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SPECIFIC DISTRIBUTION OF MINERALS IN SELECTED UNIFLORAL BEE POLLEN

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Abstract- Concentrations of selected macro and microelements in bee pollen with specific floral origin, harvested from Transylvania (Romania), were determined by flame atomic spectrometry (F-AES/AAS), considering the little information available on all over the world, particularly in this country. Potassium occurred at the highest concentrations in all samples (2483.51-7619.57mg kg^{-1}), followed by calcium (552.89-2797.85mg kg^{-1}) and magnesium (3554.96-205.14mg kg^{-1}). The microelements presented average values of 18.83-134.70mg kg^{-1} (iron) and 18.77-60.51mg kg^{-1} (zinc). The mineral levels in unifloral bee pollens analysed were found to vary due to differences in the floral origin. Unifloral bee pollen samples were classified in three main clusters, based on their Ca, Mg, Fe and Zn content. The clusters analysis of the data reveals the classification of unifloral bee pollen in three main clusters, based on the quantification of their Ca, Mg, Fe and Zn content. These results confirm that Romanian bee pollen can be used by man as a natural source of nutritionally essential minerals and contribute for a better balanced diet or for special therapeutic applications.
Keywords- bee pollen, unifloral source, minerals, atomic absorption spectroscopy
Abbreviations- AAS: Atomic Absorbtion Spectrometry, K: Potassium, Ca: Calcium, Mg: Magnesium, Fe: Iron, Zn: Zinc

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Introduction

Honeybee collected pollen (bee pollen) is the flower pollen collected by *Apis mellifera* L. with the purpose of feeding its larvae in the early stage of development, being a major source of proteins, amino acids, fatty substances, minerals and vitamins. Collected flower pollen is accumulated as a pellet or pollen load (corbicular pollen) in pouches on the rear legs of the bee and it is the mixture of these pellets that comprises pollen load [1-2]. Pollen pellets are removed from the bees before they enter in the hive by the beekeepers using different types of traps, so-called “bee pollen” [3]. The importance of the diet for a healthy life has been amply demonstrated. Individual nutrients have received variable attention, minerals are among them. They are essential protective nutrients for the maintenance of nutritional and health status of the body [4]. Deficiency of specific minerals may lead to various chronic diseases [5-6]. Minerals cannot be synthesized in the human body and, therefore, they must be obtained through the diet. Foods containing minerals in adequate amounts should be included in the daily diet to ensure adequate supply of minerals in the body. A wide range of minerals occur in feedstuffs as naturally occurring and purposely added elements, as well as by adventitious contamination. Twenty-six of the naturally occurring elements are known to be essential for human life. Mineral elements can generally be classified as nutritionally essential major elements (egg. Ca, Cl, K, Mg, N, Na, P and S), nutritionally essential minor and trace elements (egg. Fe, B, Br, I and Si) and those regarded as toxic or with an essential/ toxic duality, which at excessive levels may exhibit toxicity (egg. Zn, Se, Mn, Mo) [7]. For humans, calcium plays an important role in building and maintaining the bones and teeth, blood clotting, transmitting of the nerve impulses. Magnesium is as a co-factor of many enzymes involved in energy metabolism, protein, RNA and DNA synthesis, and maintenance of the electrical potential of nervous tissues and cell membranes [8]. Potassium functions in the maintenance of water balance and distribution, kidney and adrenal function [9]. Iron serves as a carrier of oxygen by red blood cell hemoglobin, as a transport medium for electrons within cells [8]. The participation of Zn and Fe in many kinds of enzymatic composition might play an important role in promoting the metabolism of organisms to strengthen immune ability and the control of disease [10]. Bee pollen is consumed for api-therapeutic purposes for their nutritional and medicinal properties. Considering its nutritional composition and according to non-scientific studies, dried bee pollen has been used as food in human diets providing a well-being sensation and contributing to functional and harmonious balance of the body

[11]. In this work, selected macro (K, Ca, Mg) and micro-mineral elements (Fe, Zn) were determined in honeybee-collected pollen of selected floral species, harvested from Transylvania-Romania, by flame atomic absorption spectrometry after dry ashing and wet digestion.

Materials and Methods

Materials

Standard stock solutions of each evaluated elements, containing 1001 ± 2 mg/l for K, Ca, Mg, Zn and 1000 mg/l for Fe in 0.5 mol/l HNO_3 purchased from Merck, hydrochloric acid (37%) from Merck, nitric acid (65%) from Fluka, deionized water (18.2 MΩ/cm resistivity). All reagents used in the experiments were of analytical grade. All the plastic and glassware were cleaned by soaking in dilute HNO_3 (10%) and rinsed with distilled water prior to use.

Botanical Origin Identification of the Pollen Pellets

Pollen loads samples were obtained from March to September 2010 in pollen traps installed in *Apis mellifera* (L.) hives located in the north-vest and central counties of Transylvania area (Romania). To maintain pollen quality, pollen was removed every 2 -3 days, hand cleaned and stored in a freezer (-18ºC), until analysis. The floral origin of bee pollen pellets was identified by colour and light microscope examination. Melissopalynological preparation was based on the European standard [12], without acetolysis application, adapted for pollen load [11]. The microscope examination was performed under normal lighting at 400 X magnification by using Nikon Eclipse 50*i* microscope. Pollen types were identified by comparison with pollen reference slides made by the authors of the present work, and then compared with pollen descriptive images [13-15]. The pollen reference slides were prepared from anthers of flowers and the plant taxon was identified upon the botanic atlas [16].

Samples Preparation

Bee pollen samples (2g) were placed in a quartz crucible and ashen in a muffle furnace at 450°C over the night. Ash was digested by treating with hydrochloric acid 6M (5ml) and H_2O_2 (3ml) which were then evaporated. The resulting white ash was dissolved with 25ml nitric acid 0.1M in a calibrated flask [17]. To assay pollen samples for Zn and Fe after digestion non-diluted solutions were used. The solutions were diluted 50 times for Ca assays, 200 times formg and K assays. Lanthanum nitrate [La $(NO_3)_3x6H_2O$] (1% w/v) were used as matrix modifier for the determination of potassium, calcium and magnesium. Analytical blanks (acids used for samples digestion) employed during AAS measurements were prepared for each batch of digestion set.

Atomic Absorption Spectroscopy Measurements

Elemental analysis was carried out on Atomic Absorption Spectrophotometer Schimadzu AA-6300 in air/acetylene flame. The spectrophotometer was equipped with deuterium lamp for background correction and hallow-cathode lamps for each of the element studied (single element lamps), burner system for flame analysis with a suitable acetylene cylinder and air compressor and auto sampler ASC-6100F. The device working parameters (air, acetylene, optics and electronics) were adjusted for maximum absorption for each element. The flame atomic spectrometric measurements were performed using the optimal instrumental parameters for each element**.** The following wavelengths were used for the studied metals: 766.5 nm (K), 422.7 nm (Ca), 285.2 nm (Mg), 248.3 nm (Fe) and 213.9 nm (Zn). Acetylene was of 99.99% purity. Under the optimum established parameters, standard calibration curves were constructed by plotting absorbency against concentration, with five concentration points. In a definite range for each metal, a good linearity was observed. The standard solutions used containing 1001 ± 2 mg/l for K, Ca, Mg, Zn and 1000 mg/l for Fe in 0.5 mol/l HNO_3 (Merck-Germany). The correlation coefficient for the calibration curves (r^2) ranged between 0.9990-0.9999. All reagents used in the experiments were of analytical grade. Hydrochloric acid (37%) was from Merck, nitric acid (65%) from Fluka-Germany. All solutions were prepared using deionised water (18.2 MΩ/cm) produced in our laboratory. Plastic and glassware were cleaned by soaking in dilute HNO_3 (1:9) and were rinsed with deionised water prior to use, to avoid potential contamination.

Statistics

All determinations were performed in triplicate and results are expressed as mean ± standard deviation calculated using spreadsheet software Microsoft Excel. Significance of difference was calculated by Duncan's multiple range test and results with $p<0.05$ were considered statistically significant. The relationship between different mineral content of the samples was analyzed by Pearson correlation coefficients. Cluster analysis was conducted on the values obtained using Euclidian similarity measure and paired group method. The results were processed by STATPlus2008 software.

Results and Conclusions

Botanical origin Identification of the Pollen Pellets

The pollen loads studied here, were first separated by color from the complex mixtures of pollen pellets from different species of plants, resulting monochromatic pollen loads with uniform color. The microscopic examination was the principal tool for the selection of the bee pollen pellets coming from one species. In each microscopic preparation, pollen was separated, when possible, into genus, species or family. The selected pollen load samples presented the following colours: red orange (*Helianthus annuus* L., *Taraxacum officinale* Web., *Inula helenium* L.), light green (*Crataegus monogyna* J., *Calluna vulgaris* L. Huill.), light yellow (*Pinus* sp., *Brassica* sp.), violet (*Carduus* sp.), brown (*Onobrychis viciifolia* Scop., *Verbascum phlomoides* L.), grey (*Centaurea cyanus* L., *Cichorium intybus* L.), pink (*Knautia arvensis* (L.) Coulter), maroon (*Salix* sp., *Prunus* sp.), green maroon (*Epilobium augustifolium* L.).

Mineral Content of Unifloral Bee Pollen

Potassium was the highest mineral determined in all unifloral bee pollen samples analyzed [Table-1]. Higher significant potassium levels ($p< 0.05$) were found in *Knautia arvensis* (L.) Coulter bee pollen (7619.57mg kg^{-1}) followed by *Onobrychis viciifolia Scop.*, *Crataegus monogyna* J. and *Inula helenium* L. bee pollens (6748.72, 6224.17 and 6078.28mg kg^{-1}, respectively). Significant low value ($p< 0.05$) was determined for *Taraxacum officinale* Web. (2483.51mg kg^{-1}). Similarly, *Helianthus annuus* L. and *Carduus* sp. bee pollen contained low levels of potassium, 3246.50 and

3865.27mg kg^{-1}, respectively [Table-1]. These values were similar with those previously reported: 4000mg kg^{-1}[18], 5530mg kg^{-1} [19], 4950-5131mg kg^{-1} [20] and 2843-5976mg kg^{-1}, respectively [21]. In addition, a mean potassium content of 3868-4274mg kg^{-1} was previous reported [21] for *Brassicaceae* bee pollen. The *Prunus* sp. bee pollen contained much higher potassium levels (8200mg kg^{-1}) [19].

Table 1- The macro and micromineral content of unifloral bee pollen

Botanical name of floral species	Mineral content (mg kg^{-1})**				
	K	Ca	Mg	Fe	Zn
Knautia arvensis (L.) Coulter	7619.57±91.43^{p}	932.25±18.65^{b}	3554.96±71.10^{n}	62.25±3.11^{e}	49.39±2.47^{h}
Helianthus annuus L.	3246.50±38.96^{b}	1409.79±28.20^{g}	376.94±4.83^{c}	27.42±1.37^{b}	31.61±1.58^{c}
Salix sp.	5421.85±65.06^{j}	2630.67±52.61^{n}	1008.28±20.17^{j}	122.87±6.14^{j}	40.06±2.00^{ef}
Brassica sp.	5930.38±71.16^{k}	1252.69±25.05^{c}	749.62±14.99^{g}	52.90±2.65^{c}	31.85±1.59^{c}
Epilobium augustifolium L.	4723.85±56.69^{g}	1419.91±28.40^{g}	1075..85±21.52^{k}	57.80±2.89^{d}	29.61±1.48^{c}
Taraxacum officinale Web.	2483.51±29.80^{a}	1941.98±38.84^{l}	205.14±4.10^{a}	51.44±2.57^{c}	21.25±1.06^{b}
Carduus sp.	3865.27±46.38^{c}	2205.82±44.12^{m}	782.32±15.65^{h}	100.39±5.02^{i}	21.74±1.09^{b}
Pinus sp.	4348.19±52.18^{d}	552.89±11.06^{a}	348.52±6.97^{b}	18.83±0.94^{a}	18.77±0.94^{a}
Crataegus monogyna J.	6224.17±74.69^{n}	1879.44±37.59^{k}	840.79±16.82^{i}	72.01±3.60^{g}	43.35±2.17^{g}
Calluna vulgaris L. Huill.	5974.04±71.69^{l}	2797.85±55.96^{n}	639.67±12.79^{i}	51.73±2.59^{c}	34.65±1.73^{d}
Inula helenium L.	6078.28±72.94^{m}	1277.52±25.55^{d}	1322.41±26.45^{l}	62.96±3.15^{e}	60.51±3.03^{i}
Onobrychis viciifolia Scop.	6748.72±80.98^{o}	1633.99±32.68^{j}	1547.97±30.96^{m}	134.70±6.74^{l}	43.09±2.15^{g}
Centaurea cyanus L.	4525.97±54.31^{f}	1849.87±37.00^{j}	496.60±9.93^{d}	66.65±3.33^{f}	31.71±1.59^{c}
Cichorium intybus L.	4459.60±53.52^{e}	1607.67±32.15^{h}	596.01±11.92^{e}	76.46±3.82^{h}	42.00±2.10^{fg}
Verbascum phlomoides L.	5237.76±62.85^{h}	1334.63±26.69^{e}	804.50±16.09^{h}	99.59±4.98^{i}	40.40±2.02^{ef}
Prunus sp.	5292.68 ±63.51^{i}	1380.29±27.61^{f}	988.98±19.78^{j}	126.90±6.35^{k}	38.79±1.94^{e}

**Samples analyzed in triplicate.*

*** Means followed by the same letters are not significantly different ($p \leq 0.05$)*

In respect of the calcium content, there is a significant difference, within a confidence level of 95%, between the results obtained for the samples analyzed, with the exception of *Helianthus annuus* L. and *Epilobium augustifolium* L. bee pollen (1409.79 and 1419.91mg kg^{-1}, respectively). The significant lowest levels of calcium (p< 0.05) were determined in *Pinus* sp. (552.89mg kg^{-1}) followed by *Knautia arvensis* (L.) Coulter bee pollen (932.25mg kg^{-1}). The unifloral bee pollen from *Calluna vulgaris* L. Huill. and *Salix* sp. contained the highest levels of calcium, with values of 2797.85 and 2630.67mg kg^{-1}, respectively, followed by *Carduus* sp. (2205.82mg kg^{-1}). Similar calcium level for multifloral bee pollen (1146mg kg-1) has been reported [19]. The determined calcium levels reported previously [21] present a high variability, ranged between 105-1080mg kg^{-1}. The average calcium content determined in this work is higher than some data previously reported [19], namely 422-448mg kg^{-1} and similar with other [22], namely 1600mg kg^{-1}. The *Brassica* sp. bee pollen analyzed previously contained slightly higher [19] or much less [21] calcium content, namely 1750mg kg^{-1} and 782mg kg^{-1}, probably were different species from the same genera. Magnesium concentrations vary between large limits (205.14-3554.96mg kg^{-1}), depending on the sample floral origin. The magnesium contents of *Carduus* sp. and *Verbascum phlomoides* L. bee pollen were not significantly different (782.32 and 804.50mg kg^{-1}, respectively) and similar with *Prunus* sp. and *Salix* sp. bee pollen (988.98 and 1008.28mg kg^{-1}, respectively). The magnesium content was significantly (p≤0.05) higher in *Knautia arvensis* (L.) Coulter than in *Taraxacum officinale* Web. bee pollen. Low levels of magnesium were determined also in *Pinus* sp. and *Helianthus annuus* L. bee pollen (348.52 and 376.94mg kg^{-1}, respectively). The magnesium levels of multifloral bee pollen harvested from Poland, China and Korea, present high variability, ranged between 53.2-429.8mg kg^{-1}[21]. Our results are much higher than those reported for multifloral bee pollen produced in Columbia (81.6-98.5mg kg^{-1}) [20]. In addition, the magnesium content determined in this work, with the exception of *Calluna vulgaris* L. Huill., *Centaurea cyanus* L. and *Cichorium intybus* L. bee pollens, which contained between 496.60 and 639.67mg kg^{-1} magnesium, is situated within the range reported for multifloral bee pollen produced in Australia [19] and Japan [22] with values of 716 and 950mg kg^{-1}, respectively. Higher magnesium content for *Brassica* sp. bee pollen has been previously reported (1400 and 1352mg kg^{-1}, respectively) [19, 21]. Minimum and maximum values of iron were 18,83 and 134,70mg kg^{-1} in *Pinus* sp. and *Onobrychis viciifolia Scop.*, respectively. Iron concentration were silimar in *Taraxacum officinale* Web., *Calluna vulgaris* L. Huill., *Brassica* sp., *Knautia arvensis* (L.) Coulter and *Inula helenium* L. bee pollen (51.44, 51.73, 52.90mg kg^{-1}, 62.25 and 62.96mg kg^{-1}, respectively). In the same way were positioned the results obtained for *Verbascum phlomoides* L. (99.59mg kg^{-1}) and *Carduus* sp. (100.39mg kg^{-1}) bee pollen. Iron values in multifloral bee pollen have been reported in the range of 40.4-136.1mg kg^{-1} in Poland, 74.3-365.9mg kg^{-1} in South Korea and 59.0-182.3 in China samples [21]. Similar results are obtained previously [18] for the commercial multifloral bee pollen produced in Spain (40mg kg^{-1}). The present results are not in concordance with the iron values for bee pollen samples reported by [20] and [22] (2221-2576 and 300mg kg-1, respectively). Analogous iron levels were obtained for *Brassica* sp. bee pollen [21] with average values of 59.3mg kg^{-1}, in comparison with other lower value reported previously [19] (27mg kg^{-1}). The zinc levels determined in this work show small variability between samples, ranged between 18.77 (*Pinus* sp.) and 60.51mg kg^{-1} (*Inula helenium* L.) [Table-1]. Not significantly different zinc values were determined for *Taraxacum officinale* Web. and *Carduus* sp. bee pollen (21.25 and 21.74mg kg^{-1}, respectively), and also for *Epilobium augustifolium* L., *Helianthus annuus* L., *Centaurea cyanus* L. and *Brassica* sp. bee pollen (29.61, 31.61, 31.71 and 31.85mg kg^{-1}, respectively). In the same whay, *Prunus* sp., *Salix* sp., *Verbascum phlomoides* L., *Cichorium intybus* L., *Onobrychis viciifolia Scop.* and *Crataegus*

monogyna J. bee pollen contained like average zinc values, ranged between 38.79 and 43.09mg kg^{-1}. Our present results are in concordance with the zinc values in multifloral bee pollen previously [18-19, 21], namely 23.7-60.7, 58 and 36.6mg kg^{-1}, respectively. In addition, our samples contain much lower zinc levels than those reported for Columbian bee pollen [20] (162-311mg kg^{-1}). The values for the zinc contents of our samples were in line with the literature levels. Comparable average zinc levels were reported for *Brassica* sp. bee pollen [21], namely 35.7mg kg^{-1}. Detection limit values of elements were 0.002mg kg^{-1} for K, 0.0025mg kg^{-1} for Ca, 0.0007mg kg^{-1} for Mg, 0.0057mg kg^{-1} for Fe and 0.0024 for Zn. Detection limits corresponded to the concentration associated with three times higher the standard deviation of the background noise recorded in 10 assays of a sample with 3 times higher than the concentration of the expected detection limit. The accuracy of the measurements was evaluated with respect to the certified values by standard addition method with tree different concentration levels (0.1, 0.15 and 0.2mg kg^{-1}. Mean recoveries of 96.77%, 97.42%, 97.99%, 93.29% and 93.34%, respectively, was obtained for K, Ca, Mg, Fe and Zn. The relative standard deviations were less than 10% for all investigated elements. In order to reveal the hidden relationship between data and samples, we perform the chemometric analysis (cluster analysis) of them, using the following options: paired linkage and Euclidean distance between the variables (elements and samples). Taking account of the linkage distances between mineral elements, they were stepwise eliminated in descending order of the distance between them. The biggest linkage distances are between K and other elements: Ca >mg > Fe> Zn (in descending order) [Fig-1]. The K elimination does not modify either the position of the remaining elements in the cluster tree or the linkage distance. The classification of the samples carried out by the cluster analysis was performed by keeping only the Ca, Mg, Fe and Zn averages concentrations for calculation, which permit the small-scale classification of the samples. In the dendrograme resulted [Fig-2], the samples are grouped into three main clusters. Single branches are represented by the *Knautia arvensis* (L.) Coulter and *Pinus* sp. bee pollen, witches are well distinguished from the others. Small linkage distances are found between samples of unifloral bee pollen belonging to the *Compositae* Family, namely *Helianthus annuus* L., *Centaurea cyanus* L., *Cichorium intybus* L., *Taraxacum officinale* Web and *Carduus* sp. bee pollen. The *Inula helenium* L. bee pollen does not present similar mineral profile with others *Compositae* unifloral bee pollens, being grouped in a separate main cluster, along with *Onobrychis viciifolia Scop., Prunus* sp., *Epilobium augustifolium* L., *Brassica* sp. and *Verbascum phlomoides* L. bee pollen, with a similar mineral profile in terms of the five selected elements analysed here. Similar mineral profile presented \the *Salix* sp. and *Calluna vulgaris* L. Huill. bee pollen in terms of the four selected elements taking in to calculation. Strong positive correlation [Table-2] was found only between potassium and magnesium content (r^2=0.744) and potassium and zinc levels (r^2=0.713). In addition, the magnesium and zinc levels determined in the unifloral honeybee-collected pollen samples analyzed were good correlated (r^2=0.580). Our study starts from previous literature data, which indicate bee pollen as a major source of minerals, among others valuable nutrients such as proteins, vitamins, and antioxidants. The mineral levels of selected unifloral bee pollens can be used as quality criteria tool to indicate the most suitable bee pollen samples to be used by humans, depending on specific mineral nutrition purpose. The mineral levels in bee pollen were found to vary considerably due to differences in the floral origin of the pollen. This was true for potassium, magnesium, calcium, manganese and iron, while the zinc content of pollen appeared to be more constant. However, other factors such as soil characteristics such as content of organic matter, pH, and clay mineralogy, which can affect the bioavailability of mineral elements. Geographical conditions are also expected to affect the mineral content of bee pollen. Future analysis is required, not only in determining other mineral element concentrations, but also in analyzing samples collected from different areas, in order to elucidate the potential differences due to substrate, such as soil and water mineral particularities, geographic conditions and environmental factors. The comparison of our results with those from the literature data is difficult specially because the majority of available reported data refers to multifloral bee pollen, a large part of the selected floral sources of the bee pollen analyzed in this work have not been studied before. The results of this study provide valuable information about the mineral contents of Romanian unifloral bee pollen and show that this can be considered a potential source of minerals and contribute for a better balanced diet or for special therapeutic applications. Our preliminary results will give an important contribution, considering that little information on this matrix is available all over the world and in this region of Romania, the supply of bee pollens with specific flora origin can be an add values for the product.

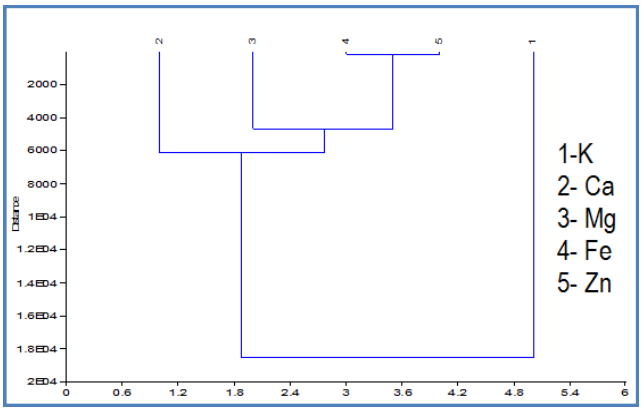

Fig. 1- Dendrogram from Clusters analysis for the mineral elements

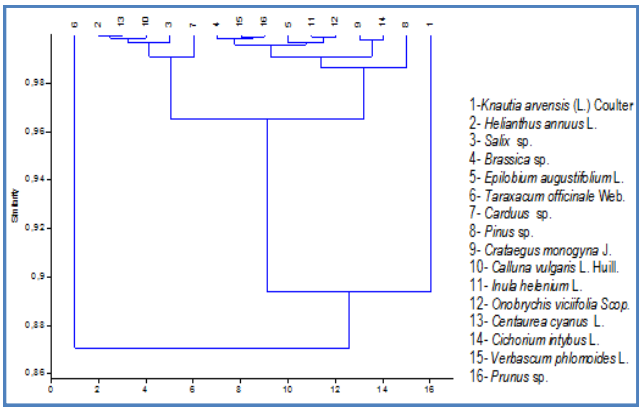

Fig. 2- Dendrogram from Clusters analysis for the mineral content of the unifloral bee pollen

Table 2- Pearson correlation matrix

	K	Ca	Mg	Fe	Zn
K	1				
Ca	-0.106**	1			
Mg	0.744	-0.272**	1		
Fe	0.315**	0.341**	0.218**	1	
Zn	0.713	-0.075**	0.58	0.327**	1

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