measured by SLS).
Conclusions. The present findings may help in the formulation of oils that possess health protecting properties with an increased daidzein content and in the elucidation of daidzein antioxidant action mechanisms in bulk oils.

Keywords: daidzein, soybean oil, association colloids, reverse micelles, phosphatidylcholine, dynamic light scattering

INTRODUCTION

Refined soybean oil has a high nutritional value, good organoleptic properties and a relatively low market price resulting from the ease of soybean cultivation (Hill et al., 2008; Kozłowska and Gruczyńska, 2018; Zhang et al., 2017). Soybean oil is rich in unsaturated fatty acids and contains surface-active compounds which, due to their amphiphilic nature, are capable of forming association colloids above critical micelle concentrations (CMC) in the presence of trace amounts of water. These structures are considered to

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be autoxidation centers in which, among other things, the decomposition of hydroperoxides to free radicals takes place in the presence of transition metal ions. The result of this process is a decrease in the sensory and nutritional quality of the product (Chen, 2012; Kittipongpittaya et al., 2014).

Oxidation of lipids in oil can be prevented with antioxidants, but, due to regulatory requirements and consumer expectations, natural antioxidants are preferred. Such compounds include isoflavones present in soybeans: daidzein, genistein, glycitein and their glycosides. In addition to antioxidant properties, they have a number of health-promoting effects, such as the ability to reduce the risk of cardiovascular disease, cancer and osteoporosis, and to mitigate the symptoms of menopause (Dwiecki et al., 2009). However, the solubility of isoflavones in fats is limited, and their average content in crude soybean oil is 0.2 mg/g (Yue et al., 2007). The solubility of phenolic compounds, as well as their antioxidant potential, may change depending on the environment, so it is important to recognize their interactions with the association colloids present in oil. Recognition of such interactions may allow effective utilization of the antioxidant potential of these compounds and the design of oils with increased content of health-promoting compounds, such as isoflavones. Therefore, the aim of this study is to characterize the interactions of isoflavone daidzein (DAI) with phosphatidylcholine association colloids (reverse micelles) and get insights into the aggregates formed from daidzein itself in soybean oil. In this investigation, static and dynamic light scattering techniques (SLS, DLS) were used to take measurements. Association colloids were formed from DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) in soybean oil from which the amphiphilic minor components were removed.

MATERIALS AND METHODS

Preparation of purified soybean oil

Refined soybean oil (Heuschen & Schrouff) was dissolved in *n*-hexane (1:1 v/v) and purified using a three-stage method using a glass chromatography column (10 × 50 cm) packed with layers of (I stage) silicic acid, activated charcoal and second layer of silicic acid. The solvent was evaporated from stripped oil using a rotary evaporator R-215 (Büchi Labortechnik, Switzerland) and nitrogen stream.

Determination of tocopherol content

The content of tocopherols (expressed in mg/kg) in the soybean oil was analyzed using liquid chromatography (HPLC Waters Asc.) in a normal phase system. Purified oil (1 g) was dissolved in *n*-hexane in a 10 ml volumetric flask. The solutions were transferred to chromatographic vials and an analysis was performed. The mobile phase was *n*-hexane mixed with 1,4-dioxane (96:4 v/v), and a 1.0 ml/min flow was applied to the LiChrosorb Si 60 column (5 μm; 4.6 × 250 mm). Fluorescence detection of tocopherols was performed at excitation and emission wavelengths equal to 295 nm and 330 nm, respectively.

Determination of sterol content

Extraction of free and esterified sterols. Separation of free and esterified sterols was carried out using a glass chromatographic column packed with 15 g of silica gel. Purified oil (1.5 g) was dissolved in *n*-hexane. The esterified sterols were eluted with a 75 ml mixture of *n*-hexane and ethyl acetate (90:10; v/v). This was followed by elution of free sterols with 75 ml of *n*-hexane/diethyl ether/ethanol (25:25:50; v/v) solution. Then, after evaporation of the solvents from the eluate, saponification of lipids was carried out with 1 M solution of KOH in methanol (18 h). The unsaponifiable fraction was extracted with ethyl ether and silylated with BSTFA (N,O-bis-trimethylsilyl-trifluoroacetamide) reagent with 1% TMCS (trimethylchlorosilane).

Sterol analysis. Sterol content was determined using a GC (Hewlett-Packard 6890) equipped with a capillary column (Alltech EC5 30 m × 0.25 mm × 0.25 μm) and an FID detector (helium was the carrier gas). The initial oven temperature during analysis was 290°C, which was maintained for 20 minutes. Then it was increased to 300°C for 1 minute and maintained for 10 minutes. Phytosterols were identified by comparing the obtained retention data with relevant standards and expressed in milligram per gram – mg/g.

Determination of chlorophyll and β-carotene

The content of chlorophyll was determined according to AOCS official method Cc13d-55. β-Carotene was determined spectrophotometrically by measuring the absorbance at a wavelength of 450 nm. The concentration of pigments was expressed in milligram per kilogram – mg/kg.

Determination of acid value and peroxide value

Acid value [mg KOH/g] and peroxide value [meq O_2/kg] were determined according to PN-EN ISO 660:2021 and PN-EN ISO 3960:2017.

Determination of water content in soybean oil

Water in oil [mg/kg] was determined using Karl Fischer titration according to PN-EN ISO 8534:2017.

Preparation of oil samples

The solutions of DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) in chloroform and daidzein in methanol were pipetted into conical flasks, and the solvents were evaporated using a rotary evaporator. The film formed was dissolved in 5 ml of stripped soybean oil. Samples were mixed with a magnetic stirrer for 12 hours at room temperature and then at 55°C for 1 hour. The final concentration of DOPC was 500 μmol/kg oil and daidzein 0–600 μmol/kg oil.

Static and dynamic light scattering measurements (SLS, DLS)

Size measurements of aggregates were made using the SLS and DLS methods with a Zetasizer Nano ZS--90 (Malvern, UK) at 23.9°C in polystyrene cuvettes. The results were expressed as the mean value of static light scattering [kilocounts of photons per second – kcps], *z*-average (the intensity weighted mean hydrodynamic size of aggregates) and the polidispersity index (PDI).

Statistical analysis

The analysis was performed using one-way analysis of variance (ANOVA) with Tukey's test. All tests were considered significant at $p < 0.05$. The calculations were performed in the Statistica program (TIBCO Software 2017).

RESULTS AND DISCUSSION

Research concerning association colloids in bulk oils requires the removal of antioxidants and other minor oil components in order to form micelles and/or associates under controlled conditions. In this study, the chromatographic method of oil stripping was used, as a result of which the chlorophyll dyes and tocopherols were completely removed. The content of carotenoids, expressed as β-carotene, was reduced by nearly 92%, to the level of 0.0638 mg/kg. The content of phytosterols was lowered by nearly 70%, and the level of amphiphilic free sterols was equal to 0.23 mg/g. The low acid value and peroxide value recorded (0.037 mg KOH/g and 0.50 meq O_2/kg respectively) indicate that the oil stripping process did not significantly affect its quality, including the content of free fatty acids and lipid oxidation products. The water level in the oil used in this experiment was 280 mg/kg.

Static light scattering (SLS) is proportional to molecular weight and may show aggregate sizes (diameter) in oil. The oils with the addition of DAI (10–600 μmol/kg of oil) and with DAI and DOPC (500 μmol/kg oil) were analyzed (Fig. 1). In the samples containing DAI, a statistically significant increase in the SLS value was observed at isoflavone concentrations above 50 μmol/kg and in the samples with DOPC above 400 μmol/kg. This means that aggregates are formed in the oil at DAI concentrations above 50 μmol/kg, while in the presence of DOPC, isoflavones are incorporated into the structure of the existing reverse micelles, and only above 400 μmol/kg do they cause changes in their structure, leading to an increase in the size of association colloids which are already present.

Further data concerning the investigated structures was provided by dynamic light scattering (DLS) measurements. Dynamic light scattering is one of the most effective methods used to determine particle size in emulsions, including reverse micelles in hydrophobic media. The main advantage of this method is that the result of the analysis is obtained immediately after the measurement, which does not require a large amount of time to develop (Khan et al., 2016). Figure 2 shows the hydrodynamic diameter of association colloids expressed as *z*-average (intensity weighted mean hydrodynamic diameter). The hydrodynamic diameter (*z*-average) in the case of the oil containing DOPC

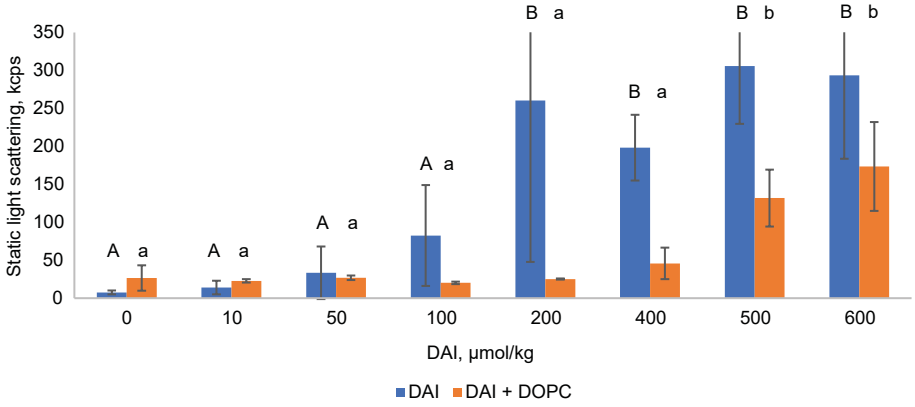

Fig. 1. Static light scattering [kilocounts of photons per second – kcps] of samples containing daidzein (DAI) and DAI + DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) in stripped soybean oil as a function of DAI concentration. Values denoted with different letters differ statistically significantly ($p \leq 0.05$)

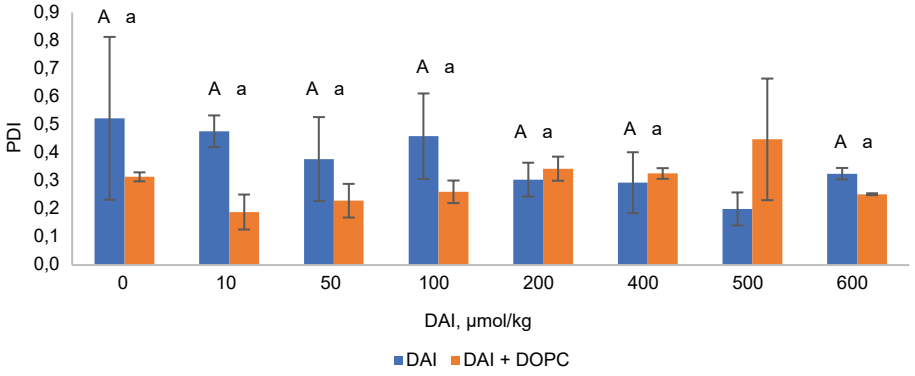

Fig. 2. Hydrodynamic diameter [nm] of association colloids in the samples containing daidzein (DAI) and DAI + DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) expressed as *z*-average. Values denoted with different letters differ statistically significantly ($p \leq 0.05$)

micelles was in the range 201.4–366.7 nm (when DAI was in the concentration range of 0–600 μmol/kg), and only the sample containing 600 μmol/kg DAI differed statistically significantly from the others. In the samples of the oil without DOPC, the hydrodynamic diameter of DAI associates was in the range of 96.8–246.9 nm, with samples containing 400–500 μmol/kg DAI being statistically significantly different from the control sample and the sample containing 10 μmol/kg of this isoflavone. This confirms the results obtained using SLS measurements: DAI influences the increase in DOPC micelle size at high concentrations (600 μmol/kg), while isoflavone in oil without phospholipids forms association colloids itself at lower concentrations (400–500 μmol/kg). The diameter of the structures formed by DAI depends on the concentration of isoflavone.

A deeper insight into the diameter of the colloidal structures formed is given by the particle size distribution (Fig. 3a, 3b). The hydrodynamic diameter of associates formed in bulk oil from DAI at a concentration of 10 μmol/kg is in the wide range of 40–650 nm, while at

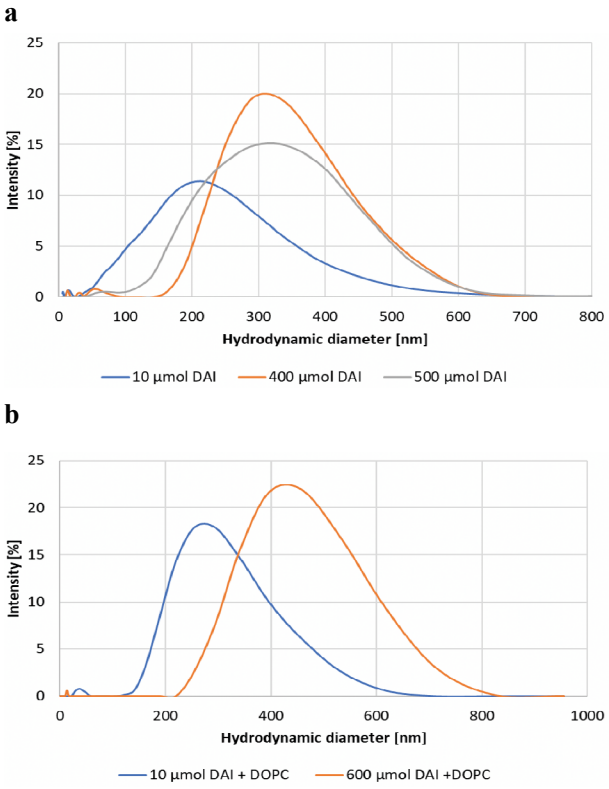

Fig. 3. Particle size distribution by intensity in the oil samples containing: **a** – daidzein (DAI), **b** – DAI + DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine)

concentrations of 400 µmol/kg and 500 µmol/kg, this range is 150–650 nm and 100–650 nm, respectively. The increase in the hydrodynamic diameter of DOPC reverse micelles as a result of interactions with DAI was observed and was dependent on the isoflavone concentration. At a DAI concentration of 10 µmol/kg, the hydrodynamic diameter of DOPC micelles was in the range of 120–710 nm and at 600 µmol/kg, it was 220–825 nm (Fig. 3b).

The polydispersity index (PDI), which reflects the degree of non-uniformity of aggregate size distribution in the samples, was also determined (Fig. 4). The PDI value in the samples without DOPC ranged between 0.2–0.52, while for oils containing DAI with DOPC, it was in the range of 0.19–0.45. Only the sample containing 500 µmol/kg DAI differed statistically significantly from the others in both variants. A PDI smaller than 0.05 is mainly recorded in highly monodisperse samples, whereas values higher than 0.7 indicate that a sample has very broad particle size distribution and is probably not suitable for DLS analysis (Danaei et al., 2018).

Interactions of phenolic antioxidants with reverse micelles in medium-chain triglycerides were investigated by Chatzidaki et al. (2017). Using DLS, the authors recorded that the average hydrodynamic diameter of the reverse micelles was 20 nm. The same value was noted when the micelles were loaded with

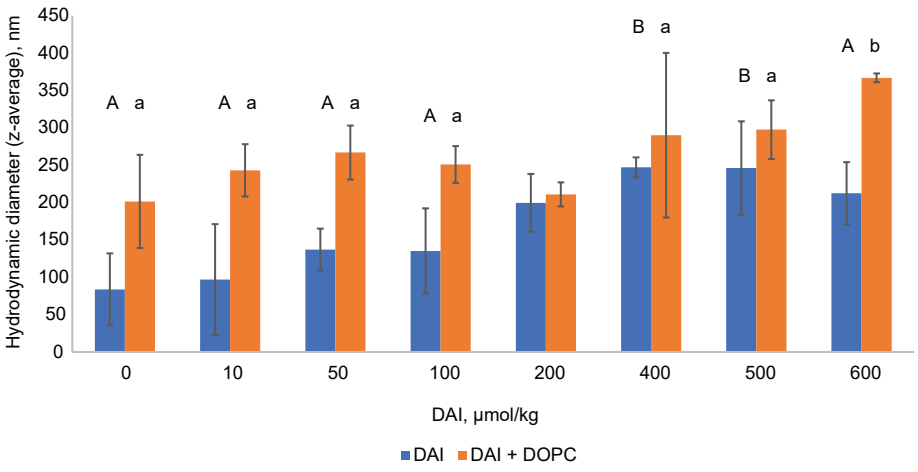

Fig. 4. Polydispersity index (PDI) of association colloids in the samples containing daidzein (DAI) and DAI + DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine). Values denoted with different letters differ statistically significantly ($p \leq 0.05$)

hydroxytyrosol, but in the presence of gallic acid their diameter increased to 30 nm. This has been attributed to the encapsulation of the antioxidant at the hydrophilic core of the reverse micelles. Applying fluorescence spectroscopy (Rokosik et al., 2020a; 2020b) found that sinapic acid, ferulic acid and canolol are able to interact with DOPC reverse micelles in rapeseed oil. Additionally, in the absence of DOPC, canolol is able of form structures (associates) with enhanced rigidity at the water-oil interface. However, measurements of the hydrodynamic diameter were not performed. In the current study, daidzein was found to self-assemble and form associates in soybean oil, as well as incorporating itself into DOPC reverse micelles. This antioxidant increases the hydrodynamic diameter of DOPC micelles only above a specified critical concentration (600 μmol/kg according to DLS measurements), while structures composed of DAI are formed at its lower concentrations (above 50 μmol/kg as measured by SLS). This indicates the different nature of associates formed in the presence and absence of phospholipids. The conclusions of the present experiments may help in the formulation of oils that possess health protecting properties with an increased daidzein content and in the elucidation of daidzein antioxidant action mechanisms in bulk oils.

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