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Bee Pollen in Transylvania (Romania): Palynological Characterization and $ORAC_{FL}$ Values of Lipophilic and Hydrophilic Extracts of Monofloral Pollen Pellets

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Abstract: A melissopalynological study carried out on 35 bee pollen samples from Romania (Transylvania) shows predominant species as *Taraxacum officinale*, *Tilia* sp., *Verbascum* sp., *Zea mays* L., *Onobrychis viciifolia* Scop., *Geranium sanguineum* L., *Filipendula ulmaria* L., *Cydonia oblonga* L., *Calluna vulgaris* L. and *Brassica* sp.. This is the first time for the contribution of total phenolic (TP) and total carotenoid (TC) content to the modified oxygen radical absorbance capacity ($ORAC_{FL}$) values in 29 monofloral samples. Hydrophilic $ORAC_{FL}$ (H-$ORAC_{FL}$) values ranged between 7.13-10.12 μmol TE/g and 6.94-22.46 μmol TE/g, whereas lipophilic $ORAC_{FL}$ (L-$ORAC_{FL}$) values were between 2.19-7.79 μmol TE/g and 1.65-9.96 μmol TE/g, in multifloral and monofloral samples, respectively. It has proved the complex involvement of botanical origins on the antioxidant features with a specific occurrence. Some monofloral samples presented particular high antioxidant potential, such as *Salix* sp., *Taraxacum officinale*, *Matricaria chamomilla*, *Cichorium intybus* and the poorly studied *Cirsium arvense* (L.) Scop. and *Scilla bifolia* L.. New variables, such as soil characteristics, climates together with botanical origins, were introduced in a multivariate analysis of antioxidant data matrix, given a possible important involvement of all of them in affecting, not only the phytochemical composition, but thereafter the antioxidant capacity. All these data could be crucial to a new way of gathering, by beekeepers, depending on the market demand and the purposes for the product that has a potential for further therapeutic bioactivities investigations, and added value in the enrichment of certain products.

Key words: Pollen, ORAC, antioxidant, flavonoids, carotenoids.

1. Introduction

Bee pollen is a valuable apiary product widely used by humans as a natural food supplement and for certain floral sources. It is also explored as herbal medicine in strengthening the body's resistance against diseases [1]. It is nutritionally well balanced, given its low fat content, relatively high dietary fiber and protein contents, and includes important amounts of essential free amino acids and minerals [1]. Other phytochemicals, such as phenolic compounds (e.g., flavonoids, anthocyanins and phenolic acids) and carotenoids, have a wide range of important and well-documented biological activities, which are attributed to their antioxidant potential [2, 3]. As pollen includes many kinds of polyphenols, carotenoids and vitamins, it has been suggested that it may be a useful supplement to prevent oxidation [4, 5].

Reports of the human health benefits of bee pollen have encouraged researchers to examine factors that influence the content of phytochemical antioxidants in bee pollen. Furthermore, the antioxidant capabilities of phenolic containing extracts made from bee pollen have been extensively studied, in regard to their free radical scavenger inhibition capacity, ferric-reducing

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capacity and lipid peroxidation inhibition capacity [6, 7].

Oxygen radical absorbance capacity (ORAC) assay, both in the original form [8] and in the modified one $ORAC_{FL}$ [9], was developed with the aim to solve some practical problems in regard to the antioxidant capacity assessment. Firstly, the method is suitable for lipophilic measurements, since most popular *in vitro* antioxidant assays were designed primarily for hydrophilic components [10]. Second, it measures the chain-breaking antioxidant capacity of both lipophilic and hydrophilic components for a given sample separately, allowing better assessment of total antioxidant capacity (TAC) of a given sample [11]. Thirdly, it uses specific biologically relevant free radical sources, usually the same peroxyl radical, which plays a key role in lipid oxidation in food and biological systems and which is generated by using different radical sources, in order to obtain the relative response of different dietary antioxidants [12]. Given this, ORAC method is more significant from this point of view among the majorities of *in vitro* antioxidant assays [10]. Fourthly, it is the only method that combines both time and degree of inhibition into one value using area under the curve (AUC) [11]. This approach unifies the lag time method and initial rate method, which applies equally well for both antioxidants that exhibit distinct lag phases and those samples that have no lag phases, being particularly useful for food samples, which often contain multiple ingredients and have complex reaction kinetics [12]. ORAC assay has been criticized for the need of expensive equipment (e.g., fluorometer) and the long analysis time. Nevertheless, ORAC assay is to date, the only method that responds to the main need for TAC, which is critical for possible health benefits evaluation of a specific food or dietary supplement [11]. In fact, an antioxidant database of common foods has been generated by applying the ORAC assay in combination with the total phenols assay [10, 11].

The natural variability supports current results for both the antioxidant composition, with phenolic compounds as the most studied, and antioxidant capabilities of monofloral and multifloral samples [7], mainly linked to the floral sources of the pollen loads. However, this type of information is very limited for bee pollen, especially for samples with selected floral origin and their $ORAC_{FL}$ values.

The purpose of this study was to provide the palynological spectrum of bee pollen harvested from Transylvania region, Romania. In addition, it is the first time for the authors to show data on the lipophilic, hydrophilic and total antioxidant capacities evaluated by $ORAC_{FL}$ for 29 monofloral pellets coming from 35 multifloral bee pollen samples. All these together with the contribution of the phenolic and carotenoid pigments to the total antioxidant capacities will complete the information.

2. Materials and Methods

2.1 Chemicals

2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), Folin Ciocalteu's phenol reagent, gallic acid and rutin were purchased from Sigma Aldrich (Germany); fluorescein disodium salt (FL) was from Fluka (Switzerland); randomly methylated-β cyclodextrin (RMCD) was from Cyclolab (Hungary).

2.2 Bee Pollen Samples and Botanical Origin Identification

Bee pollen samples were collected by beekeepers from March to September 2013, in pollen traps installed in *Apis mellifera* L. hives located in the Northwest and Central counties of Transylvania area, Romania. To maintain bee pollen quality, samples were removed every 2-3 d, hand cleaned and stored in a freezer (-18 °C) until they were sent to the laboratory. They were purchased at regular intervals (weekly) to ensure a supply of fresh samples throughout the period of the study.

The palynological content of multifloral bee pollen

samples was determined by light microscope examination, performed under normal lighting at 40× magnification (microscope Nikon Eclipse 50i). The floral origin of monofloral pellets was determined by color and microscopic examination. Melissopalynological preparation was based on the European standard [13], without acetolysis application adapted for pollen loads [14]. For the identification of pollen types, both reference pollen slides prepared by the authors, were used along with pollen atlas [15, 16]. The pollen reference slides were prepared from anthers of flowers and the plant taxon was identified upon the botanic atlas [17].

The taxonomic identification was done at the lowest level possible, plant family, genus or species, depending on the difficulty level encountered. In the case of multifloral samples, after identifying the pollen types present on the slides, at least 1,200 pollen grains were counted in 100 microscopy fields to establish the occurrence percentages of each pollen type.

2.3 Samples Extraction

Bee pollen samples (0.5 g), previously grounded, were extracted in two stages to obtain lipophilic and hydrophilic constituents. Hexane:dichloromethane (HD 1:1) was initially used for the lipophilic constituents, followed by acetone:water:acetic acid (AWA 70:29.5:0.5) for the hydrophilic constituents, which were found to be appropriate to separate lipophilic and hydrophilic components [10, 11].

Firstly, samples were mixed with HD solvent (2.5 mL) for 1 h. After centrifugation (4,000 rpm, 4 °C, 20 min), the supernatants from two repeated extractions were carefully collected and combined, evaporated at room temperature (23 °C) under vacuum to dryness and then re-dissolved in acetone (5 mL). The HD extract was used to measure lipophilic $ORAC_{FL}$ (L-$ORAC_{FL}$) and total carotenoids (TC). For the lipophilic antioxidant assay, the HD extract was re-dissolved with 7% RMCD solution (50% acetone/50%water, v/v).

The solid residue was subjected to a second extraction with 2.5 mL AWA. The AWA extracts were diluted with AWA to total volume 5 mL. This solution was used to measure the hydrophilic $ORAC_{FL}$ (H-$ORAC_{FL}$) and total phenolic (TP) content.

2.4 TP Content Analysis

Soluble TP in AWA solvent were determined with Folin-Ciocalteu reagent by the method of Singleton et al. [18], adapted for the 96 well microplate reader (Synergy™ HT BioTek Instruments, USA) [19]. The extracts were oxidized with Folin-Ciocalteu reagent, and the reaction was neutralized with sodium carbonate. Briefly, 125 µL Folin-Ciocalteu reagent (0.2 N) was added to 25 µL of bee pollen extracts and mixed for 5 min. After the addition of 100 µL sodium carbonate (Na_2CO_3) solution (75 g/L), the extracts were incubated for 2 h. The absorbance of the resulting blue color was measured at 760 nm. The results were expressed as milligrams of gallic acid equivalents per gram fresh matter sample (mg GAE/g FW) based on a standard curve prepared with gallic acid concentrations of 0.005-0.1 mg/mL.

2.5 TC Content Analysis

Soluble TC in HD solvent were determined by visible absorption spectrophotometry at an absorbance of 450 nm [20], using a standard curve of β-carotene, prepared for the concentrations range of 0.001-0.02 mg/mL. The wavelength was chosen based on the spectrum registration maxima of the β-carotene in HD solvent (data not shown). TC content was expressed based on β-carotene equivalents per gram fresh matter sample (mg βCE/g FW). Additional dilution was done, if the absorbance value measured was over the linear range of the standard curve.

2.6 Determination of Antioxidant Capacity by ORAC Assay

The ORAC assay measures the antioxidant

scavenging function against peroxyl radical generated *in situ* at constant rate by the thermal decomposition of AAPH at 37 °C. Fluorescein was used as a fluorescent compound that is prone to be oxidized by the reactive species into a non-fluorescent product. The extent of the protection against the loss of FL fluorescence emission, which reflects the decreased rate of the oxidative damage induced by the peroxyl radicals ($ROO^{\cdot}$), is used as a measure of the antioxidant capacity of the sample added in the reaction mixture [8]. The $ORAC_{FL}$ assay was conducted on the basis of a report by Ou et al. [9], adapted to manual handling and to the use of a fluorescent plate reader used with fluorescence filters for an excitation wavelength of 485 ± 20 nm and an emission wavelength of 520 ± 25 nm. The plate reader was controlled by Gen5^{TM} data analysis software.

Fluorescein stock solution #1 was prepared by dissolving 0.0225 g FL in phosphate buffer (75 mM, pH = 7.4) and stored at -20 °C. Aliquot (2 mL) of FL stock solution #2 (5.98 µM) was stored at -20 °C until use. FL working solution (95.68 nM) was prepared daily from stock solution #2. Before usage, the FL working solution was incubated in the water bath at 37 °C. The AAPH solution reagent (79.6 µM) was prepared in pre-incubated phosphate buffer (25 °C) immediately before the start of the assay. AAPH was kept in the refrigerator at 4 °C before adding the warm buffer. AAPH, FL working solution and Trolox calibration solutions were prepared daily. Phosphate buffer (75 mM, pH = 7.4) and 7% RMCD solution were used as a blank and solvent for the Trolox standards, in the hydrophilic and lipophilic assay, respectively. The AAPH reaction is temperature-dependent, since AAPH undergoes spontaneous decomposition and produces peroxyl radicals with a rate primarily determined by temperature [11]. Timing of the addition of the AAPH is critical and the time to add all columns should be kept to a convenient minimum. In order to avoid possible positional errors, a "forward-then-reverse" was used.

For the reaction, 200 µL FL working solution and 20 µL of antioxidant solution were incubated at 37 °C (20 min) in the plate reader. Then, 20 µL of AAPH was added rapidly to start the reaction. The fluorescence was recorded every 1 min for 35 min, and the microplate was automatically shaken prior each reading. A blank instead of the antioxidant and calibration solutions of Trolox (0, 6.25, 12.5, 25, 50 and 100 µM) as antioxidant was also carried out in each assay.

The relative area under curve (AUC) of the standards and samples was calculated as Eq. (1):

$$AUC = (f_1/f_1 + f_2/f_1 + f_3/f_1 + f_4/f_1 + \ldots + f_{35}/f_1) \quad (1)$$

where, f_i = fluorescence reading at cycle 1 and f_{35} = fluorescence reading at cycle 35.

The net AUC corresponding to the sample was calculated by subtracting the AUC corresponding to the blank, as Eq. (2):

$$\text{Net AUC} = AUC_{antioxidant} - AUC_{blank} \quad (2)$$

The standard curve was obtained by plotting Trolox concentrations against the net AUC derived from at least three measurements for each concentration in each plate. The final $ORAC_{FL}$ values were calculated by using a quadratic regression equation ($y = a + bx + cx^2$) between Trolox concentration (µM) and the net area under the FL decay curve (net AUC) [10]. Data are expressed as micromoles of Trolox equivalents per of fresh weight of samples (µmol TE/g FW). Data were analyzed by Microsoft Excel (Microsoft, Roselle, IL). A quality control standard of Gallic acid was included in each run.

2.7 Statistical Analysis

All analytical determinations were repeated with three independent samples, and obtained data are expressed as mean values ± standard deviation and are expressed on "as is" fresh weight basis (FW). Statistical differences between samples were assessed by one-way analysis of variance (ANOVA). Significance of difference was calculated by least

significant differences test (LSD) and results with $P \leq 0.01$ were considered statistically significant. The relationship between the antioxidant capacities and pigment content of the samples was analyzed by Pearson correlation coefficients. The principal component analysis (PCA) was subsequently applied on the antioxidant descriptors. All data matrix were analyzed by using the StatSoft Statistica 10 software.

3. Results

3.1 Botanical Origin Identification of the Bee Pollen Samples

The floral composition of the 35 multifloral bee pollen samples is presented in Table 1. Trapped pollen samples usually contained between five (in nine samples) to six (in 12 samples) pollen types, with some cases (13 samples) were one being dominant (> 45%), in accordance with the pollen classes established for honey sediment analyses [13]. In these samples, the predominant species were Tar (BP5, BP26, BP30 and BP31), Ti (BP11), Ver (BP29), Ze (BP27), On (BP10), Ge (BP16), Fi (BP6), Cy (BP32), Cal (BP7) and Br (BP13). In other multifloral pollen samples, one or two species occurred with frequencies between 16% and 45%, as secondary pollen types (Table 1).

In spite of the high diversity of plants, 67 taxa identified in samples (Table 2), pollen from fewer than 10 plant species has been detected in each multifloral sample. The most heterogeneous ones were samples BP2 and BP16 with the highest pollen types (nine and eight species, respectively), reflecting the flower diversity specific to the harvest period of June and July. In contrast, some samples contained only two (BP11, BP27) or three (BP31) pollen types. These results in this paper are in agreement with previous reports on the high pollen foraging selectivity of the bees, which showed that multifloral bee pollen samples comprise flower pollens from few of the plant species potentially available in the surroundings [21, 22].

In relation to the distribution of pollen taxa identified in the multifloral samples, it can be observed that nearly 49% of these occurred only once. In some cases, they contribute very little (< 3%) to the total pollen spectrum: Cor (1.1%, BP16), Ir (1.4%, BP34), Ae (1.5%, BP24), Sa (1.6%, BP14), Ai (2.6%, BP24), Care (2.7%, BP14) and Ta (2.9%, BP26). Generally, they occurred as important minor pollens, such as Cart (3.3%, BP6), As (3.4%, BP28), Col (3.5%, BP16), Ab (3.9%, BP16), Pl (5.2%, BP29), In (5.7%, BP34), Si (5.8%, BP2), Pl (6.8%, BP12), Am (7.6%, BP9), Ni (7.9%, BP26), Eu (8.9%, BP9), So (12.2%, BP23), Tu (12.8%, BP26) and Con (13.2%, BP9). Others represented secondary pollens: Po (22.9%, BP11), Carp (24.0%, BP24), Sc (27.5%, BP10), Py (31.7%, BP17), Ros (33.2%, BP9), Ch (42.8%, BP26), Car (43.5%, BP18) and Rob (44.8%, BP25). Only two of them were predominant pollens, namely, On (45.2%, BP10) and Ze (81.7%, BP27). Some species seems to be abundant sources for the honeybees, such as *Taraxacum officinale* Web. (Tar.), which occurred in 26 samples. Among these, *Salix* and *Prunus* species were the most frequented by the honeybees, being identified in nine samples. These results also indicate the importance of pollen from Ac, Br, Cra, Fi along with Ep, Pa and Ra for honeybees as pollen sources, occurring in six and five, respectively, multifloral samples.

After identifying the pollen types present in the multifloral samples, 29 pollen loads with homogenous color were selected resulting monochromatic pollen samples. A pellet's color and morphology depend on the floral species of origin [13]. The microscopic examination was the principal tool for the selection of the bee pollen pellets coming from one species. The color of pollen loads provides only a preliminary indication of species composition, particularly for unknown or closely related species [22]. Therefore, for this study, only the bee pollen loads with monofloral homogenous microscopic composition (100%) were selected, since some of the monochromatic loads showed two and sometimes three different floral grains in their composition.

Table 1 Palynological content of multifloral bee pollen (BP) samples.

Sample	Floral composition (botanical name, %)
BP1	*Oxalis* sp. (42.1%), *Geranium* sp. (27.6%), *Taraxacum officinale* Web. (21.7%), *Crataegus monogyna* Jacq. (5.1%), *Verbascum phlomoides* L. (2.8%), *Anthyllis* sp. (0.7%)
BP2	*Filipendula ulmaria* L. (39.8%), *Hypericum perforatum* L. (25.6%), *Linaria* sp. (10.1%), *Epilobium angustifolium* L. (9.3%), *Silene dioica* (5.8%), *Taraxacum officinale* Web. (3.1%), *Rubus* sp. (2.7%), *Cirsium arvense* (L.) Scop. (1.9%), *Arctium minus* (Hill) Bernh. (1.7%)
BP3	*Filipendula ulmaria* L. (42.8%), *Epilobium angustifolium* L. (27.1%), *Hypericum perforatum* L. (23.7%), *Linaria* sp. (4.3%), *Arctium minus* (Hill) Bernh. (2.1%)
BP4	*Calluna vulgaris* L. (28.4%), *Matricaria chamomilla* L. (17.5%), *Epiloboium angustifolium* L. (18.3%), *Filipendula ulmaria* L. (16.7%), *Helianthus annuus* L. (15.6%), *Cichorium intybus* L. (3.2%)
BP5	*Taraxacum officinale* Web. (54.2%), *Linaria* sp. (27.9%), *Trifolium repens* L. (12.2%), *Arctium minus* (Hill) Bernh. (5.7%)
BP6	*Filipendula ulmaria* L. (47.5%), *Calluna vulgaris* L. (39.3%), *Taraxacum officinale* Web. (4.2%), *Carthamus lanatus* L. (3.3%), *Lycopodium cernuum* spores (5.7%)
BP7	*Calluna vulgaris* L. (87.4%), *Taraxacum officinale* Web. (8.7%), *Epilobium angustifolium* L. (3.9%)
BP8	*Helianthemum nummularium* L. (34.7%), *Arctium minus* (Hill) Bernh. (24.8%), *Taraxacum officinale* Web. (21.2%), *Matricaria chamomilla* L. (12.1%), *Carduus* sp. (5.2%), *Filipendula ulmaria* L. (1.5%)
BP9	*Rosa canina* L. (33.2%), *Taraxacum officinale* Web. (25.6%), *Conium maculatum* L. (13.2%), *Trifolium incarnatum* L. (11.2%), *Euphorbia cyparissias* L. (8.9%), *Amorpha fruticosa* L. (7.6%)
BP10	*Onobrychis viciifolia* Scop. (45.2%), *Scabiosa atropurpurea* L. (27.5%), *Lycopodium cernuum* spores (11.4%), *Prunus* sp. (8.9%), *Taraxacum officinale* Web. (5.3 %), *Filipendula ulmaria* L. (1.7%)
BP11	*Tilia* sp. (77.1%), *Potentilla reptans* L. (22.9%)
BP12	*Taraxacum officinale* Web. (30.2%), *Crataegus monogyna* Jacq. (28.3%), *Salix* sp. (26.1%), *Platanus* sp. (6.8%), *Prunus* sp. (5.9%), *Brassica* sp. (2.7%)
BP13	*Brassica* sp. (63.2%), *Taraxacum officinale* Web. (19.7%), *Salix* sp. (17.1%)
BP14	*Salix* sp. (37.1%), *Prunus* sp. (27.3%), *Taraxacum officinale* Web. (23.8%), *Malus domestica* Borkh. (7.5%), *Carex distans* L. (2.7%), *Sambucus ebulus* L. (1.6%)
BP15	*Quercus* sp. (39.7%), *Ranunculus* sp. (25.1%), *Crataegus monogyna* Jacq. (18.2%), *Salix* sp. (14.5%), *Papaver rhoeas* L. (2.5%)
BP16	*Geranium* sp. (51.2%), *Acer* sp. (34.7%), *Abies* sp. (3.9%), *Colutea arborescens* L. (3.5%), *Ranunculus* sp. (2.7%), *Taraxacum officinale* Web. (2.1%), *Cornus sanguinea* L. (1.1%), *Brassica* sp. (0.8%)
BP17	*Prunus* sp. (39.5%), *Pyrus* sp. (31.7%), *Salix* sp. (20.4%), *Taraxacum officinale* Web. (6.7%), *Acer* sp. (1.7%)
BP18	*Cardamine* sp. (43.5%), *Taraxacum officinale* Web. (29.2%), *Scilla bifolia* L. (12.4%), *Papaver rhoeas* L. (12.3%), *Ailanthus* sp. (2.6%)
BP19	*Prunus spinosa* L. (43.9%), *Taraxacum officinale* Web. (27.4%), *Prunus* sp. (15.9%), *Salix* sp. (12.8%)
BP20	*Salix* sp. (35.3%), *Taraxacum officinale* Web. (27.8%), *Antthylis* sp. (20.7%), *Crataegus monogyna* Jacq. (16.2%)
BP21	*Brassica* sp. (37.8%), *Prunus* sp. (29.5%), *Taraxacum officinale* Web. (27.3%), *Quercus* sp. (5.4%)
BP22	*Brassica* sp. (33.2%), *Acer* sp. (26.8%), *Ranunculus* sp. (21.6%), *Sambucus ebulus* L. (7.3%), *Pinus* sp. (4.7%), *Salix* sp. (3.7%), *Crataegus monogyna* Jacq. (2.7%)
BP23	*Matricaria chamomilla* L. (44.7%), *Taraxacum officinale* Web. (37.5%), *Solidago virgaurea* L. (12.2%), *Cichorium intybus* L. (3.7%), *Rubus* sp. (1.2%), *Trifolium* sp. (0.7%)
BP24	*Brassica* sp. (33.5%), *Carpinus* sp. (24.0%), *Trifolium* sp. (19.9%), *Taraxacum officinale* Web. (15.8%), *Papaver rhoeas* L. (5.3%), *Aesculus hippocastanum* L. (1.5%)
BP25	*Robinia* sp. (44.8%), *Acer* sp. (32.7%), *Ranunculus* sp. (11.7%), *Trifolium* sp. (9.1%), *Pinus* sp. (1.7%)
BP26	*Chrysanthemum* sp. (42.8%), *Papaver rhoeas* L. (21.7%), *Tusilago* sp. (12.8%), *Linaria* sp. (11.9%), *Nigella* sp. (7.9%), *Tamarix* sp. (2.9%)
BP27	*Zea mays* L. (81.7%), *Helianthus annuus* L. (18.3%)
BP28	*Taraxacum officinale* Web. (53.5%), *Cirsium arvense* (L.) Scop. (21.2%), *Carduus* sp. (11.3%), *Papaver rhoeas* L. (7.7%), *Aster* sp. (3.4%), *Trifolium* sp. (2.9%)
BP29	*Verbascum phlomoides* L. (48.9%), *Taraxacum officinale* Web. (43.2%), *Plantago lanceolata* L. (5.2%), *Acer* sp. (2.7%)
BP30	*Taraxacum officinale* Web. (61.5%), *Cydonia oblonga* L. (18.7%), *Trifolium pratense* L. (12.5%), *Verbascum phlomoides* L. (5.2%), *Trifolium repens* L. (2.1%)
BP31	*Taraxacum officinale* Web. (35.6%), *Cichorium intybus* L. (41.1%), *Brassica* sp. (23.3%)
BP32	*Cydonia oblonga* L. (45.7%), *Ranunculus* sp. (29.3%), *Taraxacum officinale* Web. (19.1%), *Crataegus monogyna* Jacq. (5.9%)
BP33	*Malus domestica* Borkh. (50.4%), *Taraxacum officinale* Web. (18.6%), *Prunus* sp. (15.1%), *Hypericum perforatum* L. (11.9%), *Acer* sp. (4.0%)
BP34	*Arctium minus* (Hill) Bernh. (38.3%), *Epilobium angustifolium* L. (35.6%), *Filipendula ulmaria* L. (17.8%), *Inula helenium* L. (5.7%), *Helianthemum nummularium* L. (1.2%), *Iris pseudoacorus* (1.4%)
BP35	*Taraxacum officinale* Web. (36.3%), *Salix* sp. (33.5%), *Oxalis* sp. (12.7%), *Prunus* sp. (12.5%), *Scilla bifolia* L. (3.3%), *Pinus* sp. (1.7%)

Table 2 Floral taxa identified in the multifloral bee pollen samples.

Botanical name	Botanical family	Codification	Botanical name	Botanical family	Codification
Acer sp.	Aceraceae	Ac	*Matricaria chamomilla* L.	Compositae	Mat
Abies sp.	Pinaceae	Ab	*Nigella* sp.	Ranunculaceae	Ni
Aesculus hippocastanum L.	Hippocastanaceae	Ae	*Onobrychis viciifolia* Scop.	Leguminosae	On
Ailanthus sp.	Simaroubaceae	Ai	*Oxalis* sp.	Oxalidaceae	Ox
Amorpha fruticosa L.	Fabaceae	Am	*Papaver rhoeas* L.	Papaveraceae	Pa
Anthyllis sp.	Leguminosae	An	*Pinus* sp.	Pinaceae	Pi
Arctium minus (Hill) Bernh.	Compositae	Ar	*Plantago lanceolata* L.	Plantaginaceae	Pl
Aster sp.	Asteraceae	As	*Platanus* sp.	Platanaceae	Pl
Brassica sp.	Cruciferae	Br	*Potentilla reptans* L.	Rosaceae	Po
Calluna vulgaris (L.) Huill.	Ericaceae	Cal	*Prunus* sp.	Rosaceae	Pr
Cardamine sp.	Brassicaceae	Car	*Prunus spinosa* L.	Rosaceae	Prs
Carduus sp.	Asteraceae	Card	*Pyrus* sp.	Rosaceae	Py
Carex distans L.	Cyperaceae	Care	*Quercus* sp.	Fagaceae	Q
Carpinus sp.	Betulaceae	Carp	*Ranunculus* sp.	Ranunculaceae	Ra
Carthamus lanatus L.	Compositae	Cart	*Robinia* sp.	Leguminosae	Rob
Chrysanthenum sp.	Asteraceae	Ch	*Rosa canina* L.	Rosaceae	Ros
Cichorium intybus L.	Compositae	Cic	*Rubus* sp.	Rosaceae	Ru
Cirsium arvense (L.) Scop.	Compositae	Cir	*Salix* sp.	Salicaceae	Sa
Colutea arborescens L.	Luguminosae	Col	*Sambucus ebulus* L.	Caprifoliaceae	Sam
Conium maculatum L.	Umbeliferae	Con	*Scabiosa atropurpurea* L.	Dipsacaceae	Sc
Cornus sanguinea L.	Cornaceae	Cor	*Scilla bifolia* L.	Hyacinthaceae	Sci
Crataegus monogyna Jacq.	Rosaceae	Cra	*Silene* sp.	Caryophyllacea	Si
Cydonia oblonga L.	Rosaceae	Cy	*Solidago virgaurea* L.	Asteraceae	So
Epiloboium augustifolium L.	Onagraceae	Ep	*Tamarix* sp.	Tamaricaceae	Ta
Euphorbia cyparissias L.	Euphorbiaceae	Eu	*Taraxacum officinale* Web.	Compositae	Tar
Filipendula ulmaria L.	Rosaceae	Fi	*Tilia* sp.	Tiliaceae	Ti
Geranium sanguineum L.	Geraniaceae	Ge	*Trifolium incarnatum* L.	Leguminosae	Tri
Helianthemum nummularium L.	Cistaceae	He	*Trifolium repens* L.	Leguminosae	Trr
Helianthus annuus L.	Compositae	Hel	*Trifolium pratense* L.	Leguminosae	Trp
Hypericum perforatum L.	Papaveraceae	Hy	*Trifolium* sp.	Leguminosae	Tr
Inula helenium L.	Asteraceae	In	*Tussilago* sp.	Asteraceae	Tu
Iris pseudacorus	Iridaceae	Ir	*Verbascum* sp.	Scrophulariaceae	Ve
Linaria sp.	Scrophulariacea	Li	*Zea mays* L.	Poaceae	Ze
Malus domestica Borkh.	Rosaceae	Mal			

3.2 TP Content

TP in AWA extracts of multifloral samples ranged between 1.26 GAE/g (BP27) and 8.43 mg GAE/g (BP21), with average values of 4.21 mg GAE/g (Table 3).

These results are in the range of previous values [10], obtained for fruits and vegetables, when where analyzed 100 different kinds of foods, including also nuts, dried fruits, spices and cereals, by using the same extraction procedure for lipophilic and hydrophilic fractions. For fruits, they reported TP values in the range of 0.47 mg GAE/g FW (peaches) and 7.09 mg GAE/g FW (cranberry), with low values in honeydew honey (0.72 mg GAE/g FW). In addition, the vegetables contained between 0.27 mg GAE/g FW and 12.47 mg GAE/g FW, in cucumber and beans samples, respectively, with the highest values obtained for the nuts (20.16 mg GAE/g FW).

The data in this paper are in part agreed with other previous reports, in which multifloral bee pollen was analyzed using different solvents for extraction. Similar TP values were reported by Carpes et al. [6] for the

Table 3 $L\text{-}ORAC_{FL}$, $H\text{-}ORAC_{FL}$, total antioxidant capacity (TAC), TP and TC of multifloral bee pollen[a].

Samples	Antioxidant capacity			TP	TC
	$L\text{-}ORAC_{FL}$ (μmol TE/g)	$H\text{-}ORAC_{FL}$ (μmol TE/g)	TAC[b] (μmol TE/g)	(mg GAE/g)	(mg βCE/g)
BP1	3.10 ± 0.71[c]	9.85 ± 0.65[c]	12.95	4.40 ± 0.72	1.953 ± 0.368[c]
BP2	2.91 ± 0.73[c]	9.14 ± 0.64	12.05	7.44 ± 1.14[c]	1.252 ± 0.225
BP3	3.96 ± 1.03	9.69 ± 0.61[c]	13.65	4.85 ± 0.80	0.955 ± 0.173
BP4	4.65 ± 1.21	9.19 ± 0.61	13.84	4.35 ± 0.66	0.275 ± 0.049[c]
BP5	3.37 ± 0.85	9.77 ± 0.66[c]	13.14	3.09 ± 0.46[c]	1.571 ± 0.286
BP6	3.70 ± 0.97	9.01 ± 0.60	12.71	5.99 ± 1.00[c]	0.837 ± 0.146
BP7	3.27 ± 0.85	9.45 ± 0.93	12.71	4.46 ± 0.65	0.189 ± 0.039[c]
BP8	4.72 ± 1.25	9.08 ± 0.62	13.80	3.57 ± 0.60[c]	1.251 ± 0.229
BP9	4.87 ± 1.18	9.73 ± 0.71[c]	14.59	6.83 ± 1.12[c]	0.665 ± 0.116[c]
BP10	4.29 ± 1.23	9.43 ± 0.72	13.71	3.91 ± 0.71	0.652 ± 0.118[c]
BP11	6.68 ± 1.79[c]	9.79 ± 0.66[c]	16.47	7.40 ± 1.19[c]	1.083 ± 0.194
BP12	2.34 ± 0.64[c]	10.12 ± 0.68[c]	12.46	8.12 ± 1.30[c]	0.400 ± 0.068[c]
BP13	2.89 ± 0.76[c]	8.71 ± 0.57[c]	11.60	6.85 ± 1.08[c]	1.037 ± 0.186
BP14	3.19 ± 0.86	8.78 ± 0.44[c]	11.97	5.37 ± 0.85	2.585 ± 0.469[c]
BP15	3.89 ± 0.98	9.60 ± 0.66	13.49	3.29 ± 0.48[c]	1.117 ± 0.206
BP16	4.71 ± 1.17	10.04 ± 0.64[c]	14.75	3.21 ± 0.54[c]	0.201 ± 0.038[c]
BP17	3.59 ± 0.70	9.34 ± 0.73	13.03	6.37 ± 1.03[c]	0.030 ± 0.010[c]
BP18	3.57 ± 0.91	9.18 ± 0.62	12.75	5.47 ± 1.03	0.823 ± 0.146
BP19	3.21 ± 0.89	9.20 ± 0.62	12.41	7.44 ± 1.17[c]	1.981 ± 0.361[c]
BP20	2.19 ± 0.56[c]	9.11 ± 0.57	11.30	5.16 ± 0.98	1.280 ± 0.225
BP21	3.43 ± 0.84	9.03 ± 0.63	12.45	8.43 ± 1.45[c]	1.541 ± 0.280
BP22	4.90 ± 1.38	9.60 ± 0.74	14.50	2.86 ± 0.45[c]	0.490 ± 0.089[c]
BP23	5.13 ± 1.32	9.56 ± 0.68	14.69	2.57 ± 0.45[c]	4.285 ± 0.795[c]
BP24	5.54 ± 1.39[c]	9.17 ± 0.62	14.71	4.95 ± 0.71	0.382 ± 0.624[c]
BP25	4.52 ± 1.10	9.36 ± 0.70	13.88	3.44 ± 0.50[c]	0.101 ± 0.025[c]
BP26	5.73 ± 1.52[c]	9.25 ± 0.62	14.98	3.01 ± 0.44[c]	3.381 ± 0.624[c]
BP27	3.13 ± 0.89[c]	8.52 ± 0.64[c]	11.65	1.26 ± 0.20[c]	0.095 ± 0.020[c]
BP28	7.79 ± 2.07[c]	8.56 ± 0.61[c]	16.35	5.57 ± 0.84	2.580 ± 0.487[c]
BP29	5.54 ± 1.46[c]	9.09 ± 0.58	14.63	3.98 ± 0.62	1.469 ± 0.271
BP30	5.94 ± 1.51[c]	9.06 ± 0.61	15.00	3.93 ± 0.51	1.375 ± 0.253
BP31	3.01 ± 0.74[c]	7.13 ± 0.50[c]	10.14	2.43 ± 0.36[c]	0.468 ± 0.079[c]
BP32	2.90 ± 0.80[c]	9.78 ± 0.62[c]	12.68	5.98 ± 0.90	1.965 ± 0.373[c]
BP33	5.04 ± 1.36	9.68 ± 0.61	14.72	2.05 ± 0.31[c]	0.693 ± 0.123[c]
BP34	6.05 ± 1.55[c]	9.26 ± 0.65	15.31	4.93 ± 0.78	3.364 ± 0.623[c]
BP35	3.69 ± 0.96	9.59 ± 0.59	13.27	6.49 ± 1.06[c]	1.520 ± 0.290

[a] Samples analyzed in triplicate and data represent the average values and are expressed on "as is" fresh weight basis (FW); [b] TAC represents the sum of the average values of $L\text{-}ORAC_{FL}$ and $H\text{-}ORAC_{FL}$; [c] significant at $P \leq 0.01$.

ethanol (70%) extracts of bee pollen samples from Brazil (8.1 mg GAE/g dry weight). Slightly higher values were obtained by Morais et al. [23] for the methanol extracts of bee pollen from Portugal (10.5-16.8 mg GAE/g dry weight). Higher TP concentrations were determined in the ethanol (70%) extracts of Brazilian bee pollen (19.28-48.90 mg GAE/g dry weight) [1].

The monofloral bee pollen analyzed here showed wide variability with regard to the TP of their AWA extracts (Table 4), being divided into three groups, namely, high (> 8 mg GAE/g), medium (2-8 mg GAE/g) and low (< 2 mg GAE/g) phenolic content. The significantly highest TP values ($P \leq 0.01$) were

determined in bee pollen from Hy (13.39 mg GAE/g), Card (13.36 mg GAE/g) and Cor (13.33 mg GAE/g), which were included in the high phenolic content group. The majority of the monofloral bee pollen (20 samples) contained medium values of TP, including Tar and Cra (5.43 mg GAE/g and 5.49 mg GAE/g). Tu and Ze bee pollen contained significant ($P \leq 0.01$) lower levels of TF (1.66 mg GAE/g and 1.24 mg GAE/g), representing the lower phenolic content group.

Amongst the Compositae family, the TP values ranged from 5.52 mg GAE/g to 13.38 mg GAE/g, in Ar and Card bee pollen. In the case of the Rosaceae family, the TP levels of monofloral bee pollen ranged between 5.49 mg GAE/g and 11.14 mg GAE/g, in Cra and Col, respectively.

The palynologic composition of the multifloral samples influences their TP values. This observation is valid for the samples, where the monofloral pellets values are available by comparing their frequencies percentages. The high TP values of some of the multifloral samples is reflected by the strong content

Table 4 $L\text{-}ORAC_{FL}$, $H\text{-}ORAC_{FL}$, total antioxidant capacity (TAC), TP and TC of monofloral bee pollen[a].

Botanical name of floral species	Antioxidant capacity			TP (mg GAE/g)	TC (mg βCE/g)
	$L\text{-}ORAC_{FL}$ (μmol TE/g)	$H\text{-}ORAC_{FL}$ (μmol TE/g)	TAC[b] (μmol TE/g)		
Taraxacum officinale Web.	8.88 ± 2.40	18.34 ± 1.35	27.22	5.43 ± 0.92	3.266 ± 0.609
Geranium sanguineum L.	2.38 ± 0.69	13.93 ± 1.07	16.31	5.66 ± 0.98	0.056 ± 0.014[c]
Calluna vulgaris (L.) Huill.	6.27 ± 1.68	14.09 ± 1.07	20.36	7.22 ± 1.24	0.351 ± 0.061[c]
Carduus sp.	3.86 ± 1.05	12.93 ± 0.96	16.79	13.36 ± 2.18[c]	1.738 ± 0.328
Cichorium intybus L.	3.01 ± 0.81	20.98 ± 1.69	23.99	7.78 ± 1.37	0.469 ± 0.085[c]
Prunus sp.	3.22 ± 0.84	14.94 ± 1.12	18.16	7.57 ± 1.37	0.137 ± 0.024[c]
Brassica sp.	4.79 ± 1.30	11.58 ± 0.92[c]	16.38	11.62 ± 2.00[c]	0.005 ± 0.001[c]
Crataegus monogyna Jacq.	4.25 ± 1.15	14.76 ± 1.26	19.01	5.49 ± 0.94	0.115 ± 0.021[c]
Salix sp.	9.96 ± 2.52[c]	14.40 ± 1.17	24.37	9.09 ± 9.09	4.194 ± 0.774[c]
Helianthus annuus L.	6.29 ± 1.85	15.61 ± 1.18	21.90	6.82 ± 1.18	3.395 ± 0.631
Tussilago sp.	8.62 ± 2.26	10.22 ± 0.85[c]	18.84	1.66 ± 0.30[c]	0.589 ± 0.111
Scilla bifolia L.	9.24 ± 2.57 [c]	19.54 ± 1.52[c]	28.78	9.33 ± 1.61[c]	0.129 ± 0.025[c]
Matricaria chamomilla L.	6.56 ± 1.80	19.77 ± 1.48[c]	26.33	5.88 ± 1.00	12.532 ± 2.329[c]
Cirsium arvense (L.) Scop.	8.18 ± 2.17	22.46 ± 1.77[c]	30.64	6.79 ± 1.16	0.046 ± 0.013[c]
Filipendula ulmaria L.	2.21 ± 0.60	13.08 ± 1.06[c]	15.29	5.94 ± 1.03	0.019 ± 0.006[c]
Silene sp.	5.49 ± 1.49	19.15 ± 1.44[c]	24.64	3.92 ± 0.69 [c]	0.184 ± 0.030[c]
Oxalis sp.	4.61 ± 1.21	10.17 ± 0.75[c]	14.78	5.46 ± 0.92	6.744 ± 1.249[c]
Verbascum sp.	4.43 ± 1.09	12.00 ± 0.90[c]	16.42	7.83 ± 1.33	1.961 ± 0.368
Arctium minus (Hill) Bernh.	4.90 ± 1.39	18.10 ± 1.41[c]	23.00	5.52 ± 1.03	0.168 ± 0.031[c]
Hypericum perforatum L.	5.93 ± 1.65	16.20 ± 1.36	22.13	13.39 ± 2.26[c]	4.470 ± 0.826[c]
Trifolium incarnatum L.	5.40 ± 1.48	13.68 ± 1.09	19.08	4.00 ± 0.66[c]	0.900 ± 0.164
Tilia sp.	7.96 ± 2.18	16.06 ± 1.17	24.02	7.14 ± 1.07	0.182 ± 0.031[c]
Ranunculus sp.	3.54 ± 0.87	14.56 ± 1.18	18.10	5.53 ± 0.95	2.286 ± 0.422
Cornus sanguinea L.	2.34 ± 0.70	13.30 ± 1.06	15.64	13.33 ± 2.25[c]	0.010 ± 0.005[c]
Colutea arborescens L.	1.65 ± 0.46	11.06 ± 0.82[c]	12.71	11.14 ± 1.89[c]	2.682 ± 0.497
Solidago virgaurea L.	5.79 ± 1.51	18.43 ± 1.45[c]	24.22	6.14 ± 1.04	6.778 ± 1.255[c]
Papaver rhoeas L.	7.79 ± 1.97	16.26 ± 1.19	24.05	6.58 ± 1.11	2.344 ± 0.437
Zea mays L.	2.08 ± 0.69	14.09 ± 1.15[c]	16.17	2.13 ± 0.34[c]	0.072 ± 0.012[c]
Helianthemum nummularium L.	1.47 ± 0.40	4.53 ± 0.52[c]	6.00	1.42 ± 0.29[c]	1.510 ± 0.278

[a] Samples analyzed in triplicate. Data represents the average values and are expressed on "as is" fresh weight basis (FW); [b] TAC represents the sum of the average values of $L\text{-}ORAC_{FL}$ and $H\text{-}ORAC_{FL}$; [c] significant at $P \leq 0.01$.

of the monofloral pellets rich in polyphenols, namely, Br, Pr species (in BP21), Ti (in BP11), Br (in BP13), Fi and Hy (in BP2). Furthermore, the reduced values of the multifloral samples BP5, BP16 and BP27 may be explained by the high content of monofloral samples with relatively low TP content (Tar and Ge) and very low TF values (Ze), respectively.

3.3 TC Content

The multifloral samples contained mean TC values of 1.253 mg βCE/g, in the range of 0.030-4.285 mg βCE/g, with high variability between the samples (Table 3).

The comparison with previous literature data is difficult, since few data are available, and all of them, according to available data by the authors in this study, were obtained by using chromatographic techniques. Nevertheless, similar high variation in the carotenoid values for multifloral samples was obtained by other authors. TC values of 0.49-242.6 mg/g dry weight were obtained by Muniategui et al. [24] for multifloral Spanish bee pollen, by using high performance liquid chromatography. Almeida-Muradian et al. [14] registered TC values ranged between 12.38 μg/g and 126.4 μg/g for multifloral Brazilian samples analyzed by open column chromatography. Similar TC values were reported by Dominguez-Valhondo et al. [5], but they analyzed only two different types of bee pollen, one monofloral (*Cistus ladanifer* L.) and one multifloral sample, both as fresh, lyophilized and hot-dried form. The TC of fresh samples were 20.40 μg/g and 5.51 μg/g, respectively. The lack of β-carotene in all dried samples was suggested to be caused by the thermal decomposition during the dehydration process of bee pollen.

TC content of the monofloral bee pollen investigated in this study varied between very large limits (Table 4). Mat bee pollen had significantly ($P \leq 0.01$) higher TC content (12.532 mg βCE/g). It is noteworthy that all monofloral samples, which contain 3.202-6.778 mg βCE/g (So, Ox, Hy, Sa, Hel and He bee pollen), show variation of orange coloration, since it is well known that the carotenoid pigments are generally responsible for the orange color in the vegetables [25]. Nine of the monofloral samples contained 0.100-0.600 mg βCE/g, and for the six of them, the TC content was significantly lower ($P \leq 0.01$), being situated below 0.100 mg βCE/g. The lowest levels were found in Br, Cor and Fi bee pollen (0.005, 0.010 and 0.019 mg βCE/g, respectively).

Amongst the Compositae family, the TC values ranged from 0.046 to 12.532 mg βCE/g in Cir and Mat bee pollen. In the monofloral bee pollen from the Rosaceae family investigated here, the TC levels ranged between 0.019 mg βCE/g and 2.682 mg βCE/g.

The high variability of TC may be explained by the botanic differences between the foraging plants. Moreover, at least one plant species per foraging month possesses carotenoid pigments in the pollen loads, while others do not present carotenoids [25].

In the current study, the obtained differences found between the multifloral samples may be explained in part by the variation of their palynologic composition. The highest value was obtained in BP23 with strong content of Mat and Tar, along with So bee pollen, each of them with individual high TC values (Tables 3 and 4). The high TC content of other multifloral samples can be explained by the high percentages of monofloral pellets rich in carotenoids, such as Tar (BP28 and BP14) and Sa (BP14). Similarly, the poorest multifloral samples were generally those that contained high percentages of monofloral pollens with very low content of TC. In this contest, BP17 and BP27 have registered low values, since their floral composition were predominantly from plants with similarly lower carotenoid content, such as Pr and Ze, respectively.

3.4 ORAC Antioxidant Values

The ORAC assay was used in this study to assess TAC of bee pollen because of the difficulty in measuring each antioxidant component separately and

the interactions among different antioxidant components in the product. Furthermore, the AUC technique combines both inhibition percentage and the length of inhibition time of free radical action by an antioxidant into a single quantity, as the reaction goes to completion, which makes the ORAC assay superior to other similar methods for assessing antioxidant capacity [11]. The ORAC approach has been previously used for the evaluation of the antioxidant activity of a wide variety of samples, such as foods, dietary supplements, botanicals, human plasma [10, 11], but the literature contains very few studies regarding the bee pollen.

The L-$ORAC_{FL}$ values of multifloral samples ranged between 2.19 μmol TE/g and 7.79 μmol TE/g (BP20 and BP28), with average values of 4.25 μmol TE/g (Table 3). The majority of the multifloral samples (19 samples) contained between 2-4 μmol TE/g. The next interval (4-6 μmol TE/g) was determined in 14 samples, while only two of them contained more than 6 μmol TE/g (BP28 and BP11). The H-$ORAC_{FL}$ values of the multifloral samples were situated in 8-10 μmol TE/g, except BP3 (7.13 μmol TE/g), BP 16 (10.04 μmol TE/g) and BP 12 (10.12 μmol TE/g), which registered significantly ($P \leq 0.01$) lower and higher H-$ORAC_{FL}$ values, respectively. The majority of multifloral samples registered TAC values ranged in 11-14 μmol TE/g (23 samples), while 12 samples contained higher values (14-17 μmol TE/g) and only one sample showed significantly ($P \leq 0.01$) lower values of 10.14 μmol TE/g (BP31).

With regard to the L-$ORAC_{FL}$ values, the monofloral samples showed the reduced variability, with significantly higher antioxidant potential ($P \leq 0.01$) determined in two samples, namely, Sa and Sci (9.96 μmol TE/g and 9.24 μmol TE/g, respectively) (Table 4). The liphophilic extracts of bee pollen from Col and Ze species exhibit the lower antioxidant potentials (1.65 μmol TE/g and 2.08 μmol TE/g). With regard to the H-$ORAC_{FL}$ values, significantly ($P \leq 0.01$) higher antioxidant potential was exhibited by Cir bee pollen (22.46 μmol TE/g), followed by Cic, Mat, Sci, Si, So, Tar and Ar bee pollen (20.9, 19.77, 19.54, 19.15, 18.43, 18.34 and 18.10 μmol TE/g). In contrast, the hydrophilic extracts of bee pollen from Ze and He registered the lowest $ORAC_{FL}$ values (6.94 μmol TE/g and 9.06 μmol TE/g). In accordance with the above-mentioned results, the added values of both lipophilic and hydrophilic fractions (TAC values) have shown great variability in the monofloral samples. As indicated for different plant products in previous studies, the botanical source influences the antioxidant capacity; the authors in this study have observed > 3 fold difference between those monofloral bee pollen with a high antioxidant capacity and those with low potential.

Comparing the palynologic spectrum of multifloral samples BP11 and BP27 with their predominant monofloral pellets (> 70%), Ti and Ze, respectively, similar behaviour were determined both for TF versus H-$ORAC_{FL}$ and TC versus L-$ORAC_{FL}$ relationship, which sustain more the main role of the floral origin into the variability of the biochemical composition and consecutively the antioxidant potential of bee pollen (Tables 1, 3 and 4).

The resulting ORAC values in this paper for monofloral and multifloral bee pollen were difficult to compare to other species and/or samples because of absent reliable literature data.

3.5 Relationship between the Antioxidant Content and the ORAC Values

Good association between the phenolic content and the hydrophilic antioxidant activity was determined in the case of Hy and Sci bee pollen from one side, and Ze, He and Tu to the other side. These samples registered high (TP: 13.39 GAE/g and 9.33 mg GAE/g; H-$ORAC_{FL}$: 16.20 TE/g and 19.54 μmol TE/g), and low (TP: 2.13, 1.42 and 1.66 mg GAE/g; H-$ORAC_{FL}$: 14.09, 4.53 and 10.22 μmol TE/g) values, respectively, for both of the parameters. In contrast, anomalous

behavior presented Si and Mat, where low TF values (3.92 mg GAE/g and 5.88 mg GAE/g) do not clearly support the H-ORAC$_{FL}$ values (19.15 μmol TE/g and 19.77 μmol TE/g). Similarly, the higher H-ORAC$_{FL}$ scavenging activity (22.46 μmol TE/g and 20.98 μmol TE/g) was not reflected by the medium TF values (6.79 mg GAE/g and 7.78 mg GAE/g) obtained for Cir and Cic bee pollen. In contrast, Col and Br bee pollen demonstrated low H-ORAC$_{FL}$ values (11.06 μmol TE/g and 11.58 μmol TE/g), which are not in accordance with high TF content (11.14 mg GAE/g and 11.62 mg GAE/g).

Looking at Pearson's correlation coefficient between TC/L-ORAC$_{FL}$ and TC/TAC, a weak relationship was observed for monofloral samples, explicitly r = 0.192 and r = 0.177 (Table 5). These findings suggest that antioxidant components, other than carotenoids, contribute most to the lipophilic antioxidant capacity of bee pollen. The lack of good association between TC content and the lipophilic antioxidant activity of monofloral samples, is due to the anomalous behaviour of Ti, Cir and Sci bee pollen, where high L-ORAC$_{FL}$ values (7.96, 8.18 and 9.24 μmol TE/g) were not clearly supported by the TC content (0.182, 0.046 and 0.129 mg βCE/g). Furthermore, Mat, So and Ox bee pollen demonstrated opposite behaviour, where high TC content (12.523, 6.778 and 6.774 mg βCE/g) was not been reflected by the medium L-ORAC$_{FL}$ values (6.56, 5.79 and 4.61 μmol TE/g). In contrast, high TC values (4.194, 3.266 and 2.344 mg βCE/g) are accompanied by high L-ORAC$_{FL}$ values (9.96, 8.88 and 7.79 μmol/TE g) in the case of Sa, Tar and Pa bee pollen. Moreover, the TC (0.019, 0.056 and 0.072 mg βCE/g) and L-ORAC$_{FL}$ (2.21, 2.38 and 2.08 μmol TE/g) values manifested similar tendencies in the case of Fi, Ge and Ze bee pollen.

Due to the existence of different factors (botanical origins, soil characteristics, climates) that could affect phytochemical composition and consecutively the antioxidant capacity of bee pollen samples, the multivariate analysis of data matrix was carried out. A principal component analysis (PCA) was subsequently applied on the antioxidant descriptors to produce an antioxidant map of the samples. It has been tried to find the principal component in such a way that best describes all the different samples in the attempt to investigate the classification of them according to their affiliation to the same botanical family (Tables 5 and 6).

Table 5 Pearson correlation matrix of monofloral bee pollen.

	L-ORAC$_{FL}$	H-ORAC$_{FL}$	TAC	TF	TC
L -ORAC$_{FL}$	1		0.776	-0.056	0.192
H-ORAC$_{FL}$	0.453	1	0.914	0.128	0.127
TAC	0.776	0.914	1	0.1065	0.177
TF	-0.056	0.128	0.065	1	0.001
TC	0.192	0.127	0.177	0.001	1

L-ORACFL: lipophilic antioxidant capacity; H-ORACFL: hydrophilic antioxidant capacity; TAC: total antioxidant capacity; TF: total phenolics; TC: total carotenoids.

Table 6 Pearson correlation matrix of multifloral bee pollen.

	L-ORAC$_{FL}$	H-ORAC$_{FL}$	TAC	TF	TC
L-ORAC$_{FL}$	1	0.040	0.924	-0.237	0.287
H-ORAC$_{FL}$	0.040	1	0.418	0.130	-0.006
TAC	0.924	0.418	1	-0.166	0.258
TF	-0.237	0.130	-0.166	1	-0.022
TC	0.287	-0.006	0.258	-0.022	1

L-ORAC$_{FL}$: lipophilic antioxidant capacity; H-ORAC$_{FL}$: hydrophilic antioxidant capacity; TAC: total antioxidant capacity; TF: total phenolics; TC: total carotenoids.

Table 7 Eigenvalues for the principal components factors (PCs) of monofloral bee pollen.

Variables	PC1	PC2	PC3	PC4
Eigenvalue	2.503	1.040	0.942	0.515
Variability (%)	50.063	20.791	18.848	10.298
Cumulative (%)	50.063	70.854	89.702	100.000

Table 8 Factor loading table of the principal components factors (PCs) for monofloral bee pollen.

Factor	PC1	PC2	PC3	PC4
L-ORAC$_{FL}$	0.810	-0.217	-0.078	0.539
H-ORAC$_{FL}$	0.878	0.180	-0.122	-0.426
TAC	0.991	0.029	-0.122	-0.055
TF	0.088	0.927	0.314	0.182
TC	0.294	-0.315	0.899	-0.082

L-ORAC$_{FL}$: lipophilic antioxidant capacity; H-ORAC$_{FL}$: hydrophilic antioxidant capacity; TAC: total antioxidant capacity; TF: total phenolics; TC: total carotenoids.

The eigenvalues in Table 7 indicates that first component PC1 explain only 50% of the variability between the samples, while similar percents of contribution correspond to the PC2 and PC3 components, namely 20% and 18%, respectively. In this case, PC1 and PC2 allow to describe only 70% of the initial variability of data, and therefore some of the information might be hidden in the next factor PC3. For these reasons, the confident description of the complex relationship between the data has to take into account all three components, PC1, PC2 and PC3, in order to achieve a cumulative variability close to 100%, namely, 89.7% in this case. From the factor loading table (Table 8), the contribution of the variables to the PCs indicates that PC1 is represented by the ORAC values (L-ORAC$_{FL}$, H-ORAC$_{FL}$ and TAC), PC2 by the TF levels and PC3 by the TC amounts. For these reasons, the variability of the monofloral bee pollen samples can be explained in part by the TF content, but also by the TC levels, in relation to their antioxidant capacities.

According to the above-mentioned results of the multivariate data matrix, the bi-plots PC1/PC2 (Fig. 1) and PC1/PC3 (Fig. 2) were obtained, where both samples and variables (antioxidant features) are mapped together into the bi-dimensional graphics of the corresponding principal components factors (PCs). This approach has the main objective to investigate the influence of the affiliation of the monofloral samples to the same botanical family on their antioxidant features. The bi-plot PC1/PC2 indicates that majority of the monofloral samples of Compositae family (Cic, Cir, He, Mat and Tar) seem to have similar antioxidant features, namely, good ORAC values and low TF levels. For Card (*Asteracea*) bee pollen, of which high TF content was not accompanied by similar ORAC values. Some of the differences between the samples were explained from the second bi-plot PC1/PC3, since some of the samples manifested high TC levels (Mat, Hel and So). When analyzing the Rosaceae Family, the monofloral samples were positioned more closely on both of the bi-plots, given their similar antioxidant features related to the low ORAC values, medium TF levels and low TC contents, but without being well separated on the map among other samples.

4. Discussion

The present study confirmed previous findings that the botanical origin influences the polyphenol and carotenoid content of bee pollen and the specific occurrence of them in the monofloral pollen loads. Considering all the bee pollen samples analyzed here, the relation between the antioxidant content and their performed antioxidant capacities showed a very week correlation. The results support previous conclusions

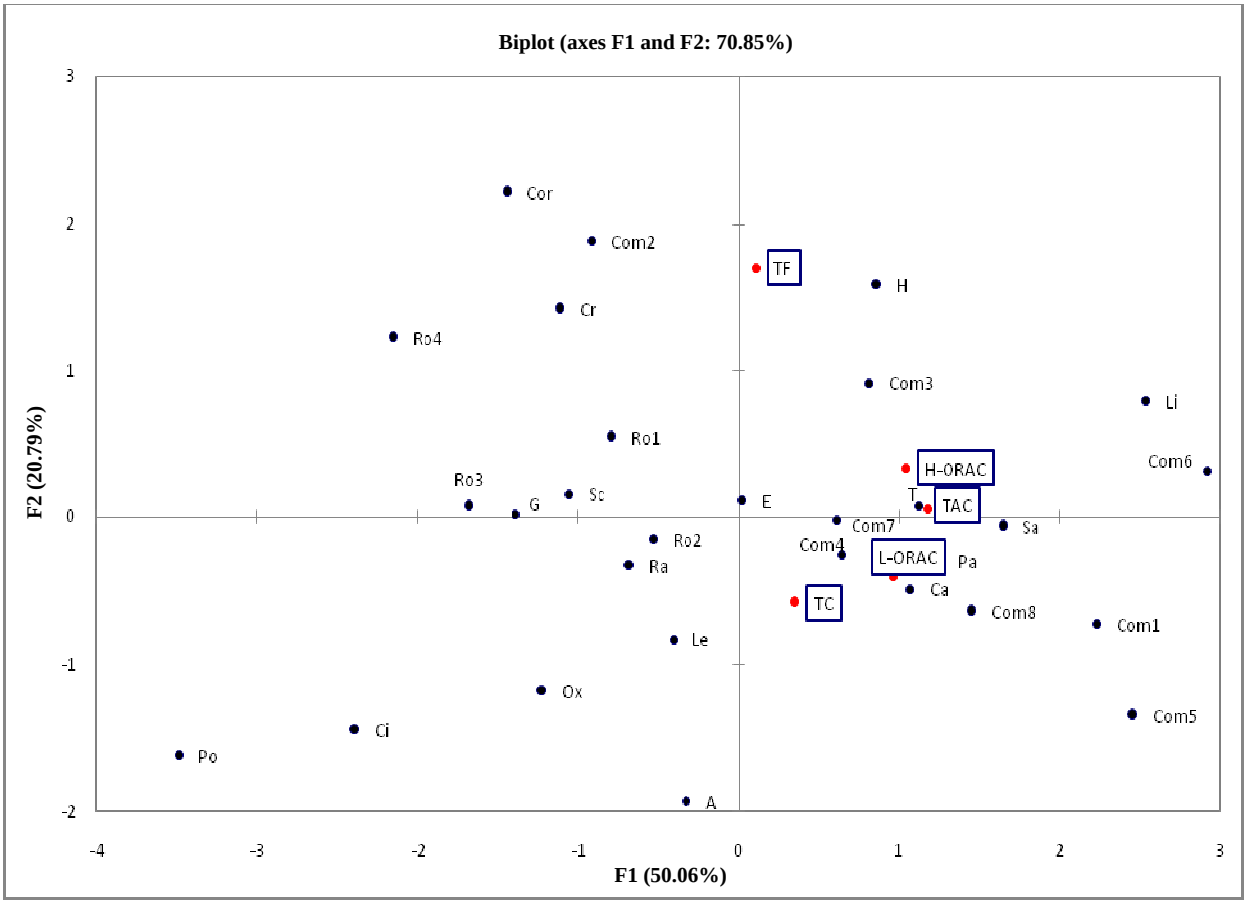


Fig. 1 Bi-plot graph of PC1/PC2 components for the monofloral bee pollen samples.

A: Asteraceae; Ca: Caryophyllaceae; Ci: Cistaceae; Com: Compositae; Cor: Cornaceae; Cr: Crucifeare; E: Ericaceae; G: Geraniaceae; H: Hypericaceae; Le: Leguminosae; Li: Liliaceae; Ox: Oxalidaceae; Pa: Papaveracea; Po: Poaceae; Ra: Ranunculaceae; Ro: Rosaceae; Sa: Salicaceae; Sc: Scrophulariaceae; T: Tiliaceae; Com1: *Taraxacum officinale* Web.; Com2: *Arctium minus* (Hill) Bernh.; Com3: *Cichorium intybus* L.; Com4: *Helianthuus annuus* L.; Com5: *Matricaria chamomilla* L.; Com6: *Carthamus lanatus* L.; Com7: *Cirsium arvense* L.; Ro1: *Prunus* sp.; Ro2: *Crataegus monogyna* J.; Ro3: *Filipendula ulmaria* L.; Ro4: *Colutea arborescens* L..

that the free radical scavenging effectiveness is determined by its particularly phenolic/carotenoid or non-phenolic/non-carotenoid constituents with their variable structure and actions. New variables, such as soil characteristics, climates together with botanical origins, were introduced in a multivariate analysis of antioxidant data matrix, given a possible important involvement of all of them in affecting, not only the phytochemical composition, but thereafter the antioxidant capacity.

To achieve this data, 29 monofloral pellets coming from 35 multifloral bee pollen harvested from Romania (Transylvania region) were studied here for palynological spectrum, TP and TC content of these samples and their contribution to the lipophilic, hydrophilic and total antioxidant capacities, evaluated by $ORAC_{FL}$. Prior to this report, no data exist on Romanian bee pollen and especially on monofloral pellets.

All 35 multifloral samples were found to be heterofloral, a total of 67 pollen types being identified along with *Lycopodium cernuum* spores (Table 2). None of them were found in all of the samples, mainly due to their different geographical origins. The pollen spectrum reflects the available surrounding bee pasture in the apiary vegetation, as well as on the climate conditions for flowering [26, 27].

Results in the present study agree with previous

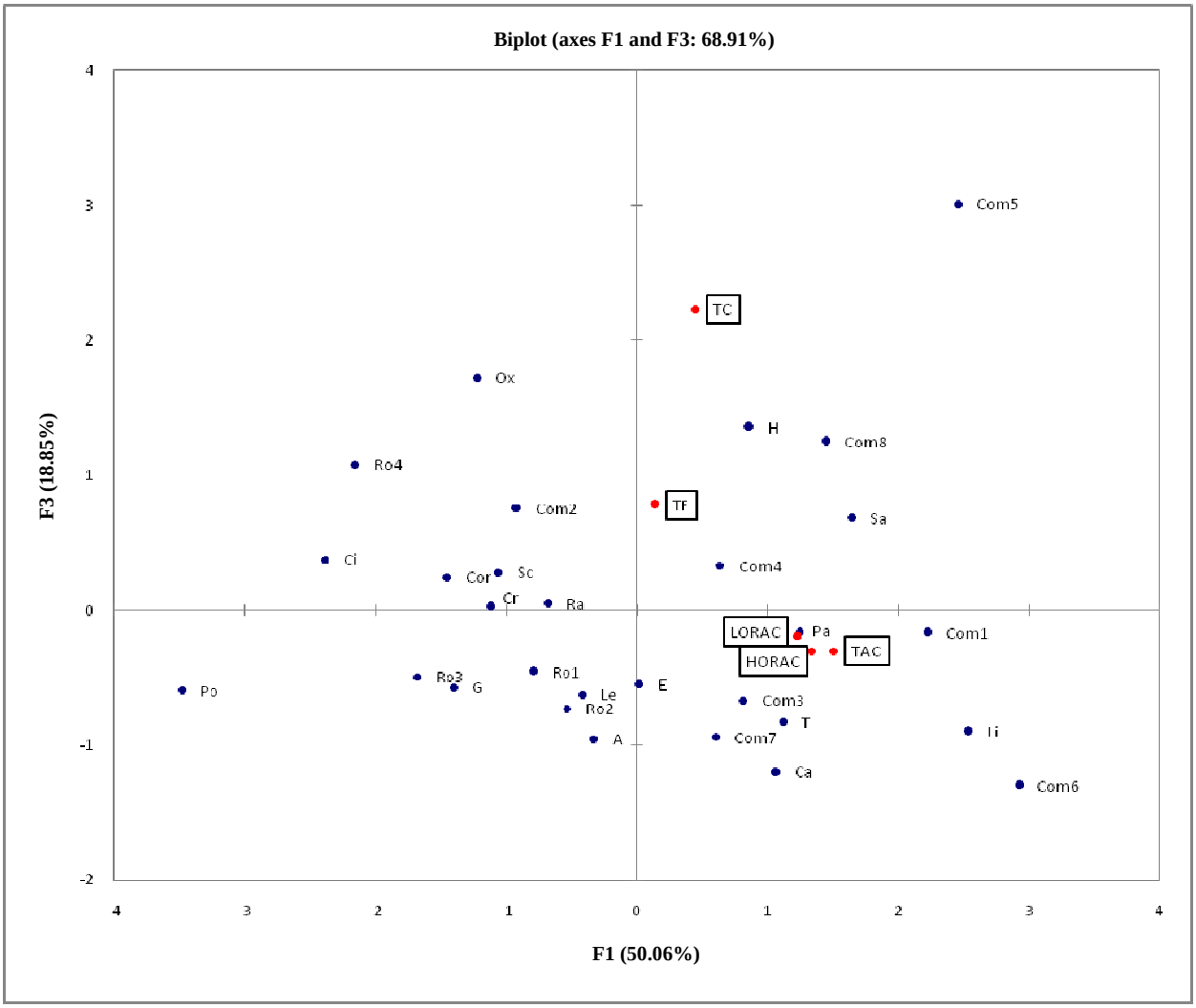


Fig. 2 Bi-plot graph of PC1/PC3 components for monofloral bee pollen samples.

A: Asteraceae; Ca: Caryophyllaceae; Ci: Cistaceae; Com: Compositae; Cor: Cornaceae; Cr: Crucifeare; E: Ericaceae; G: Geraniaceae; H: Hypericaceae; Le: Leguminosae; Li: Liliaceae; Ox: Oxalidaceae; Pa: Papaveracea; Po: Poaceae; Ra: Ranunculaceae; Ro: Rosaceae; Sa: Salicaceae; Sc: Scrophulariaceae; T: Tiliaceae; Com1: *Taraxacum officinale* Web.; Com2: *Arctium minus* (Hill) Bernh; Com3: *Cichorium intybus* L.; Com4: *Helianthuus annuus* L.; Com5: *Matricaria chamomilla* L.; Com7: *Cirsium arvense* L.; Ro1: *Prunus* sp.; Ro2: *Crataegus monogyna* J.; Ro3: *Filipendula ulmaria* L.; Ro4: *Colutea arborescens* L..

literature reports about the prevalence of the pollen from entomophilous species in the pollen foraging habits of the honeybees [22]. The most important family plant as pollen sources for honeybees was Compositae, since it was the most present in multifloral samples usually with frequencies between 16% and 45%. Other important ones were the Rosaceae, Leguminosae and Asteraceae families, given their distribution among the samples and their frequencies in a given sample. Other ones, such as Papaveraceae, Ranunculaceae and Scrophulaceae, were also found in more samples, but with little contribution to the total pollen spectrum. The high variety of the plant families' recorder in this study may be considered as a good indication of the flora diversity of this specific area of Romania, which can be considered as valuable assets of the multifloral bee pollen harvested from here.

The phenolic content varied greatly amongst the different types of monofloral bee pollen, similarly with the authors' previous reports [19], where the

methanolic extracts (80%) of monofloral samples were analyzed. By comparing the obtained results from this two works, the methanol solvent extracted more phenolic compounds, given previous TF values of Tar, Hel, Cra, Mat, Card and Sa bee pollen (16.2, 11.4, 15.3, 14.0, 12.9 and 16.4 mg GAE/g).

Botanical origin, genetic factors, taxonomic variety, soil, environmental conditions, time of the year, age of plant at pollen development, nutritional status of the plant, method of pollen extraction, etc., greatly influence the formation of secondary metabolites in plants. Other data is in line with the literature, that refers the presence and distribution of polyphenols and carotenoids in vegetables and fruits vary accordingly [5, 27, 28]. Each pollen type has its own specificity, mainly linked to the floral species or cultivars [26]. Furthermore, the organoleptic and biochemical properties of the monofloral bee pollen are similar to the original plant. When the pollen originates from several botanical species, the loads are heterofloral and possess various properties [28].

Bees foraging preference of bees is still discussed, the nutritional quality of pollen, mainly linked to the protein and amino acid content, being only in part the main influencing factor, other non-nutritional ones being cited to be involved in, such as, plant resource availability, color, odor and morphology of flowers. In effect, honeybees do collect and consume pollen and pollen-like substances with little or no nutritive value [22]. In addition, the anemophilus plants also benefit from other pollinators, such as bees that visit flowers for pollen and possibly nectar [27]. The male flowers of maize (*Zea mays*), characterized by anemophilus pollination, are not of great interest for bee foragers when there are other good sources. Despite their low protein content, they are visited by bees for pollen, and sometimes bees come to feed almost exclusively on corn pollen in case of scarcity or when apiaries are located in areas with large plantations of corn [27]. One of samples studied in this paper was almost pure in this taxon (81.7%).

According to Carpes et al. [6], the pollen collected by bees generally shows characteristic amounts of total polyphenols due to its botanical and geographical origin. This situation can explain the differences between these samples collected in this study, since it is well known that genetics, geographic and environmental conditions may significantly influence the content of plant secondary metabolites and consecutively the bioactive phyto-compound levels in the foods of vegetable origin [10].

The main end-point of the present study was to investigate the relationship between the antioxidant content and potential of both monofloral and multifloral bee pollen, with special attention on the floral spectrum of the samples.

The results indicated that the lipophilic fraction of multifloral bee pollen has the highest antioxidant capacity than the majority of the fruits and vegetables tested by ORAC assay in the previous study of Wu et al. [10] who studied with over of 100 different kinds of foods. Between the tested fruits and vegetables, only some of the samples registered higher L-ORAC$_{FL}$ values (above 1 μmol/TE g), which, in comparison with results in this study, are lower (blueberry, raspberry, asparagus, broccoli, corn, lettuce) or similar (avocado, spinach and dry mature beans). When comparing the H-ORAC$_{FL}$ values, similar results were obtained for sweet potatoes, radishes, potatoes, peppers, onions, lettuces, carrots, cabbages and some broccoli varieties.

The hydrophilic extracts of other fresh vegetables exhibited higher antioxidant potential, usually 2-4 fold higher (spinach, pears, red cabbages, raab broccoli, beets, spinach, pears), with some exceptions in the case of dry mature beans (8-12 fold). In contrast, some of the vegetables, such as cucumber, celery, cauliflower and fresh beans, presented reduced hydrophilic antioxidant potential (1-3 fold lower) than multifloral bee pollen. Among the fresh fruits tested by the above-mentioned researchers, similar results were obtained for pineapples, nectarines, mango and

bananas, while the majorities presented higher H-$ORAC_{FL}$ values (1-4 fold higher), except for plums, blueberry and blackberry (5-7 fold higher) and cantaloupe and watermelon (3 and 7 fold lower).

Compared with H-$ORAC_{FL}$, the L-$ORAC_{FL}$ values of bee pollen samples were generally lower (from 1.1 to 6.7 fold lower), which agree with previous reports for common foods [10]. In addition, TAC paralleled H-$ORAC_{FL}$ in bee pollen, because hydrophilic ORAC made up 52%-88% of TAC. These results differ slightly from those of Wu et al. [10], which reported above 90% of the H-$ORAC_{FL}$ contribution for all the common tested foods. These differences may be related to the high known carotenoid content of bee pollen, as lipophilic pigments with antioxidant properties.

From the current results, a linear correlation is not found between the antioxidant content (phenolic and carotenoid compounds) of bee pollen and the performed antioxidant capacities (Tables 5 and 6). These agree with the authors' previous study, where the antioxidant capacities, tested by DPPH, TEAC and FRAP assays, were different for each floral species (12 samples) and were not clearly associated to their TF content [19].

Relationships between the content and the antioxidant capacity are complex; association among phenolic compounds also affects the overall antioxidant activity [29]. The constituents responsible for the hydrophilic antioxidant activity are primarily phenolic compounds, anthocyanins, vitamin C, whereas carotenoids and tocopherols are the main antioxidant constituents in the lipophilic extracts [2, 24].

Antioxidative ability of foods seems to be due in great part to the phenolic compounds [2]. Generally, the flavonoid/phenolic content was closely related to bee pollen antioxidant activity [7]. From current results, it was found that this may not be true across all types of monofloral bee pollen analyzed. Among the monofloral samples, only five presented high TP and H-$ORAC_{FL}$ values, with H-$ORAC_{FL}$/TP ratio around 1, namely Car, Br, Hy, Cor and Col bee pollen.

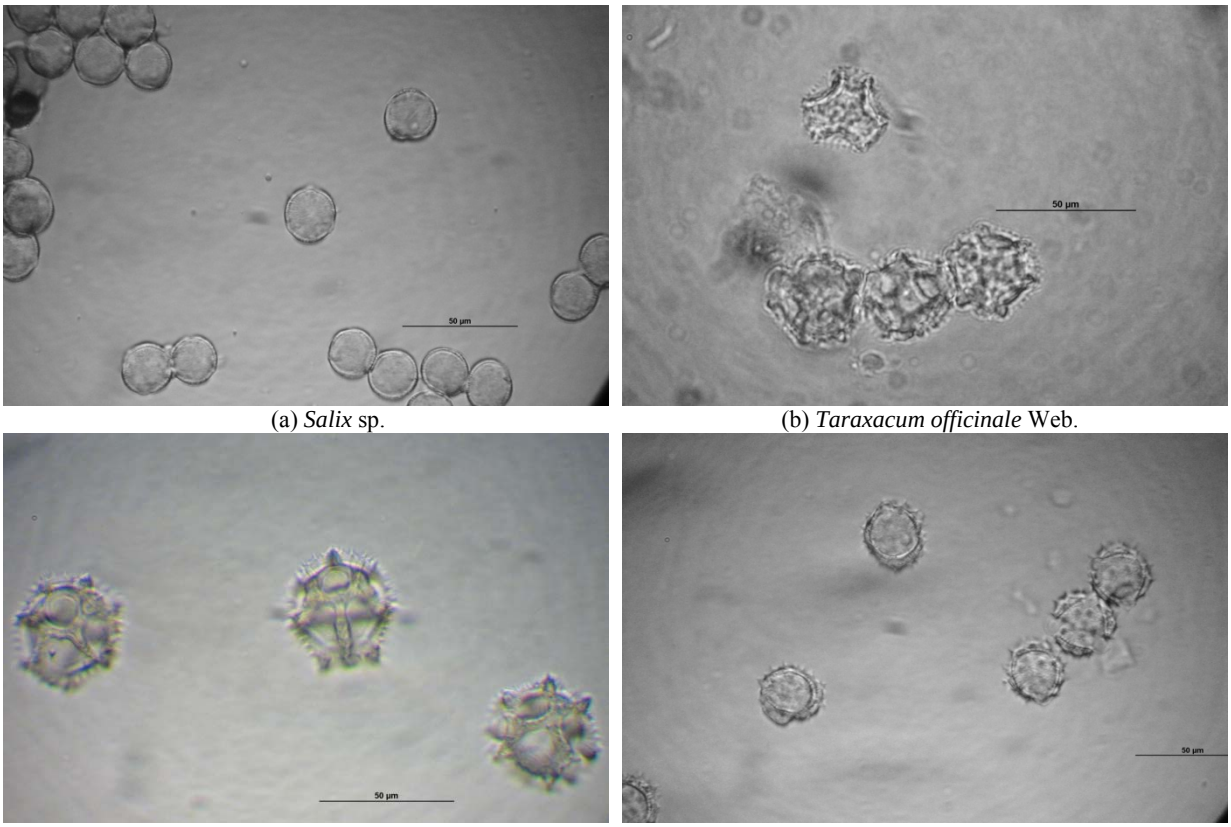
One of the problems involves the use of the Folin-Ciocalteu reagent that reacts with any reducing substance, and measures the total reducing capacity of a sample, not only the phenolic compounds, but also ascorbic acid, sugars, organic acids, amino acids and proteins [10, 12]. In addition, phenolics respond differently to the Folin-Ciocalteau reagent depending on the structure. In spite of these criticisms, the methodology continues to be widely used for determination of phenolic content in foods and dietary supplements [21].

The results support the previous suggestion that measurement of TP alone may not be a good indicator of the antioxidant capacity. Similarly, a non-linear correspondence was previously found for most of the foods analyzed by Wu et al. [10], in which samples with high antioxidant capacity tended to have higher H-$ORAC_{FL}$/TP ratios. The questionable relationship between these parameters was also found for some of the monofloral bee pollen analyzed previously [7].

The current study supports previous reports, which indicate that the antioxidant activity of a given sample is not limited to phenolics or carotenoids, or some phenolic/carotenoid compounds are more "effective" than others or have higher reactivity with the free radicals [29].

In order to establish more accurate relations between phenolic content and antioxidant activity of bee pollen, performing cross studies between pollen flavonoid/phenolic, carotenoids, volatile oils and antioxidant vitamin content (vitamins C and E) could be beneficial. These substances interact synergistically in human organism and their eventual concerted action could, perhaps, explain some controversial results. In addition, special attention must be paid to the concept of the "antioxidant paradox", which claims that antioxidant species, in certain conditions and/or concentrations, can have a pro-oxidant activity, meaning oxidant behaviour [7].

Further studies need to be performed for identification of the specific compounds contained in



(a) *Salix* sp. (b) *Taraxacum officinale* Web.

(c) *Cichorium intybus* L. (d) *Matricaria chamomilla* L.

Fig. 3 Pollen grains with high antioxidant potential.

the bee pollen, especially in regard to those monofloral samples that presented high antioxidant potential. The full elucidation of the individual phytochemicals in the samples and the involvement on the bioactivity, alone and/or in association of them, will give a better insight into who is responsible for this activity.

5. Conclusions

Studies of the palynologic spectrum of bee pollen are still few in Romania, and have only been sparsely reported. The present work provides important additional data about the pollen flora collected by honeybees in the Transylvania region of Romania. This characterization of the native apicultural flora is important information for beekeepers to know the better places to locate the hives. The generalized information about pollen collection patterns allows comparisons with other areas with different plant communities. Pollen types of the Compositae and Rosaceae family occurred very frequently in pollen analyses undertaken in the present study, and demonstrated the importance of this plant group to honeybees.

This investigation further supports the view that some monofloral bee pollens are good sources of natural antioxidants and potential crude materials for therapeutic bioactivities investigations. Thus some promising monofloral pellets have been identified with particular high antioxidant potential, such as *Salix* sp., *Taraxacum officinale* Web., *Matricaria chamomilla* L. and *Cichorium intybus* L. (Fig. 3). In addition, antioxidant values for some of monofloral bee pollen poorly studied are reported, such as *Cirsium arvense* (L.) Scop. and *Scilla bifolia* L., which have presented high antioxidant capacities. The results show that high phenolic and carotenoid content along with antioxidant capacity provide useful information, such as the potential use of some of

monofloral samples as a natural source of antioxidants and as a value-added product in the preparation of functional food ingredients and/or for enrichment of certain products. Furthermore, both TP and TC content, as well as the composition of individual phytochemicals in bee pollen is important to assess TAC of different samples.

Acknowledgments

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