Composition of Sunflower Seed Extracts Obtained with Sub-critical Water: Kinetics Study and Analysis of Extracts

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ABSTRACT

The kinetics of extraction of polar and nonpolar compounds and simultaneous hydrolysis of water soluble extracts from sunflower seeds using subcritical water was studied. The extractions were performed at temperatures 100 °C, 130 °C and 160 °C using a high-pressure batch extractor. Yields of extracted oil and water soluble phases were determined. The content of total proteins and carbohydrates in water phase were analyzed using the Bradford and phenol/sulfuric acid colorimetric methods, respectively and based on the obtained results the degree of hydrolysis of extract was studied. Products of hydrothermal degradation were quantified using high performance liquid chromatography. Extracts were also analyzed for total phenolic compounds and their antioxidant activities using the Folin-Ciocalteu and radical scavenging colorimetric methods, respectively.

INTRODUCTION

The aqueous oil extraction is an alternative and greener extraction method that was intended to replace conventional oil extraction methods using hexane as solvent. Its unique ability of simultaneous oil and protein extraction made it an attractive idea in the past. Due to the low acquired yields of oil and protein and long agitation times required, as well as a need to change the pH of the media, made its use implausible and conventional methods of extraction remained the main method of oil extraction to date [1]. A possible increase of yield and decrease of agitation time could be achieved at temperatures higher than the boiling point of water (in the subcritical region), since that is where diffusivity rates increase drastically [2]. The question of product quality arises since high extraction temperatures are applied and hydrothermal degradation of extracts might occur. One phase that is particularly vulnerable is the water soluble phase, since there is a possibility of formation of toxic products after treatment with subcritical water.

In this study the kinetics of subcritical water extraction of polar and nonpolar compounds and simultaneous hydrolysis of water soluble extracts was observed. The extractions were performed in temperature range of 100-160 °C in a high-pressure batch extractor. Total

amounts of extracted oil and water soluble phase were determined. Water soluble phase was analyzed for total proteins and carbohydrates using the Bradford and phenol/sulfuric acid colorimetric methods, respectively and based on obtained results the degree of hydrothermal degradation was studied. Products of hydrothermal degradation were studied and quantified using high performance liquid chromatography (HPLC). Extracts were analyzed for total phenolic compounds and their antioxidant activities using the Folin-Ciocalteu and radical scavenging colorimetric methods, respectively.

MATERIALS AND METHODS

Materials

Dehulled sunflower seeds (Natura, Slovenia) were ground prior every experiment.

All reagents, standards and solvents were of analytical grade. Coomasie brilliant blue, albumin bovine serum (≥96 %), phenol, glucose (≥98 %), sodium carbonate and 2,2-diphenyl-1-pikrylhidrazil (DPPH) were purchased from Sigma-Aldrich (Slovenia). Gallic acid (≥98 %) was purchased from Fluka (Germany). Sulfuric acid, Follin-Ciocalteu phenol reagent, acetic acid and ethanol were purchased from Merck (Germany). 5-hydroxymethyl furfural (≥98 %), caffeic acid (≥99 %) and chlorogenic acid (≥98 %) were purchased from Acros Organics (Belgium). Phosphoric acid was purchased from Kemika (Croatia) and hexane was purchased from Carlo Erba (Italy). Methanol was purchased from J.T. Baker (Netherlands).

Subcritical water extraction

For extraction experiments a 60 mL cylindrical stainless steel high-pressure vessel (Autoclave Engineers, USA) was used. Temperature regulation was performed with a heating cable and stirring of the extraction media was performed by using a magnetic stirrer. The extraction pressure applied for all experiments was equal to 30 bar.

Kinetics of extraction was studied at temperatures 100 °C, 130 °C and 160 °C for extraction times ranging from 5 min to 120 min at a material to solvent ratio of 1/20 g/mL. After extraction the suspension was filtered and the obtained liquid extract was introduced into a separation funnel and 50 mL of hexane were added. The mixture was shaken rigorously for 3 min and then the phases were separated by using centrifuge (11000 rpm for 2 min). Both phases were collected and evaporated until dryness.

Analysis of extracts

Total proteins concentration in water-soluble extracts (WSE) was determined with the Bradford colorimetric method [3]. Briefly, to a 1 mL aliquot of a protein solution 1 mL of prepared Bradford reagent (100 mg of Coomasie Blue was mixed in a 1000 mL glass flask

with 50 mL of 95 % ethanol and 100 mL 85 % (v/v) phosphoric acid and diluted with deionized water) was mixed in a test tube and vortexed for 30 s before the absorbance of the solution was measured at 595 nm on a UV-Vis spectrophotometer (Varian, USA). A reference solution was prepared in identical manner as explained above, except that the 1 mL aliquot of protein solution was replaced by deionized water. The quantification was done based on a calibration curve obtained with BSA (Albumin Bovine Serum). Total proteins content in water soluble extracts (w_{BSA}) was expressed in mg of BSA per g of extract (mg BSA/g ext.).

Total carbohydrates concentration in WSE was determined with the phenol/sulfuric acid colorimetric method [4]. Briefly, to a 2 mL aliquot of a carbohydrate solution 1 mL of 5 % aqueous solution of phenol was mixed in a test tube. Subsequently, 5 mL of concentrated sulfuric acid was added rapidly to the mixture. Test tubes were shaken in an ultrasonic bath for 10 min and then left to stand at room temperature for 20 min for color development. The absorbance of the acquired solution was measured at 490 nm on a UV-Vis spectrophotometer. A reference solution was prepared in identical manner as explained above, except that the 2 mL aliquot of carbohydrate solution was replaced by deionized water. The quantification was done based on a calibration curve obtained with glucose. Total carbohydrates in water soluble extracts (w_{GLU}) were expressed in mg of glucose per g of extract (mg GLU/g ext.).

Total phenolic compounds in WSE were determined according to the Follin-Ciocalteu colorimetric method [5]. Briefly, to a 0.5 mL aliquot of water-soluble sample solution 2.5 mL of Follin-Ciocalteu reagent (diluted ten times with water) and 2 mL of 75 g/L Na_2CO_3 were added. The temperature of the solution was then elevated in a water bath at 50 °C for 5 min. After cooling at room temperature for 30 min, the absorbance of the solution was measured at 760 nm by an UV-Vis spectrophotometer. A reference solution was prepared in identical manner as explained above, except that the 0.5 mL aliquot of extract solution was replaced by deionized water. The quantification was done by calibration curve prepared with gallic acid. Total phenolics in water soluble extracts (w_{GA}) were expressed in mg of gallic acid per g of extract (mg GA/g ext.).

HPLC analysis. A HPLC method was developed for determination of products of hydrothermal degradation present in the WSE, namely 5-hydroxymethyl furfural (5-HMF), caffeic acid (CA) and chlorogenic acid (CHA). Chromatographic analysis was performed on a Varian system, equipped with a Prostar 210 binary pump, a column heater, a Prostar 410 autosampler and a Prostar 310 variable wavelenght detector (VWD), connected to a ChemStation software. The separation was achieved on column Agilent Zorbax SB-C18 150 x 4.6 mm with 5 μm particle size at 25 °C and a flow rate of 1 mL/min. The mobile phase consisted of two solvents, A: 2 % acetic acid in water, and B: methanol. The gradient was: 0 min 15 %B, 20 min 35 %B, 22 min 15 %B. Detection of the compounds was performed at 280 nm and the quantification was done using calibration curves. Amount of a compound was expressed in mg of compound per g of extract (mg/g ext.).

Radical scavenging activity of WSE was determined using the DPPH (1,1-diphenyl-2-picrylhydrazyl) colorimetric method. A $6 \cdot 10^{-5}$ M solution of DPPH in methanol was prepared. 3 mL of this solution was added to 77 μL of extract solution and incubated in a dark room for 15 min. After incubation the absorbance at 515 nm was measured. The reference solution was prepared similarly but instead of sample solution, methanol was used with absorbance being measured immediately at 515 nm. Antioxidant activity is expressed as % of inhibition towards the reference solution and is calculated using eq. 1 [6]:

$$\%\ inhibition = 100 \cdot \left(\frac{A_c^0 - A_s^{15}}{A_c^0} \right) \quad (1)$$

where A_c^0 represents the absorbance of the blank at 0 min and A_s^{15} the absorbance of the sample after 15 min of incubation.

RESULTS

Extraction yields of oil soluble and water soluble phase

The highest yield of oil (44.3 %) was achieved at 130 °C after 30 min of extraction. At 160 °C a similar maximum (43.9 %) was achieved after 120 min of extraction, while at 100 °C lower yield was obtained (max. 27.1% after 10 min).

The highest yield of WSE was obtained at 160 °C after 60 min of extraction and was approximately 30 %. At temperatures 130 °C and 100 °C max. yields were 18.9% (after 120 min) and 12.1% (after 5 min), respectively. The increase of WSE yield at temperatures higher than 100 °C over longer period of time can be a consequence of hydrolysis reactions of proteins producing water-soluble peptides and amino acids or hydrolysis of carbohydrates producing water-soluble oligosaccharides and simple sugars. Both of these reactions can contribute to the total amount of WSE. It is also possible that other products may have been formed during extraction.

Total proteins and carbohydrates in water soluble extracts

Total contents of proteins and carbohydrates in WSE are presented in **Fig. 1a** and **Fig. 1b**, respectively. From **Figure 1a** it can be observed that total protein content is the highest at 100 °C after 10 min of extraction and afterwards the content slowly decreases with extraction time. At temperatures higher than 100 °C overall proteins content in WSE decreases with increasing temperature and at 160 °C the concentration of proteins decreases rapidly with extraction time. The decrease of proteins concentration with increasing temperature is probably a consequence of lower solubility of proteins at higher temperatures, while the rapid decrease with extraction time at 160 °C is possibly due to hydrolysis of proteins.

For total carbohydrates a quite similar pattern can be observed. At 100 °C, again only little influence of extraction time can be observed, since carbohydrate concentration remains

almost constant after 10 min of extraction. At 130 °C concentration of carbohydrates starts to decrease with time and at 160 °C the decrease is the highest.

The carbohydrate profile of sunflower seeds consists mainly from hexose derivatives (glucose, sucrose, cellulose etc.) [7] and it can be assumed that after dehydration of the sugars mostly 5-hydroxymethyl furfural (5-HMF) is formed [8]. By further dehydration of 5-HMF subsequently levulinic acid and formic acid can also be formed.

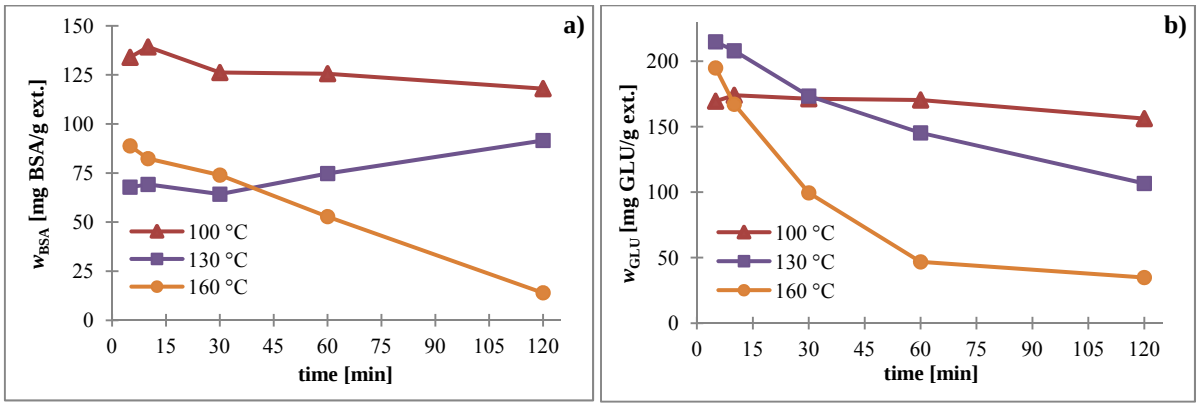

Figure 1: Total content of proteins (a) and carbohydrates (b) in WSE.

Total phenolics of WSE samples and their radical scavenging activities

Total phenolics content in WSE is presented in **Fig. 2a**. Interestingly, overall content is the highest at 100 °C, whereas at 130 °C and 160 °C the content is significantly lower. Results also show that at higher temperatures (130 °C and 160 °C) content of total phenolics decreases with extraction time. The higher amounts of total phenolics extracted at lower temperatures are probably due to the presence of non-hydrolyzed glycosides and CHA in the samples, which are phenolics typically found in sunflower extracts [9]. Also, these compounds are generally more soluble in water at ambient conditions. Decrease of phenolics content with extraction time at 130 °C and 160 °C is probably a consequence of degradation of phenolics at the applied temperatures and longer exposition times.

Radical scavenging activities of WSE are presented in **Fig. 2b.** It can be seen that there is a strong relation between total phenolics content and radical scavenging activities of WSE. The highest radical scavenging activities can be observed for extracts obtained at 100 °C, whereas lower activities can be observed for extracts obtained at 130 °C and 160 °C. Similar as in the case of total phenolics content, radical scavenging activity decreases with extraction time at temperatures higher than 100 °C.

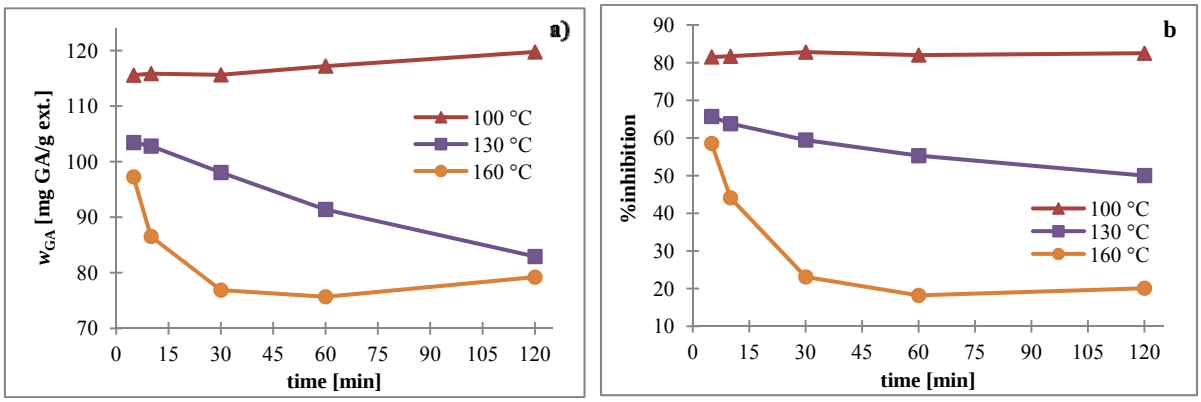

Figure 2: Content of total phenolics (a) and radical scavenging activities (b) of WSE.

Content of 5-HMF, CHA and CA

Degree of hydrothermal degradation in WSE was assessed by analyzing the concentration of two phenolic compounds typically present in sunflower seeds, namely CHA and one of its hydrolytic derivatives, i.e. CA (**Fig. 3**). Furthermore, the decomposition (dehydration) of sugars was studied by measuring the amount of 5-HMF formed during extraction (**Fig. 4**)

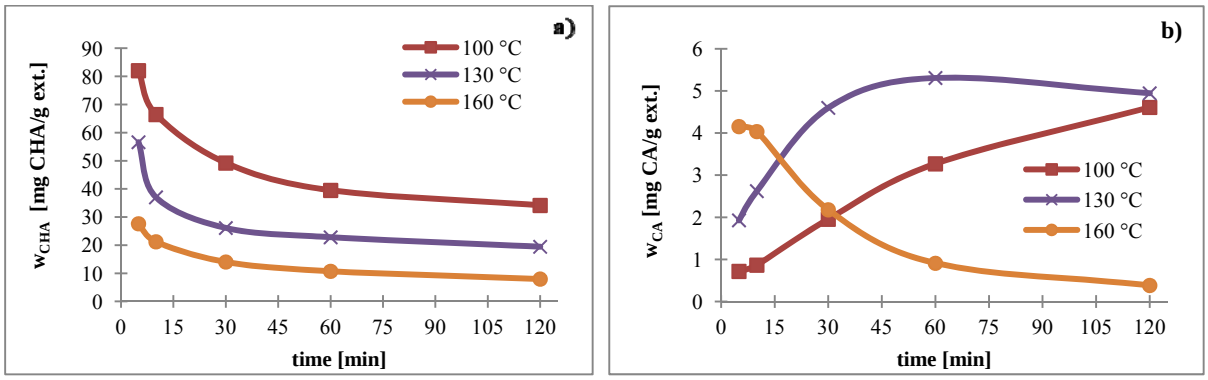

Figure 3: Concentration of chlorogenic acid (w_{CHA}) (a) and caffeic acid (w_{CA}) (b) in WSE in mg/g ext.

Compared to the total phenolics content, it can be observed that CHA is the main phenolic compound in WSE, however its content decreases rapidly with extraction time and increasing temperature. It seems that the ester bond present in CHA is not very stable in subcritical water at these conditions. Consequently, the increase of CA concentration with extraction time and increasing temperature from 100 °C to 130 °C can be observed, what indicates that hydrolysis of CHA to CA takes place during extraction at these conditions. At 160 °C the highest decomposition rate of CHA can be observed and at the same time the concentration of CA after 5 min of extraction is the highest, compared to the concentrations obtained in the same time at lower temperatures, and afterwards it starts to decrease rapidly

with time. This is probably a consequence of further hydrothermal degradation of CA at longer exposition times.

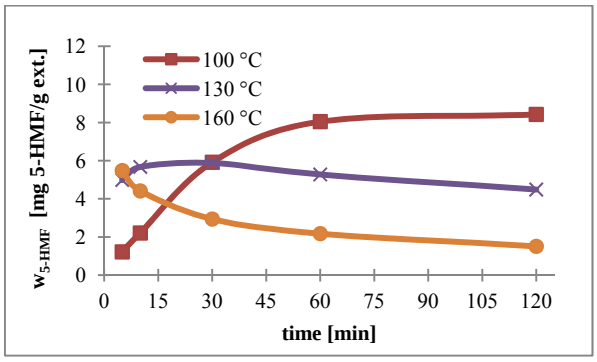

Figure 4: Concentration of 5-hydroxymethyl furfural ($w_{5\text{-HMF}}$) in WSE in mg/g ext.

From **Fig. 4** it can be also observed that 5-HMF is formed during extraction at the studied extraction conditions. At 100°C the concentration of 5-HMF increases with time and after 60 min of extraction it reaches the maximum value. At higher temperatures initially higher concentrations of 5-HMF are obtained in WSE, however the concentration decreases with extraction time. At 130 °C the decrease of 5-HMF concentration is observed after 30 min while at 160 °C already after 5 min of extraction. This decrease in concentration could indicate further hydrothermal degradation of 5-HMF to other products, such as organic acids (levulinic acid and formic acid).

CONCLUSIONS

In this study the kinetics of extraction of polar and nonpolar compounds and simultaneous hydrolysis of water soluble extracts from sunflower seeds using subcritical water was studied. The highest amount of oil (44.3 %) was obtained at 130 °C after 30 min of extraction, whereas the highest amount of water soluble extract (30 %) was obtained at 160 °C after 60 min of extraction. Results showed that at 130 °C and 160 °C hydrothermal degradation (hydrolysis) of water soluble phase started to occur, since extraction yield increased over longer periods of time and concentrations of total carbohydrates and total proteins started to decrease with time. Similarly, total phenolics, as well as radical scavenging activity of WSE decreased with time at temperatures higher than 100 °C. HPLC analysis showed that concentration of CHA decreases rapidly with increasing temperature and with extraction time at all temperatures investigated, while oppositely the concentration of CA increased with increasing temperature and extraction time in temperature range from 100 °C to 130°C. At 160°C rapid hydrothermal degradation of CA with prolonged extraction time is observed. The decomposition product of carbohydrates, i.e. 5-HMF was also found in all obtained extracts.

At 100°C the concentration of 5-HMF was initially the lowest and increased with extraction time, while at higher temperatures its concentration started to decrease with time (after 30 min at 130 °C and after 5 min at 160 °C). This decrease of 5-HMF concentration indicates hydrothermal degradation of 5-HMF to other products, such as organic acids.

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