International Journal of Food Science +Technology

Institute of Food Science +Technology ifst

Original article

Performance of green and conventional techniques for the optimal extraction of bioactive compounds in bee pollen

Volkan Aylanc,[1] Seymanur Ertosun,[1] Paulo Russo-Almeida,[2] Soraia I. Falcão[1*] & Miguel Vilas-Boas[1*]

1 Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, Bragança 5300-253, Portugal
2 Laboratório Apícola – LabApis, Departamento de Zootecnia, Universidade de Trás-os-Montes e Alto Douro (UTAD), Vila Real 5000-801, Portugal

(Received 17 December 2021; Accepted in revised form 22 February 2022)

Summary The exploitation of phenolic compounds in different fields has motivated researchers to explore eco-friendly and efficient extraction techniques. This study aimed to comparatively reveal that green extraction techniques (microwave- and ultrasound-assisted) are alternative to conventional extraction (maceration and magnetic stirring) with positive impact on the phenolic content, antioxidant activity, and bioactive profile of bee pollen extracts. The highest total phenolic and flavonoid content was reached using the microwave-assisted technique (MAE) with equivalent values of 28 and 8 mg g^{-1}, with magnetic stirring and maceration showing a lower value. The composition profile of the extracts revealed the presence of twenty-six bioactive compounds, including thirteen phenolics and thirteen phenylamides. Although the extraction technique had little impact on the chemical diversity, the amount of bioactive compounds raised significantly with the use of the green extraction techniques, with gains between 40% and 60% for phenolics and up to 200% for phenylamides. The radical scavenging activity and the reducing power of the extracts confirmed that bee pollens are potent antioxidant source, with the most bioactive extracts corresponding to green extraction techniques. Consequently, all findings recommend the use of MAE as the technique most effective for the extraction of bioactive compounds from naturally encapsulated structures such as bee pollen.

Keywords Antioxidants, bee pollen, extraction techniques, green extraction, phenolic profile.

Introduction

Honeybees are social insects belonging to the genus *Apis*, producing valuable natural products such as honey, apitoxin, bee pollen, propolis, royal jelly, and wax. Bee products have been collected and used by humans for centuries with the thought of treating many diseases and enhancing the nutritional status (Aylanc *et al.*, 2021a). Nowadays, with the progress of modern medicine and the development of nutritional characterisation techniques, bee pollen has emerged as a natural product that can be used as a potential therapeutic and supplementary food (Feás *et al.*, 2012; Denisow & Denisow-Pietrzyk, 2016; Tomás *et al.*, 2017).

Bees, while harvesting pollen in the flower, mix the grains with their own secretions, agglutinating it as pellets (1–4 mm in size) on the insect's hind legs, which are then transported to the hive. This pellet is called bee pollen and is as valuable as other bee products (Aylanc *et al.*, 2021a). The worthiness of bee pollen is attributed to its high nutritional value and especially to the richness of bioactive compounds that have positive effects on health (Denisow & Denisow-Pietrzyk, 2016). The bioactive compounds of bee pollen are strongly dependent on the pollen's botanical origin and geographical conditions (Muñoz *et al.*, 2020). The consumption of bee pollen has increased worldwide due to its inclusion in food as an health ingredient, taking advantage of its numerous functional properties, including anticancer, antiobesity, antimicrobial, anti-inflammatory, antioxidant, and antihyperpigmentation (Maruyama *et al.*, 2010; Cheng *et al.*, 2019; Kaškonienė *et al.*, 2020; Tuoheti *et al.*, 2020). Moreover, bee pollen can regulate hypolipidaemic activity by decreasing the content of total lipids and cholesterol (Denisow & Denisow-Pietrzyk, 2016), and improving liver function (Uzbekova *et al.*, 2003).

*Correspondent: E-mail: sfalcao@ipb.pt (S.I.F.); mvboas@ipb.pt (M.V.-B.)

doi:10.1111/ijfs.15672

© 2022 Institute of Food, Science and Technology (IFSTTF).

For the application of nutraceutical sources in food and health systems, it is important to assess the complete range of bioactive compounds present, which frequently requires suitable extraction techniques for their separation and characterisation. Selection of the extraction technique and optimisation of practical parameters are also critical for scale-up purposes. Regarding bee pollen specificities, there are some difficulties in effectively removing bioactive compounds from the natural matrix. The most important of these is the bee pollen wall structure, which is arranged in a double layer resistant to physical and chemical factors, unlike many other plant and plant-based products (Aylanc *et al*., 2021a). Besides, since bee pollen contain many types of pollen grain coming from different plants, the wall structures (intine and exine thickness, etc.) of the pollen types may also be affected by the applied extraction technique at different levels (Aylanc *et al*., 2021a). Therefore, the selection and optimisation of suitable techniques for the extraction of bioactive compounds in bee pollen are crucial for the effectiveness of their biological activity assessment.

The most preferred extraction techniques for bee pollen are based on conventional methods, including maceration, percolation, hot continuous and magnetic stirring (Carpes *et al*., 2007; Feás *et al*., 2012; El-Ghouizi *et al*., 2020). Conventional extraction techniques require, in general, more energy input, time, and labour (Biesaga, 2011). Then, the global awareness for more sustainable processes triggers the research for 'greener extraction techniques' such as microwave-assisted and ultrasound-assisted extraction, because they can significantly reduce the above-mentioned limitations and are also accessible (Biesaga, 2011; Ameer *et al*., 2017; Carbone *et al*., 2020). These techniques are also eco-friendly due to the reduction of chemical inputs, without compromising their efficiency when compared to conventional extraction techniques (Ameer *et al*., 2017; Dhanani *et al*., 2017). Despite the benefits of microwave-assisted and ultrasound-assisted extraction over conventional extraction techniques being known (less processing time, cost-effective, and less solvent usage), natural capsulated products such as bee pollen have been poorly studied under those techniques and should therefore be clarified.

The aim of this study was to assess the impact of different extraction techniques on the bioactive compound content and antioxidant activity of bee pollen and to select the most appropriate technique for bee pollen characterisation. Conventional extraction techniques, such as maceration (MR), magnetic stirring (MS), and green extraction techniques like microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE), were investigated regarding the total phenolic content (TPC), total flavonoid content (TFC), bioactive compounds' profile, and antioxidant activity.

Materials and methods

Chemicals and reagents

Ethanol, methanol, acetonitrile, formic acid, trichloroacetic acid, gallic acid, ferric chloride, and potassium ferricyanide were purchased from Fisher Scientific (Pittsburgh, PA, USA). Folin–Ciocalteu's reagent and glacial acetic acid were purchased from Panreac Applichem (Barcelona, Spain). Aluminium chloride, potassium persulfate, and naringenin were purchased from Acros Organics (Pittsburgh, PA, USA), and sodium carbonate anhydrous was purchased from Labkem (Barcelona, Spain). 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-di-(3-ethylbenzothiazoline sulphonic acid (ABTS), quercetin, chrysin, and *p*-coumaric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Kaempferol was purchased from Extrasynthese (Genay, France).

Sample collection and preparation

Three different bee pollen samples were collected using pollen traps from *Apis mellifera iberiensis* hives in May and June 2019. The bee pollen samples, named as BP1, BP2, and BP3, were collected from the west of Portugal (Leiria), north of Portugal (Vila Real), and east of Portugal (Nisa), respectively. Bee pollen samples were cleaned from debris of wax and dead bee parts. All samples were freeze-dried using a lyophiliser (FreeZone 4.5 model 7750031, Labconco, Kansas City, MO, USA) and stored at −20 °C until further analysis.

Palynological analysis

The homogenised samples, about 2 g, were placed in separate vials with distilled water and vortexed vigorously. Then, 200 μL was taken from the resulting mixture and centrifuged at 1000 ***g*** for 15 min. The obtained pellet was subjected to acetolysis according to the method reported previously (Louveaux *et al*., 1978). Pollen identification and counting were performed using an optical microscope (Nikon Microphot-FXA, Melville, NY, USA). More than 1200 grains per preparation were counted following the criteria of Vergeron (Vergeron, 1964).

Extraction procedures

Previous to extraction, the freeze-dried bee pollen samples were powdered using a lab-type blender. Three parallel samples of 2 g were prepared for each extraction technique and $EtOH/H_2O$ (80:20, v/v) was used for all analyses. The temperatures and times of all extraction methods were made according to the applied methods and results of previously reported studies in the literature (Trusheva *et al*., 2007;

© 2022 Institute of Food, Science and Technology (IFSTTF).

Dhanani *et al.*, 2017; Galan *et al.*, 2017; Aylanc *et al.*, 2021b), by considering factors such as maximum yield and minimum degradation of bioactive compounds.

Maceration extraction
The MR extraction was performed by modification of a previously reported method (Trusheva *et al.*, 2007). Briefly, the pollen samples (2 g) were combined with 40 mL of 80% EtOH in an Erlenmeyer flask and placed in the dark for 72 h. During the incubation, the samples were agitated occasionally. The samples were then vacuum filtered and evaporated at 40 °C in a rotary evaporator (model Hei-VAP, Heidolph, Schwabach, Germany). The extracts were freeze-dried and stored at −20 °C until further analysis.

Magnetic stirring extraction
The MS extraction was carried out according to the method reported by Tomás *et al.* (2017). Briefly, 2 g of each sample was mixed with 40 mL of 80% EtOH and then kept in agitation with a magnetic stirrer (Multimatic 9-N, Selecta, Barcelona, Spain) for 6 h. The resulting mixture was vacuum filtered and the residue was re-extracted under the same conditions with additional 40 mL of solvent. After the extracts were combined and the solvent was evaporated under vacuum at 40 °C. Finally, bee pollen extracts were lyophilised and stored at −20 °C until further analysis.

Ultrasound-assisted extraction
The UAE was performed according to the method described by Carbone *et al.* (2020), with some modifications. Two grams of bee pollen sample was mixed with 40 mL of 80% EtOH in a glass beaker and placed in an ultrasonic homogeniser (Model CY-500, Optic Ivymen System, Barcelona, Spain) under continuous mode, at 400 W, for 20 min. The ultrasound titanium probe (diameter: 1 cm) was placed in the centre of the beaker containing the sample/solvent, 2 cm above the base. The energy density applied was 12 $kJcm^{-3}$. During the ultrasonic process, the glass beaker containing the sample solution was immersed in a water bath. The temperature of the extraction solution was monitored with a digital thermometer, Figure S1A, and kept constant at ~25 °C, adding ice when necessary to the water bath. After this period, the extracts were vacuum filtered and evaporated under vacuum at 40 °C. The extracts were then lyophilised and stored at −20 °C until further analysis.

Microwave-assisted extraction
MAE was performed in a Microwave Synthesis/Extraction System professional multi-mode microwave oven (Model: NuWav-Uno, Nutech Analytical Technologies, Kolkata, India) with a maximum capacity of 1200 W. The temperature was monitored by a platinum probe (Pt100 sensor, diameter: 3 mm) inside the microwave oven while stirring of the sample was provided by a magnetic stirrer integrated with the microwave oven. This device was also equipped with software that allowed both time and energy to be programmed. Two grams of bee pollen sample was mixed in 40 mL of 80% EtOH in a round-bottom angled three-neck flask and extraction was performed at 200 W microwave power for 10 min. The energy density applied was set to 3 $kJcm^{-3}$. During the extraction of the samples, the maximum temperature was set to 80 °C through the software integrated with the microwave extraction system, Figure S1B, so that in case the solution temperature reaches the set maximum, the device automatically reduces the energy to control the temperature. After extraction, the reactor was cooled before opening. Thereafter, the extracts were vacuum filtered and evaporated under vacuum at 40 °C. The extracts were lyophilised and stored at −20 °C until further analysis.

Phenolic content

Total phenolic content
TPC was determined by the Folin–Ciocalteu method (Falcão *et al.*, 2013). According to the procedure, 0.5 mL of ethanolic extract (1 mg/mL) was mixed with 0.25 mL of Folin–Ciocalteu's reagent. After 3 min, 1 mL of 20% Na_2CO_3 was added and the final volume was adjusted to 5 mL with deionised water. The solution was left in a water bath at 70 °C for 10 min and then cooled in the dark for 30 min. The absorbance was read at 760 nm using a spectrophotometer (Analytikijena 200–2004 Spectrophotometer, Analytik Jena, Jena, Germany). The TPC value of the bee pollen samples was expressed as milligram of gallic acid equivalent per g of the dry weight of bee pollen sample (mg GAE g^{-1}).

Total flavonoid content
TFC was recorded spectrophotometrically according to Aylanc *et al.* (2021b). First, a 2% $AlCl_3$ solution (w/v) was prepared by dilution in 5% glacial acetic acid: methanol (5/95, v/v). Then, 0.2 mL of this solution was mixed with 0.2 mL of the ethanolic extract (5 mg/mL) and 2.8 mL of 5% acetic acid/methanol. After 30 min at room temperature, the absorbance was read at 415 nm. The TFC value of the bee pollen samples was expressed as milligram of quercetin equivalent per g of the dry weight of bee pollen sample (mg QE g^{-1}).

LC/DAD/ESI-MS^n bioactive compounds' analysis

For chromatographic analysis, 20 mg of each extract was mixed with 2 mL of 80% EtOH and thoroughly dissolved. The solution was then filtered through a 0.22 μm nylon membrane and used for analysis.

A Dionex UltiMate 3000 ultra-pressure liquid chromatography instrument connected to a diode array

© 2022 Institute of Food, Science and Technology (IFSTTF).

and attached to a mass detector was used for liquid chromatography/diode array detection/electrospray ionisation-mass spectrometry (LC/DAD/ESI-MS^n) analyses (Thermo Fisher Scientific, San Jose, CA, USA). LC was run in a Macherey-Nagel Nucleosil C18 column (250 mm × 4 mm id; particles' diameter of 5 mm, end-capped) and its temperature kept constant at 30 °C. The conditions applied in the liquid chromatography (LC) were based on a previous work (El-Ghouizi *et al.*, 2020); the flow rate was set at 1 mL min^{-1}, and an injection volume of 10 μL was used. The final spectral data were accumulated in the wavelength interval of 190–600 nm.

The LTQ XL linear ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with an electrospray ionisation (ESI) source was set in the negative ion mode. The ESI conditions were set in accordance with the previously reported (El-Ghouizi *et al.*, 2020). Mass spectra were acquired by full-range acquisition covering 100–1000 m/z. For the fragmentation study, a data-dependent scan was performed by deploying collision-induced dissociation (CID). The normalised collision energy of CID cell was set at 35 (arbitrary units). Data acquisition was carried out with the Xcalibur® data system (Thermo Scientific).

The elucidation of the phenolic compounds was achieved by comparison of the chromatographic behaviour, ultraviolet (u.v.) spectra, and MS information, to those of reference compounds. When standards were not available, the structural information was confirmed with u.v. data combined with MS fragmentation patterns reported in the literature. Quantification was achieved using calibration curves for *p*-coumaric acid (0.00925–0.4 mg/mL; $y = 1.9 \times 107x - 12927$; $R^2 = 0.996$), quercetin (0.037–1.6 mg/mL; $y = 4.0 \times 106x - 10216$; $R^2 = 0.997$), kaempferol (0.037–1.6 mg/mL; $y = 4.3 \times 106x - 13\,567$; $R^2 = 0.998$), chrysin (0.0185–0.8 mg/mL; $y = 1.2 \times 107x - 51\,265$; $R^2 = 0.999$), and naringenin (0.0185–0.8 mg/mL; $y = 8.0 \times 106x - 10\,998$; $R^2 = 0.998$). When the specific standard was not available, the compounds were quantified using the calibration curve of the structurally closest standard, and the final result was given in equivalent terms, expressed as mg/g extract.

Antioxidant activity

DPPH radical scavenging assay

The DPPH radical scavenging capacity of bee pollen extracts was determined according to the method previously described by Brand-Williams *et al.* (1995). As much as 1.93 mL of $DPPH^{\bullet}$ working solution in methanol (6×10^{-5} M), daily prepared, was mixed with 70 μL of ethanolic solutions of bee pollen extracts (concentration; 5 mg/mL). The mixture was then incubated at room temperature for 30 min in the dark. The absorbance was measured at 515 nm using a spectrophotometer. The percentage of radical scavenging activity was calculated using the following equation:

$$\%\,\text{Inhibition} = [(A_C - A_E) / A_C]\, x\, 100 \qquad (1)$$

Where A_C is the absorbance of the control and A_E is the absorbance of the bee pollen extract.

ABTS radical scavenging activity

The ABTS radical scavenging activity assay was performed according to a method previously reported (Re *et al.*, 1999). The $ABTS^{\bullet+}$ stock solution was prepared by reacting ABTS (7 mM in deionised water) with 2.45 mM potassium persulfate. The mixture was left at room temperature for 16 h in the dark until it reached a stable oxidative state. $ABTS^{\bullet+}$ working solution was prepared by dilution with ethanol to give an absorbance of 0.70 ± 0.05 at 734 nm. Then, 980 μL of the working solution was mixed with 20 μL of the ethanolic extract (concentrations: 5 mg/mL) and the absorbance was measured at 734 nm. The percentage of ABTS radical scavenging activity was calculated using the above equation (Eqn 1).

Reducing power assay

The reducing power assay was performed according to Falcão *et al.* (2013). An aliquot of 0.25 mL of ethanolic sample extract (1 mg mL^{-1}) was mixed with 1.25 mL of phosphate buffer (0.2 M, pH 6.6) and 1.25 mL of 1% potassium ferricyanide. The mixture was left in a water bath at 50 °C for 20 min. Then, 1.25 mL of 10% trichloroacetic acid was added to the mixture and centrifuged at 3000 ***g*** for 10 min. As much as 1.25 mL of the upper layer was mixed with 1.25 mL of deionised water and 0.25 mL of 0.1% $FeCl_3$, and the absorbance was read at 700 nm. Gallic acid was used as standard and the results were expressed as milligram of gallic acid equivalent per g of the dry weight of bee pollen sample (mg GAE g^{-1}).

Data analysis

All analyses were performed in triplicate and the results were denoted as mean ± standard deviation (SD). The obtained data were analysed using GraphPad Prism version 8 (San Diego, CA, USA). One-way analysis of variance, followed by Tukey's multiple comparison test, was conducted to see whether there is a statistical significance. A value of $P < 0.05$ was considered as significant.

Results and discussion

Botanical origin of bee pollen

The palynological profile of three different bee pollen samples collected in different regions in Portugal can

© 2022 Institute of Food, Science and Technology (IFSTTF).

be observed in Table 1. Only pollen types with a relative frequency >1% are discriminated. *Eucalyptus* sp. (52%) was found to be the dominant pollen in the BP1 sample, followed by *Salix* sp. (30%) and *Cytisus striatus type* (15%). These three pollen types represented 97% of the total relative frequency of pollen species in bee pollen samples from the west region of Leiria. In the case of BP2 sample from the north region of Vila Real, *Rubus* sp. (55%) from Rosaceae family was determined as the dominant pollen type, while *Castanea sativa* (22%) and *Jasione montana* (13%) showed a distribution at the accessory or isolated pollen level. The latter botanical representative of Campanulaceae family, *Jasione montana*, was the dominant pollen type in BP3 sample from the east of Portugal, with a large relative frequency of 74%, accompanied by other pollen species such as *Rubus* sp., *Halimium* sp., *Olea europaea*, and *Crepis capillaris type* at isolated level. Since no major taxa in the analysed bee pollen samples had a relative frequency greater than 80%, all samples were classified as multifloral (Campos *et al*., 2008). Nevertheless, the chemical composition will be greatly dependent on the main pollens found in each sample, Figure 1. All the pollen types determined in the present study have been commonly found in bee pollen collected in the central and northern regions of Portugal (Estevinho *et al*., 2012; Anjos *et al*., 2017; Aylanc *et al*., 2021b). Nevertheless, their distinct botanical profile is an important factor to test the reliability of the extraction methods towards probable distinctive phenolic profiles, dependent on botanical origin.

Table 1 Botanical origin of bee pollen samples

		Relative frequency (%) of pollen types		
Family	**Pollen**	**BP1**	**BP2**	**BP3**
Asteraceae	*Galactites tomentosa*	—	—	2.30
	Crepis capillaris type	—	—	2.6
Brassicaceae	*Raphanus raphanistrum type*	1.2	—	—
Campanulaceae	*Jasione montana*	—	12.6	74.3
Cistaceae	*Halimium* sp.	—	—	3.5
Crassulaceae	*Sedum* sp.	—	—	1.7
Fabaceae	*Cytisus striatus type*	14.9	1.5	—
	Trifolium sp.	—	1.4	—
Fagaceae	*Castanea sativa*	—	21.8	—
Myrtaceae	*Eucalyptus* sp.	52.2	—	—
Oleaceae	*Olea europaea*	—	—	2.8
Rosaceae	*Prunus* sp.	1.0	—	—
	Rubus sp.	—	54.6	7.7
Salicaceae	*Salix* sp.	30.1	—	—
Scrophulariaceae	*Scrophularia canina type*	—	1.2	—
Vitaceae	*Vitis vinifera*	—	1.1	1.3
Classification		Multifloral		

Dominant pollen (>45%); Accessory pollen (15–45%); Isolated pollen (3–15%). BP1, BP2, and BP3 represent different bee pollen (BP) samples 1, 2, and 3, respectively.

Extraction yield

Maceration and magnetic stirring are among the most commonly used conventional techniques for the extraction of bioactive compounds from bee pollen matrices, but recently, green extraction techniques such as ultrasounds and micro-wave assisted are being employed with increasing frequency, with the understanding of the working principles and their effectiveness on the bioactive compounds (Lv *et al*., 2015; Muñoz *et al*., 2020). Extraction efficiency depends directly on the technique, the structure and particle size of the samples, as well as the solvent, temperature, and time (Do *et al*., 2014). These factors affect in particular the extraction of phenolic compounds due to the specificity and fragility of their chemical nature. In this study, the conventional and green extraction techniques were compared, keeping constant parameters such as sample, solvent, and concentration.

According to the results given in Table 2, the extraction yield is sensitive to the bee pollen sample and extraction technique. The MAE was the most effective extraction technique towards BP1, while MS was the most effective for both BP2 and BP3. The lowest extraction yield for all three samples was obtained in the MR technique with an average extraction yield of 50%. This can probably be explained by the increment in the mass transfer rate from the sample to solvent due to the continuous agitation imposed on all procedures but MR, leading to an increase in extraction efficiency. On the other hand, and even with differences in the energy densities, there was no statistically significant ($P > 0.05$) difference between the two green extraction techniques, no matter whatever the bee pollen samples. Even though, green techniques resulted in a lower extraction yield than the MS technique, but with not being statistically significant between samples, except BP2. In a general comparison, the average yields of the different extraction techniques decreased in the following order: MS (57.0%) > MAE (54.4%) > UAE (53.2%) > MR (50.4%). The results of this study resemble those reported for conventional and green extraction techniques, using other plant matrices (Aspé & Fernández, 2011; Dhanani *et al*., 2017; Farahmandfar *et al*., 2019).

Total phenolic and flavonoid content

Table 2 shows the total phenolic and flavonoid content of the bee pollen extracts according to the different

© 2022 Institute of Food, Science and Technology (IFSTTF).

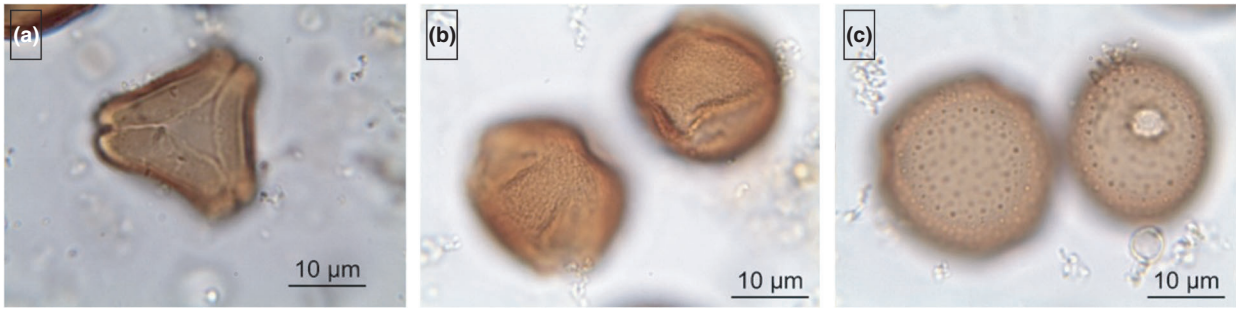

Figure 1 Light micrographs of the major pollens found in the samples under study: (a) *Eucalyptus* sp.; (b) *Rubus* sp.; and (c) *Jasione montana*.

extraction techniques. The TPC and TFC of the bee pollen extracts differed remarkably, depending on both the sample and the extraction technique. Besides, the techniques with the highest extraction yield do not consequently reveal the highest phenolic content, what may suggest that the extracts contain other chemical substances, soluble in ethanol 80%, besides phenolics.

The highest TPC for BP1 was obtained in the MAE with a value of 28.0 mg GAE g^{-1}, and the lowest for MR (13.5 mg GAE g^{-1}). There was no statistically significant difference between the green extraction techniques for the BP1, but they differed significantly ($P < 0.05$) by comparison with the conventional ones. A similar pattern was observed for the other bee pollen samples, with the highest TPC observed for MAE and the lowest for the MR technique, however, for these samples, there was no statistical difference between UAE and MS.

In respect to the total flavonoid content efficiency of the extraction techniques, BP1 and BP2 samples reached the highest values in the MAE with values of 8.0 mg QE g^{-1} and 3.6 mg QE g^{-1}, respectively, while with ultrasounds the outputs decreased on average around 14 to 16%. For the BP3 sample, the highest TFC value was obtained with the MS (6.8 mg QE g^{-1}), but with differences not significant to the remaining techniques explored.

Overall, it is interesting to notice that, with a few exceptions, the highest TPC and TFC were achieved in microwave-assisted extraction and the lowest in maceration. The highest performance of the MAE technique can be attributed to the rupture of the rigid multilayered pollen wall due to the microwave irradiation that accelerates the cell's burst with a sudden increase in temperature and internal pressure of the cell, and thus it causes the chemical compounds in the pollen to be released more effectively towards the solvent (Castro-López *et al.*, 2017). Previous studies on different plant matrices reported that MAE is generally a more effective technique to enhance the extraction of phenolic compounds than UAE or conventional techniques (Aspé & Fernández, 2011; Castro-López *et al.*, 2017; Carbone *et al.*, 2020), what is in line with our results. In addition, the UAE technique gave a higher phenolic content for BP1 than the MS technique, while for BP2 and BP3 the values did not differ between those extraction methods. These results revealed that exposing the plant-based samples to long-term stirring in the solvent may be enough to release phenolic compounds from pollen grains as with ultrasounds.

Table 2 Extraction technique, extraction yield, TPC, and TFC of bee pollen

Extraction technique	Extraction yield (%)			TPC (mg GAE g^{-1})			TFC (mg QE g^{-1})		
	BP1	BP2	BP3	BP1	BP2	BP3	BP1	BP2	BP3
MR	46.2 ± 0.7^{Bb}	52.3 ± 1.2^{Ab}	52.6 ± 2.5^{Ab}	13.5 ± 0.4^{Ac}	13.8 ± 0.9^{Ab}	11.9 ± 0.2^{Bb}	5.4 ± 0.2^{Ac}	2.8 ± 0.1^{Bb}	6.2 ± 0.5^{Aa}
MS	52.2 ± 1.1^{Ba}	59.1 ± 3.0^{Aa}	59.8 ± 3.7^{Aa}	18.7 ± 0.4^{Ab}	15.5 ± 1.0^{Bba}	13.1 ± 0.7^{Cba}	5.6 ± 0.1^{Bc}	2.9 ± 0.2^{Cb}	6.8 ± 0.1^{Aa}
UAE	51.0 ± 0.4^{Ba}	54.7 ± 1.3^{Ab}	54.0 ± 0.2^{Aab}	26.7 ± 0.7^{Aa}	14.8 ± 0.4^{Bba}	11.8 ± 0.2^{Cb}	6.7 ± 0.3^{Ab}	3.1 ± 0.2^{Bb}	6.4 ± 0.2^{Aa}
MAE	52.4 ± 0.7^{Ba}	55.6 ± 0.2^{Aab}	55.2 ± 0.5^{Aab}	28.0 ± 1.3^{Aa}	16.6 ± 0.6^{Ba}	14.2 ± 1.0^{Ba}	8.0 ± 0.3^{Aa}	3.6 ± 0.0^{Ca}	6.3 ± 0.1^{Ba}

For extraction yield, total phenolic content (TPC), and total flavonoid content (TFC), capital letters (A–C) in each row indicate significant differences between samples, while lowercase letters (a–c) in each column indicate differences between extraction techniques. Here, BP1, BP2, and BP3 represent different bee pollen (BP) samples 1, 2, and 3, respectively, while MR, MS, UAE, and MAE represent maceration, magnetic stirring, ultrasound-assisted extraction, and microwave-assisted extraction techniques. Here, mg GAE g^{-1} denotes milligram of gallic acid equivalent per g of the dry weight of bee pollen sample, while mg QE g^{-1} denotes milligram of quercetin equivalent per g of the dry weight of bee pollen sample.

© 2022 Institute of Food, Science and Technology (IFSTTF).

Besides the differences between extraction techniques, the outputs allow us to highlight some differences among bee pollen samples linked to their botanical diversity (Muñoz *et al*., 2020). The *Eucalyptus* sp. pollen species, which is dominant in the BP1 sample, seems richer in bioactive compounds than *Rubus* sp. (BP2) and *Jasione montana* (BP3) pollens. Nevertheless, this difference may not be due to differences in the content of each pollen, but may be linked to an easier release of the compounds from the pollen shell, which may vary according to characteristics such as wall structure, the aperture number, the nanochannels, or micropores in the pollen wall. The physical characteristics of each pollen can be affected differently by the solvent–sample interaction, the effect of stirring, frequency of sound waves, or microwave extraction conditions such as the temperature and delivered energy (Aylanc *et al*., 2021b). Overall, it can be clearly distinguished that MAE is more effective than the other techniques for extracting phenolic compounds.

Bioactive compounds' profile

LC/DAD/ESI-MS^n fingerprint is a reliable technique to access the chemical identity of the bioactive compounds in plant extracts and is used here to detect if the variability observed in the phenolic and flavonoid content for the different extraction techniques affects all the compounds or some specific classes only. In the present study, the chemical identification was ensured by comparing the MS^n spectrums of the extracts with those available in the literature, in a combination with the spectral information gathered from MS and u.v. data.

A total of twenty-six different compounds were identified in the samples, including thirteen phenolics and thirteen phenylamides. The mass spectra and retention times of all the compounds identified in the bee pollen extracts, using the negative ion mode, can be found in Table S1. Bee pollen extracts were dominated by flavonoid derivatives, such as quercetin, kaempferol, and herbacetin, bonded to sugar moieties assigned to rutinosides, hexosides, and glucosides. Similar results were reported in bee pollen samples from different botanical origins (El-Ghouizi *et al*., 2020; Aylanc *et al*., 2021b), including the presence of the polyamine derivatives, which are described as plant-specific secondary metabolites with multiple functional roles, with evidence on drought tolerance (Handrick *et al*., 2010). In the extracts, the most widely distributed acidic parent compounds of phenylamides were caffeic, ferulic, and *p*-coumaric acids, whereas the aliphatic polyamines spermidine and spermine were the predominant amine fragments detected (Edreva *et al*., 2007). These compounds have been described in high concentrations for several pollen families, including Asteraceae, Campanulaceae, Myrtaceae, Rosaceae, and Fabaceae (Urcan *et al*., 2018; Aylanc *et al*., 2021b).

The individual amount of each compound is shown in Tables 3 and 4. The profile of each sample extract was kept constant independently of the technique applied, with two minor exceptions for BP2 and BP3. For BP3, the presence of feruloyl dicoumaroyl spermidine was not detected for all extraction techniques, while for BP2 the presence of N^1,N^5,N^{10}-*tri-p*-coumaroylspermidine was detected with all extraction techniques. Nevertheless, the major differences were, by far, in terms of quantity, with the amount of spermidines increasing up to 200%, when comparing MAE and MR, while the phenolics increased around 40 to 60% when changing from conventional to green extraction technics (BP2 is an exception with the phenolic content unchangeable). This behaviour was not transversal to all compounds. For instance, the concentration of phenolics, such as quercetin-diglucoside (m/z 625), methyl herbacetin-*O*-dihexoside (m/z 639), quercetin-*O*-hexosyl-*O*-rutinoside (m/z 771), kaempferol-3-O-rutinoside (m/z 593), quercetin-3-O-glucoside (m/z 463), or apigenin-O-hexoside (m/z 431), is almost constant independently of the extraction techniques, while others, such as kaempferol-*O*-derivative (m/z 800), luteolin (m/z 285), or herbacetin-methyl-ether (m/z 271), were detected in higher quantities with the application of green extraction techniques. Yet, the most significant increments were observed for phenylamides such as N^1, N^5, N^{10}-*tri*-caffeoylspermidine (m/z 630), N^1, N^5, N^{10}-*tri-p*-coumaroylspermidine (isomer, m/z 582), tetracoumaroyl spermine (isomer, m/z 785), and polyamide derivative (m/z 672), some of them rising up to three times more.

Even though there were relevant differences in the bioactive profile according to the botanical origin of the sample, BP1 extracts showed a similar trend to those of BP2, that is, a high concentration of phenylamides (27.25 and 33.54 mg g^{-1}, respectively, for MAE) and a relatively lower phenolic compound content (4.62 and 2.03 mg g^{-1}, respectively, for MAE), Figure S2. Sample BP3, from *Jasione montana*, in contrast to the other samples, had a higher concentration of phenolic compounds compared to the phenylamide content. The absent or low contribution of the major spermidines observed in the other samples justifies this difference, together with the high content in kaempferol-*O*-malonyl hexoside (m/z 533) that accounts for 65% of the total phenolic content in BP3.

As discussed above, the microwave energy used during extraction increases the pressure inside the cell, promoting the degradation of the cell wall and pushing the cell components into the solvent, at the same time that the energy from the microwave generates heat as it disperses through polar molecules such as the phenolic compounds, which causes the bioactive compounds to be released from the cell wall more

© 2022 Institute of Food, Science and Technology (IFSTTF).

Table 3 Quantification of the phenolic and phenylamide compounds (mg g^{-1}) in bee pollen samples obtained by MR and MS extraction techniques

Compounds	MR			MS		
	BP1	BP2	BP3	BP1	BP2	BP3
Quercetin-diglucoside	0.46 ± 0.00	ND	0.06 ± 0.00	0.54 ± 0.01	ND	0.06 ± 0.00
Methyl herbacetin-*O*-dihexoside	0.17 ± 0.00	1.22 ± 0.02	ND	0.22 ± 0.00	1.10 ± 0.04	ND
Quercetin-*O*-hexosyl-*O*-rutinoside	0.05 ± 0.00	ND	ND	0.04 ± 0.00	ND	ND
Methyl herbacetin-3-*O*-rutinoside	ND	0.50 ± 0.01	ND	ND	0.36 ± 0.00	ND
Kaempferol-*O*-derivative	1.12 ± 0.00	ND	ND	1.04 ± 0.03	ND	ND
Kaempferol-3-*O*-rutinoside	ND	ND	0.27 ± 0.01	ND	ND	0.26 ± 0.00
Quercetin-3-*O*-glucoside	ND	ND	0.11 ± 0.00	ND	ND	0.10 ± 0.00
Quercetin-*O*-malonyl hexoside	ND	ND	0.31 ± 0.01	ND	ND	0.51 ± 0.00
Kaempferol-*O*-hexoside	ND	ND	0.29 ± 0.01	ND	ND	0.23 ± 0.00
Apigenin-*O*-hexoside	ND	ND	0.62 ± 0.01	ND	ND	0.63 ± 0.01
Kaempferol-*O*-malonyl hexoside	ND	0.25 ± 0.00	1.51 ± 0.12	ND	0.24 ± 0.00	3.20 ± 0.01
N^1, N^5, N^{10}-*tri*-caffeoylspermidine	1.05 ± 0.01	0.25 ± 0.02	ND	0.61 ± 0.00	0.21 ± 0.02	ND
N^1, N^5, N^{10}-*tri*-caffeoylspermidine (isomer)	2.75 ± 0.04	1.45 ± 0.02	ND	5.21 ± 0.04	1.46 ± 0.03	ND
N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine	0.50 ± 0.03	0.75 ± 0.03	ND	0.55 ± 0.00	0.91 ± 0.08	ND
N^1, N^5-*di-p*-coumaroyl-N^{10}-caffeoylspermidine	ND	1.38 ± 0.02	0.27 ± 0.00	ND	1.27 ± 0.06	0.24 ± 0.01
N^1, N^5, N^{10}-*tri-p*-coumaroylspermidine	ND	1.57 ± 0.01	ND	ND	1.70 ± 0.13	ND
Luteolin	1.08 ± 0.01	ND	ND	0.91 ± 0.01	ND	ND
N^1, N^5-*di-p*-coumaroyl-N^{10}-caffeoylspermidine	0.24 ± 0.00	2.45 ± 0.03	ND	0.18 ± 0.00	3.34 ± 0.04	ND
N^1, N^5, N^{10}-*tri-p*-coumaroylspermidine (isomer)	ND	2.37 ± 0.06	0.25 ± 0.01	ND	2.65 ± 0.17	0.25 ± 0.01
Herbacetin-methyl-ether	0.34 ± 0.00	ND	ND	0.76 ± 0.01	ND	ND
N^1, N^5, N^{10}-*tri-p*-coumaroylspermidine (isomer)	ND	3.65 ± 0.02	ND	ND	4.40 ± 0.04	ND
N^1, N^5, N^{10}-*tri-p*-coumaroylspermidine (isomer)	ND	1.68 ± 0.01	ND	ND	1.85 ± 0.00	ND
N^1, N^5, N^{10}-*tri-p*-coumaroylspermidine (isomer)	1.18 ± 0.00	10.07 ± 0.24	1.37 ± 0.00	4.24 ± 0.00	11.94 ± 0.53	2.10 ± 0.01
Feruloyl dicoumaroyl spermidine	0.46 ± 0.01	ND	ND	0.38 ± 0.00	0.70 ± 0.09	ND
Tetracoumaroyl spermine (isomer)	0.83 ± 0.02	ND	1.22 ± 0.01	2.30 ± 0.00	ND	1.67 ± 0.00
Polyamide derivative	1.91 ± 0.00	0.75 ± 0.03	ND	5.38 ± 0.01	0.86 ± 0.04	ND
Total amount of phenolic compounds	3.22	1.98	3.16	3.51	1.70	4.99
Total amount of phenylamides	8.92	26.37	3.11	18.84	31.30	4.26

Values expressed as milligram of each compound per gram sample. Here, BP1, BP2, and BP3 represent different bee pollen (BP) samples 1, 2, and 3, respectively, while MR and MS represent maceration and magnetic stirring techniques, respectively. ND, not detected

effectively (Castro-López *et al*., 2017). The results confirmed, however, that those effects do not impact all classes of compounds in the same magnitude, since, when shifting from MR to MAE, the upturn is higher for phenylamides. In plants, these compounds are responsible for protecting the genetic material from u.v. radiation, displacing Ca^{2+} from cell wall pectin, producing supramolecular conformational changes, and blocking the activity of Ca^{2+}-bound α-1,4-oligogalacturonides, accumulating in the vacuole and, mostly, in the cell wall of the pollen grains (Edreva *et al*., 2007; Vogt, 2018). Considering the higher ability of microwaves to disrupt the cell wall, it is expectable to achieve a selective increment of phenylamines when using the MAE technique. Besides, based on the disruption theory, cell components dissolve better in the solvent as a result of the heating effect (Pak-Dek *et al*., 2011). Previously, Carbone *et al*. (2020) and Biesaga (2011) reported that the *Humulus lupulus* and maize extracts obtained with different extraction techniques resulted in a higher bioactive compound content with microwave-assisted procedures rather than the conventional and in most cases UAE technique.

Impact of extraction technique on the antioxidant activity

Several antioxidant activity assays have been explored to attest the protective ability of molecules available in natural matrix, towards a specific molecular target. Nonetheless, due to the diversity of antioxidant mechanisms in natural systems, there is no method with unequivocal results, so, for a better discrimination of the antioxidant activity, it is recommended to use a broader approach with a combination of different antioxidant assays (Carocho & Ferreira, 2013). In this work, three complementary assays were applied to assess the impact of the extraction techniques on the antioxidant capacity of the bee pollen samples: DPPH, ABTS, and reducing power. Even though both DPPH

© 2022 Institute of Food, Science and Technology (IFSTTF).

Table 4 Quantification of phenolic and phenylamide compounds (mg g^{-1}) in bee pollen samples obtained by UAE and MAE extraction techniques

	UAE			MAE		
Compounds	**BP1**	**BP2**	**BP3**	**BP1**	**BP2**	**BP3**
Quercetin-diglucoside	0.38 ± 0.01	ND	0.11 ± 0.00	0.38 ± 0.00	ND	0.08 ± 0.00
Methyl herbacetin-*O*-dihexoside	0.12 ± 0.00	1.24 ± 0.01	ND	0.13 ± 0.00	1.30 ± 0.04	ND
Quercetin-*O*-hexosyl-*O*-rutinoside	0.04 ± 0.00	ND	ND	0.05 ± 0.00	ND	ND
Methyl herbacetin-3-*O*-rutinoside	ND	0.47 ± 0.01	ND	ND	0.51 ± 0.06	ND
Kaempferol-*O*-derivative	1.05 ± 0.02	ND	ND	1.49 ± 0.01	ND	ND
Kaempferol-3-*O*-rutinoside	ND	ND	0.28 ± 0.00	ND	ND	0.26 ± 0.00
Quercetin-3-*O*-glucoside	ND	ND	0.10 ± 0.00	ND	ND	0.11 ± 0.00
Quercetin-*O*-malonyl hexoside	ND	ND	0.44 ± 0.00	ND	ND	0.37 ± 0.02
Kaempferol-*O*-hexoside	ND	ND	0.24 ± 0.00	ND	ND	0.36 ± 0.00
Apigenin-*O*-hexoside	ND	ND	0.66 ± 0.00	ND	ND	0.61 ± 0.01
Kaempferol-*O*-malonyl hexoside	ND	0.22 ± 0.01	3.34 ± 0.05	ND	0.21 ± 0.01	3.39 ± 0.16
N^1, N^5, N^{10}-*tri*-caffeoylspermidine	1.46 ± 0.05	0.18 ± 0.01	ND	2.04 ± 0.00	0.23 ± 0.02	ND
N^1, N^5, N^{10}-*tri*-caffeoylspermidine (isomer)	5.58 ± 0.11	1.16 ± 0.10	ND	8.15 ± 0.14	1.23 ± 0.09	ND
N^1-*p*-coumaroyl-N^5, N^{10}-dicaffeoylspermidine	1.40 ± 0.05	0.60 ± 0.09	ND	1.64 ± 0.00	0.75 ± 0.03	ND
N^1, N^5-*di*-*p*-coumaroyl-N^{10}-caffeoylspermidine	ND	0.86 ± 0.05	0.22 ± 0.00	ND	1.13 ± 0.08	0.28 ± 0.00
N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine	ND	1.26 ± 0.02	ND	ND	1.23 ± 0.01	ND
Luteolin	1.10 ± 0.05	ND	ND	1.54 ± 0.03	ND	ND
N^1, N^5-*di*-*p*-coumaroyl-N^{10}-caffeoylspermidine	0.62 ± 0.01	3.62 ± 0.29	ND	0.75 ± 0.00	3.62 ± 0.16	ND
N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine (isomer)	ND	3.54 ± 0.14	0.27 ± 0.00	ND	3.61 ± 0.02	0.37 ± 0.00
Herbacetin-methyl-ether	0.84 ± 0.03	ND	ND	1.03 ± 0.01	ND	ND
N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine (isomer)	ND	4.45 ± 0.07	ND	ND	5.02 ± 0.09	0.31 ± 0.00
N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine (isomer)	ND	1.33 ± 0.12	ND	ND	1.61 ± 0.04	ND
N^1, N^5, N^{10}-*tri*-*p*-coumaroylspermidine (isomer)	3.41 ± 0.08	12.46 ± 0.06	1.59 ± 0.04	4.14 ± 0.09	13.77 ± 0.11	2.22 ± 0.00
Feruloyl dicoumaroyl spermidine	1.30 ± 0.04	0.44 ± 0.02	ND	1.57 ± 0.01	0.65 ± 0.01	ND
Tetracoumaroyl spermine (isomer)	2.38 ± 0.06	ND	1.39 ± 0.01	2.75 ± 0.01	ND	1.37 ± 0.00
Polyamide derivative	5.55 ± 0.04	0.63 ± 0.03	ND	6.21 ± 0.07	0.69 ± 0.08	ND
Total amount of phenolic compounds	3.54	1.94	5.18	4.62	2.03	5.18
Total amount of phenylamides	21.70	30.56	3.47	27.25	33.54	4.56

Values expressed as milligram of each compound per gram sample. Here, BP1, BP2, and BP3 represent different bee pollen (BP) samples 1, 2, and 3, respectively, while UAE and MAE represent ultrasound-assisted extraction and microwave-assisted extraction techniques, respectively. ND, not detected.

and ABTS assays are used to measure the free radical scavenging capacity of plant materials, their working principles are different: the ABTS test can be applied to both hydrophilic and hydrophobic antioxidant systems, while the DPPH test employs a radical dissolved in an organic medium, and hence it is applicable only to hydrophobic systems (Floegel *et al*., 2011). For the other side, the reducing power assay attests the electron transfer ability of the natural molecules, towards the reductive process of Fe^{3+}/Fe^{2+} (Carocho & Ferreira, 2013; Do *et al*., 2014).

Figure 2a,b show the antiradical capacity of bee pollen extracts against DPPH and ABTS chromogenic radicals, expressed in inhibition percent. Both DPPH (94.2%) and ABTS (98.6%) radical activity of BP1 extract were maximum for MAE followed closely by UAE, with no significant differences, however, the differences become significant when compared with the conventional extraction approaches, with the lowest values observed at MR (DPPH = 83.9% and ABTS = 66.1%), respectively. The same trend between extraction techniques was observed for the other two extracts, however with much less radical scavenging activity, with values around 50% to 65% for BP2 pollen extract and 30% to 40% for BP3. In general, the strong antioxidant activity of MAE extracts, followed by UAE and MS, could be correlated to the higher TPC and TFC content, due to the effect of the hydroxyl groups, which may act as electron donors (Castro-López *et al*., 2017). These results are similar to the findings of bee pollen extracts obtained by green and traditional techniques (Feás *et al*., 2012; Gabriele *et al*., 2015; Fadzilah *et al*., 2017; Aylanc *et al*., 2021b).

The results ranged from 5.4 to 11.5 mg GAE g^{-1} in BP1, 3.5 to 4.7 mg GAE g^{-1} in BP2, and 3.8 to 4.8 mg GAE g^{-1} in BP3, Figure 2c, with the highest activity recorded again for MAE and the lowest for

© 2022 Institute of Food, Science and Technology (IFSTTF).

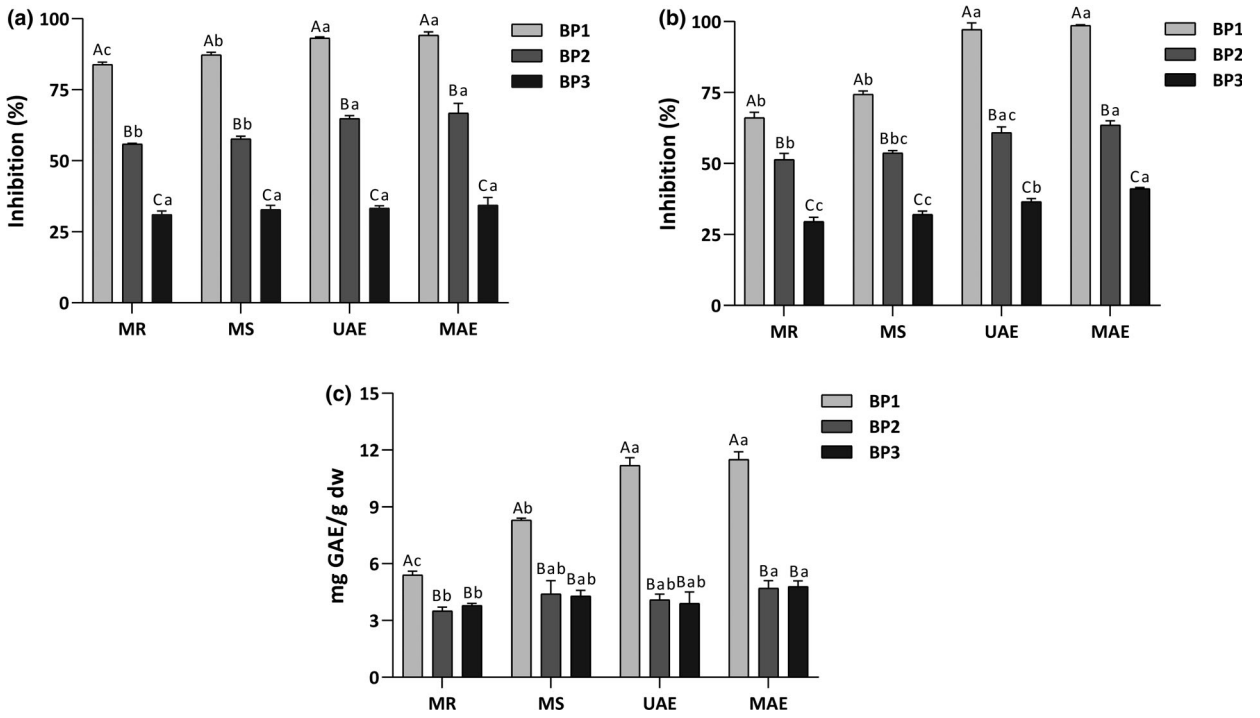

Figure 2 Antioxidant behaviour of bee pollen: (a) 2,2-diphenyl-1-picrylhydrazyl (DPPH), (b) 2,2′-azino-di-(3-ethylbenzothiazoline sulphonic acid (ABTS), and (c) reducing power activity of extracts obtained with different extraction techniques (MR, maceration; MS, magnetic stirring; MAE, microwave-assisted extraction; and UAE, ultrasonic-assisted extraction). The different capital letters (A–C) indicate significant differences ($P < 0.05$) between samples in each extraction technique and different lowercase letters (a–c) indicate significant differences ($P < 0.05$) between techniques for each bee pollen sample.

the MR extract. Nevertheless, the differences between green and conventional extraction techniques only showed statistical significance for the most active extract, BP1, highlighting the importance of the botanical origins in the bioactive performance of pollen (Fatrcová-Šramková *et al.*, 2013; Tomás *et al.*, 2017; Aylanc *et al.*, 2021b).

The antiradical capacity of plant-derived matrixes is well proven and linked to the presence of bioactive compounds such as phenolics. The high concentration of these chemical compounds provides a large number of hydroxyl groups in the reaction medium, thus, the probability of hydrogen donation to free radicals increases, and this is reflected in the antiradical result of the tested sample (Castro-López *et al.*, 2017; Carbone *et al.*, 2020). For example, the hydrogen atoms of adjacent hydroxyl groups (o-diphenol) in various positions of the A, B, and C rings in quercetin, the double bonds of the benzene, and the double bond (-C = O) of the oxo functional group ensure the compound to exhibit higher antioxidant capacity (Aylanc *et al.*, 2020), due to its ability for delocalisation of the radical. The findings given above confirmed as well that microwave-assisted extraction may be a promising technique for removing antioxidant compounds from natural resources including from resistant structures like bee pollen.

Conclusion

With the increasing request not only on products with natural ingredients, but also the concern for sustainable production, there has been a demand for using extraction techniques with lower impact on the environment but showing high efficacy to extract the desired compounds from the natural matrixes. Undoubtedly, bee pollen may serve as an excellent source of natural ingredients due to its rich composition, but the specificities of its structure may limit the ability to obtain bioactive substances. In this study, we confront the traditional techniques frequently used to extract these valuable compounds from bee pollen with greener extraction techniques, using different bee pollen samples.

Overall, and independently of the botanical origin of the samples, the techniques with higher performance in total phenolic and flavonoid content were microwave-assisted extraction, followed by ultrasound.

© 2022 Institute of Food, Science and Technology (IFSTTF).

Besides, MAE was also found to be a better approach in terms of the concentration of individual bioactive compounds, particularly in respect to phenylamides. The richness of the extracts obtained under those techniques was evidenced through the higher radical scavenging capacity, compared to those obtained conventionally. Green extraction techniques, especially MAE, demonstrated many advantages such as time-saving, minimum solvent consumption, richer phenolic extracts, and energy-saving compared to the conventional ones. Considering all these findings, MAE was the most convenient technique for extracting valuable compounds from plant origin samples resistant to physical and chemical conditions such as pollen grains, and it showed that green extraction techniques could replace traditional techniques.

Funding

The authors are grateful to the Foundation for Science and Technology (FCT, Portugal) for financial support by national funds FCT/MCTES to CIMO (UIDB/00690/2020). Thanks to the Programa Apícola Nacional 2020-2022 (National Beekeeping Program) for funding the project 'Standardization of production procedures and quality parameters of bee products'. Thanks to the European Regional Development Fund (ERDF) through the Regional Operational Program North 2020, within the scope of Project PDR2020-1.0.1-FEADER-031734: 'DivInA-Diversification and Innovation on Beekeeping Production' and project GreenHealth, Norte-01-0145-FEDER-000042. The authors acknowledge the National funding by FCT-Foundation for Science and Technology, through the institutional scientific employment program-contract with Soraia I. Falcão.

Author contributions

Volkan Aylanc: Conceptualization (equal); investigation (lead); methodology (lead); writing – original draft (lead). **Seymanur Ertosun:** Investigation (supporting); methodology (supporting). **Paulo Russo-Almeida:** Investigation (supporting); methodology (supporting); validation (supporting); writing – review and editing (supporting). **Soraia I. Falcão:** Conceptualization (supporting); methodology (supporting); validation (lead); writing – original draft (supporting); writing – review and editing (supporting). **Miguel Vilas-Boas:** Conceptualization (equal); project administration (lead); resources (lead); supervision (lead); writing – review and editing (lead).

Conflict of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Ethical approval

Ethics approval was not required for this research.

Peer review

The peer review history for this article is available at https://publons.com/publon/10.1111/ijfs.15672.

Data availability statement

All data of this study are presented within the manuscript.

References

Ameer, K., Shahbaz, H.M. & Kwon, J.H. (2017). Green extraction methods for polyphenols from plant matrices and their byproducts: a review. *Comprehensive Reviews in Food Science and Food Safety*, **16**, 295–315.

Anjos, O., Santos, A.J., Dias, T. & Estevinho, L.M. (2017). Application of FTIR-ATR spectroscopy on the bee pollen characterization. *Journal of Apicultural Research*, **56**, 210–218.

Aspé, E. & Fernández, K. (2011). The effect of different extraction techniques on extraction yield, total phenolic, and anti-radical capacity of extracts from *Pinus radiata* Bark. *Industrial Crops and Products*, **34**, 838–844.

Aylanc, V., Eskin, B., Zengin, G., Dursun, M. & Cakmak, Y.S. (2020). In vitro studies on different extracts of fenugreek (*Trigonella spruneriana* BOISS.): phytochemical profile, antioxidant activity, and enzyme inhibition potential. *Journal of Food Biochemistry*, **44**, e13463.

Aylanc, V., Falcão, S.I., Ertosun, S. & Vilas-Boas, M. (2021a). From the hive to the table: nutrition value, digestibility and bioavailability of the dietary phytochemicals present in the bee pollen and bee bread. *Trends in Food Science & Technology*, **109**, 464–481.

Aylanc, V., Tomás, A., Russo-Almeida, P., Falcão, S.I. & Vilas-Boas, M. (2021b). Assessment of bioactive compounds under simulated gastrointestinal digestion of bee pollen and bee bread: bioaccessibility and antioxidant activity. *Antioxidants*, **10**, 651.

This article describes the extraction method of bee pollen and bee bread and the quantification of flavonoids by the spectrophotometric assay. It also provided important information for us to understand and interpret the obtained results in the Results and discussion part of this study.

Biesaga, M. (2011). Influence of extraction methods on stability of flavonoids. *Journal of Chromatography A*, **1218**, 2505–2512.

Brand-Williams, W., Cuvelier, M.-E. & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT-Food Science and Technology*, **28**, 25–30.

Campos, M.G., Bogdanov, S., Almeida-Muradian, L.B. *et al*. (2008). Pollen composition and standardisation of analytical methods. *Journal of Apicultural Research*, **47**, 154–161.

Carbone, K., Macchioni, V., Petrella, G. & Cicero, D.O. (2020). Exploring the potential of microwaves and ultrasounds in the green extraction of bioactive compounds from *Humulus lupulus* for the food and pharmaceutical industry. *Industrial Crops and Products*, **156**, 112888.

Carocho, M. & Ferreira, I.C. (2013). A review on antioxidants, prooxidants and related controversy: natural and synthetic

© 2022 Institute of Food, Science and Technology (IFSTTF).

compounds, screening and analysis methodologies and future perspectives. *Food and Chemical Toxicology*, **51**, 15–25.

Carpes, S.T., Begnini, R., Alencar, S.M.D. & Masson, M.L. (2007). Study of preparations of bee pollen extracts, antioxidant and antibacterial activity. *Ciência e Agrotecnologia*, **31**, 1818–1825.

Castro-López, C., Ventura-Sobrevilla, J.M., González-Hernández, M.D. *et al.* (2017). Impact of extraction techniques on antioxidant capacities and phytochemical composition of polyphenol-rich extracts. *Food Chemistry*, **237**, 1139–1148.

This study explores different extraction techniques to extract bioactive compounds from *Punica granatum*, *Juglans regia*, *Moringa oleifera*, and *Cassia fistula* plant and discusses the possible mechanisms that may play a role in extraction. Therefore, this paper helped us clarify the effects of extraction methods on pollen fractions.

Cheng, N., Chen, S., Liu, X., Zhao, H. & Cao, W. (2019). Impact of SchisandraChinensis bee pollen on nonalcoholic fatty liver disease and gut microbiota in highfat diet induced obese mice. *Nutrients*, **11**, 346.

Denisow, B. & Denisow-Pietrzyk, M. (2016). Biological and therapeutic properties of bee pollen: a review. *Journal of the Science of Food and Agriculture*, **96**, 4303–4309.

Dhanani, T., Shah, S., Gajbhiye, N. & Kumar, S. (2017). Effect of extraction methods on yield, phytochemical constituents and antioxidant activity of *Withania somnifera*. *Arabian Journal of Chemistry*, **10**, S1193–S1199.

This research is cited because it describes the methods for obtaining phenolic compounds from *Withania somnifera* extract using green and traditional extraction techniques and gives important information about the extraction efficiency.

Do, Q.D., Angkawijaya, A.E., Tran-Nguyen, P.L. *et al.* (2014). Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila aromatica*. *Journal of Food and Drug Analysis*, **22**, 296–302.

Edreva, A., Velikova, V. & Tsonev, T. (2007). Phenylamides in plants. *Russian Journal of Plant Physiology*, **54**, 287–301.

This review article discusses the presence and functions of phenylamides in plants. Therefore, the article provided important information for us to evaluate the structure of phenylamide compounds and their presence in pollen.

El-Ghouizi, A., El-Menyiy, N., Falcão, S.I., Vilas-Boas, M. & Lyoussi, B. (2020). Chemical composition, antioxidant activity, and diuretic effect of Moroccan fresh bee pollen in rats. *Veterinary World*, **13**, 1251.

Estevinho, L.M., Rodrigues, S., Pereira, A.P. & Feás, X. (2012). Portuguese bee pollen: palynological study, nutritional and microbiological evaluation. *International Journal of Food Science & Technology*, **47**, 429–435.

Fadzilah, N.H., Jaapar, M.F., Jajuli, R. & Wan Omar, W.A. (2017). Total phenolic content, total flavonoid and antioxidant activity of ethanolic bee pollen extracts from three species of Malaysian stingless bee. *Journal of Apicultural Research*, **56**, 130–135.

Falcão, S.I., Freire, C. & Vilas-Boas, M. (2013). A proposal for physicochemical standards and antioxidant activity of Portuguese propolis. *American Oil Chemists' Society*, **90**, 1729–1741.

Farahmandfar, R., Esmaeilzadeh Kenari, R., Asnaashari, M., Shahrampour, D. & Bakhshandeh, T. (2019). Bioactive compounds, antioxidant and antimicrobial activities of *Arum maculatum* leaves extracts as affected by various solvents and extraction methods. *Food Science & Nutrition*, **7**, 465–475.

Fatrcová-Šramková, K., Nôžková, J., Kačániová, M., Máriássyová, M., Rovná, K. & Stričík, M. (2013). Antioxidant and antimicrobial properties of monofloral bee pollen. *Journal of Environmental Science and Health*, **48**, 133–138.

Feás, X., Vázquez-Tato, M.P., Estevinho, L., Seijas, J.A. & Iglesias, A. (2012). Organic bee pollen: botanical origin, nutritional value, bioactive compounds, antioxidant activity and microbiological quality. *Molecules*, **17**, 8359–8377.

Floegel, A., Kim, D.-O., Chung, S.-J., Koo, S.I. & Chun, O.K. (2011). Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods. *Journal of Food Composition and Analysis*, **24**, 1043–1048.

Gabriele, M., Parri, E., Felicioli, A. *et al.* (2015). Phytochemical composition and antioxidant activity of Tuscan bee pollen of different botanic origins. *Italian Journal of Food Science*, **27**, 248.

Galan, A.-M., Calinescu, I., Trifan, A. *et al.* (2017). New insights into the role of selective and volumetric heating during microwave extraction: investigation of the extraction of polyphenolic compounds from sea buckthorn leaves using microwave-assisted extraction and conventional solvent extraction. *Chemical Engineering and Processing: Process Intensification*, **116**, 29–39.

Handrick, V., Vogt, T. & Frolov, A. (2010). Profiling of hydroxycinnamic acid amides in *Arabidopsis thaliana* pollen by tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*, **398**, 2789–2801.

Kaškonienė, V., Adaškevičiūtė, V., Kaškonas, P., Mickienė, R. & Maruška, A. (2020). Antimicrobial and antioxidant activities of natural and fermented bee pollen. *Food Bioscience*, **34**, 100532.

Louveaux, J., Maurizio, A. & Vorwohl, G. (1978). Methods of melissopalynology. *Bee World*, **59**, 139–157.

Lv, H., Wang, X., He, Y., Wang, H. & Suo, Y. (2015). Identification and quantification of flavonoid aglycones in rape bee pollen from Qinghai-Tibetan Plateau by HPLC-DAD-APCI/MS. *Journal of Food Composition and Analysis*, **38**, 49–54.

Maruyama, H., Sakamoto, T., Araki, Y. & Hara, H. (2010). Anti-inflammatory effect of bee pollen ethanol extract from *Cistus* sp. of Spanish on carrageenan-induced rat hind paw edema. *BMC Complementary and Alternative Medicine*, **10**, 1–11.

Muñoz, E., Velásquez, P., Rodriguez, K., Montenegro, G. & Giordano, A. (2020). Influence of *Brassica campestris* and *Galega officinalis* on antioxidant activity of bee pollen. *Revista Brasileira de Farmacognosia*, **30**, 444–449.

Pak-Dek, M.S., Osman, A., Sahib, N.G. *et al.* (2011). Effects of extraction techniques on phenolic components and antioxidant activity of Mengkudu (*Morinda citrifolia* L.) leaf extracts. *Journal of Medicinal Plants Research*, **5**, 5050–5057.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, **26**, 1231–1237.

Tomás, A., Falcão, S.I., Russo-Almeida, P. & Vilas-Boas, M. (2017). Potentialities of beebread as a food supplement and source of nutraceuticals: botanical origin, nutritional composition and antioxidant activity. *Journal of Apicultural Research*, **56**, 219–230.

Trusheva, B., Trunkova, D. & Bankova, V. (2007). Different extraction methods of biologically active components from propolis: a preliminary study. *Chemistry Central Journal*, **1**, 1–4.

Tuoheti, T., Rasheed, H.A., Meng, L. & Sheng Dong, M. (2020). High hydrostatic pressure enhances the anti-proliferative properties of lotus bee pollen on the human prostate cancer PC-3 cells via increased metabolites. *Journal of Ethnopharmacology*, **261**, 113057.

Urcan, A.C., Criste, A.D., Dezmirean, D.S., Mărgăoan, R., Caeiro, A. & Graça Campos, M. (2018). Similarity of data from bee bread with the same taxa collected in India and Romania. *Molecules*, **23**, 2491.

Uzbekova, D., Makarova, V., Khvoynitskaya, L. & Slepnev, A. (2003). Evaluation of bee-collected pollen influence on lipid peroxidation, antioxidant system and liver function in old animals. *Journal of Hepatology*, **38**, 203.

Vergeron, P. (1964) Interprétation statistique des résultats en matière d'analyse pollinique des miels, translated by EDP Sciences, 349–364.

Vogt, T. (2018). Unusual spermine-conjugated hydroxycinnamic acids on pollen: function and evolutionary advantage. *Journal of Experimental Botany*, **69**, 5311–5315.

© 2022 Institute of Food, Science and Technology (IFSTTF).

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Temperature profile during extraction (A) ultrasound-assisted extraction and (B) microwave-assisted extraction.

Figure S2. Total amount of phenolic (A) and phenylamide compounds (B) in bee pollen samples.

Table S1. Identification of phenolic and phenylamide compounds in bee pollen samples.

© 2022 Institute of Food, Science and Technology (IFSTTF).