Application of enzymes for press oil production from pumpkin seeds

Hanna Vovk[1], Kwankao Karnpakdee[2], Olga Golubets[3], Iryna Levchuk[3], Roland Ludwig[2], Tamara Nosenko[1]

1 – National University of Food Technologies, Kyiv, Ukraine;
2 – University of Natural Resources and Life Sciences (BOKU), Vienna, Austria;
3 – State Enterprise "Ukrmetrteststandard", Kyiv, Ukraine

Keywords:

Enzymatic pretreatment
Press oil
Pumpkin seeds
Protease
Cellulase
Pectinase

Article history:

Received 29.10.2022
Received in revised form 16.01.2023
Accepted 31.03.2023

Corresponding author:

Tamara Nosenko
E-mail:
tamara_nosenko@ukr.net

DOI: 10.24263/2304-974X-2023-12-1-4

Abstract

Introduction. The aim of this research was to study the effect of pretreatment of pumpkin seed with proteolytic, cellulolytic, and pectolytic enzymes on pressed oil production.

Materials and methods. Enzymes, papain, pectinase, cellulase, pepsin, and cellulolytic enzyme mixture Viscozyme L were used in the study. The number of disrupted cells was determined by the amount of easily extractable pumpkin seed oil as result of immediate shaking. To study the cell microstructure, ultramicrotome slices of pumpkin seeds were treated with individual enzymes and enzyme mixtures. The antioxidant activity of the pumpkin seed oil was determined using (DPPH) 2,2-diphenyl-1-picrylhydrazylradical radical scavenging activity.

Results and discussion. The evaluation of cell integrity by immediate hexane extraction (shaking method) showed that all samples treated with different enzymes had a higher oil yield, ranging from 33.2 to 34.1% of seed weight, than the control samples (32.1%). The number of disrupted cells in enzyme treated samples was also higher than the control (64.4%), ranging from 67.6 to 69.5%. The highest amount of damaged cells, 71.0 and 71.1%, was found in the samples treated with mixtures (a) pepsin, Viscozyme L, pectinase and (b) pepsin, cellulase, with the pepsin+ViscozymeL+pectinase mixture was by 7.0% higher than that of the control sample. The enzymatic pretreatment did not significantly affect the free fatty acid content, peroxide value, fatty acid, and phytosterols composition. The antioxidant activity expressed as DPPH radical scavenging capacity of the pressed oil obtained from enzymatically pretreated pumpkin seeds was by 2.7% higher than of control oil.

Conclusion. Pretreatment of pumpkin seeds with a mixture of cellulolytic and proteolytic enzymes allowed increasing the yield of oil with high quality characteristics.

Introduction

Vegetable oils are the source of such essential substances for humans as polyunsaturated fatty acids, fat-soluble vitamins, phytosterols, and others. It is important to include in diet various vegetable oils with different fatty acid compositions and content of valuable micronutrients. Among vegetable oils, pumpkin seed oil is especially valuable due to its biologically active substances, which are useful in the treatment and prevention of many diseases (Dotto et al., 2020; Shaban et al., 2017). In particular, it contains natural antioxidants, ω-6 and ω-3 acids represented by linoleic and linolenic fatty acids, respectively, as well as squalene, which is a precursor for the synthesis of sterols, steroid hormones, and vitamin D (Dotto et al., 2020; Nosenko et al., 2019a). Carotenoids, tocopherols, and phenolic compounds, as well as oleic acid, are contained in relatively high amounts in pumpkin seed oil (Dotto et al., 2020; Procida et al., 2013; Shaban et al., 2017). Tocopherols of pumpkin seed oil have antihyperglycemic properties (Shaban et al., 2017; Sharma et al., 2013). Phytosterols contained in pumpkin seed oil prevent the development of cardiovascular disease due to the reduction of the level of the low-density lipoprotein cholesterol in the blood. At the same time, phytosterols are able to reduce the risk of some kinds of cancer (Dotto et al., 2020; Shaban et al., 2017).

Nowadays, two methods are used to obtain oil from oilcontaining seeds: pressing and solvent extraction. Solvent extraction is considered more efficient allowing the release of almost all oil from oil containing material. However, in addition to explosive and flammable solvents, numerous studies have found them dangerous to human health, as regular inhalation of its vapors can lead to diseases, such as peripheral neuropathy and sensory loss (Herskowitz et al., 1971; Kutlu et al., 2009). The release of organic solvent vapors into the environment is also negative due to its reaction with air pollutants and ozone formation (Montero-Montoya et al., 2018). Another disadvantage of solvent extraction is that obtained oil has to be refined, which causes the loss of most of its biologically valuable compounds. On the other hand, the pressing, especially cold pressing, is more environmentally friendly, as well as allows to the preservation of valuable natural components in the oil (Yakymenko et al., 2022). However, the pressing never removes the oil from the oil material completely and a significant oil content remains in the pressed cake.

Therefore, the development of pretreatment processes capable of increasing the oil yield during pressing and keeping the oil quality is very important. A promising method is the use of enzymes for disrupting plant cell walls. This potentially increases oil yield, but can also increase the nutritional value and antioxidant activity through the enhanced extraction of oil biologically active substances, such as phytosterols, tocopherols, and phenolic compounds (Kaseke et al., 2021; Latif et al., 2007).

The aim of this work was to study the influence of enzymatic pretreatment of pumpkin seeds on the cell integrity, pressed oil yield as well as its composition.

Materials and methods

Materials

Hull-less pumpkin seeds (*Cucurbita pepo*) with an oil content of 49.05% (determined according to ISO, 2009) were supplied by a farm at Doberndorf, Horn, Lower Austria. For pretreatment of pumpkin seeds pepsin, papain, pectinase, cellulase, and Viscozyme L were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pepsin (P7000) produced from

porcine gastric mucosa was purchased from Carl Roth (Karlsruhe, Germany). The declared activity at pH 2.0 and 37 °C is 800–2500 U/mg of solids, where 1 U will produce a change in A 280 of 0.001 per min measured as soluble in trichloroacetic acid products using hemoglobin as substrate. Papain (P3375) wasproduced from papaya latex crude powder. The declared activity at pH 6.2 (optimum) and 25 °C is 2.1 U/mg of solids, where 1 U will hydrolyze 1 µmol/min of N-α-benzoyl-l-arginine ethyl ester per minute. Cellulase (C2605) is produced by *Aspergillus sp*. The declared activity at pH 4.5 and 50 °C is 1000 CU/g, where 1 CU (cellulase unit) corresponds to the amount of enzyme which produces 1 µmol/min of glucose from carboxymethylcellulose. Pectinase (P2611) is produced from *Aspergillus aculeatus*. The declared activity at pH 3.5 (optimum) and 20 °C is 3800 polygalacturonic units/ml of suspension. The standard activity is determined by measuring the viscosity reduction of a pectic acid solution. Viscozyme L (V2010) is produced by *Aspergillus sp.* (a multi-enzyme mixture containing cellulases, β-glucanase, and hemicellulases like arabinase and xylanase). The declared activity at pH 5.0 and 50 °C is 100 fungal β-glucanase units/g.

Enzymatic pretreatment

To study the enzymatic pretreatment effect on the pumpkin seed cell structure, the seeds were ground using a laboratory mill to a particle size ≤5.0 mm. Ten g of ground seeds were placed in a 250-ml Erlenmeyer flask, suspended in 10 ml of 100 mM phosphate buffer, pH 5.5, and treated with the enzyme (0.6% w/w). The obtained suspensions were incubated in a water bath for 2.0 h at 48–52 °C with a manual stirring every 20 min. At the end of the incubation, the enzymes were inactivated at 80 °C during 15 min. The control samples were treated under the same conditions as the experimental ones,but without application of enzymes. The initial cell integrity was examined inground seeds before the treatment.

In some experiments, elevated enzyme amounts (1.8, 3.0 and 6.0% w/w) such as pepsin+Viscozyme L+pectinase, pepsin+cellulase+pectinase, papain+Viscozyme L+pectinase, or papain+cellulase+pectinase in a ratio of 1:1:1 were used under the conditions described above. Each variation of the treatment was performed in triplicate. After incubation and enzyme inactivation, all experimental and control samples were transferred from the flasks into evaporating porcelain cups and dried first in an oven at 100–110 °C for 3.0–3.5 h, and then overnight at room temperature, until moisture content was reached 0–2.0%. The dried material was re-ground in a laboratory mill and used to determine the number of disrupted cells.

Evaluation of disrupted cells by the method of immediate oil extraction

This method is used to determine the amount of easily extractable pumpkin seed oil and can be used to determine the fraction of disrupted plant cells. Therefore, 10 g of the re-ground samples (pretreated or reference samples) was placed in a 250-ml Erlenmeyer flask, mixed with 100 ml of n-hexane, and the contents of the flask were shaken for exactly 3 s. Then the flask was left for exactly 10 s and afterward, the obtained extract was immediately filtered into a weighed Erlenmeyer flask. The filter was washed with several portions of n-hexane and all portions were added to the weighed flask with the obtained extract. The hexane was distilled from the extracts on a rotary evaporator at a rotation speed of 50–54 rpm and at 45 °C. The obtained oil was dried in an oven at 100–105 °C for 1.0 h and then was left overnight under a fume hood for finally solvent removal. Afterward, the flask with dried oil was

weighed. The oil yield determined by the method of immediate extraction (a_1, %) was calculated using Equation 1:

$$a_1 = \frac{m_1 \cdot 100}{m_2} \quad (1)$$

where m_1 is a mass of the obtained oil (g); m_2 is a mass of the seed sample (g).

The number of disrupted cells (x, %) was calculated using Equation 2:

$$x = \frac{a_1 \cdot 100}{a_2} \quad (2)$$

where a_2 is the total oil content in pumpkin seeds (%).

From the results of three experimental replicates the mean value and standard deviation were calculated.

Pumpkin seed microstructure analysis

A cross-section of pumpkin seeds was sliced on a microtome. Pumpkin seed slices of 20 µm thickness were treated with pepsin, Viscozyme L, or pectinase as well as the enzyme mixture pepsin+Viscozyme L+pectinase in a microwell plate (same amount of enzymes as in previous experiments was dissolved in 1 ml of a phosphate buffer solution with a pH of 5.5 in each well). All samples were incubated for 2.0 h at 48–52 °C in a microwell plate placed in a petri dish with water in a laboratory oven. Some wells of the plate contained control samples, which were treated under the same conditions, but did not contain enzymes in the buffer solution. After incubation, all enzyme-treated and control samples were transferred to slides and fixed by drying at 65 °C in an oven for 1 h. All fixed samples were stained with Nuclear Fast Red for 5 min, afterward were examined by light microscopy using a microscope Leica DM750 with the camera Leica ICC50 and software Leica Application Suite version 3.0.0. Ten different locations on every sample were imaged and representative images are shown in Figure 3.

Pressing of pretreated pumpkin seeds

For the study of the effect of pumpkin seeds enzymatic pretreatment on the press oil yield, a 200 g of seeds were ground in a laboratory mill to the particle size ≤10.0 mm. Each seed portion was treated with the 1.8% enzyme mixture pepsin+Viscozim+pectinase in a ratio of 1:1:1 dissolved in 70 ml of a phosphate buffer solution with pH 5.5. The obtained mixture was placed in a glass jar and incubated at 48–52 °C for 2.0 h under constant shaking. The experiment was performed in triplicate. The control samples also were incubated in triplicate under the same conditions as the experimental samples, but without the presence of enzymes in the added buffer solution. After incubation, all samples were transferred from the jars to metal trays and dried in an oven at 100–110 °C for 1.5–2.0 h with thorough manually stirring every 30 min to a moisture content of 3.0–4.3%. Afterward, all driedsamples were weighed and pressed on a laboratory screw press at 100–125 °C and a nozzle hole diameter of 6.0 mm. The pressed oil yield in terms of seed weight was calculated using Equation 1. The oil yield in terms of total oil content in the pumpkin seeds was calculated using Equation 2. From the results of three experimental replicates of each control or experimental treatment, a mean value and standard deviation was calculated.

Analysis of pumpkin seed residues

Determination of the oil content in residual pressed cake was performed in the Soxhlet apparatus according to ISO 659:2009. The experiment was performed in triplicate with further calculation of the mean value and standard deviation.

Analysis of extracted oil

Quality parameters

To evaluate the quality of pressed pumpkin seed oils, free fatty acid (FFA) content and peroxide value (PV) were determined according to the ISO 660:2020, and ISO 3960:2007, respectively. From the results of three replicates of each experiment, the mean value and standard deviation were calculated.

DPPH radical scavenging activity and kinetic analysis

Determination of the antioxidant activity of the press pumpkin seed oils was carried out by the method of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (Broznic et al., 2016). The solution of 2,2-diphenyl-1-picrylhydrazyl at a concentration of 3 mg/100 ml ofethyl acetate, which had an optical density in the range of 0.7–0.9 at a wavelength of 520 nm. To prepare the reaction mixture 15 ml of DPPH solution were added to 100 mg of oil, mixed thoroughly, and the initial absorbance of the reaction mixture was determined at 520 nm (A_0). The reaction mixture was kept in the dark place and the absorbance was measured at 520 nm (A_t) at a specified time interval (every 5 min) for 25 min. DPPH radicals scavenging effect of the oil solutions ($AA_{t\%}$) at specified time intervals was calculated according to Equation 3:

$$AA_t = \frac{(A_0 - A_t) \cdot 100}{A_0} \qquad (3),$$

where A_0 is an absorbance of the freshly prepared DPPH oil solution;

A_t is an absorbance of the DPPH oil solution at time t.

Antioxidant activity ($AA_{25\%}$) was the total DPPH scavenging for 25 min. The experiment at each time interval was performed in triplicate, and the mean values and standard deviations were calculated.

Fatty acid composition analysis

Determination of the fatty acid composition of the press pumpkin seed oils was carried out by gas-liquid chromatography of fatty acid methyl esters (Nosenko et al., 2014). For the preparation of fatty acid methyl esters, 100 mg of each oil sample was dissolved in a 2 ml solution (0.5 g/l) of butylated hydroxytoluene in heptane. Then 100 µl of sodium in methanol solution (46 mg/ml) was added, solution have been mixed for 2 min and exposed for 15 min. 1 to 2 g of sodium hydrosulfate was added. The samples were filtrated through the anhydrous sodium sulfate, and then 2 ml of butylated hydroxytoluene heptane solution was added. The solution obtained was filtrated one more time through a 0.45 µm membrane cellulose filter, and the filter was washed with 1 ml of the same solvent. The two filtrates were combined and the solution was used for analysis. The obtained fatty acid methyl esters were analyzed on Hewlett Packard gas chromatograph model HP 6890 with capillary column HP-88 (88%-

cyanopropyl aryl-polysiloxane; 100 m × 0.25 mm; 0.25 µm film thickness (Agilent Technologies)). The temperature of the injector was 280 °C, the detector had a temperature of 290 °C. The temperature steps and heating rates were as follows: holding the temperature at 60 °C for 4 min; heating to 150 °C at 4 °C/min, holding at for 10 min; heating to 180 °C at 3 °C/min, holding for 5 min; heating to 190 °C at 3 °C/min, holding for 2 min, heating to 230 °C at 3 °C/min, holding for 2 min; heating to 260 °C at 4 °C/min, holding for 2 min. The flow rate of carrier gas was 1.2 ml/min, and the sample volume was 1.0 µl. Identification of the fatty acids was performed by comparison of the retention times with a standards mixture of fatty acid methyl esters (37 Component FAME Mix, SUPELCO). Reported data are the mean value and standard deviation of three analytical replicates.

Phytosterols composition

Analysis of phytosterols composition was performed according to ISO 6799:1991. 10 ml of standard betulin solution and 100 ml of alcoholic KOH solution were added to the 100 mg oil sample and boiled in a water bath for 1 h. After cooling, the sample was transferred to a separatory funnel by adding 200 ml of distilled water and 100 ml of diethyl ether, shaken vigorously, and separatedthe upper ether layer. The washing was repeated 3 times. The combined ether extracts were transferred to another separatory funnel by adding 100 ml of distilled water and shaken gently. After delamination, the aqueous layer was drained, and the washing was repeated 3 times. The upper ether solution was filtered through a pad of sodium sulfate. The solvent was evaporated on a rotary evaporator to give 1 ml of solution. The solution was applied with a micro syringe to the prepared silicon wafer and placed in the developer tank. The plate was removed after distillation and the solvent was allowed to evaporate. Silicon containing the sterol fraction was collected with a micro spatula. The silicon was placed in an Erlenmeyer flask and boiled with 5 ml of diethyl ether in a water bath for 15 min. The solution was cooled and filtered through a paper filter. The silicon from the filter paper was re-extracted and filtered twice more. The residue was dissolved in a minimum amount of solvent for development and analyzed by gas phase chromatography. The measurement was performed on a gas chromatograph CP-3800 (Varian), equipped with a flame ionization detector, electronic gas flow control system, universal injector for the introduction of samples in the split and non-separation modes, and autosampler (CP-8410 Varian). A capillary column MET-Biodiesel with a built-in pre-column (14 m × 0.53 mm; 0.16 µm film thickness) was used under the following conditions: carrier gas flow rate, 5.0 ml/min; flow separation factor 1:10; evaporator temperature, 360 ºC; detector temperature, 390 ºC; column temperature mode: gradual heating from 160 ºC to 340 ºC. The sample injection volume was 1 µl. Reported data are the mean value and standard deviation of three analytical replicates.

Results and discussion

Effect of the enzyme pretreatment on the cell integrity of pumpkin seeds

All pumpkin seeds samples, treated with single enzymes, such as pepsin, papain, Viscozyme L, celulase, and pectinase, gave a higher oil yield with immediate oil extraction, ranging from 33.2 to 34.1%, compared with the oil yield from control samples, 32.1%, which was incubated only with 100 mM phosphate buffer, pH 5.5 (Figure 1). It was found that seed samples treated with different enzymes had also a higher number of disrupted cells, varing from 67.6 to 69.5% (control samples had 64.4%).

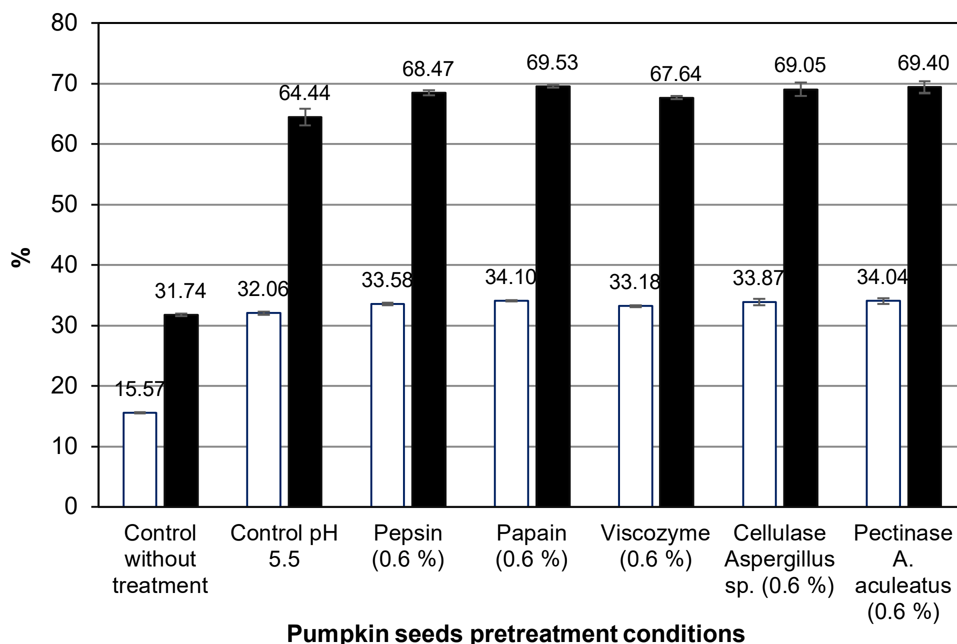

Figure 1. Oil yield ($a_{1,\%}$) and number of disrupted cells (x, %) of control samples and samples pretreated with single enzyme, estimated by the method of immediate oil extraction

It was shown that pretreatment of milled hulled pumpkin seeds with acid protease at pH 5.2 for 2.0 h at 48–52 °C leads to an increase in the number of disrupted cells by 10.4% compared to control samples (Nosenko et al., 2019b). However, the percentage of disrupted cells in control and enzyme-treated samples were 46.7 and 57.1%, respectively, that are lower than it was received in the present study (64.4 and 69.5%). This can be explained by the presence of the husk covering pumpkin seeds in the previous research.

In a study of rapeseed enzymatic pretreatment with cellulase (from *Bacillus subtilis*, 300 units/g, from Enzyme, Ukraine) in the amount of 2.0% (w/w) for 2 h at 40–42 °C a relatively low percentage of disrupted cells in both control and enzyme-treated samples also were found, that was 35 and 50%, respectively (Cherstva et al., 2016). In the present study, the higher percentage of disrupted cells can be associated with significantly higher hydrolysis temperature, while a smaller difference between the number of disrupted cells in the pretreated and control samples may be due to the use of less amount of enzyme by seed weight.

At the same time, the greatest difference in the percentage of disrupted cells was observed between control samples incubated in phosphate buffer at pH 5.5 for 2 h and control samples without this treatment, which indicates that swelling is an important factor to consider in the pretreatment, even without the use of enzymes. A similar result was obtained during the study of the different sunflower seed pretreatment methods effect on the n-hexane oil extraction. Due to the treatment of dehulled seeds with convective heat or steam, the oil yield was by 11% higher than during the extraction of crude seeds for 1.0 h, while the treatment of seeds with Viscozyme L in combination with heat or steam resulted in increased oil yield compared to crude control by 12 and 14%, respectively (Danso-Boateng, 2011).

Effect of the pretreatment with the enzyme mixtures on the cell integrity

All variations of the pumpkin seed pretreatment with different enzyme mixtures lead to an increase in oil yield during immediate oil extraction and to an increase in the number of disrupted cells in comparison to the control samples (Figure 2). Initially, four different enzyme mixtures were used for seeds treatment for 2.0 h with the amount of mixture for each sample of 1.8% (w/w). The highest amount of disrupted cells 71.0 and 71.1% were detected in samples treated with pepsin+ Viscozyme L+pectinase and pepsin+cellulase+pectinase mixtures, respectively, which was by 6.6% higher than in control samples. A similar result was achieved during the treatment of milled pumpkin seeds with hulls with a mixture of acid protease and cellulase (from *Trichoderma reesei*) in different ratios at pH 5.2, the number of disrupted cells reached 60%, which was by 3% more than in control samples (Nosenko et al., 2019b).

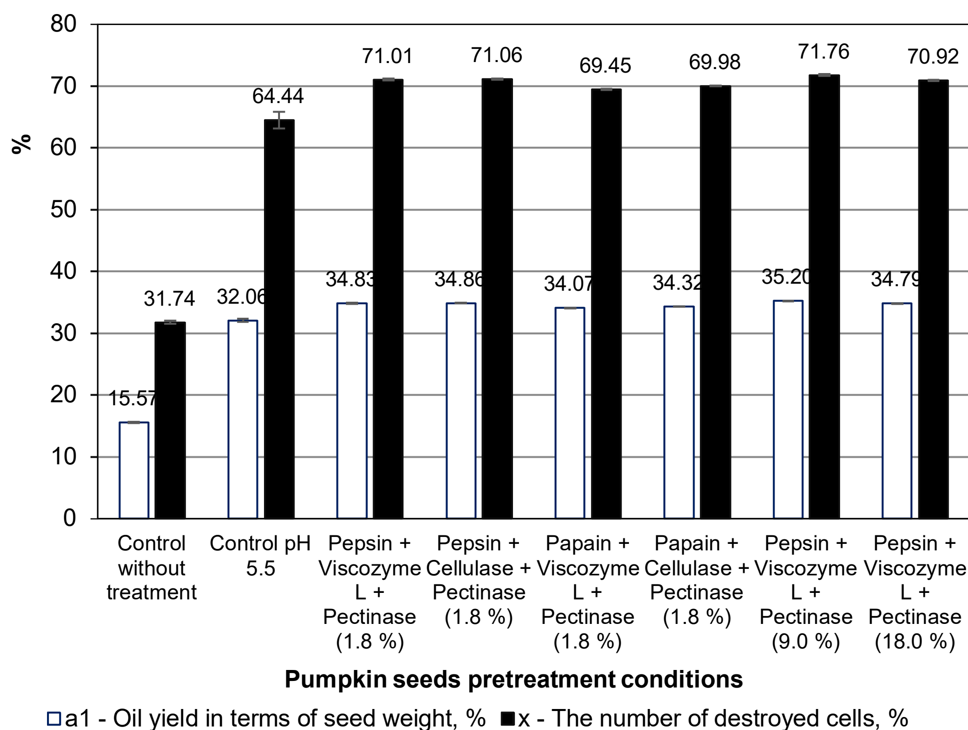

Figure 2. Oil yield ($a_{1,\%}$) and number of disrupted cells (x, %) of control samples and samples pretreated with enzyme mixtures, estimated by the method of immediate oil extraction

For study of the effect of added enzyme amount, the ground pumpkin seeds were treated with a pepsin+Viscozyme L+pectinase mixture. However, no significant change in the percentage of disrupted cells was observed when the amount of added enzyme mixture was raised 5- or 10-times (9.0 and 18.0% w/w, respectively). An explanation for the ineffectiveness of increased enzyme concentrations is, that the high concentration already saturates available cleavage sites and that an increase beyond a monolayer coverage of the substrate does not lead to a higher activity. Similarly, the soybean- and sunflower seeds were

treated by enzyme mixtures with cellulolytic and pectolytic activities in the amounts from 0.01 to 5.0% by seed weight before oil extraction with n-hexane (Dominguez et al., 1993). It was found that increasing of the enzyme mixture concentration above 1.0% during soybean pre-treatment and more than 2.0% for sunflower seeds did not lead to intensification of oil extraction. In another study of enzymatic pretreatment of rapeseeds with a mixture of protease (from *Bacillus subtilis*, 70 units/g) and cellulase (from *Bacillus subtilis*, 300 units/g) the concentration of enzyme mixture was changed from 0.4 to 1.4% w/w. The optimal enzyme concentration was found to be 0.6%, which allowed achieving a pressed oil yield of 43.1% (Cherstva et al., 2016). Excessive increases in the added enzyme amount and in the enzymatic pretreatment duration are not only economically unprofitable but can also lead to hydrolysis of polysaccharides with the formation of free reducing sugars, which will be quickly caramelized at further drying and prevent the release of oil (Kumar et al., 2017; Latif, 2009).

Influence of the enzymatic pretreatment on the pumpkin seed cells microstructure

To observe the effect of the enzymatic pretreatment on the cell walls and oil bodies within the pumpkin seed cells, microtome slices of pumpkin seeds were enzymatically treated and compared to non-treated samples (Figure 3). Oil bodies are protected by the cell walls and all cells are intact in the control samples of pumpkin seed slices treated with buffer without the addition of enzymes (Figure 3a). Pepsin-treated samples show intact cell walls but also oil bodies which are less stained indicating that the oleosin-containing protein membrane covering the oil body has been partially degraded by the protease (Figure 3b). Pectinase hydrolyzes the pectin gluing cells together, which is visible (Figure 3c). The thickness of the cell walls of pectinase-treated slices was also lower than in the control and pepsin-treated slices, which indicates the presence of cellulases and other cell wall hydrolyzing enzymes. Some ruptured cells have lost their oil bodies, which are visible outside the cells (upper left corner). The effect of Viscozyme L was the largest observed since it is already a mixture of different enzymes and contains cellulases, β-glucanase, and hemicellulases, which have a much bigger effect on the cell wall structures, disrupted a large portion of the cells and released most of the oil bodies from the cells (Figure 3d). A similar result was observed in the treatment of sunflower kernels by Viscozyme L (Danso-Boateng, 2011). The most effective pretreatment of pumpkin seed slices was obtained with a mixture of pepsin+Viscozim+pectinase (Figures 3e and 3f). The cell walls completely disappeared and only residues are observed. Some of the oil bodies are still intact, but also large spots of oil from coagulated oil bodies with some intact oil bodies are visible that indicate a large number of ruptured oleosomes (Figure 3f). In two studies of the pretreatment effect of pomegranate seeds with Pectinex Ultra SPL, Flavorzyme 100 L, and cellulase crude enzymes, as well as the enzymatic treatment of palm fruit fiber with Cellic CTec2, Cellic HTec2, and Pectinex Ultra SPL mixture, significant destruction of cells walls and oleosomes was achieved too (Kaseke et al., 2021; Silvamany and Jamaliah, 2015).

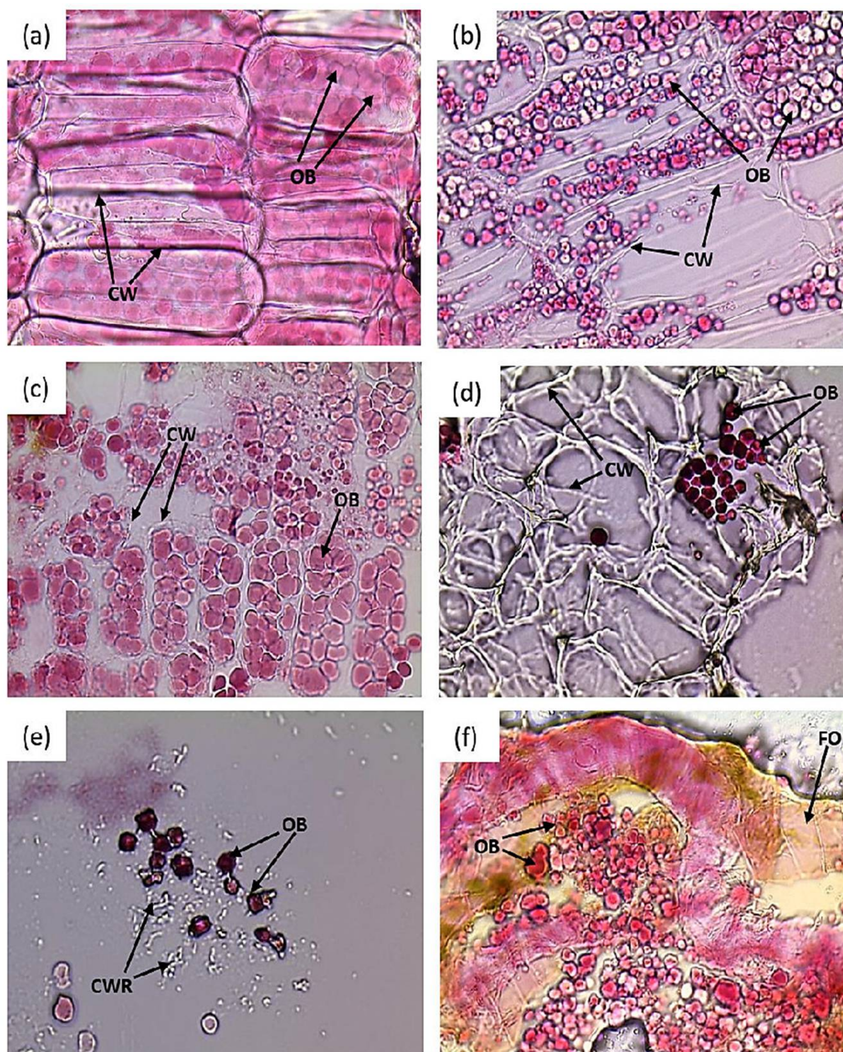

Figure 3. Pumpkin seed microtome slices stained with Nuclear Fast Red.
The slices treated without the addition of enzyme (a), pepsin treated slices (b), pectinase treated slices (c), Viscozyme L treated slices (d), the slices treated with pepsin+Viscozyme L+pectinase mixture (e, f).
Magnification = 100 ×, CW – cell walls, OB – oil bodies, CWR – cell wall residues, FO – free oil

During *in vitro* digestion of a walnut oil body dispersion with pepsin, large oil bodies were revealed indicating their coalescence because of surface peptide destruction (Gallier et al., 2013). The effect of pepsin in combination with the cellulolytic, hemicellulolytic, and pectinolytic enzymes becomes clear in the experiment with the enzyme mixture (Fig. 3, e, f), demonstrating that the proteolytic activity is needed for the destruction of membrane protein of oil bodies leading to the coagulation into large oil droplets.

At the same time, it is important to mention that such a complete degradation of the cell wall structure is only possible in the case of microtome slices with a thickness of 20 µm. It is almost impossible to achieve such a degree of destruction with the large particle sizes commonly used for mechanical pressing (0.5 to 5 mm or even intact pumpkin seeds), as enzymes will have a greater effect on smaller particles with a higher specific surface area. Cells inside a large particle are inaccessible to enzymes until the cells around them are degraded.

Effect of enzymatic pretreatment on press oil yield

The press oil yield from seeds treated with pepsin+Viscozyme L+pectinase mixture was 15.5% (Figure 4), which is by 7.0% higher than in the control samples, while the oil yield calculated to the base of the total oil content in pumpkin seeds is by 14.3% higher compared to control samples (31.6%). A similar result was found in a study of the enzyme-assisted cold pressing of cotton seeds with the highest press oil yield of 12.89% (by seed weight) obtained from samples treated with Kemzyme compared to an oil yield of 8.50% in control samples (Latif et al., 2007). The increase in oil yield by 5.5 and 6.1% compared to the control samples was achieved because of flax- and hemp seed treatment with Viscozyme L, respectively.

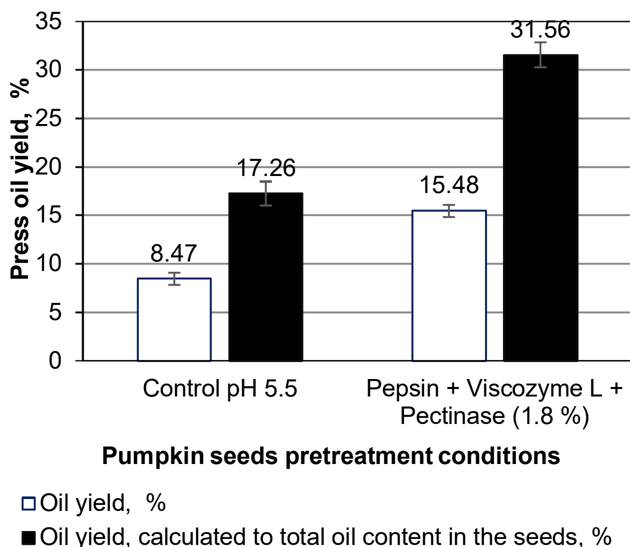

Figure 4. Press oil yield of control samples and enzymatically pretreated samples

Low oil yields in both enzyme-treated and control samples may be due to the used parameters of pressing, such as press temperature, nozzle hole diameter, particle size, and moisture content of oil material, which may be not optimally selected for this type of seeds. The residual cake oil content in terms of seed weight decreased from 39.5% in the control samples to 31.8% in samples treated with a pepsin+Viscozyme L+pectinase mixture (Figure 5).

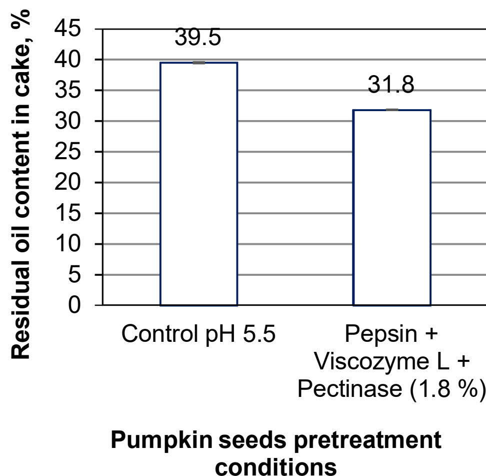

Figure 5. Residual cake oil content of control samples and enzyme pretreated samples

Oil quality and antioxidant activity

Characteristics oil quality and antioxidant activity of pumpkin seed oil are shown in Table 1. The content of free fatty acids in pressed oil from seeds treated with pepsin+Viscozyme L+pectinase mixture was slightly higher compared to the control sample, while its peroxide value is slightly lower than in the control. Similar results were observed after the treatment of cotton seeds with Phytezyme, Kemzyme, and Feedzyme, which led to some increase of free fatty acids in the obtained pressed oil (3.80, 3.90, and 3.60 respectively) compared with the oil obtained from the control (3.22), as well as in flaxseeds treated by Viscozyme L, Kemzyme, and Feedzyme, the peroxide value was slightly lower (1.90, 2.25, and 2.19 meq/kg, respectively) than in the control (2.35 meq/kg) (Anwar et al., 2013;Latif et al., 2007). As a result of hemp seed pre-treatment with Kemzyme, Protex 7L, Viscozyme L, Feedzyme, and Natuzyme, slight variations of free fatty acids (1.73–1.87% as oleic acid) and peroxide value (1.54–1.62 meq/kg) were observed relative to the control (1.75% and 1.57 meq/kg, respectively) (Latif et al., 2009). The decrease of the peroxide value in the oil pressed from enzymatically pretreated seeds may be due to the higher release of tocopherols and increased antioxidant properties compared to the control oil. At the same time, in a study of enzyme-assisted cold-press oil of borage seeds, it was found that the effect of enzymatic pretreatment of seeds on the content of free fatty acids and peroxide values was insignificant (Soto et al., 2007).

Table 1

Free fatty acids, peroxide value, and DPPH scavenging capacity of control oil and oil from enzyme-treated pumpkin seeds

Sample	Acid value, mg KOH/g oil	Peroxide value, meq O/kg oil	Antioxidant activity, AA_{25} , %
Control	0.94±0.13	1.7±0.04	17.6±0.1
Press oil from enzyme-treated seeds	1.03±0.13	1.6±0.1	20.2±0.1

Enzymatic pretreatment of pumpkin seeds had a positive effect on the oil antioxidant activity, which expressed as a DPPH radical disappearance. The results show that the total DPPH scavenging effect of pressed oil from enzyme-treated seeds was by 2.7% higher than that of control press oil (Table 1). A similar result was obtained during enzymatic treatment of flax seeds, DPPH radical scavenging of press oil from samples treated with Viscozyme L, Feedzyme, and Kemzyme (50.03, 45.30, and 43.01% respectively) was significantly higher compared to DPPH radical disappearance of oil from control seed samples (35.20%) (Anwar et al., 2013). Antioxidant activity of oil from seeds pomegranate cultivar was increased by enzymatic pretreatment from 1.60 to 2.91 mmol Trolox/g of pomegranate seed oil as well as DPPH scavenging effect (Kaseke et al., 2021). DPPH radical disappearance of cold press perilla seed oil was 50.6%, while the perilla seeds oil treated with ultrasound-assisted aqueous enzymatic extraction in combination with cellulase, neutral proteinase, and pectinase had a DPPH scavenging capacity of 70.6% (Li et al., 2017). At the same time, total DPPH radical scavenging of both enzyme-assisted and control pressed pumpkin seed oils was much lower in the current study than reported in other previous studies of this oil's antioxidant properties, where DPPH scavenging activity of pumpkin seed oil obtained by n-hexane extraction was 35.5%, while in unrefined pressed oils from roasted pumpkin seeds, this value ranged from 32.28 to 65.33% (Agustina et al., 2019; Andjelkovic et al., 2010).

The effect of enzymatic pre-treatment of pumpkin seeds on its oil DPPH scavenging capacity over time is shown in Figure 6. DPPH scavenging activity of enzyme-assisted pressed oil is significantly higher at each time point than that of the control pressed oil. The fast, initial phase of the reaction shows a fast disappearance of DPPH radicals within the first 5 min. Afterward, the reaction slows down. In an analogous study of the biphasic DPPH disappearance kinetics in pumpkin seed oil, the first, fast phase occurred between 4.5–6.5 min in oil samples obtained from roasted seed paste, while for cold-pressed oils from unroasted seeds the duration of the first phase was 8.5–10.5 min (Broznic et al., 2016).

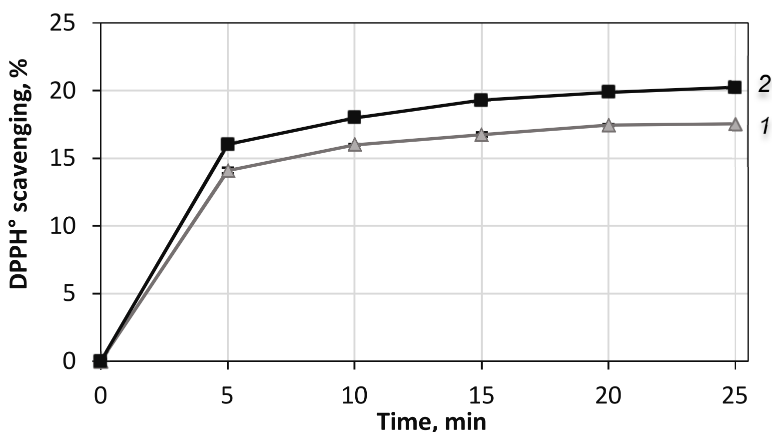

Figure 6. The kinetics of DPPH scavenging by pumpkin seed oil:
1 – control press pumpkin seed oil, 2 – enzyme pretreated press pumpkin seed oil

Fatty acid composition of pumpkin seed oil

The fatty acid composition of control and enzyme-assisted pressed pumpkin seed oils are shown in Table 2. The most abundant fatty acids among unsaturated fatty acids for both control and enzyme-treated samples were linoleic acid (56.2–57.0%) and oleic acid (23.2–24.3%), while the predominant saturated fatty acids were palmitic acid and stearic acid with an amount of 12.1 and 6.0%, respectively. Similarly, the study of the fatty acid composition of 100 different Styrian pumpkin seed oils from different breeding lines showed that the main pumpkin seed oil fatty acids, such as linoleic-, oleic-, palmitic-, and stearic acids, were found in amounts of 43.8–52.4, 28.6–38.1, 11.4–13.3, and 4.8–6.7% respectively (Frühwirth et al., 2007;Procida et al., 2013). The content of these fatty acids varied in the ranges of 44.30–51.58, 33.60–42.59, 9.13–13.35, and 0.27–0.55%, respectively when twelve pumpkin seed oil samples of different origins were analyzed.

Table 2

Fatty acid composition of control oil and oil from enzyme-treated pumpkin seeds

Fatty acid	Content of fatty acid, % of the total content	
	Control press oil	Press oil from enzyme-treated pumpkin seeds
Palmitic acid (C 16:0)	12.07±0.15	12.14±0.15
Palmitooleic acid (cis-9-C 16:1)	0.11±0.05	0.12±0.05
Stearic acid (C 18:0)	5.98±0.10	5.96±0.10
Oleic acid (cis-9-C 18:1)	24.28±0.20	23.23±0.20
Octadecenoic acid (cis-11-C 18:1)	0.51±0.15	0.61±0.15
Linoleic acid (cis, cis-9,12-C 18:2)	56.15±0.20	57.03±0.20
α-Linolenic acid (cis,cis,cis-9,12,15-C 18:3)	0.16±0.20	0.16±0.20
Arachidic acid (C 20:0)	0.38±0.05	0.37±0.05
Behenic acid (C 22:0)	0.11±0.05	0.12±0.05
Saturated fatty acids	18.70	18.75
Monounsaturated fatty acids	24.98	24.03
Polyunsaturated fatty acids	56.32	57.22
Unsaturated fatty acids:Saturated fatty acids	4.35	4.33

Because of enzymatic pretreatment of pumpkin seeds, the content of linoleic acid in the pressed oil increased slightly, while the amount of oleic acid reduced, but also not significantly (Table 2). In spite of the higher polyunsaturated fatty acids amount and lower monounsaturated fatty acids content were observed, however, the unsaturated fatty acids: saturated fatty acids ratio did not change significantly. There also was no significant difference in the amount of palmitic-, palmitoleic-, stearic-, octadecenoic-, α-linolenic-, arachidic-, and behenic acids in the control and enzyme-treated samples. In line with the present study, the research of cotton-, flax-, hemp-, and borage seed cold pressing revealed no significant impact of enzymatic pretreatment on its oil fatty acid profiles (Anwar et al., 2013; Latif et al., 2007; 2009; Soto et al., 2007).

Phytosterols composition of pumpkin seed oil

The phytosterols composition of pumpkin seed oils is presented in Table 3.

Table 3

Phytosterol composition of of control oil and oil from enzyme-treated pumpkin seeds

Phytosterol	Content of phytosterol, % of the total content	
	Control press oil	Press oil from enzyme-treated pumpkin seeds
Cholesterol	0.77± 0.05	0.82± 0.05
Campesterol	1.74± 0.10	1.78± 0.10
Stigmasterol	1.95±0.15	2.88±0.15
24-Methylcholest-7-enol	0.91±0.05	0.76±0.05
α-Spinasterol+Δ7,22,25-Stigmastatrienol	48.69±0.20	47.46±0.20
Δ5-Avenasterol	1.75±0.10	2.56±0.10
Δ7,25-Stigmastadienol	22.47±0.20	20.71±0.20
Δ7-Stigmastenol	4.81±0.10	4.52±0.10
Δ7-Avenasterol	16.90±0.15	18.51±0.15

α-Spinasterol and Δ7,22,25-stigmastatrienol accounted for almost 50% of the total sterol content and were found to be the predominant phytosterols of oils from control and enzyme pretreated pumpkin seed, while Δ7,25-stigmastadienol and Δ7-avenasterol were also present in relatively high amounts 20.7–22.5 and 16.9–18.5%, respectively. Previous studies of the pumpkin seed oil phytosterols composition reported that Δ7-sterols, such as α-spinasterol, Δ7,22,25-stigmastatrienol, Δ7,25-stigmastadienol, Δ7-avenasterol, and Δ7-stigmastenol, significantly predominate over Δ5-sterols (Dotto et al., 2020; Fruhwirth et al., 2007).

A slight decrease of α-spinasterol, Δ7,22,25-stigmastatrienol, and Δ7,25-stigmastadienol content was observed in the enzyme-assisted press pumpkin seed oil, while the content of stigmasterol, Δ5-avenasterol, and Δ7-avenasterol slightly increased. Similarly, the stigmasterol content of the oil obtained from enzyme-pretreated pomegranate seed samples was significantly higher (29.8–52.0mg/100 g oil), compared to control pomegranate seed oil samples (20.5–45.8 mg/100 g oil), that might be due to reduced complexation of the phytosterols with the seed polysaccharides and further enhancement of their mass transfer into the oil phase (Kaseke et al., 2021).

Conclusions

1. Proteolytic enzymes are needed to degrade the oleosin-rich membranes around the oil bodies in seed cells. In the mixtures with cellulolytic, hemicellulolytic and pectinolytic enzymes, proteolytic enzymes help to disrupt pumpkin seed cells synergistically leading to the most prominent increase of oil yield.
2. The yield of press pumpkin seed oil was about 14.3% higher from seeds incubated with a pepsin+Viscozyme L+pectinase mixture at 48–52 °C for 2.0 h at pH 5.5 compared to the seeds pretreated at the same conditions, but without adding enzymes.

3. There was no significant influence of enzyme treatment on the press oil quality and composition, but a slightly higher total DPPH scavenging capacity of oil from enzyme-pretreated seeds was detected. This oil had also higher stigmasterol content.

.

Acknowledgment. The authors gratefully acknowledge the financial support provided by the Ministry of Education and Science of Ukraine (Contract № M/51-2022, 24. 05. 2022). Funding has also been provided by the Austrian Agency for Education and Internationalisation through a Scientific and Technological Cooperation Grant the Ukraine and Austria (WTZ grant Nr. UA 05/2020).

References

Agustina N.A., Elisabeth J., Julianti E. (2019), A comparison studies on the chemical characteristics of pumpkin seed oil with extra virgin olive oil and palm olein, *International Conference on Agriculture, Environment, and Food Security 2018 24–25 October 2018, Medan, Indonesia*, 260(1), 012091, https://doi.org/10.1088/1755-1315/260/1/012091

Andjelkovic M., Van Camp J., Trawka A., Verhe R. (2010), Phenolic compounds and some quality parameters of pumpkin seed oil, *European Journal of Lipid Science and Technology,* 112(2), pp. 208–217, https://doi.org/10.1002/ejlt.200900021

Anwar F., Zreen Z., Sultana B., Jamil A. (2013), Enzyme-aided cold pressing of flaxseed (*Linumusitatissimum*L.): Enhancement in yield, quality and phenolics of the oil, *GrasasAceites*, 64, pp. 463–471, https://doi.org/10.3989/gya.132212

Broznic D., CanadiJuresic G., Milin C. (2016), Involvement of α-, γ- and δ-tocopherol isomers from pumpkin (*Cucurbita pepo* L.) seed oil or oil mixtures in the biphasic DPPH disappearance kinetics, *Food Technology and Biotechnology*, 54(2), pp. 200–210, https://doi.org/10.17113/ftb.54.02.16.4063

Cherstva A., Lastovetska A., Nosenko T. (2016), Using of enzymes to extract of rapeseed oil by pressing, *Ukrainian Journal of Food Science*, 4 (1), pp. 85–92.

Danso-Boateng E. (2011), Effect of enzyme and heat pretreatment on sunflower oil recovery using aqueous and hexane extractions, *International Journal of Chemical, Materials and Biomolecular Sciences*, 4(1), pp. 28–34, https://doi.org/10.5281/zenodo.1327718

Dominguez H., Nunez M.J., Lema J.M. (1993), Oil extractability from enzymatically treated soybean and sunflower: range of operational variables, *Food Chemistry*, 46(3), pp. 277–284, https://doi.org/10.1016/0308-8146(93)90119-Z

Dotto J. M., Chacha J. S. (2020), The potential of pumpkin seeds as a functional food ingredient: A review, *Scientific African*, 10, e00575, https://doi.org/10.1016/j.sciaf.2020.e00575

Fruhwirth G.O., Hermetter A. (2007), Seeds and oil of the Styrian oil pumpkin: Components and biological activities, *European Journal of Lipid Science and Technology,* 109(11), pp. 1128–1140, https://doi.org/10.1002/ejlt.200700105

Gallier S., Tate H., Singh H. (2013), *In vitro* gastric and intestinal digestion of a walnut oil body dispersion, *Journal of Agriculture and Food Chemistry,* 61(2), pp. 410–417, https://doi.org/10.1021/jf303456a

Herskowitz A., Ishii N., Schaumburg H. (1971), n-Hexane neuropathy: A syndrome occurring as a result of industrial exposure, *The New England Journal of Medicine*, 285, pp. 82–85, https://doi.org/10.1056/NEJM197107082850204

Kaseke T., Opara U. L., Fawole O. A. (2021), Effects of enzymatic pretreatment of seeds on the physicochemical properties, bioactive compounds, and antioxidant activity of pomegranate seed oil, *Molecules*, 26(15), 4575, https://doi.org/10.3390/molecules26154575

Kumar S.P.J., Prasad S.R., Banerjee R., Agarwal D.K., Kulkarni K.S., Ramesh K.V. (2017), Green solvents and technologies for oil extraction from oilseeds, *Chemistry Central Journal,* 11(9), pp. 1–9, https://doi.org/10.1186/s13065-017-0238-8

Kutlu G., Gomceli Y. B., Sonmez T., Inan L. E. (2009), Peripheral neuropathy and visual evoked potential changes in workers exposed to n-hexane, *Journal of Clinical Neuroscience,* 16 (10), pp. 1296–1299, https://doi.org/10.1016/j.jocn.2008.12.021

Latif S., Anwar F., Ashraf M. (2007), Characterization of enzyme-assisted cold-pressed cottonseed oil, *Journal of Food Lipids*, 14(4), pp. 424–436, https://doi.org/10.1111/j.1745-4522.2007.00097.x

Latif S., Anwar F. (2009), Physicochemical studies of hemp (*Cannabis sativa*) seed oil using enzyme-assisted cold-pressing, *European Journal of Lipid Science and Technology,* 111(10), pp. 1042–1048, https://doi.org/10.1002/ejlt.200900008

Li H., Zhang Z., He D., Xia Y., Liu Q., Li X. (2017), Ultrasound-assisted aqueous enzymatic extraction of oil from perilla seeds and determination of its physicochemical properties, fatty acid composition and antioxidant activity, *Food Science and Technology*, 37(1), pp. 71–77, https://doi.org/10.1590/1678-457X.29116

Montero-Montoya R., Lopez-Vargas R., Arellano-Aguilar O. (2018), Volatile organic compounds in air: Sources, distribution, exposure and associated illnesses in children, *Annals of Global Health*, 84(2), pp. 225–238, https://doi.org/10.29024/aogh.910

Nosenko T., Kot T., Kichshenko V. (2014), Rapeseeds as a source of feed and food proteins, *Polish Journal of Food and Nutrition Science*, 64(2), pp. 109–114, https://doi.org/10.2478/pjfns-2013-0007

Nosenko T., Koroluk T., Usatuk S., Vovk G., Kostinova T. (2019a), Comparative study of walnut and pumpkin seeds oils biological value and oxidative stability, *Food Science and Technology,* 13(1), pp. 60–65, https://doi.org/10.15673/fst.v13i1.1311

Nosenko T., Vovk G., Koroluk T. (2019b), Effect of hydrolytic enzymes pretreatment on the oil extraction from pumpkin seeds, *Ukrainian Food Journal*, 8(1), pp. 80-88, https://doi.org/10.24263/2304-974X-2019-8-1-9

Procida G., Stancher B., Catenia F., Zacchignaa M. (2013), Chemical composition and functional characterisation of commercial pumpkin seed oil, *Journal of the Science and Food Agriculture,* 93(5), pp. 1035–1041, https://doi.org/10.1002/jsfa.5843

Shaban A., Sahu R. P., (2017), Pumpkin seed oil: An alternative medicine, *International Journal of Pharmacogn and Phytochemical Research,* 9(2), pp. 223–227, https://doi.org/10.25258/phyto.v9i2.8066

Sharma A., Sharma A. K., Chand T., Khardiya M., Yadav K. C. (2013), Antidiabetic and antihyperlipidemic activity of *Cucurbita maxima* duchense (pumpkin) seeds on streptozotocin induced diabetic rats, *Journal of PharmacognPhytochemistry,* 1(6), pp. 108–116.

Silvamany H., Jamaliah M. J. (2015), Enhancement of palm oil extraction using cell wall degrading enzyme formulation, *Malaysian Journal of Analytical Science*, 19(1), pp. 77–87.

Soto C., Chamy R., Zuniga M. E. (2007), Enzymatic hydrolysis and pressing conditions effect on borage oil extraction by cold pressing, *Food Chemistry*, 102(3), pp. 834–840, https://doi.org/10.1016/j.foodchem.2006.06.014

Yakymenko I., Bublienko N., Salavor O., Nychyk O., Shapovalov Ye. (2022), Energy security of the European Union: challenges of russian energy resources, *Selected Papers of IV International Conference on European Dimensions of Sustainable Development*, *October 20–21, 2022, Kyiv, NUFT*, *Kyiv.*