

PROTEOMIC ANALYSIS AND ANTIOXIDANT EVALUATION OF ULTRASOUND - ASSISTED POLLEN EXTRACTS

Ancuța-Veronica Lupaescu^a, Laura Ion-Darie^b,
Brîndușa Alina Petre^{b,c,*}, Florin Ursachi^d and Mircea Oroian^d

^a*Department of Biomedical Sciences, Stefan cel Mare University of
Suceava, 13 University, Suceava 720229, Romania*

^b*Department of Chemistry, "Al.I. Cuza" University Iasi, 11 Carol I Bd
Iasi 700506, Romania*

^c*Center for Fundamental Research and Experimental Development in
Translation Medicine – TRANSCEND, Regional Institute of Oncology,
2-4 General Henri Mathias Berthelot, Iași 700483, Romania*

^d*Department of Food Technologies, Safety of Food Production and the
Environment, Stefan cel Mare University of Suceava Romania,
13 University, Suceava 720229, Romania*

Abstract: The identification of compounds with antioxidant activity is considered to be of great interest to both the pharmaceutical and cosmetic industries. Bee products such as pollen, propolis, beeswax and royal jelly have long been used for their medicinal and health-enhancing properties. Bee pollen is a raw product rich in secondary metabolites and high concentrations of phytochemicals and nutrients. Protein is contained in the pollen matrix at an average of 22.7% and its content varies among plant species. The present study aimed to analyze the protein content of linden and hawthorn pollen. After a delipidation step, pollen samples were extracted using ultrasonic energy and three different extraction buffers. After quantification by the Bradford colorimetric method, protein extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Fourier transform infrared spectroscopy. Finally, we evaluated the total antioxidant capacity of pollen extracts using the iron(III) reduced antioxidant capacity assay.

Keywords: pollen; proteins; antioxidant activity; ultrasounds

* Brîndușa Alina, Petre, *e-mail:* brindusa.petre@uaic.ro

Introduction

Apiculture products have been used medicinally since ancient times. In traditional medicine, people consume bee products for their therapeutic effects. The ability of bee products to improve health properties is due to their high levels of bioactive compounds.¹ Numerous studies showed promising reports on the antibacterial, antifungicidal, antioxidant and anti-inflammatory potential of natural bee products such as pollen, propolis, bee wax and royal jelly.^{1,2} Bee pollen is a raw product rich in secondary metabolites and high concentrations of phytochemicals and nutrients. Bee pollen is produced by flowering plants and collected by bees, who mix it with nectar and bee secretions to form small granules. In addition to nutritional components such as proteins, amino acids, lipids and carbohydrates, , bee pollen is rich in bioactive compounds.³ Quality and quantity of pollen protein are known to vary among plant species, its levels ranging from 10 to 40% of dry mass.⁴

Nowadays, natural substances capable of neutralize the effects of free radicals and oxidative stress underlying the pathogenesis of various diseases have become highly attractive for the pharmaceutical and cosmetic industry. Among polyphenols, proteins play an important role in the nutraceutical potential of pollen. Previous studies have confirmed the antioxidant activity of proteins and peptides. For example, proteins contribute to the endogenous antioxidant capacity of various foods by inhibiting lipid oxidation, indicating excellent potential as antioxidant additives.⁵ Moreover, Maqsoudlou *et. al* suggested that the antioxidant activity of pollen proteins can be increased through controlled enzymatic hydrolysis following which bioactive peptides with high antioxidant and ACE2 inhibitory potential can be generated.⁶ Although the polyphenolic

composition of bee products has been characterized in numerous studies, the proteomic matrix is still under investigation. Therefore, this study is intended for providing new information on the protein composition and antioxidant capacity of linden and hawthorn pollen samples.

Ultrasound-assisted extraction (UAE) is a conventional technique that uses acoustic waves in order to provide a higher solvent penetration into the sample matrix. The ability of ultrasound to improve extraction efficiency has been associated to the appearance of cavitation phenomenon generated when an extraction solvent in contact with a sample is subjected to ultrasonic wave. Due to its multiple advantages, such as reduced time and laboratory accessibility, this green technique is increasingly used in the extraction of biological active compounds.⁷⁻¹⁰ The present study compares the ability of carbonate and ammonium buffer systems to extract peptide compounds from the pollen matrix and allows the identification of the optimal solvent to be used in further qualitative research.

Results and Discussion

The current study uses linden and hawthorn pollen as raw starting material. Following extraction procedure, the protein content was assessed using the time-tested colorimetric method. Table 1 shows the protein content extracted from pollen samples by the UAE technique. The highest protein content was observed in pollen extracted with carbonate buffer. Besides, the concentration of the linden extract proved to be higher than the one obtained using the hawthorn sample. In general line, the linden sample extracted with carbonate buffer presents the highest concentration of proteins while the lowest protein content was observed on hawthorn pollen extracted on ammonium buffer. Furthermore, by examining the

morphological changes in pollen samples after UAE treatment, it is evident that the ultrasound waves caused disruption to the cell wall of pollen grains, allowing for high-efficiency protein extraction.

Table 1. Protein content ($\mu\text{g}/\text{mL}$) of pollen extracts in different buffer.

<i>Pollen</i>	<i>Buffer</i>	<i>Carbonate-Bicarbonate</i> <i>0.1 M pH 8.5</i>	<i>Ammonia-ammonium</i> <i>acetate 0.1 M pH 8.5</i>
<i>Linden</i>		883 ± 0.04	796 ± 0.09
<i>Hawthorn</i>		780 ± 0.12	660 ± 0.06

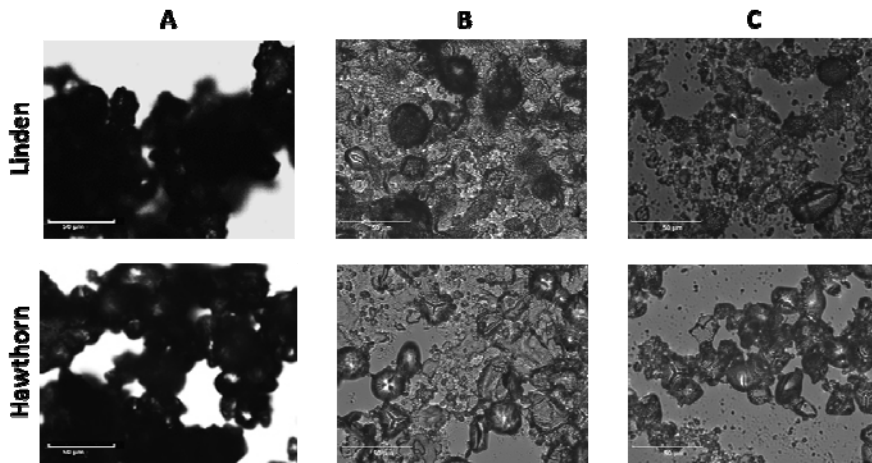


Figure 1. Morphologies of pollen samples captured by the Celena S Digital Imaging System before extraction (A), after extraction on carbonate buffer (B), after extraction on ammonium buffer (C). Scale bar, 50 μM .

In order to provide information about the molecular size, amount, and diversity of a pollen proteins, the extracts were subjected to one-dimensional gel electrophoresis.¹¹ The obtained gels were further stained using Coomassie and silver staining procedures. As seen in Figure 2 (right), pollen extracts from the samples contain a mixture of different proteins as indicated by the numerous bands in each lane. Although the signals for the

pollen samples extracted in different buffers are similar, visible differences can be easily observed between the two pollen samples. For example, in the case of linden samples most of the proteins have a molecular mass situated in the range of 70-40 kDa, with the exception of the signal from approximately 18 kDa. In the case of hawthorn samples, the molecular masses of the extracted proteins are found in a narrower range: 60-40 kDa. This result confirms the diversity of the pollen matrix within the native species. However, the lack of a difference between the composition of samples extracted in carbonate medium and those obtained in acetate solution may be due to the pH value, which is 8.5 in both buffers.

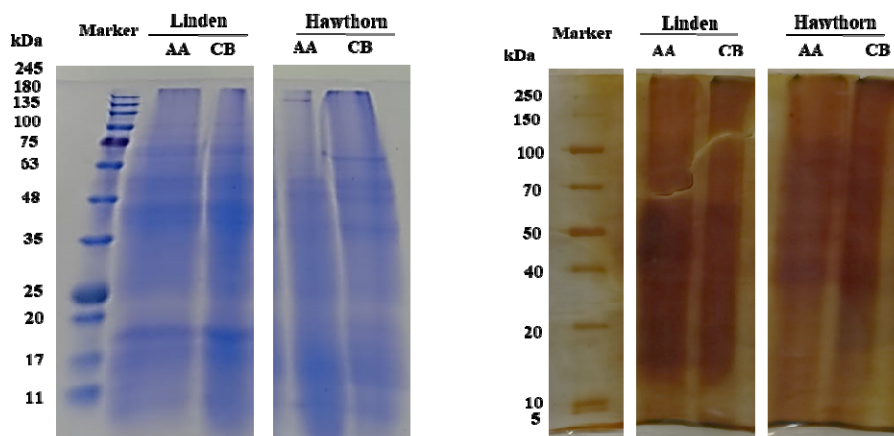


Figure 2. One-dimensional gel electrophoresis of pollen protein extracts: Coomassie-stained (**left**) and silver-stained (**right**) 10% SDS polyacrylamide gel. The samples extracted contained 80 μg (**left**) and 30 μg (**right**) of protein mixture purified by acetone precipitation. **AA**: Ammonia-Ammonium acetate buffer; **CB**: Carbonate-Bicarbonate buffer.

The silver staining method is a more sensitive method than Coomassie that allows detection of protein samples as small as 0.25–5 ng.¹² This allows visualizing bands that are unstained with Coomassie blue dye. Thus, as observed in Figure 2 (left), the hawthorn extract contains small amounts of proteins whose molecular weight is found in the range

60-100 kDa. Following electrophoresis, the separated proteins can be recovered from Coomassie stained gels for subsequent characterization by a variety of secondary techniques, such as mass spectrometry to determine the amino acid sequence.¹³

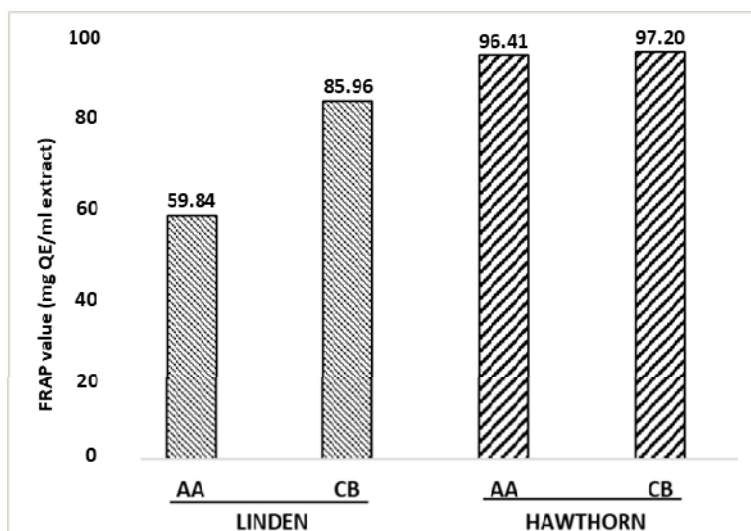


Figure 3. Total FRAP value of pollen extracts expressed in mg quercetin (QE) per mL extract. **AA:** Ammonia-Ammonium acetate buffer; **CB:** Carbonate-Bicarbonate buffer.

Extraction techniques and solvent nature can mediate the total antioxidant capacity of a sample. The ferric reducing antioxidant power (FRAP) assay is a common method that measures the reduction of Fe^{3+} -tripyridyltriazine complex to the Fe^{2+} -ligand complex by antioxidants in an acidic medium. The antioxidant activity detected at 593 nm can be expressed as micromolar ferrous (Fe^{2+}) equivalents, relative to an antioxidant standard or reducing activity percentage.¹⁴⁻¹⁶ As observed in Figure 3, the strongest antioxidant activity was found in the carbonate extracts of pollen samples: 97.2 mg QE/mL hawthorn sample and 85.96 mg QE/mL linden extract. Interestingly, hawthorn pollen demonstrated the strongest reduction response, indicating that, despite having less protein than the linden matrix, it contains a greater number of natural reducing compounds.

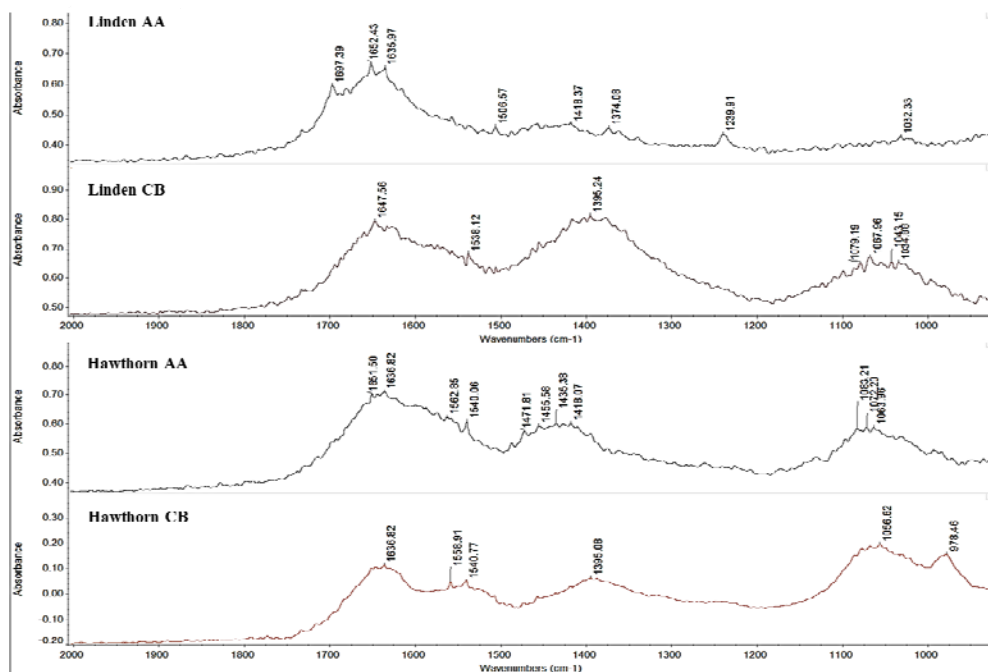


Figure 4. FT-IR spectra of linden and hawthorn pollen samples extracted in ammonium buffer (AA) and carbonate buffer (CB).

Fourier transform infrared spectroscopy – attenuated total reflectance (FTIR–ATR) is a widely used analytical technique. This method provides essential information necessary for structural characterization of various compounds. Figure 4 presents the FT-IR spectra of linden and hawthorn pollen extract that was obtained in carbonate and ammonium buffer. Although the spectra were recorded in the range $4000\text{--}400\text{ cm}^{-1}$, the most relevant peaks were found between 1800 and 900 cm^{-1} . The range between 1700 and 1500 cm^{-1} contains peaks assigned to the vibration of peptide bonds (CO-NH) such as Amide I ($\nu\text{ C=O}$, $\nu\text{ C-N}$) in 1640 cm^{-1} and Amide II in 1550 cm^{-1} ($\delta\text{ N-H}$, $\nu\text{ C-N}$).¹⁷ These peaks are closely related to the amount of protein found in the samples.

The region located at $1200\text{--}900\text{ cm}^{-1}$ is dominated by polysaccharide peaks. For example, the peak at approximately 1080 cm^{-1} observed in

hawthorn extracts that is associated to OH stretch highlights the presence of carbohydrates in the samples. The intensity of the carbohydrate region proved to be lower for the linden samples, the peak from 1030 cm^{-1} (ν C-O of alcohols) having a more intense signal in carbonate buffer. Other signals observed in the FTIR spectra can be attributed either to the extraction medium or to other compounds found in the pollen matrix.

Experimental

Materials: Crude pollen samples were acquired from a local beekeeper supplier (Suceava County, Romania) in 2020 and were stored in a freezer at $-20\text{ }^{\circ}\text{C}$ until further use. All reagents were of analytical grade and were used without further purification. Ethanol absolute, sodium bicarbonate, sodium hydroxide, ammonium acetate and ammonium hydroxide were purchased from Merck (Darmstadt, Germany) while the protein ladder was obtained from Thermo Fisher Scientific (Massachusetts, USA). Furthermore, methanol, acetone and 2,4,6-Tris(2-pyridyl)-s-triazine were procured from Sigma–Aldrich (St. Louis, USA) while the Bradford Assay Kit, Bradford solution and albumin were purchased from Carl Roth (Karlsruhe, Germany)

Sample preparation: Prior to protein extraction the freshly milled pollen ($<125\text{ }\mu\text{m}$) was subjected to a combined pre-treatment for the removal of lipids impurities and polyphenols compounds. In this regard, 7 g of crude pollen were transferred into a conical tube with 21 mL ethanol absolute and stirred at room temperature (RT) for 3 hours at 400 rpm. After centrifugation at 4°C for 10 min and 5000 rpm the defatting step was repeated. The defatted samples were air dried overnight at RT.

Ultrasonic Extraction Procedure: Protein extractions from linden and hawthorn pollen samples were carried out using two extraction buffers: i) bicarbonate buffer 0.1 M pH 8.5 and ii) ammonia-ammonium acetate buffer solution 0.1 M pH 8.5. The extraction step was performed using 4.5 mL solution buffer for 150 mg pollen. The samples were subjected to ultrasonic extraction for 30 minutes on a Homogenizer (type CY- 500, JP SELECTA Co, Spain) set to pulse width 5 seconds and an ultrasonic power (amplitude) of 45%. After centrifugation, at 4°C for 10 min and 5000 rpm, the supernatant was separately collected and stored in the fridge before further analyses.

The morphological characterization was performed using a Celena S Digital Imaging System on 10x and 40x objective lenses. The images were processed using Mountains premium 9 software (Digital surf, Lavoisier, France).

Determination of protein content: The exact protein concentration of the samples was determined by Bradford colorimetric method using Bradford solution kit. The calibration curve (20 - 200 µg/mL) was generated according to the product guidance using dilutions of a standard albumin protein stock solution (400 µg/mL) dissolved in bi-distilled water. Prior to quantification, the extracted samples were diluted 1:10 (v/v) with bi-distilled water. Explicitly, 50 µL of sample buffer, standard albumin protein dilution or diluted samples were pipetted in 96-well microplates and mixed with 200 µL of diluted protein assay dye reagent (2 mL concentrated reagent + 5,5 mL bi-distilled water) and incubated for 5 minutes at RT. Finally, the absorbance was measured at 595 nm using a multi-mode microplate reader (BioTeck, Argilent, USA).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out under reducing conditions according to the Laemmli procedure from 1970. Acetone precipitation of proteins mixture (~ 80 µg), in a fourfold excess volume followed by incubation for 2h at -20°C, was performed prior to SDS-PAGE analysis. The samples were centrifuged for 10 min at 12,000 rpm and 4°C and left to dry overnight at RT. The pellet was redissolved in 20 µl SDS sample buffer and the mixture was heat-denatured at 55 °C for 5 min prior to loading the sample wells. The SDS gel was run for 30 min at 40 V and at 110 V for another 2 h. Finally, the gel was incubated overnight in Coomassie brilliant blue staining solution and destained the next day with Coomassie destaining. For evaluating the molecular weight of unknown proteins, a protein ladder ranging from 11 to 245 kDa (Thermo Scientific, USA) was used as reference. The silver staining was performed using a different protocol on another polyacrylamide gel that contained a protein ladder ranging from 5 to 250 kDa. Following the first step of fixation, the gel was incubated with protein treatment solution and washed for 5 minutes with MilliQ before adding the coloring solution containing 0.1% silver nitrate. Finally, the gel was subjected to the developing step. Once the desired intensity was achieved, the stop solution was added.

Ferric reducing antioxidant power (FRAP) assay was realized according to Benzie and Strain method, with small modifications were made to adjust the assay on a 96-well microplate.¹⁸ FRAP reagent was obtained by mixing 5 mL acetate buffer (0.3 M, pH 3.6) with 0.5 mL 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) 10 mM solution dissolved in 40 mM HCl and 0.5 mL of 20 mM Fe(NO₃)₃. 25 µL of investigated protein extract solution were added to the 275 µL of FRAP reagent freshly prepared. The samples were incubated for 30 min at 37°C before reading the plate at 593 nm. All

measurements were made in triplicate and averaged. The results were shown in mg quercetin equivalents per 1 mL pollen extract.

Fourier-transform infrared spectroscopy (FT-IR) analysis was performed using a Nicolet i-20 spectrophotometer (ThermoScientific, USA). The precipitated samples (2 mg protein mixture) were placed on the ATR crystal and the spectra were recorded in transmission mode within the wavenumber range of 4000–400 cm^{-1} at a resolution of 4 cm^{-1} . Spectra Gryph–spectroscopy software (Version 1.2.11) was used for spectra analysis.

Conclusions

Natural products, including bee products, are used by consumers as alternative medicine, especially for curative purposes. This study suggests that the protein matrix of pollen samples can be easily extracted using conventional and ultrasonic techniques and alkaline medium. Alkaline treatment accelerates matrix degradation and allows extraction of active compounds. Differences in protein composition observed in electrophoretic separations affected the antioxidant capacity produced by each pollen sample. As expected, both linden and hawthorn extracts give positive FRAP reactions and can be considered as promising sources of bioactive compounds. However, further studies are needed to fully characterize the pollen-extracted antioxidant proteins and elucidate the mechanisms of action that may be activated upon free radical scavenging.

Acknowledgements

DECIDE - Development through entrepreneurial education and innovative doctoral and postdoctoral research, project code POCU / 380/6/13/125031, project co-financed from the European Social Fund through the 2014 – 2020 Operational Program Human Capital.

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