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Investigating the Antioxidant and Color Properties of Bee Pollens of Various Plant Sources

Rita Végh,[a] Gizella Sipiczki,[b] and Mariann Csóka*[a]

In our study, Central and Eastern European bee pollens of different botanical origins were compared, based on their antioxidant and color properties. Total phenolic content (TPC), total flavonoid content (TFC), and *in vitro* antioxidant capacity (by FRAP, CUPRAC, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ assays) were determined spectrophotometrically. Besides, Relative Antioxidant Capacity Indexes (RACI) were calculated. CIELAB color parameters (L*, a*, b*, chroma) were determined by using a tristimulus-based instrument. Potential correlations between the investigated parameters were also identified. Based on the results of the preliminary study, ethanol:distilled water (60:40) was chosen as an extraction solvent. The total phenolic content of our samples ranged between 9.41 and 27.49 mg GAE/g dw. Pollens showed TFC:TPC ratios between 9 and 44%. RACI values indicate that rapeseed (*Brassica napus*), traveller's joy (*Clematis vitalba*) and phacelia (*Phacelia tanacetifolia*) pollens have relatively high, while pollens of certain plants of the Asteraceae family possess low antioxidant potential. Antioxidant properties correlated significantly in most cases. RACI values showed strong positive correlation with each of the other antioxidant capacity parameters, suggesting that this approach is well applicable for comparing the antioxidant potential of bee pollens. No clear correlation was found between the antioxidant and color parameters.

Introduction

Due to their potential health benefits, dietary antioxidants have become to the focus of consumers' attention and scientific research as well.[1] Antioxidants are chemical compounds that inhibit free radical reactions, thus delay cellular damage. Free radical formation occurs continuously in the cells as a part of normal metabolic processes, however, they may also be produced as a result of diet, smoking, exercise, inflammation, exposure to sunlight, air pollutants, stress, alcohol and drugs.[2] The increased formation of free radicals causes excessive oxidative stress, which contributes to the aging process and can result in an incidence of various diseases.[3] Epidemiological studies and meta-analyses have suggested that a long-term consumption of antioxidant-rich foods may help to reduce the risk of different types of cancers, autoimmune disorders, osteoporosis as well as cardiovascular and neurodegenerative diseases.[1,4,5] The most important sources of dietary antioxidants are fruits, vegetables, whole grains, tea, coffee, wine, beer, herbs and spices.[6] In addition, an increasing demand can be observed for functional foods and nutritional supplements that naturally contain antioxidants.[7]

Apicultural products, especially bee pollens are rich in antioxidant phytochemicals.[5] Bee pollens consist of small, granular-looking pellets formed by honeybees (*Apis mellifera L.*), which contain sugars, fibers, amino acids, fatty acids, minerals, vitamins and a wide range of bioactive metabolites, mainly phenolic compounds and carotenoids.[8,9,10,11] The quantity and quality of nutrients vary greatly between pollens belonging to different taxonomic groups and are also affected by geographical origin, climatic conditions, bee species, soil type, beekeeper's activity, preservation methods and storage conditions.[12,13,14] Bee pollens are used in complementary and alternative medical practices as they potentially pose antioxidant, immunostimulating, antimicrobial, antiinflammatory, antiallergic, hepatoprotective and anticarcinogenic activities.[15] Medical properties of bee pollen are attributed to a wide range of secondary plant metabolites, primarily to phenolic compounds.[5,8,9,13] Based on more than a hundred studies, Thakur & Nanda found that the total phenolic content (TPC) vary widely (0.69–213.20 mg GAE/g) in bee pollens.[16] This great variance can be attributed to the differences between plant sources,[4,8,12] but results are also influenced significantly by the extraction conditions.[17] In addition, sample processing and storage may also have a significant impact on this parameter.[18,19] Phenolic compounds account for 1.6% of the products on average and include mainly flavonoids and phenolic acids.[20] Previous studies suggest that flavonoid glycosides, particularly derivatives of kaempferol, quercetin, and isorhamnetin are usually present in significant amounts in pollen.[13,21,22] The most common phenolic acids are chlorogenic, gallic, ferulic, cinnamic and caffeic acids, as well as hydroxycinnamic, *o*-coumaric and *p*-coumaric acids.[23,24] Several studies indicate that phenolic compounds are potential taxonomic

[a] *R. Végh, M. Csóka*
Department of Nutrition, Institute of Food Science and Technology, Hungarian University of Agriculture and Life Sciences, 1118 Budapest, Somlói út 14-16., Hungary
E-mail: Csoka.Mariann@uni-mate.hu

[b] *G. Sipiczki*
Department of Bioengineering and Alcoholic Drink Technology, Institute of Food Science and Technology, Hungarian University of Agriculture and Life Sciences, 1118 Budapest, Ménesi út 45., Hungary

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markers of pollen, which induced an increasing scientific interest recently.[23,25–27]

Besides phenolic compounds, other antioxidants, such as ascorbic acid, tocopherols, carotenoids, fatty acids, polysaccharides and active peptides are also found in bee pollen.[4,16,28] More methods including the FRAP (ferric reducing antioxidant power), CUPRAC (cupric ion reducing antioxidant capacity), $ABTS^{\bullet+}$ (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), $DPPH^{\bullet}$ (2,2-diphenyl-1-picryhydrazyl) and ORAC (oxygen radical absorbance capacity) assays have been used to assess the *in vitro* antioxidant capacity of bee pollens.[4,12,25,26,27,29] Antioxidant capacity tests can be divided into two main groups. Single electron transfer (SET) reaction-based methods, such as the FRAP, CUPRAC, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ assays involve a redox reaction with the oxidant as an indicator of the reaction endpoint, while hydrogen atom transfer (HAT) reaction-based methods including the ORAC assay generally measure the ability of an antioxidant to neutralize free radicals by hydrogen donation and are based primarily on reaction kinetics.[30,31] The FRAP method is based on the reduction of ferric-tripyridyltriazine (Fe^{III}-TPTZ) to Fe^{II} at acidic pH (pH = 3.6), which results in a violet solution. The color change can be monitored spectrophotometrically at 593 nm.[32] As the Fe^{III}-TPTZ complex has higher affinity toward the aqueous phase than towards organic solvents, the determination of lipophilic antioxidants is limited.[33] This method is unable to accurately detect the antioxidant activity of slow reacting polyphenols, such as caffeic acid, tannic acid, ferulic acid and quercetin.[30] Furthermore, the FRAP assay is not sensitive to thiol-type antioxidants, such as glutathione and proteins.[34] The CUPRAC assay is based on the increase in absorbance of bis(neocuproine)copper(II) containing solution upon the reduction of Cu^{II} to Cu^{I} at 450 nm. The reaction takes place at a neutral pH, so it better simulates the physiological action of antioxidants compared to the FRAP assay.[33] The CUPRAC chromophore is soluble in both aqueous and organic solvents, enabling the determination of hydrophilic and lipophilic antioxidants as well.[35] The $ABTS^{\bullet+}$ assay is based on the generation of a bluish-green $ABTS^{\bullet+}$ that can be reduced by antioxidants, whereas the $DPPH^{\bullet}$ assay is based on the reduction of the purple $DPPH^{\bullet}$. These tests are convenient in their application and thus popular among researchers, however they are limited as they use non-physiological radicals.[35] $DPPH^{\bullet}$ is a lipophilic radical with limited accessibility to the hydrophilic components, thereby requiring alcohol in the reaction mixture to ensure maximum solubility.[36] Results of previous studies indicate that the $ABTS^{\bullet+}$ assay better reflects the antioxidant capacity of plant foods containing hydrophilic and highly pigmented antioxidants compared to the $DPPH^{\bullet}$ assay.[36,37] The ORAC assay is based on the inhibition of the oxidation of a fluorescent substrate by peroxyl radicals. This assay can be adapted to detect both hydrophilic and hydrophobic antioxidants.[33]

The antioxidant capacity assays are based on different mechanisms, consequently, they generate unique results. Currently, there is no single universally accepted assay that is adequate for the quantification of the total antioxidant capacity. Thus, a combination of different experiments is generally used by researchers in order to obtain more information on the antioxidant potential of a given matrix.[35] An integrated approach proposed by Sun and Tanumihardjo is applicable to evaluate food samples based on their antioxidant capacity. Relative antioxidant capacity index (RACI) is calculated as a mean value of standard scores transformed from initial data generated with different *in vitro* methods for each sample. RACI represents the antioxidant capacity determined by each method and provides an accurate rank of antioxidant potential among foods.[31] This approach has been applied to a wide range of foods, but not yet to bee pollen.

CIELAB color scales are commonly used for characterizing the color of bee pollen.[8,38,39] The CIELAB color coordinates are L* (absolute white – absolute black), a* (red – green) and b* (yellow – blue). Chroma (C*) is a quantitative attribute of colorfulness (saturation).[40] Bees collect pollen from a single plant species during one foraging trip, therefore, a pollen pellet is generally monochromatic. However, as members of a bee colony usually forage on several plant species at a given period of time, pollen samples harvested by beekeepers consist of pellets of various colors.[13,20] Pollen loads of different botanical origin show a wide range of colors, but they are yellowish or orange in most cases.[41] The color of bee pollens is also influenced by geographical origin, climatic conditions, time of collection, age and nutritional status of the source plant, preservation method, and storage conditions.[42] The main pigments of bee pollens are flavonoids and carotenoids, which have antioxidant effect.[43] Consequently, there is a possible correlation between color and antioxidant parameters of bee pollen. To our knowledge, this relationship was examined only in one study, in which slight correlations were observed between yellowness/chroma and TPC/DPPH/ORAC values of pollens.[8]

The aim of our work was to characterize mono- and polyfloral pollens that are typical for the Central and Eastern European flora, based on their total phenolic content (TPC), total flavonoid content (TFC), antioxidant capacity determined by four different *in vitro* methods (FRAP, CUPRAC, $ABTS^{\bullet+}$, $DPPH^{\bullet}$) and color parameters. The botanical composition of our samples was determined by microscopic pollen analysis. Extraction solvent was optimized based on a preliminary experiment. Relative antioxidant capacity index (RACI) values were calculated by integrating data obtained in the four antioxidant capacity determinations. Possible correlations between the tested parameters were also investigated.

Results and Discussion

Botanical origin of pollen samples

The results of the microscopic pollen analysis are summarized in Table 1. The predominant pollen of fourteen samples exceeded 80%, therefore, they can be considered as monofloral.[44] Mainly pollens of wildflowers and trees were included among them, but rapeseed (*Brassica napus* L.), phacelia (*Phacelia tanacetifolia* Benth.), sunflower (*Helianthus*

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Table 1. Botanical composition of bee pollen samples.

Sample name	Geographical origin	Predominant pollen (>45%) Species	%	Secondary pollen (15–45%)	Important minor pollen (3–15%)	Minor pollen (<3%)
dandelion	Baia Mare, Romania	*Taraxacum officinale*	84	–	*Salix* *Brassica napus*	Different fruits
dropwort	Kimle, Hungary	*Filipendula vulgaris*	95	–	–	*Trifolium repens* *Helianthus annuus* *Chenopodium* *Carduus nutans* *Papaver rhoeas* *Impatiens* *Leucanthemum vulgare*
honey locust	Máza, Hungary	*Gleditsia triacanthos*	99	–	–	*Tilia*
musk thistle	Kimle, Hungary	*Carduus nutans*	94	–	*Helianthus annuus*	*Calluna vulgaris* *Impatiens* *Cyanus segetum*
phacelia	Kimle, Hungary	*Phacelia tanacetifolia*	100	–	–	-
rapeseed-1	Kimle Hungary	*Brassica napus*	96	–	*Frangula*	*Acer* *Loranthus europaeus*
rapeseed-2	Esztergom, Hungary	*Brassica napus*	94	–	–	*Salix* Different fruits
rapeseed-3	Baia Mare, Romania	*Brassica napus*	96	–	–	*Scripus lacustris* *Juglans regia* *Acer*
red poppy	Mezőkövesd, Hungary	*Papaver rhoeas*	94	–	*Phacelia tanacetifolia*	Different fruits *Ligustrum* *Tilia* *Convolvulus arvensis*
rock-rose	Baia Mare, Romania	*Cistus incana*	97	–	*Calluna vulgaris* *Taraxacum officinale* *Thymus*	-
sunflower	Kimle, Hungary	*Helianthus annuus*	97	–	–	*Taraxacum officinale*
sweet cherry	Kimle, Hungary	*Prunus avium*	96	–	*Salix*	-
traveller's joy	Esztergom Hungary	*Clematis vitalba*	89	–	*Plantago*	*Tilia* *Taraxacum officinale* *Trifolium repens*
wild blackberry	Esztergom, Hungary	*Rubus fruticosus*	98	–	–	not identified
polyfloral-1	Kimle, Hungary	*Salix caprea*	60	*Prunus avium*	*Prunus domestica* *Brassica napus*	*Quercus* *Trifolium* *Taraxacum officinale*
polyfloral-2	Kimle, Hungary	*Sophora japonica*	46	*Zea mays*	*Solidago*	*Chenopodium* *Carduus nutans* *Helianthus annuus* *Polygonaceae* *Taraxacum officinale* *Betula pendula* *Centaurea* *Impatiens*
polyfloral-3	Máza, Hungary	*Cornus sanguinea*	62	*Brassica napus*	Different fruits *Trifolium* *Helianthus annuus*	*Frangula* *Carduus nutans* *Papaver rhoeas*
polyfloral-4	Baia Mare, Romania	*Acer campestre*	66	*Vicia*	*Frangula* *Brassica napus*	*Cornus sanguinea* *Pinaceae* *Phacelia tanacetifolia*
polyfloral-5	Baia Mare, Romania	*Trifolium sp.*	51	–	*Filipendula vulgaris* *Leucanthemum vulgare* *Centaurea* *Rosa canina* *Plantago* *Calluna vulgaris*	*Epilobium angustifolium* *Thymus* *Tilia* *Brassica napus* *Carduus nutans* *Dianthus* *Taraxacum officinale*
polyfloral-6	Baia Mare, Romania	*Calluna vulgaris*	47	–	*Filipendula ulmaria* *Trifolium repens* *Rosa canina* *Poaceae*	*Lotus corniculatus* *Thymus* *Plantago lanceolata* *Taraxacum officinale* *Epilobium angustifolium*
polyfloral-7	Baia Mare, Romania	*Rosa canina*	68	*Salix sp.*	–	Different fruits *Scripus lacustris* *Taraxacum officinale*

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annuus L.) and sweet cherry (*Prunus avium* L.) were also identified. Three of the samples were originated from rapeseed, but they were harvested in different locations. In polyfloral samples, also a single plant species dominated, the ratio of which varied between 46 and 68%. The sources of the predominant pollen (>45%) of most samples belonged to the natural flora of Central and Eastern Europe. These include trees (*Acer campestre* L., *Salix caprea* L., *Sophora japonica* L.), shrubs (*Calluna vulgaris* L. Hull., *Cornus sanguinea* L., *Rosa canina* L.) and wildflowers (members of the genus *Trifolium*).

Optimal extraction solvent

The phenolic composition of bee pollens of diverse botanical sources show great variances according to previous studies.[25,45,46] Phenolic compounds exhibit a wide range of solubilities, and are usually more soluble in solvents less polar than water.[47] For these reasons, the solvent which is optimal for the extraction of phenolic compounds from our bee pollen samples was identified prior to the total phenolic content, total flavonoid content and antioxidant capacity determinations. A representative pollen sample was used for this purpose, which contained each of the above-described products (fourteen monofloral and seven polyfloral pollens) in equal proportions. Extractions were carried out under the same conditions by using distilled water and three types of organic solvents (methanol, ethanol and acetone) at different concentrations. Followingly, the total phenolic content of the extracts was determined by the Folin-Ciocalteu method.

Total phenolic contents obtained for the mixed pollen sample prepared by using different solvents are presented in Figure 1. Based on our results, the tested solvents showed great variances regarding extraction efficiency ($p < 0.05$). Ethanol: distilled water (60:40) was the most effective to extract phenolic compounds of bee pollen among the tested solvents. Aqueous solutions of ethanol and acetone at 60–80% concentration levels appeared to be very effective, but methanolic extracts resulted in significantly lower TPC values. The usage of each solvent and their aqueous solutions resulted in significantly higher TPC values compared to distilled water, except for the pure acetone, which appear to present very low extraction efficiency. In general, it can be concluded that organic solvents at 60–80% concentration levels provide adequate extraction efficiency for bee pollen samples. This observation is in accordance with the findings of previous studies.[17,48,49,50] With regard to these results, aqueous solution of ethanol at 60% concentration level was used as the extraction solvent for further determinations considering the environmental, economic and occupational safety reasons.

Dry matter content of bee pollen samples

In Figure 2, the dry matter content of the examined bee pollen samples are presented. This parameter varied between 91.75 and 95.21% and showed significant differences ($p < 0.05$). The pollen sample originating from musk thistle (*Carduus nutans* L.) had the lowest, whereas pollens of dropwort (*Filipendula vulgaris* Moench.), honey locust (*Gledistia triacanthos* L.), sunflower (*Helianthus annuus* L.) as well as polyfloral samples originating mainly from the Japanese pagoda tree (*Sopohora japonica* L.) and common dogwood (*Cornus sanguniea* L.) had high dry matter content. The reason behind the differences between dry matter contents may be that our samples had variable initial moisture content. According to literature data,

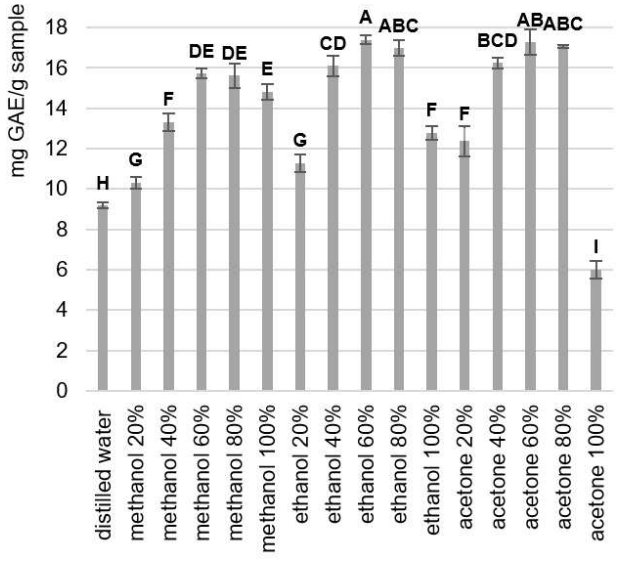


Figure 1. Total phenolic content of extracts of the mixed bee pollen prepared by different solvents. Different letters indicate significant differences ($\alpha = 0.05$).

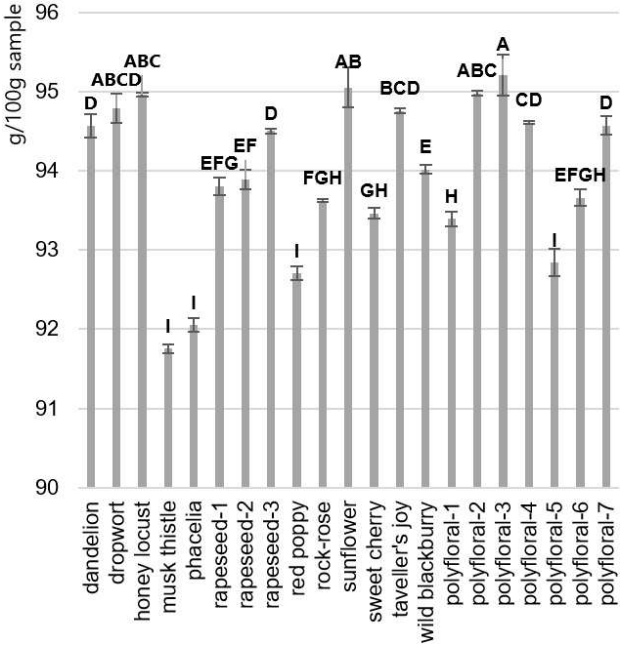


Figure 2. Dry matter content (g/100 g) of the bee pollen samples. Different letters indicate significant differences ($\alpha = 0.05$).

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the moisture content of freshly collected bee pollens ranges usually between 20 and 30%, which is favorable for the growth of microorganisms.[16,44] Moisture contents of our samples ranged between 5 and 8%, which ensure adequate microbiological stability and acceptable sensory properties.[44] Hence, it can be concluded that the drying of our samples was carried out properly.

Total phenolic and total flavonoid contents of bee pollen samples

Results of the total phenolic content (TPC) and total flavonoid content (TFC) are expressed on a dry weight basis and presented in Table 2. The total phenolic content of the samples ranged between 9.41 and 27.49 mg GAE/g dw. The obtained data are in accordance with the results of a comprehensive literature review of Thakur & Nanda, which reported that the TPC of bee pollens vary between 0.69 and 213.20 mg GAE/g.[16] The total flavonoid content of our samples varied between 0.88 and 11.09 mg QE/g dw. Statistically significant differences were observed between samples of different plant sources ($p < 0.05$) regarding both parameters. Small differences can be observed between the mean and median values of the TPC and TFC, indicating that no outstandingly low or high value has been detected.

The TPC and TFC values of rapeseed pollens harvested in different locations of Hungary showed relatively low differences, suggesting that botanical origin has a greater effect on these parameters than geographical origin. Bee pollens showed heterogeneous TFC:TPC ratios, which is in compliance with the results of previous studies.[25,29,46,51,52,53,54] Flavonoids accounted for 20% of the total phenolic contents in our samples on average. The TFC:TPC ratio was exceptionally high (44%) in the case traveller's joy (*Clematis vitalba* L.) pollen. Among polyfloral samples, the highest TFC:TPC ratio was observed for the product containing pollen grains mainly from the Japanese pagoda tree. The flower of this plant is used as a medicinal herb in some Asian countries and reported to be a rich source of flavonoids including rutin, quercetin, isorhamnetin and kaempferol.[55]

Antioxidant capacity of bee pollen samples

Results of the four antioxidant capacity assays (FRAP, CUPRAC, $ABTS^{\bullet+}$, $DPPH^{\bullet}$) are presented in Table 2. Each of the applied antioxidant capacity assays resulted in values with statistically significant differences between pollens of diverse botanical sources ($p < 0.05$). The rapeseed pollens harvested in different locations showed relatively low differences regarding their antioxidant capacity. The FRAP assay resulted in antioxidant capacities between 2.22 and 10.59 mg AAE/g dw (5.98 mg AAE/g dw on average), while results of the CUPRAC assay ranged between 7.90 and 44.89 mg TE/g dw (25.58 mg TE/g dw on average). Differences may be attributed to the limited ability of the FRAP assay to measure lipophilic antioxidants, thiol-type antioxidants and slow-reacting phenolic compounds.[3,33,34] Results of the $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ assays fell within a comparable range (between 3 and 20 mg TE/g dw), but the $ABTS^{\bullet+}$ assay resulted in higher values for most of the samples, probably due to the fact that the $DPPH^{\bullet}$ assay is less sensitive to the hydrophilic and highly pigmented antioxidants.[36,37] Results of these two assays differed to the greatest extent in the case of the wild blackberry (*Rubus fruticosus* L.) pollen, suggesting that this product may contain significant amounts of hydrophilic and/or pigmented antioxidants. Nevertheless, roughly similar $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ values were obtained for dropwort, honey locust, rapeseed, rock-rose and a polyfloral sample containing mainly pollen grains of common dogwood. Very small differences were observed between the mean and median values of each antioxidant capacity assay, suggesting that the obtained results are balanced.

Relative Antioxidant Capacity Indexes of bee pollen samples

Relative Antioxidant Capacity Indexes (RACI) of bee pollens were calculated according to Sun and Tanumihardjo,[31] considering the results of the FRAP, CUPRAC, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ assays. The calculated values of the samples are presented numerically in Table 2. Followingly, samples were ranked based on their RACI values and plotted on a bar graph (Figure 3). Based on these results, sunflower (*Helianthus annuus* L.), musk thistle (*Carduus nutans* L.) and dandelion (*Taraxacum officinale* Weber.) pollens have lower antioxidant potential compared to other monofloral samples. These plant species belong to the Asteraceae family. Members of this taxonomical group was also found to have relatively low TPC, TFC and antioxidant capacity in previous research: recently, fourteen Croatian monofloral bee pollens were characterized based on their antioxidant proper-

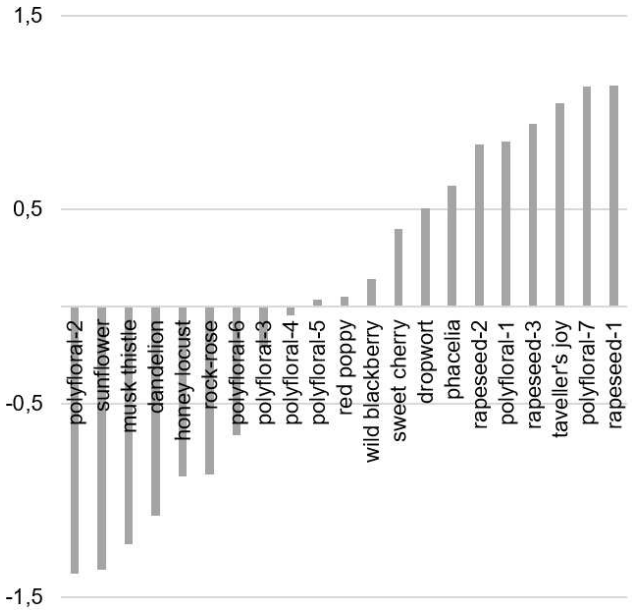


Figure 3. Ranking of pollen samples based on their Relative Antioxidant Capacity Index (RACI).

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Table 2. Total phenolic content (TPC), total flavonoid content (TFC), antioxidant capacity (FRAP, CUPRAC, ABTS$^{\bullet+}$, DPPH$^{\bullet}$) and Relative Antioxidant Capacity Index (RACI) of bee pollen samples (n = 21).

Origin of pollen samples Botanical	Geographical	TPC (mg GAE/g dw)	TFC (mg QE/g dw)	FRAP (mg AAE/g dw)	CUPRAC (mg TE/g dw)	ABTS$^{\bullet+}$ (mg TE/g dw)	DPPH$^{\bullet}$ (mg TE/g dw)	RACI[a]
dandelion (> 80 % Taraxacum officinale)	Baia Mare, RO	18.14 ± 0.20^{fgh}	3.31 ± 0.05^{ij}	3.58 ± 0.05^{l}	10.47 ± 1.07^{fg}	10.82 ± 1.20^{h}	3.39 ± 0.23^{g}	-1.08
dropwort (> 80 % Filipendula vulgaris)	Kimle, HU	18.43 ± 0.25^{fg}	6.82 ± 0.07^{b}	9.42 ± 0.24^{bc}	29.50 ± 0.75^{c}	12.08 ± 1.14^{gh}	11.70 ± 0.40^{b}	0.51
honey locust (> 80 % Gleditsia triacanthos)	Máza, HU	10.35 ± 0.70^{h}	0.88 ± 0.18^{p}	4.77 ± 0.06^{hijk}	17.84 ± 0.18^{e}	6.23 ± 0.90^{lm}	6.40 ± 0.36^{f}	-0.88
musk thistle (> 80 % Carduus nutans)	Kimle, HU	17.76 ± 0.69^{h}	1.97 ± 0.10^{n}	2.87 ± 0.05^{m}	10.28 ± 0.74^{fg}	8.81 ± 0.57^{ij}	3.93 ± 0.27^{g}	-1.23
phacelia (> 80 % Phacelia tanacetifolia)	Kimle, HU	27.49 ± 0.73^{a}	3.58 ± 0.08^{hi}	8.16 ± 0.24^{d}	28.26 ± 1.20^{cd}	18.14 ± 0.53^{bc}	10.39 ± 0.32^{c}	0.63
rapeseed-1 (> 80 % Brassica napus)	Kimle, HU	26.03 ± 0.72^{ab}	3.01 ± 0.06^{jk}	9.93 ± 0.26^{b}	44.89 ± 3.04^{a}	14.69 ± 0.56^{def}	13.45 ± 0.09^{a}	1.14
rapeseed-2 (> 80 % Brassica napus)	Esztergom, HU	24.42 ± 0.72^{cd}	3.08 ± 0.10^{jk}	8.33 ± 0.23^{d}	39.57 ± 0.41^{b}	14.06 ± 0.35^{ef}	13.35 ± 0.25^{a}	0.84
rapeseed-3 (> 80 % Brassica napus)	Baia Mare, RO	25.19 ± 0.65^{bc}	2.96 ± 0.05^{kl}	9.27 ± 0.48^{c}	43.34 ± 0.94^{a}	$13.59 \pm 0.90e^{fg}$	12.84 ± 0.03^{a}	0.94
red poppy (> 80 % Papaver rhoeas)	Mezőkövesd, HU	19.61 ± 0.67^{ef}	4.90 ± 0.13^{de}	8.40 ± 0.55^{d}	18.05 ± 1.31^{e}	13.25 ± 0.87^{fg}	$8.87 \pm 0.52d^{e}$	0.05
rock-rose (> 80 % Cistus incana)	Baia Mare, RO	14.06 ± 0.45^{i}	4.20 ± 0.02^{f}	5.93 ± 0.06^{fg}	13.10 ± 0.81^{f}	6.74 ± 0.52^{klm}	5.87 ± 0.52^{f}	-0.87
sunflower (> 80 % Helianthus annuus)	Kimle, HU	12.29 ± 0.40^{j}	2.65 ± 0.14^{lm}	2.54 ± 0.09^{mn}	9.03 ± 0.57^{g}	8.30 ± 0.90^{jkl}	3.30 ± 0.42^{g}	-1.36
sweet cherry (> 80 % Prunus avium)	Kimle, HU	23.44 ± 0.57^{d}	5.33 ± 0.07^{c}	6.27 ± 0.14^{f}	29.17 ± 0.73^{c}	16.47 ± 0.49^{cd}	10.85 ± 0.55^{bc}	0.40
traveller's joy (> 80 % Clematis vitalba)	Esztergom HU	25.08 ± 0.40^{bc}	11.09 ± 0.21^{a}	10.59 ± 0.28^{a}	36.37 ± 2.12^{b}	15.27 ± 0.53^{de}	13.23 ± 0.19^{a}	1.05
wild blackberry (> 80 % Rubus fruticosus)	Esztergom, HU	21.07 ± 0.26^{e}	2.80 ± 0.19^{klm}	4.14 ± 0.23^{kl}	25.20 ± 1.63^{d}	20.31 ± 0.88^{a}	7.96 ± 0.35^{e}	0.14
polyfloral-1 (mostly Salix caprea)	Kimle, HU	23.31 ± 0.24^{d}	4.45 ± 0.03^{f}	5.39 ± 0.13^{gh}	39.21 ± 0.87^{b}	19.89 ± 0.30^{ab}	12.81 ± 0.52^{a}	0.85
polyfloral-2 (mostly Sophora japonica)	Kimle, HU	9.41 ± 0.23^{k}	2.54 ± 0.08^{m}	2.22 ± 0.09^{n}	7.90 ± 0.33^{g}	8.14 ± 0.60^{jklm}	3.93 ± 0.32^{g}	-1.38
polyfloral-3 (mostly Cornus sanguinea)	Máza, HU	14.28 ± 0.54^{i}	1.43 ± 0.09^{o}	4.50 ± 0.18^{jk}	25.84 ± 1.57^{d}	10.48 ± 0.82^{hi}	10.41 ± 0.46^{c}	-0.22
polyfloral-4 (mostly Acer campestre)	Baia Mare, RO	20.12 ± 0.57^{e}	4.16 ± 0.15^{fg}	4.35 ± 0.29^{k}	26.91 ± 0.46^{cd}	16.07 ± 0.71^{d}	8.06 ± 0.47^{de}	-0.04
polyfloral-5 (mostly Trifolium sp.)	Baia Mare, RO	20.16 ± 0.74^{e}	3.81 ± 0.31^{gh}	5.03 ± 0.32^{hij}	25.66 ± 1.11^{d}	15.57 ± 0.34^{de}	9.04 ± 0.48^{d}	0.03
polyfloral-6 (mostly Calluna vulgaris)	Baia Mare, RO	17.95 ± 0.69^{gh}	4.84 ± 0.18^{e}	2.82 ± 0.07^{mn}	12.85 ± 0.67^{f}	14.89 ± 1.12^{def}	6.26 ± 0.37^{f}	-0.67
polyfloral-7 (mostly Rosa canina)	Baia Mare, RO	23.52 ± 0.60^{d}	5.21 ± 0.14^{cd}	6.98 ± 0.25^{e}	43.84 ± 1.29^{a}	19.75 ± 0.61^{ab}	13.45 ± 0.45^{a}	1.13
minimum		9.41	0.88	2.22	7.90	6.23	3.30	-1.38
maximum		27.49	11.09	10.59	44.89	20.31	13.45	1.14
mean		19.62	3.95	5.98	25.58	13.50	9.02	0.00
median		20.12	3.58	5.39	25.84	14.06	9.04	0.05

[a]Relative antioxidant capacity indexes (RACI) were calculated according to Sun and Tanumihardjo (2007),[31] based on the results of four antioxidant capacity assays. Means with different superscripts in the columns indicate significant difference between samples ($p < 0.05$).

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ties. Two of these samples, namely *Taraxacum officinale* Weber. and *Crepis biennis* L. belonged to the Asteraceae family, which had very low total phenolic content and antioxidant capacity, however, their total flavonoid content was roughly similar to other samples.[54] Another study conducted in Romania also found relatively low antioxidant capacity ($ABTS^{\bullet+}$, $DPPH^{\bullet}$) values in bee pollens of *Carduus sp.*, *Taraxacum officinale* and *Helianthus annuus*.[50] In addition, Asteraceae-type bee pollens are reported to have relatively low antioxidant potential also in Italian, Chinese, Slovakian and Finnish studies.[13,46,56,57] Among our samples, the lowest RACI value was observed for a polyfloral pollen (polyfloral-2). Although 46% of pollen grains in this product originates from the flavonoid-rich Japanese pagoda tree,[55] it also contained significant amounts of maize (*Zea mays*) pollen which was reported to have low antioxidant potential in previous studies.[50,58,59] Besides, this sample contained pollens of some Asteraceae species with presumably low antioxidant potential, including sunflower, musk thistle, dandelion, as well as members of the genera *Solidago* and *Centaurea*.

The obtained RACI values indicate that rapeseed (*Brassica napus* L.), traveller's joy (*Clematis vitalba* L.) and phacelia (*Phacelia tanacetifolia* Benth.) pollens have high antioxidant potential compared to other samples. Pollens of the Brassicaceae plant family are major protein sources for honeybees,[60] thus, the antioxidant properties of these products have been widely investigated. Several studies reported that Brassicaceae-type pollens contain high amounts of phenolic compounds and/or can be characterized by a relatively high antioxidant capacity.[13,46,50,56,57,61] Based on previous research works, bee pollens originating from phacelia can be characterized as a moderately good source of phenolics, flavonoids and/or antioxidants.[54,58,61] It should also be noted that the results of TPC, TFC and antioxidant capacity determinations depend not only on the botanical composition of pollen pellets, but also influenced by extraction conditions.[17] For example, Kostić and co-workers found that methanol is more effective in comparison to ethanol for extracting the phenolic compounds of sunflower bee pollen.[22]

Color properties of pollen samples

CIELAB color coordinates (L*, a*, b*) and chroma (C*) values of bee pollen samples are presented in Table 3. Results for each of these parameters differed significantly for most of the sample pairs ($p < 0.05$). Yellowness (b*) and chroma (C*) of the monofloral rapeseed pollens harvested in different locations showed statistically significant differences, but lightness (L*) was not affected by the geographical origin. Regarding their redness (a*) value, significant differences were observed between rapeseed-1 (harvested in Kimle, Hungary) and rapeseed-2 (harvested in Esztergom, Hungary), however, neither of these samples differed significantly from rapeseed-3 (harvested in Baia Mare, Romania).

Most of the samples were characterized by a lightness (L*) value above 50, except for musk thistle, phacelia, red poppy and a polyfloral sample containing mainly pollen of common heather (*Calluna vulgaris* L. *Hull.*). Honey locust, traveller's joy and rapeseed pollens showed exceptionally high ($L^* > 70$) lightness values. Positive redness (a*) values were obtained for most of the samples, except for honey locust, sweet cherry, wild blackberry and two of the rapeseed pollens indicating that these samples have a slightly greenish hue. In contrary, musk thistle, dandelion, sunflower, red poppy and phacelia pollens had relatively high amounts of red pigments ($a^* > 10$). The yellowness (b*) value was positive in all cases. Relatively high yellowness ($b^* > 50$) values were observed for pollens of dandelion, rapeseed, rock-rose, sunflower and some polyfloral samples, whereas phacelia, musk thistle and wild blackberry showed low amounts of yellowness ($b^* < 20$). Chroma (C*), a calculated parameter indicating saturation, ranged between 11.93 and 79.97. Dandelion and sunflower pollens showed exceptionally high C* values of almost 80, because both a* and b* were relatively high in these samples. The reason behind this observation may be that pollen pellets of both of these plant species are rich in carotenoid pigments.[11,43] In contrary, phacelia and musk thistle pollens were characterized by low b* values, thus, the saturation of these samples is low compared to other pollens. The range of the obtained CIELAB color parameters were roughly comparable to previously reported data.[8,38,39,42] However, Salazar-González and co-workers[42] reported higher a* values, while Dulger-Altiner and co-workers[39] published lower b* values compared to the results obtained for our samples.

Correlations between the tested parameters

Table 4 shows the correlation matrix of the tested parameters, in which Pearson's correlation coefficients (r) are presented with the p values. A slight correlation can be observed between the total phenolic content and flavonoid content ($r = 0.422$), but it is not significant ($p = 0.057$). Nevertheless, TPC correlated with the results of each antioxidant capacity assay. FRAP is the only assay the results of which significantly correlated with the flavonoid content. Data obtained by the four antioxidant capacity assays correlated in most cases. RACI is the only parameter which significantly correlated with each of the other antioxidant parameters, suggesting that the method proposed by Sun and Tanumihardjo[31] is well applicable for comparing the antioxidant potential of bee pollens.

Lightness (L*) significantly correlated with the other color parameters. A very strong positive correlation ($r = 0.993$) was found between chroma (C*) and yellowness (b*), but redness (a*) did not affect chroma significantly. Our results do not support the observations of De-Melo and co-workers,[8] because the antioxidant parameters and yellowness were found to be independent for our samples. However, significant negative correlations were found between redness and CUPRAC/DPPH/RACI values. This may be attributed to the observation that pollens with relatively high a* values (musk thistle, dandelion, sunflower) can be characterized with low antioxidant content, whereas rapeseed pollens with low a* values are rich in phenolic compounds.

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Table 3. CIELAB color parameters of bee pollen samples (n = 21).

Origin of pollen samples Botanical	Geographical	Color coordinates L* (lightness)	a* (redness)	b* (yellowness)	C* (chroma)
dandelion (> 80 % Taraxacum officinale)	Baia Mare, RO	57.90 ± 0.50^{g}	17.93 ± 0.52^{b}	77.93 ± 0.24^{a}	79.97 ± 0.18^{a}
dropwort (> 80 % Filipendula vulgaris)	Kimle, HU	57.33 ± 0.61^{g}	3.90 ± 0.14^{hi}	45.57 ± 0.33^{i}	45.73 ± 0.32^{i}
honey locust (> 80 % Gleditsia triacanthos)	Mája, HU	72.50 ± 0.36^{a}	-0.20 ± 0.25^{lm}	46.23 ± 0.52^{i}	46.23 ± 0.52^{i}
musk thistle (> 80 % Carduus nutans)	Kimle, HU	28.30 ± 0.80^{m}	18.53 ± 0.31^{a}	12.77 ± 0.52^{p}	22.50 ± 0.05^{n}
phacelia (> 80 % Phacelia tanacetifolia)	Kimle, HU	30.97 ± 0.12^{l}	10.37 ± 0.25^{e}	5.90 ± 0.16^{q}	11.93 ± 0.28^{p}
rapeseed-1 (> 80 % Brassica napus)	Kimle, HU	70.90 ± 0.14^{b}	-0.57 ± 0.12^{m}	54.23 ± 0.12^{g}	54.24 ± 0.12^{g}
rapeseed-2 (> 80 % Brassica napus)	Esztergom, HU	70.60 ± 0.37^{b}	0.27 ± 0.05^{l}	55.70 ± 0.21^{f}	55.70 ± 0.21^{f}
rapeseed-3 (> 80 % Brassica napus)	Baia Mare, RO	71.20 ± 0.36^{b}	-0.03 ± 0.16^{lm}	58.47 ± 0.08^{d}	58.47 ± 0.08^{d}
red poppy (> 80 % Papaver rhoeas)	Mezőkövesd, HU	41.37 ± 0.60^{k}	11.47 ± 0.19^{d}	38.20 ± 0.71^{l}	39.88 ± 0.70^{k}
rock-rose (> 80 % Cistus incana)	Baia Mare, RO	63.40 ± 0.33^{e}	7.53 ± 0.39^{g}	65.50 ± 0.37^{b}	65.93 ± 0.37^{b}
sunflower (> 80 % Helianthus annuus)	Kimle, HU	62.10 ± 0.29^{f}	13.03 ± 0.12^{c}	78.27 ± 0.33^{a}	79.34 ± 0.31^{a}
sweet cherry (> 80 % Prunus avium)	Kimle, HU	57.33 ± 0.76^{g}	-0.17 ± 0.08^{lm}	39.83 ± 0.25^{k}	39.83 ± 0.25^{k}
traveller's joy (> 80 % Clematis vitalba)	Esztergom HU	70.33 ± 0.53^{b}	1.17 ± 0.12^{k}	32.33 ± 0.05^{n}	32.35 ± 0.05^{m}
wild blackberry (> 80 % Rubus fruticosus)	Esztergom, HU	52.97 ± 0.52^{i}	-0.33 ± 0.05^{m}	19.97 ± 0.09^{o}	19.97 ± 0.09^{o}
polyfloral-1 (mostly Salix caprea)	Kimle, HU	64.93 ± 0.62^{d}	2.60 ± 0.24^{j}	57.17 ± 0.31^{e}	57.23 ± 0.30^{e}
polyfloral-2 (mostly Sophora japonica)	Kimle, HU	67.03 ± 0.37^{c}	2.73 ± 0.24^{j}	42.57 ± 0.39^{j}	42.65 ± 0.40^{j}
polyfloral-3 (mostly Cornus sanguinea)	Mája, HU	62.63 ± 0.25^{ef}	3.40 ± 0.08^{i}	46.00 ± 0.14^{i}	46.13 ± 0.14^{i}
polyfloral-4 (mostly Acer campestre)	Baia Mare, RO	66.73 ± 0.41^{c}	1.23 ± 0.17^{k}	40.63 ± 0.05^{k}	40.65 ± 0.05^{k}
polyfloral-5 (mostly Trifolium sp.)	Baia Mare, RO	55.70 ± 0.54^{h}	2.47 ± 0.05^{j}	51.70 ± 0.49^{h}	51.76 ± 0.49^{h}
polyfloral-6 (mostly Calluna vulgaris)	Baia Mare, RO	49.33 ± 0.75^{j}	8.30 ± 0.14^{f}	34.23 ± 0.62^{m}	35.23 ± 0.59^{l}
polyfloral-7 (mostly Rosa canina)	Baia Mare, RO	61.83 ± 0.17^{f}	4.07 ± 0.05^{h}	59.50 ± 0.29^{c}	59.64 ± 0.29^{c}
minimum		28.30	−0.57	5.90	11.93
maximum		72.50	18.53	78.27	79.97
mean		58.83	5.13	45.84	46.92
median		62.10	2.73	46.00	46.13

Means with different superscripts in the columns indicate significant difference between samples ($p < 0.05$).

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Table 4. Correlation matrix of the analyzed parameters. Significant correlations ($\alpha = 0.05$) are indicated with an asterisk.

	TPC	TFC	FRAP	CUPRAC	$ABTS^{\bullet+}$	$DPPH^{\bullet}$	RACI	L	a*	b*	C^*_{ab}
TPC	1										
TFC	0.422	1									
	p = 0.057										
FRAP	0.688*	0.534*	1								
	p = 0.001	p = 0.013									
CUPRAC	0.787*	0.307	0.0746*	1							
	p < 0.001	p = 0.177	p < 0.001								
$ABTS^{\bullet+}$	0.790*	0.364	0.318	0.650*	1						
	p < 0.001	p = 0.105	p = 0.160	p = 0.001							
$DPPH^{\bullet}$	0.762*	0.424	0.817*	0.959*	0.624*	1					
	p < 0.001	p = 0.056	p < 0.001	p < 0.001	p = 0.002						
RACI	0.865*	0.466*	0.824*	0.959*	0.741*	0.972*	1				
	p < 0.001	p = 0.033	p < 0.001	p < 0.001	p < 0.001	p < 0.001					
L	−0.104	0.070	0.187	0.382	−0.115	0.303	0.216	1			
	p = 0.655	p = 0.762	p = 0.417	p = 0.087	p = 0.621	p = 0.182	p = 0.346				
a*	−0.240	−0.138	−0.388	−0.644*	−0.338	−0.635*	−0.573*	−0.678*	1		
	p = 0.295	p = 0.550	p = 0.082	p = 0.002	p = 0.134	p = 0.002	p = 0.007	p = 0.001			
b*	−0.239	−0.094	−0.055	0.034	−0.288	−0.052	−0.103	0.630*	−0.003	1	
	p = 0.297	p = 0.685	p = 0.814	p = 0.884	p = 0.205	p = 0.822	p = 0.656	p = 0.002	p = 0.998		
C^*_{ab}	−0.247	−0.129	−0.085	−0.012	0.328	−0.105	−0.151	0.558*	0.097	0.993*	1
	p = 0.280	p = 0.578	p = 0.714	p = 0.958	p = 0.147	p = 0.651	p = 0.512	p = 0.009	p = 0.675	p < 0.001	

Conclusions

In our work, the total phenolic content (TPC), total flavonoid content (TFC), antioxidant capacity and color properties of mono- and polyfloral bee pollens were investigated. Pollens of cultivated plants, wildflowers, trees and shrubs typical for the Central and Eastern European flora were included among the samples. Different solvents were compared in terms of extraction efficiency, the results of which showed that ethanol: distilled water (60:40) is the most suitable solvent for the extraction of phenolic compounds from pollens. Our results confirmed that bee pollens are rich sources of phenolic compounds and have high antioxidant capacity. Samples were ranked based on their Relative Antioxidant Capacity Index (RACI). Rapeseed, traveller's joy and phacelia pollens appeared to have high antioxidant potential in contrast to pollens of plant species belonging to the Asteraceae family, including sunflower, musk thistle and dandelion. Our results indicate that botanical origin has a greater influence on the tested parameters of bee pollens than geographical origin. RACI was strongly correlated with other antioxidant parameters suggesting that this approach is applicable for the accurate determination of total antioxidant capacity of pollens. No correlation was found between antioxidant properties and lightness/yellowness/chroma values of our samples, but they showed a moderate negative correlation between redness and certain antioxidant capacity parameters. Nevertheless, further research is required to draw general conclusions.

Experimental Section

Bee pollen samples

Frozen bee pollen samples of unknown botanical origins were obtained from a north-western Hungarian (Kimle, Győr-Moson-Sopron; n = 8) and a northern Romanian (Baia Mare, Maramureş; n = 7) apiary. Dried samples (n = 6), treated at a temperature below 40 °C, were provided by professional Hungarian beekeepers from three settlements (Máza located in Southern Hungary, Esztergom located in Northern Hungary and Mezőkövesd located in Eastern Hungary). Frozen samples were stored at −20 ± 2 °C, then dried at 38 ± 2 °C for 20 h in a conventional oven. Followingly, pollen pellets belonging to different taxonomic groups were sorted by color, shape and size. A total of 21 sub-samples were created, the botanical origin of which were later identified. Samples were ground and stored at −20 ± 2 °C until analysis.

Palynological evaluation of pollen samples

For the identification of plant sources of the samples, microscopic pollen analysis was conducted as follows: 10 pollen pellets were suspended in 10 ml of distilled water, then dispersed by a test tube mixer. 30 µl of the suspension was transferred onto two slides. Slides were dried on a hot plate and covered with glycerine gelatine mixture or glycerine gelatine mixture stained with fuchsine. Identification of pollen grains was performed for both slides by examining an area of 20 × 20 mm. The determination was conducted by using a Delta Optical binocular light microscope (Delta Optical, Warsaw, Poland) at 400× magnification. 500 pollen grains were calculated per preparations. Pollen grains were classified as predominant (≥ 45 %), secondary (16–44 %), important minor (3–15 %), and minor (< 3 %) according to their prevalence.

Determination of dry matter content

Dry matter content of pollens was determined gravimetrically by drying 2.00 g of sample at 75 °C in a vacuum drying oven until constant weight. Calculations were conducted by the following equation (Eqn. 1), where m_1 is the weight of the pollen prior drying (g) and m_2 is the weight of pollen after drying (g):

$$Dry\ matter\ content\ (\%) = 100 - \frac{m_1 - m_2}{m_1} \times 100 \quad (1)$$

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Preparation of pollen extracts

First, a representative sample was created, which contained each of the twenty-one samples in equal proportions. This was used for the preliminary experiment, during which the optimal solvent for the extraction of phenolic compounds of pollen was identified. Aqueous solutions of organic solvents (methanol, ethanol and acetone) at 20, 40, 60, 80 and 100% concentrations were used for this purpose. Besides, an extract was prepared by using 100% distilled water as for comparison. The solvent that resulted in the highest total phenolic content was used to prepare the pollen samples of different sources. The extraction procedure was carried out as follows both for the preliminary study and for testing the samples: 0.20 g of ground bee pollen was weighed and mixed with 10 ml of solvent, then homogenized by vigorous shaking. Followingly, they were treated in an ultrasonic bath for 1 h, and centrifuged at 12000 rpm for 10 min. 1.5 ml of supernatants were transferred to Eppendorf tubes and stored at $-20\pm2\,^{\circ}C$ until use.

Determination of total phenolic content (TPC)

For the determination of the total phenolic content, the method developed by Singleton and Rossi[62] was used with modifications. 1250 µl of distilled water:Folin-Ciocalteu reagent (90:10) solution was pipetted in test tubes, then 210 µl of methanol:distilled water (80:20) was added. Followingly, 40 µl of sample extract was added to the mixture. After one min, 1000 µl of Na_2CO_3 solution (0.7 M) was also added. Test tubes were vortexed and placed at a 50 °C water bath in order to accelerate the reaction. After 5 min, the absorbances of the solutions were measured at 760 nm against a blank solution. Gallic acid standard solutions were used for calibration. The results were expressed in mg GAE (gallic acid equivalent)/g dry weight.

Determination of total flavonoid content (TFC)

The total flavonoid content of pollens was determined by the method proposed by Woisky & Salatino.[63] Reagent was prepared by mixing 3.75 ml of $AlCl_3$ solution (180.9 mg/ml), 3.75 ml of CH_3COONa solution (13.68 mg/ml), 105 ml of distilled water and 56 ml of ethanol. Followingly, 1125 µl of the reagent was pipetted in sealable test tubes, then 125 µl of sample extract was added. Prepared samples were incubated at room temperature for 30 min, and their absorbances were measured at 415 nm against a blank. Quercetin standard solutions were used for calibration. The results were expressed in mg QE (quercetin equivalent)/g dry weight.

Ferric reducing antioxidant power (FRAP) assay

The method developed by Benzie and Strain was used for the FRAP determination.[64] Firstly, the FRAP reagent was prepared, which contained the following solutions in a 10:1:1 ratio: sodium acetate buffer (300 mM/l, pH = 3.6), iron(III)chloride (20 mM/l) and 2,4,6-tri-2-pyridinyl-1,3,5-triazine (TPTZ) (10 mM/l). Followingly, 1500 µl of FRAP reagent was pipetted in test tubes, then 50 µl of sample extract was added. Test tubes were shaken and after 5 min, absorbances of the solutions were measured at 593 nm against a blank solution. Ascorbic acid standard solutions were used for calibration. The results were expressed in mg AAS (ascorbic acid equivalent)/g dry weight.

Cupric ion reducing antioxidant capacity (CUPRAC) assay

The CUPRAC assay was performed according to the method described by Apak and co-workers.[65] Briefly, 50 µl of the sample was mixed with 1 ml of copper chloride solution (10 mM), 1 ml of neocuproine alcoholic solution (7.5 mM in absolute ethanol), 1 ml of ammonium acetate buffer solution (1 M, pH 7.0) and 1 ml of distilled water. After incubation for 30 min, the absorbance was measured at 450 nm against the reagent blank. Trolox standard solutions were used for calibration. The results were expressed in mg TE (trolox equivalent)/g dry weight.

2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) assay

The method was described by Miller and co-workers.[66] As a first step, the peroxyl radical was prepared, for which 39.2 µl of potassium persulfate (125 mM) and 1960.8 µl of $ABTS^{\bullet+}$ solution (7 mM) were mixed and homogenized. The mixture was then stored in dark at room temperature for one day. The radical was diluted 80-fold with phosphate buffer (pH = 7.4), then its absorbance was adjusted to 0.700 ± 0.002 at 734 nm. Followingly, 1950 µl of $ABTS^{\bullet+}$ was pipetted into test tubes, followed by 10 µl of sample extracts. Solutions were shaken for five min, and absorbances were measured at 734 nm against the phosphate buffer. Trolox standard solutions were used for calibration. The results were expressed in mg TE (trolox equivalent)/g dry weight.

2,2-Diphenyl-1-picryhydrazyl ($DPPH^{\bullet}$) assay

A method for measuring the antioxidant capacity based on stable DPPH radical scavenging was developed by Blois.[67] To prepare the reagent, 9 mg of 2,2-diphenyl-1-picryhydrazyl ($DPPH^{\bullet}$) was dissolved in 100 ml of methanol in a dark glass bottle. Solutions were prepared by mixing 1000 µl of DPPH reagent, 1920 µl of distilled water and 80 µl of sample extract into sealable test tubes. The solutions were stored in dark for 30 min, then absorbances of the mixtures were measured at 517 nm against distilled water. Trolox standard solutions were used for calibration. The results were expressed in mg TE (trolox equivalent)/g dry weight.

Determination of Relative Antioxidant Capacity Index (RACI)

Relative Antioxidant Capacity Index (RACI) was calculated from the results of FRAP, CUPRAC, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ assays according to the method proposed by Sun and Tanumihardjo.[31] Firstly, standard scores were calculated as the following equation (Eqn. 2):

$$(x-\mu)/\sigma \tag{2}$$

where x is the raw data, μ is the mean, and σ is the standard deviation.

The standard scores obtained by data transformation will have similar normal distributions with a mean of 0 and variance of 1. Secondly, standard scores of the four assays were averaged for each sample, then samples were ranked by mean values and plotted on a bar graph.

Measurement of color parameters

Color parameters of ground pollen samples were measured using a Konica Minolta chroma meter CR-410 device (Konica Minolta, Inc., Tokyo, Japan). Before the measurement, the instrument was standardised against a reference white standard ($L = 87.2$; $a^* = 3.11$; $b^* = 3.17$). The measuring apparatus was lowered to the bottom of the sample holder, then the surface of the samples was illuminated by a xenon lamp. Results were expressed as CIELAB color coordinates,

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where L* indicates the lightness from black (0) to white (100), while a* describes the red-green color (a* > 0 indicates redness, a* < 0 indicates greenness), and b* describes the yellow-blue color (b* > 0 indicates yellowness, b* < 0 indicates blueness).[68] Chroma (C*) values were determined using the following equation (Eqn. 3):

$$C^* = (a^{*2} + b^{*2})^{0.5} \quad (3)$$

Statistical analysis

Each of the analyses were carried out in four parallel measurements, and data were expressed as mean ± standard deviation. For the determination of statistical differences, one-way ANOVA was used with Tukey post-hoc test ($\alpha = 0.05$). The linear relationship between variables was assessed by Pearson's correlation ($\alpha = 0.05$). Statistical analyses were performed using IBM SPSS Statistics version 27.0 software.

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Author Contributions

R. V. and M. C. conceptualized the project and designed the experiments. R. V., G. S. and M. C. conducted the laboratory experiments. R. V. and G. S. analyzed the data and performed statistical evaluation. R. V. wrote the manuscript and created the graphical abstract. M. C. reviewed the manuscript. All authors have read and approved the finalized manuscript.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: apicultural product · phenolics · flavonoids · relative antioxidant capacity index · CIELAB color space

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