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Optimization of extraction conditions of antioxidants from sunflower shells (*Helianthus annuus* L.) before and after enzymatic treatment

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ABSTRACT

The effects of three independent variables: solvent polarity, temperature and extraction time on the antioxidant capacity, total phenolic content and phenolic acid composition in extracts obtained from sunflower shells before and after enzymatic treatment were studied. Response surface methodology based on three-level, three-variable Box–Behnken design was used for optimization of extraction parameters and evaluation of their effect on antioxidant capacity and total phenolic content in shell extracts.

The average antioxidant capacities of extracts from sunflower shells without enzymatic treatment (368.1–1574.4 μmol TE/100 g) were higher than those for cellulolytic and pectolytic enzymes-treated shells (222.7–1419.0 and 270.7–1570.7 μmol TE/100 g, respectively). The content of total phenolic compounds ranged between 58.2–341.2 mg CGA/100 g, 26.7–277.3 mg CGA/100 g and 51.4–301.5 mg CGA/100 g for extracts obtained from shells without enzyme and treated with cellulolytic and pectolytic enzymes, respectively. Total phenolic content (TPC) in the studied shell extracts correlated significantly ($p < 0.0001$) positively with their antioxidant capacity determined by the ferric reducing antioxidant power (FRAP) method ($r = 0.9275$). Results of FRAP, TPC and phenolic acid composition in the studied shell extracts depend on the extraction conditions (solvent polarity, temperature, time), but they are independent on the addition of enzyme solutions. The antioxidant capacity and total phenolic content in the resulting extracts increased with a line in extraction temperature and solvent polarity.

1. Introduction

Sunflower (*Helianthus annuus* L.) is one of the most important oilseed crops. It is known, that extracts obtained from sunflower seeds and oils exhibit antioxidant capacity (Cevallos-Casals and Cisneros-Zevallos, 2010; Hay et al., 2006; Szydłowska-Czerniak et al., 2008; Tuberoso et al., 2007; Valavanidis et al., 2004). Moreover, sunflower meal is used primarily in ruminant feed, but its nutritional, sensory and functional properties also make it a protein and antioxidant compounds source of interest as a human food. Also, sunflower shells contain phenolic antioxidants (De Leonardis et al., 2005; Domínguez et al., 1995; Pedrosa et al., 2000; Sineiro et al., 1998; Weisz et al., 2009). The major chemical components of sunflower seed shells such as: lipids, proteins, carbohydrates along with oil, moisture content and even the average length, width, and thickness of the seeds were studied (Cancalon, 1971; Perez et al., 2007). However, quantitative analyses of total phenolic compounds (TPC) and individual phenolic acids in sunflower shell extracts were described only in few works (De Leonardis et al., 2005; Pedrosa et al., 2000; Weisz et al., 2009). Sunflower shells were extracted with methanol (Weisz et al., 2009), water and the mixture of water and alcohols (methanol or ethanol) or acetone (De Leonardis et al., 2005). Although, 94.6–99.3% of total phenolics are located in sunflower kernels, still shells contain from 0.7% to 5.4% of these compounds (Pedrosa et al., 2000), hence they can be sort of source of natural antioxidants.

Recently, high performance liquid chromatography with diode array detector (HPLC–DAD) was applied for identification and determination of individual phenolic acids in extracts obtained from sunflower kernels and shells (De Leonardis et al., 2005; Pedrosa et al., 2000; Weisz et al., 2009). Liquid chromatography with mass spectrometry (LC–MS) was also applied to identification and characterisation of phenolic acids and their derivatives in extracts from sunflower kernels and shells (Weisz et al., 2009).

The predominant phenolic acid determined in sunflower extracts was chlorogenic acid (5-caffeoylquinic acid, CGA), which content varied from 50 to almost 2800 mg/100 g of sunflower kernels (Pedrosa et al., 2000; Weisz et al., 2009) and from 1.8 to 59.1 mg/100 g of sunflower shells (Sineiro et al., 1998; Weisz et al., 2009). Chlorogenic acid constitutes about 80% of total phenolic acids in sunflower extracts determined by HPLC. The contents of o-cinnamic (o-CinA), protocatechuic (PtA), quinic (QA) and caffeic (CA) acids were much lower (5.8%, 5.2%, 4.2% and 4.1%, respectively) (De Leonardis et al., 2005; Pedrosa et al., 2000). Others pheno-

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lic acids: syringic (SyA), ferulic (FA) and *p*-coumaric (*p*-CoumA) occurred in sunflower shells in smaller amounts. Total phenolic acid content determined by HPLC varied from 40.8 to 86.0 mg/100 g of shells and depended on the variety of sunflower (Weisz et al., 2009) and extraction conditions (De Leonardis et al., 2005).

Enzymatic (Domínguez et al., 1995; Sineiro et al., 1998) and alkaline (De Leonardis et al., 2005) hydrolysis was applied to increase the amounts of individual phenolic acids in water and ethanol–water extracts obtained from sunflower seeds and shells. Antioxidants were released under alkaline treatment, especially chlorogenic acid (1.4 g/100 g of shells) and caffeic acid (0.7 g/100 g of shells) (De Leonardis et al., 2005). Content of phenolic acids in extracts from sunflower shells and kernels after hydrolysis was 80–90% higher than in those obtained without hydrolysis (De Leonardis et al., 2005; Domínguez et al., 1995; Sineiro et al., 1998). Thus, more phenolic compounds (including phenolic acids) are liberated from sunflower seeds and shells after enzymatic or alkaline treatment.

To the best of our knowledge, there are no reports on examination of the impact of an enzymatic treatment of the sunflower shells on their antioxidant capacity.

Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors, searching optimum conditions of factors for desirable responses, which has been successfully applied in many areas of biotechnology such as: optimization of media and cultivation conditions (Kumar and Gupta, 2008), biotechnological parameters (Szydłowska-Czerniak et al., 2010), lipase-catalyzed reaction (Katsoura et al., 2007), fermentation conditions (Song et al., 2007).

However, the optimum conditions of antioxidant extraction from sunflower shells before and after enzymatic treatment have not yet been reported. Besides, to the best of our knowledge, there has been no reference on the determination of antioxidant capacity of sunflower shells by ferric-reducing antioxidant power (FRAP) assay.

Therefore, this work is focused on the optimization of extraction conditions (solvent polarity, temperature and time) of compounds with high antioxidant capacities from sunflower shells without enzyme and treated with cellulolytic and pectolytic enzymes.

The response surface methodology (RSM) was used for evaluation of the effects of the three independent variables (solvent polarity, temperature and time) and their interactions on the response variables; that is, antioxidant capacity (AC) and total phenolics content (TPC) in extracts obtained from sunflower shells before and after cellulolytic and pectolytic enzymes treatment. In the presented paper, the FRAP method, after some modifications, was employed for the determination of the total antioxidant capacity of sunflower shell extracts. Besides, correlation between TPC and AC was examined and discussed.

2. Materials and methods

2.1. Plant material

Sunflower shells (*Helianthus annuus L.*) were directly supplied by Oil Factory in Martfű, Hungary. The sunflower seeds were originated from the different regions of Hungary during the August–September 2009 harvest season. The studied black shells were removed from seeds during conventional processes (crushing in the dehulling step) of traditional sunflower oil production. The composition of fresh shells, which used to gain green energy in Martfű is as follows: moisture content – 7 wt.%, oil content – 4.5 wt.%, fiber content – 53 wt.%, ash – 2.8 wt.%, C – 46.0 wt.%, O – 35.8 wt.%, H – 6.1 wt.%, N – 0.7 wt.%, S – 0.2 wt.%, Cl – 0.15 wt.%. The environmental impact of sunflower shells is caused mainly by heavy metals (Fe, Cu, Cd), pesticide residues and dioxine. Sunflower shells, which are the major byproduct of sunflower seed processing were stored in the dark closed containers at ambient temperature until treatment and further analysis.

2.2. Chemicals

All reagents were of analytical or HPLC grade. 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox (TE), 97%), 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ, 99%), Folin–Ciocalteu reagent (FC reagent, 2N), iron(III) chloride hexahydrate; sodium acetate; sodium carbonate, chlorogenic (CGA), caffeic (CA) and ferulic (FA) acids were supplied by Sigma–Aldrich (Poznań, Poland). Gallic (GA) and *p*-coumaric (*p*-CoumA) acids were purchased from POCh (Gliwice, Poland). Methanol (puriss. p.a. ≥99.8% and HPLC grade, ≥99.9%) and acetic acid (puriss. p.a. ≥99.5%) were purchased from Chempur (Piekary Śląskie, Poland).

Recently, to improvement of extraction efficiency of plant phenolic compounds, the cellulolytic and pectolytic enzymes were applied (Bonnin et al., 2002; Li et al., 2006; Zheng et al., 2009). Therefore, cellulolytic – ROHALASE® OS (ROS) and pectolytic – ROHAPECT® PTE (RPTE) enzymes were supplied by AB Enzymes GmbH, Darmstadt, Germany. ROHALASE® OS obtained from cultures of a submers produced, genetically modified *Trichoderma reesei* strain is an enzyme product that contains cellulase as main activity, β-glucanase and xylanase. ROHAPECT® PTE is a special pectolytic enzyme preparation for the processing of fruits and vegetables. The pectinase is derived from *Aspergillus*. The optimal reaction conditions are $T = 70\,^{\circ}C$, pH = 5.5 for ROS and $T = 50\,^{\circ}C$, pH = 5.8 for RPTE. Deionized water (DW) was used for the preparation of solutions.

2.3. Enzymatic treatment and extraction procedure

The sunflower shells were ground using a Retsch grinder (2.5 mm) and the flour transferred to a jacketed glass reactor connected to an oil bath. Enzymes, ROS or RPTE (0.1 vol.%) were directly sprayed on the crumbled shells and begin enzymatic reaction at 70 °C or 50 °C under mixing for 20 min. Methanol at three concentrations (c_{MeOH} = 0 vol.% – water, c_{MeOH} = 50 vol.% and c_{MeOH} = 100 vol.%) was used for extraction of antioxidant compounds from shells. Samples of shell flour (2 g) without enzymes (control samples) and after treatment with ROS or RPTE enzymes and 20 mL of solvents (c_{MeOH} = 0 vol.% – water, c_{MeOH} = 50 vol.% and c_{MeOH} = 100 vol.%) with decreasing polarity (dielectric constant, ε = 78.60, 57.60 and 32.66, respectively) were transferred into round-bottomed flasks equipped with reflux condenser and placed in a shaking water bath (Elpan 357, Wrocław, Poland) at a constant temperature: 30, 60 and 90 °C for 1, 2 and 3 h according to Box–Behnken plan (Tables 1 and 2). Each sample was extracted in triplicate and the residual flour was separated by centrifugation (4500 rpm, 15 min). The pooled extracts were filtered and stored in a refrigerator prior to analysis.

2.4. Determination of antioxidant capacity by FRAP method

Antioxidant capacity of each extract from sunflower shells was determined by spectrophotometric FRAP method (Szydłowska-Czerniak et al., 2009). In our procedure, freshly prepared FRAP reagent (2.5 mL of a 10 mmol/L TPTZ solution in 40 mmol/L HCl, 2.5 mL of 20 mmol/L $FeCl_3$ and 25 mL of 0.1 mol/L acetate buffer, pH 3.6) was incubated at 37 °C for 10 min. Then, 0.05 mL of sunflower shell extracts and 2 mL of FRAP reagent were transferred into a 10-mL volumetric flasks and made up to volume with DW and kept at room temperature for 20 min. The absorbance was measured at