Hydrothermal Degradation of Fats, Carbohydrates and Proteins in Sunflower Seeds after Treatment with Subcritical Water

M. Ravber, Ž. Knez, and M. Škerget*
University of Maribor, Faculty of Chemistry and Chemical Engineering,
Laboratory for Separation Technology and Product Design,
Smetanova 17, SI-2000, Maribor, Slovenia

doi: 10.15255/CABEQ.2015.2193

Original scientific paper
Received: February 15, 2015
Accepted: October 5, 2015

In this study, the hydrothermal degradation of fats, carbohydrates and proteins in sunflower seeds after treatment with subcritical water was observed. Sunflower seeds were subjected to subcritical water in a wide temperature range (130–240 °C) for periods from 5 to 120 minutes. The oil- and water-soluble phases were analysed for products of hydrothermal degradation. Oil stability was investigated by analysing the content of free fatty acids using gas chromatography. The water-soluble phase was analysed for the presence of any formed amino acids, and the amount of carbohydrates remaining after treatment was determined. Total amino acids and carbohydrates were determined using the ninhydrin and phenol/sulphuric acid spectrophotometric methods, respectively. The results show that oils are the most stable macronutrient present in sunflower seeds. Only small amounts of free fatty acids had formed during processing but the amount drastically started to increase at 240 °C. Proteins seem to be less stable than oils, whereas carbohydrates have proven to be very susceptible to hydrothermal degradation.

Key words:
subcritical water, hydrothermal degradation, free fatty acids, carbohydrates, proteins

Introduction

Subcritical water is an alternative and environmentally friendly processing medium with interesting applications in many fields of research[1]. Its ability to degrade natural biopolymers[2] (cellulose, protein etc.) into smaller, usually water-soluble products (sugars, amino acids etc.) has been found to be quite useful, especially in waste and biomass processing technologies. Nevertheless, for food processing, these degradation reactions can have a negative effect on the foods structure itself, resulting in an altered structure with lower quality or even formation of toxic compounds[3,4]. It is therefore important, that these hydrothermally induced reactions be studied in more detail in order to know how and when they occur.

In this study, the hydrothermal degradation of fats, carbohydrates and proteins in subcritical water was observed. For the raw material, sunflower seeds were chosen, which were subjected to subcritical water. The hexane soluble (oil) and water-soluble phases (proteins and carbohydrates) were collected and analysed for products of hydrothermal degradation. Oil stability was investigated by analysing the content of total free fatty acids. The water-soluble phase, on the other hand, was analysed for the presence of any formed amino acids. Furthermore, the total carbohydrates were analysed to evaluate any loss of carbohydrates during the treatment.

*Corresponding author: e-mail: mojca.skerget@um.si, tel: +386 2 22 94 463, fax: + 386 2 2527 774

Materials and methods

Subcritical water treatment

Raw dehulled sunflower seeds purchased from the local market (Natura, Slovenia) were ground and introduced into a high-pressure high-temperature autoclave (Parker Autoclave Engineers, PA, USA). Pure water, at a material-to-solvent ratio of 1/20 g mL^{-1}, was added to the seeds. The autoclave was sealed and purged several times with inert nitrogen (Messer, Slovenia), in order to remove any present atmospheric oxygen, which could cause unwanted side-oxidation reactions.

The reactions were carried out at five different temperatures, namely at 130 °C, 160 °C, 190 °C, 220 °C and 240 °C for five different time intervals (5 min, 10 min, 30 min, 60 min and 120 min). The pressure inside the autoclave during treatment was 35 bar.

After hydrothermal processing with subcritical water, the obtained sunflower seed/water suspension was cooled to room temperature and filtered. The supernatant, containing both a fat- and a water-soluble fraction, was placed into a separation

funnel and hexane (J.T. Baker, Netherlands) was added. The funnel was shaken rigorously and the obtained emulsion centrifuged in order to achieve separation of the phases. Both phases were then collected and evaporated until dryness using a vacuum evaporator.

Free fatty acid content

The free fatty acid (FFA) content present in the obtained oil samples was determined using gas chromatography[5]. The analyses were performed on a gas chromatograph 6890 HP model (Hewlett-Packard, CA, USA) with a flame ionization detector. The temperature of the detector was set to 300 °C and the oven time-temperature profile was as follows: 120 °C (1 min), 25 °C min^{-1} to 180 °C (1 min), 5 °C min^{-1} to 220 °C (10 min), 5 °C min^{-1} to 230 °C (30 min). The carrier gas that went through the HP-FFAP capillary column (30 m x 0.25 mm x 0.25 μm) was helium at a flow rate of 64 mL min^{-1}. Determination of FFAs was performed using calibration curves made from standards. The amounts of determined single FFAs were summed and the results presented in % of FFAs.

Total carbohydrate and furfural content

The total carbohydrate and furfural content in the water-soluble extract were determined with the UV-VIS spectrophotometric phenol/sulphuric acid method, firstly described by Dubois *et al.*[6] Solution of water-soluble extract was prepared, and 1 mL of 5 % (w/v) phenol (Sigma-Aldrich, Slovenia) was added to 2 mL of the solution. Added to the mixture was 5 mL of 95 % H_2SO_4 (Fluka, Germany), and then stirred rapidly using an ultrasonic bath for 10 minutes. After stirring, the solution was left to cool to room temperature before the absorbance was measured at 490 nm on a UV-VIS spectrophotometer (Carry 50, Varian, CA, USA). The reference solution was prepared in a similar manner using pure water instead of sunflower seed extract. Quantification was performed by a calibration curve made with glucose (>99 %, Sigma-Aldrich, Slovenia). Total carbohydrate and furfural content was expressed in mg of glucose per g of sunflower extract.

Total amino acids and amines content

The total amino acids and primary amines content in the water-soluble extract was determined with the UV-VIS spectrophotometric ninhydrin method[7]. For this purpose, the ninhydrin reagent was prepared by adding 0.5 g of ninhydrin (Sigma-Aldrich, Slovenia) to a 50 mL dark flask and diluting it with a mixture of 30 mL 2-propanol (Merck, Germany) and 20 mL 0.1 mol L^{-1} solution of acetate buffer (pH = 5.5). Solutions of water-soluble extract were prepared in 0.05 % (v/v) aqueous acetic acid (Merck, Germany). To 2 mL of prepared solution, 2 mL of ninhydrin solution was added, and gently stirred. The mixture was then put into a boiling-water bath for 15 minutes, allowing colour to develop. The boiled sample was cooled, and to it were added 3 mL of 50 % (v/v) ethanol (J.T. Baker, Netherlands). Absorption of the sample was measured at 570 nm by UV-VIS spectrophotometer. The reference solution was prepared in a similar manner using 0.05 % acetic acid instead of sunflower seed extract. Quantification was performed by a calibration curve made with L-tyrosine (>98 %, Sigma-Aldrich, Slovenia). Total amino acid and amine content was expressed in mg of L-tyrosine per g of sunflower extract.

Statistical analysis

Experimental results were expressed as means ± standard deviation (SD) of three parallel experiments (n = 3). Each data point represents the average of at least five measurements and the relative standard deviation between measurements was 1 %.

Results and discussion

Free fatty acid content

At ambient conditions, water is a polar solvent with very poor solvation properties for non-polar compounds, such as the triglycerides present in sunflower oil. In the subcritical region, the water is simultaneously heated and compressed to temperatures well above its atmospheric boiling point. At these conditions, the thermal motion of water molecules increases drastically, thus reducing the number of hydrogen bonds normally present at ambient conditions. Water, in subcritical state, therefore loses its characteristic polarity and becomes more similar to an organic solvent[8]. The decrease in the number of hydrogen bonds, on the other hand, also increases the ionic product of water, meaning that the medium becomes more acidic at increased temperature. This means that, although water, at these conditions, can be used for dissolving a non-polar substance, at the same time the question of interfering reactions of water molecules with the solute can occur[9]. Triglycerides, for example can hydrolyse to FFAs and glycerol. Nevertheless, it is possible that, due to the still high polarity of water, the non-polar triglycerides structure is not affected much, thus not allowing protons to reach the donor electron pair present at the carbonyl oxygen of the ester bond.

The percentage of formed FFAs in oils after treatment with subcritical water is presented in

Table 1 – *Total FFAs in oil soluble fractions of sunflower seeds after treatment with subcritical water*

Temperature [°C]	Time [min]	%FFAs	Temperature [°C]	Time [min]	%FFAs
	5	2.32±0.54		5	2.85±0.02
	10	2.32±0.44		10	2.91±0.88
130	30	2.30±0.81	220	30	2.95±0.06
	60	2.52±0.05		60	9.08±0.15
	120	2.62±0.51		120	19.12±0.98
	5	2.33±0.64		5	3.12±1.02
	10	2.35±0.08		10	3.46±0.06
160	30	2.32±0.09	240	30	7.80±1.11
	60	2.59±0.07		60	35.51±2.11
	120	2.56±0.91		120	72.12±5.74
	5	2.58±0.88			
	10	2.79±0.01			
190	30	2.64±0.09			
	60	2.75±0.61			
	120	3.12±0.23			

Table 1. It can be observed that indeed, the oils are very stable at the lower temperatures (≤190 °C), whereas at 220 °C, the FFA content suddenly starts to increase after 30 minutes of reaction. At 240 °C, the increase in FFA content becomes very drastic after 30 minutes as well, reaching almost complete conversion of triglycerides after 120 minutes. This induction period of FFA formation was already reported by Minami and Saka[10] and Alenezi *et al.*[11], who stated that this seem to occur only at lower (<300 °C) temperatures of subcritical water. At higher temperatures (>300 °C), this induction period could no longer be observed, meaning that high fractions of FFAs could be obtained very rapidly.

Although this is a wanted effect for biofuel production, for food applications, on the other hand, the formation of FFAs should be minimized, since the presence of FFAs in edible oils causes rancidity, which firstly increases down-stream processing costs and secondly lowers the oils quality.

From the obtained results, it can be concluded that, when using subcritical water for dissolving fats, it should not undergo temperatures higher than 190 °C. Of course, at this point it should also be mentioned that, although triglycerides are relatively stable at the mentioned conditions, it is not known how these temperatures affect the quality of naturally present antioxidants, which contribute to the overall stability of the oils. This subject has, however, already been reported in our previous work[3].

Total carbohydrate and furfural content

It is well known that subcritical water is a medium well suited for depolymerisation of polymeric carbohydrates, such as cellulose and starch[2,12,13]. Carbohydrates exhibit similar polarity as water at subcritical conditions, which means that these compounds will generally completely dissolve in the medium. Due to a homogeneous mixture of water and carbohydrates, the proton donation step is easier (as compared to an emulsion for the system subcritical water/triglycerides), which consequently enables fast conversion (hydrolysis) of complex chain carbohydrates into simple sugars. However, at this point, the degradation pathway does not stop, since sugars can react even further with the water molecules. Reactions like dehydration, decarboxylation etc. can take place, although for these reactions to occur, more contact time is needed[14]. Products, such as 5-hydroxymethylfurfural, levulinic acid, formic acid and lactic acid are commonly found in residues of subcritical water reactors, and should be removed in any food product, since these compounds are mostly non-edible and in most cases even toxic to humans in high concentrations.

The amount of total carbohydrates present in water-soluble extracts of sunflower seeds obtained after subcritical water treatment is presented in Fig. 1. Although the phenol/sulphuric acid method is primarily used for determination of total carbohydrates (poly-, oligo- and monosaccharides) it must be mentioned that all formed or already present furfurals in the extracts are also accounted by this method. Nevertheless, a decrease in total carbohydrate and furfural concentration would indicate further hydrothermal degradation of these compounds to organic acids. This phenomena can indeed be observed in Fig. 1. Already at the lowest temperature (130 °C), a decrease in total carbohydrate and furfu-

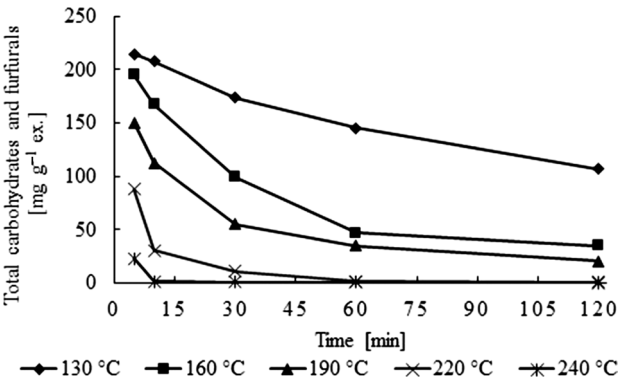

Fig. 1 – *Total carbohydrates and furfurals in water soluble fraction of sunflower seeds after treatment with subcritical water*

ral content with time can be observed. At higher temperatures, this decrease is even more drastic, and at 220 °C and 240 °C, the complete conversation of carbohydrates and furfurals can be observed in both cases. This means that, at these conditions, no more carbohydrates are present in the extract. Since the carbohydrate present in the sunflower seeds is mostly starch[15] and since the activation energy of starch hydrothermal degradation in subcritical water is quite low[2], the obtained results were, as such, expected. For this reason, the carbohydrates (in this case starch) should, not be induced in subcritical water for too long, otherwise the concentration rapidly decreases to such a point where no more carbohydrates are present.

Total amino acids and amines content

Proteins present in the sunflower seeds represent a different type of water-soluble biopolymer, compared to carbohydrates. As Rogalinski *et al.*[2] reported, proteins in subcritical water degrade a little differently than carbohydrates. Although the solubility of proteins is relatively high in ambient water, in subcritical water this changes, since the polarity of water becomes less similar to the polarity of the proteins. In addition, unlike carbohydrates, which tend to swell when in contact with hot water, proteins on the other hand exhibit the opposite effect. At increased temperature, the protein structure is usually coagulated (denaturized) and clumped into a semi-soft, solid-like substance, which consequently decreases the surface area of the protein and the water molecules, thus decreasing reaction degradation kinetics. Proteins are therefore much more stable in subcritical water, but do undergo rapid and irreversible coagulation, even at low temperatures.

From Fig. 2, it can be observed that indeed, compared to the carbohydrates, the total amount of amino acids (AA) and amines in the samples increases much slower. At 130 °C, almost no change in amino acid and amine content can be observed. Formation of free amino acids at this temperature is not expected. Concentration of total amino acids and amines in the extract is constant (8 %). This is probably due to bonding of the ninhydrin reagent to the surface of the coagulated proteins. Already at 160 °C, the content of total amino acids and amines starts to increase with time very slowly, whereas at 190 °C and higher temperatures, this increase becomes quite drastic. At 240 °C, a decrease in amino acids and amine content can be noticed, which probably indicates the formation of other amino compounds, such as ammonia[4]. Because ammonia is a gas at room temperature, it was probably removed in the solvent separation step. Although the formation of amino acids from proteins or simpler peptides is possible, it is important to note, that at these conditions, the amino acids tend to degrade quickly even further to simpler products if enough contact time with the subcritical medium is allowed. In these cases, mostly primary or secondary amines are formed, which are visible by the ninhydrin method. These compounds also cause a strong rancid odour in the food product, and their formation should be minimized as much as possible.

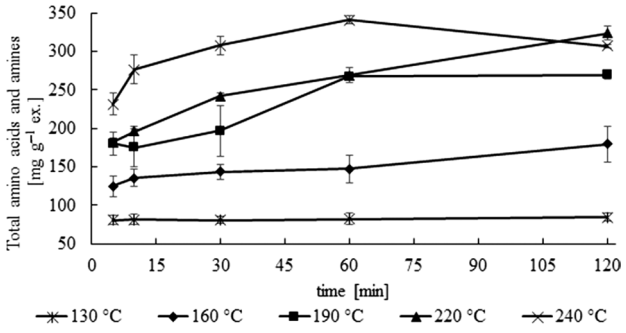

Fig. 2 – *Total AA and amines in water soluble fraction of sunflower seeds after treatment with subcritical water*

Conclusion

The results show that the oil, as the macronutrient present in sunflower seeds, is the most stable in subcritical water. Only small amounts of FFAs had formed during hydrothermal processing, but the amount drastically started to increase at 240 °C. Proteins seem to be less stable than the oils, whereas the carbohydrates have proven to be very susceptible to hydrothermal degradation, since total carbohydrate concentration rapidly decreases even at the lowest observed temperature. This decrease in total carbohydrates indicates that foods containing high amounts of carbohydrates should not be exposed to subcritical water at too high temperatures for too long; otherwise, the product is quickly degraded to non-edible or even toxic products, such as organic acids.

ACKNOWLEDGEMENTS

The authors are grateful to the Slovenian Ministry of High Education, Science and Technology for the financial support of this work. This paper was produced within the framework of the operation entitled "Centre of Open Innovation and Research of the University of Maribor (CORE@UM)".

References

1. *Pavlovič, I., Knez, Ž., Škerget, M.*, Hydrothermal Reactions of Agricultural and Food Processing Wastes in Sub- and Supercritical Water: A Review of Fundamentals, Mechanisms, and State of Research, J. Agric. Food Chem. **61** (2013) 8003. doi: http://dx.doi.org/10.1021/jf401008a
2. *Rogalinski, T., Liu, K., Albrecht, T., Brunner, G.*, Hydrolysis kinetics of biopolymers in subcritical water, J. Supercrit. Fluids **46** (2008) 335. doi: http://dx.doi.org/10.1016/j.supflu.2007.09.037
3. *Ravber, M., Knez, Ž., Škerget, M.*, Simultaneous extraction of oil- and water-soluble phase from sunflower seeds with subcritical water, Food Chem. **166** (2015) 316. doi: http://dx.doi.org/10.1016/j.foodchem.2014.06.025
4. *Sato, N., Quitain, A. T., Kang, K., Daimon, H., Fujie, K.*, Reaction Kinetics of Amino Acid Decomposition in High-Temperature and High-Pressure Water, Ind. Eng. Chem. Res. **43** (2004) 3217. doi: http://dx.doi.org/10.1021/ie020733n
5. *Kotnik, P., Škerget, M., Knez, Ž.*, Kinetics of supercritical carbon dioxide extraction of borage and evening primrose seed oil, Eur. J. Lipid Sci. Tech. **108** (2006) 569. doi: http://dx.doi.org/10.1002/ejlt.200600070
6. *DuBois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., Smith, F.*, Colorimetric Method for Determination of Sugars and Related Substances, Anal. Chem. **28** (1956) 350. doi: http://dx.doi.org/10.1021/ac60111a017
7. *Chutipongtanate, S., Watcharatanyatip, K., Homvises, T., Jaturongkakul, K., Thongboonkerd, V.*, Systematic comparisons of various spectrophotometric and colorimetric methods to measure concentrations of protein, peptide and amino acid: detectable limits, linear dynamic ranges, interferences, practicality and unit costs, Talanta **98** (2012) 123. doi: http://dx.doi.org/10.1016/j.talanta.2012.06.058
8. *Smith, R. M.*, Extractions with superheated water, J. Chromatogr. A **975** (2002) 31. doi: http://dx.doi.org/10.1016/S0021-9673(02)01225-6
9. *Kus, N. S.*, Organic reactions in subcritical and supercritical water, Tetrahedron **68** (2012) 949. doi: http://dx.doi.org/10.1016/j.tet.2011.10.070
10. *Minami, E., Saka, S.*, Kinetics of hydrolysis and methyl esterification for biodiesel production in two-step supercritical methanol process, Fuel **85** (2006) 2479. doi: http://dx.doi.org/10.1016/j.fuel.2006.04.017
11. *Alenezi, R., Leeke, G. A., Santos, R. C. D., Khan, A. R.*, Hydrolysis kinetics of sunflower oil under subcritical water conditions, Chem. Eng. Res. Des. **87** (2009) 867. doi: http://dx.doi.org/10.1016/j.cherd.2008.12.009
12. *Kumar, S., Gupta, R., Lee, Y. Y., Gupta, R. B.*, Cellulose pretreatment in subcritical water: Effect of temperature on molecular structure and enzymatic reactivity, Biores. Tech. **101** (2010) 1337. doi: http://dx.doi.org/10.1016/j.biortech.2009.09.035
13. *Zhao, Y., Wang, H.-T., Lu, W.-J., Wang, H.*, Combined supercritical and subcritical conversion of cellulose for fermentable hexose production in a flow reaction system, Chem. Eng. J. **166** (2011) 868. doi: http://dx.doi.org/10.1016/j.cej.2010.11.058
14. *Cantero, D. A., Dolores Bermejo, M., José Cocero, M.*, High glucose selectivity in pressurized water hydrolysis of cellulose using ultra-fast reactors, Biores. Tech. **135** (2013) 697. doi: http://dx.doi.org/10.1016/j.biortech.2012.09.035
15. *Grompone, M. A.*, Sunflower Oil, in *Gunstone F.* (Ed.), Vegetable Oils in Food Technology: Composition, Properties and Uses, Wiley-Blackwell, Oxford, UK, 2011, pp 137–167.