Assessment of physico-chemical properties, fatty acid, amino acid and mineral profile of bee pollen from India with a multivariate perspective

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Summary

For the first time, the bee pollen samples from Northern, North-Western and South-Western regions of India were examined for botanical origin, physico-chemical properties, fatty acid, amino acid and mineral profile. The palynological analysis identified six botanical families among which Arecaceae, Apiaceae and Brassicaceae were predominant. The physico-chemical studies found the bee pollen from all floral sources to comply with the framework of international (Brazilian, Argentinean and Swiss) regulations. The surprisingly high amounts of ω-3 fatty acids were disclosed with first-ever detection of eicosatrienoic acid, γ-linolenic acid, eicosapentaenoic acid, heptadecenoic acid, petroselinic acid and dihomo-γ-linolenic acid, establishing them as chemical markers of bee pollen from the regions under study. The amino acid analysis verified the bee pollen as a source of high-quality protein as they contained by 11 % more essential amino acids than FAO reference protein. The mineral composition showed exceptionally high levels of iron (243 $mg \cdot kg^{-1}$), i.e. more than double of FAO recommended iron intake for adults. Principal component analysis showed more than 86 % of variance in first four principal components and strong negative correlation was recorded between ω-6 and ω-3 fatty acids ($r = -0.98$). Hierarchical cluster analysis categorized all bee pollen samples successfully.

Keywords

bee pollen; composition; ω-3 fatty acids; ω-6 fatty acids; essential amino acids; essential minerals; multivariate analysis

Recently, the bee pollen has been attaining importance as an indispensable apicultural product in human diet due to its high nutritional, functional, and medicinal value [1]. It contains carbohydrates (13–55 %), proteins (10–40 %), lipids (1–10 %) and crude fibre (0.3–20 %), which vary according to the botanical source, geographic origin, beekeeping activities, collection time and environmental conditions [2–5]. Bee pollen is also rich in essential fatty acids, minerals and vitamins together with polyphenols (mainly flavonoids), carotenoids and phytosterols, which signify the role of bee pollen as a natural diet supplement [6, 7]. Bee pollen has been used in traditional medicine for centuries and recent studies exhibited its anti-inflammatory, antioxidant, anti-mutagenic, antimicrobial, immunomodulatory and anticancerous activities [8–10].

Various investigations on the palynology, chemical composition and advantages of bee pollen were conducted throughout the world and many countries like China [11], Argentina [12], Brazil [13], Poland [3], Bulgaria [3] and Switzerland [14], establishing quality guidelines of bee pollen for its use as food. The studies demonstrated substantial differences in chemical composition of bee pollen from diverse regions and countries, thus promoting the application of geographical indication.

India, being rich in polliniferous and nectariferous plant species, distinguished climatological seasons and vast geographical areas, is currently the home of about 0.25 million beekeepers and more than 3 million bee colonies producing 94 500 t of honey [15, 16]. However, no data have been reported about a comprehensive characterization of chemical constituents of bee pollen of

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various botanical origins in India [17]. Further, the present research appears to be vital because the botanical and geographical origins are the key aspect of pollen nutritional and functional quality, which determines its potential in food processing industry. Keeping in view the above circumstances, this study was proposed with the objective to evaluate, for the first time, chemical composition of bee pollen of various floral origins collected from the Northern, North-Western and South-Western regions of India.

MATERIALS AND METHODS

Standards, chemicals and reagents

The Supelco CRM47885 fatty acids methyl ester (FAME) mixture (C4 to C24), eighteen L-amino acid standards and heptane (purity 99%) were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Sodium hydroxide, sodium chloride, sodium acetate, ethylenediaminetetraacetic acid (EDTA), ethanol, *n*-hexane and petroleum ether were procured from Loba Chemie (Mumbai, India). Tridecanoic acid methyl ester (C13:0) as an internal standard, petroselinic acid methyl ester (C18:1, *cis*, ω-12) and heneicosenoic acid methyl ester (C21:1, *cis*, ω-9) were purchased from Sigma Aldrich. HPLC-grade reagents *o*-phthaldialdehyde (OPA), ethanol, 2-mercaptoethanol, methanol, triethylamine, tetrahydrofuran, acetic acid and acetonitrile were purchased from Merck (Darmstadt, Germany). Ultrapure water procured from Alfa Aesar (Haverhill, Massachusetts, USA) was used for the preparation of all solutions in HPLC analysis.

Bee pollen samples

The bee pollen samples ($n = 35$) were collected from South-Western, North-Western and Northern regions of India during January to September 2017. The beehives under study were equipped with bottom-fitted pollen traps and pollen pellets were collected twice in a week. The pollen grains were dried in shade at (28 ± 5) °C for 2 h to facilitate sorting for removing the impurities. They were then packed in glass bottles and stored at –18 °C until analysis.

Palynological analysis

The palynological analysis was performed according to the method suggested by Louveaux et al. [18] and Morais et al. [19]. A representative sample of 3 g (approximately 300 pollen grains) was categorized into sub-samples based on their colour. They were washed using 50% ethanol and the microscopic slide was prepared using the glycerin jelly and paraffin, and then identified using Olympus CX23 RFS2 microscope (Olympus, Tokyo, Japan) at 400× (for counting) and 1 000× (for identification) magnifications. A reference pollen collection of CSIR-National Botanical Research Institute (Lucknow, India) along with different pollen guides [20–22] were used for identification of botanical species. The best pollen images were captured using Magnus MIPS 5MP camera (Magnus Analytics, New Delhi, India) with Magnus Pro 3.7 software.

Physico-chemical characteristics and energy value

The moisture content, lipids, crude protein, total ash content and crude fibre content were determined using the respective AOAC methods (Method 925.10, 2003.05, 960.52, 923.03, and 992.16, respectively) [23]. Total carbohydrates, energy value, total soluble solids (*TSS*) and pH were calculated by the methods described by Feás et al. [5]. Water activity (a_w) was measured using the water activity analyser Hygrolab (Rotronic, Bassersdorf, Switzerland) [5].

Fatty acid profile

The Supelco CRM47885 FAME standard mixture was employed as a stock solution for the gas chromatography (GC) calibration. The stock solution was diluted with heptane to prepare the working standards for the calibration curves. The stock solution of the internal standard was prepared by adding 60 mg methyl tridecanoate to 25 ml of heptane. All the working standards and internal standard solutions were stored at –20 °C until analysis.

The fatty acid profile was determined by quantification of FAMEs in the pollen fat extract by GC. The dried bee pollen sample (10 g) was ground in a mortar by a pestle and extracted with *n*-hexane in a Soxhlet apparatus for 8 h. After evaporation of solvent and recovery of oil extract, an aliquot of oil was diluted in *n*-hexane (1 : 10, w/w). A volume of 50 μl of methanolic 2 mol·l^{-1} potassium hydroxide was added to the oil-hexane mixture for the preparation of FAME [11]. For GC analysis, the upper-most layer was collected after incubation for 30 min at room temperature.

FAME were analysed using Agilent 6890N gas chromatograph equipped with flame ionization detector (FID, Agilent Technologies, Santa Clara, California, USA) and SLB-IL111 capillary column (100 m × 0.25 mm internal diameter, 0.20 μm film thickness; Sigma-Aldrich). The temperature of detector and injector was 260 °C and 250 °C, respectively. A volume of 1 μl of each sample was man-

ually injected at a split ratio of 10:1. The initial temperature of oven was 80 °C for 2 min, set from 80 °C to 168 °C at 15 °C·min^{-1}, kept for 18 min at 168 °C, set from 168 °C to 186 °C at 5 °C·min^{-1} and held for 23 min at 186 °C. Helium was employed as carrier gas with a flow rate of 1.0 ml·min^{-1}. Quantification of fatty acids was done by calculating the peak area using the reference standards of Supelco 37 FAME mixture and the results were expressed as gram of fatty acids per kilogram of pollen fat.

Amino acid profile

The bee pollen sample was prepared according to the method described by González–Paramás et al. [24] where 0.1 g of pollen grains were mixed with 3 ml of ultrapure water in glass screw-cap tube and sonicated (Q125 sonicator; Qsonica, Newtown, Connecticut, USA) for up to 40 s for total dispersion of pollen loads. A volume of 3 ml of 12 mol·l^{-1} HCl was added to the dispersion and heated for 22 h at 110 °C. After cooling, filtering and rinsing, the acid hydrolysate was neutralized to pH 4–6 and its volume was made to 50 ml using ultrapure water. It was filtered through a mixed cellulose ester membrane (pore size 0.45 μm; Millipore, Billerica, Massachusetts, USA) and stored frozen until analysis.

The chromatographic analysis was performed according to the method described by da Silva et al. [25]. Agilent HPLC 1200 SL (Agilent Technologies) equipped with G1315C diode array detector (number of signals 8, sampling rate 80 Hz and wavelength 254 nm), Eclipse Plus 95 Å C18 column (250 mm × 4.6 mm, particle size 5 μm, Agilent Technologies) and Rheodyne 7725i injector (Sigma-Aldrich) were employed in the analysis. A volume of 1 ml of the pollen extract was combined with 100 μl of derivatizing mixture that was prepared by dissolving 500 mg OPA reagent in 22.5 ml of ethanol, making the volume up to 25 ml with 0.4 mol·l^{-1} borate buffer (pH 10) and 400 μl of 2-mercaptoethanol was added in the end. The flow was 0.45 ml·min^{-1} till 2 min and after that 1.5 ml·min^{-1}, at a temperature of 40 °C, the injection volume was 20 μl. Eluent A was sodium acetate (20 mmol·l^{-1}), EDTA (10 mg·l^{-1}), and triethylamine (17.2 g·l^{-1}), adjusted to pH 7.2 using tetrahydrofuran and acetic acid, whereas the eluent B consisted of sodium acetate (20 mmol·l^{-1}), adjusted to pH 7.2 using acetic acid, methanol and acetonitrile. The separation gradients were as follows: 100 % A from 0 min to 16 min; 40 % A and 60 % B from 16 min to 17 min, 100 % B from 17 min to 24 min, and then 100 % A from 24 min to 34 min. The quantitative analysis was carried out by comparing the retention times of the standards to those of the sample components.

Mineral profile

Eleven elements were determined using microwave plasma-atomic emission spectroscopy (MP-AES) with slight modifications [26]. An Agilent 4100 MP-AES (Agilent Technologies) equipped with an Agilent SPS 3 Auto Sampler and Agilent 4107 Nitrogen Generator was used for analysis, where the sample introduction system comprised a single pass glass cyclonic spray chamber, glass concentric nebulizer, orange-green for sample tubing and blue-blue for waste tubing. Other instrument conditions were 172 kPa nebulizer pressure, 3 s read time and 15 s stabilization time. All the required calibration standards were prepared using 5% HNO_3. Each sample was digested with 10 ml of concentrated HNO_3, 6.5 ml of concentrated HCl and 1 ml of H_2O_2 for 7 h at 180 °C and, after cooling, it was diluted to make the final volume of 100 ml.

Statistical analysis

All analyses were carried out in triplicate. The data obtained were statistically analysed by one-way ANOVA followed by Duncan's multiple range test (DMRT, $p < 0.05$) using Statistica v.12 software (Stat Soft, Tulsa, Oklahoma, USA) to find out the level of significance. Pearson's correlation and multivariate analysis including principal component analysis (PCA), linear discriminate analysis (LDA) and hierarchical cluster analysis (HCA) were performed using Statistica v.12 and XLSTAT (Addinsoft, New York, New York, USA). Before multivariate analysis, the whole data matrix was auto-scaled for the column, subtracting the median of each column to every sample and dividing it for their standard deviation to ensure the equal weightage for all elements in results.

RESULTS AND DISCUSSION

Palynological identification

Tab. 1 shows results on identification of six plant families among which Arecaceae, Apiaceae and Brassicaceae were recognized as the predominant constituent of pollen (> 45 %). The palynological analysis demonstrated that the pollen samples from South-Western regions of India consisted of 96 % Arecaceae (*Cocos nucifera*) and 4 % Moringaceae (*Moringa oleifera*) and were designated as a coconut sample due to the presence of *Cocos nucifera* (coconut) as a single predomi-

Tab. 1. Palynological analysis of bee pollen samples from various regions of India.

	Coconut bee pollen (n = 6)			Coriander bee pollen (n = 9)			Rapeseed bee pollen (n =10)			Multi-floral bee pollen (n =10)		
	Common name	Family	Species	Common name	Family	Species	Common name	Family	Species	Common name	Family	Species
Predominant pollen (> 45 %)	Coconut	Arecaceae	*Cocos nucifera*	Coriander	Apiaceae	*Coriandrum sativum*	Rapeseed	Brassicaceae	*Brassica napus*	–	–	–
Secondary pollen (16–45 %)	–	–	–	–	–	–	–	–	–	Maize Pearl millet	Poaceae Poaceae	*Zea mays* *Pennisetum glaucum*
Important minor pollen (3–15 %)	Drumstick	Moringaceae	*Moringa oleifera*	Cumin	Umbellifers	*Cuminum cyminum*	–	–	–	Onion weed Pigeon pea	Asphodelaceae Fabaceae	*Asphodelus tenuifolius* *Cajanus cajan*
Minor pollen (< 3 %)	–	–	–	Indian mustard	Brassicaceae	*Brassica juncea*	–	–	–	Cotton	Malvaceae	*Gossypium arboretum*

n – number of samples.

Tab. 2. Comparison of physico-chemical properties and energy value of Indian bee pollen with national standards of different countries.

Parameter	Coconut bee pollen (n = 6)	Coriander bee pollen (n = 9)	Rapeseed bee pollen (n =10)	Multi-floral bee pollen (n =10)	National regulations		
					Argentina [10]	Brazil [11]	Switzerland [12]
Moisture [g·kg^{-1}]	127.21 ± 0.90^{c}	195.93 ± 0.82^{a}	157.55 ± 0.85^{b}	182.52 ± 1.05^{a}	–	< 300	–
Carbohydrates [g·kg^{-1}]	461.58 ± 1.08^{a}	423.26 ± 0.73^{d}	448.93 ± 0.95^{abc}	460.66 ± 0.74^{ab}	–	–	130–550
Proteins [g·kg^{-1}]	253.92 ± 0.92^{a}	220.61 ± 0.83^{b}	196.27 ± 0.54^{c}	218.93 ± 0.91^{b}	150–280	> 80	100–400
Lipids [g·kg^{-1}]	104.25 ± 0.52^{b}	82.73 ± 0.71^{c}	123.82 ± 0.64^{a}	71.37 ± 0.52^{d}	–	> 18	10–100
Ash [g·kg^{-1}]	22.70 ± 0.38^{ab}	34.59 ± 0.42^{a}	32.43 ± 0.47^{a}	29.28 ± 0.73^{a}	< 4	< 40	20–60
Crude fibre [g·kg^{-1}]	30.54 ± 0.33^{ab}	43.13 ± 0.63^{a}	41.28 ± 0.56^{a}	37.50 ± 0.63^{a}	–	–	3–200
Total energy [kJ·kg^{-1}]	15894.59 ± 4.04^{a}	13888.78 ± 12.63^{b}	15458.21 ± 11.72^{a}	14056.98 ± 1.92^{b}	–	–	–
pH	5.23 ± 0.02^{b}	5.48 ± 0.05^{a}	4.74 ± 0.04^{d}	5.16 ± 0.02^{c}	4–6	–	–
Water activity	0.39 ± 0.07^{c}	0.47 ± 0.04^{a}	0.47 ± 0.03^{a}	0.41 ± 0.05^{b}	–	–	–

Results are expressed as mean values ± standard deviations. Moisture is expressed per kilogram of dry matter. Means in a row with same letter in superscript are not significantly different (p > 0.05) based on the Duncan's multiple range test.
n – number of samples.

nant plant source. Likewise, the pollen collected from Indian North-Western region during January–February 2017 contained 92% Apiaceae (*Coriandrum sativum*), 7% Umbellifers (*Cuminum cyminum*) and 1% Brassicaceae (*Brassica juncea*) and was referred to as coriander pollen sample for further analysis. The Northern region bee pollen contained 100% Brassicaceae (*Brassica napus*) and, therefore, was considered as rapeseed pollen sample. The pollen from North-Western region collected during August–September 2017 was found to be multi-floral and consisted of 42% Poaceae (*Zea mays*), 34% Poaceae (*Pennisetum glaucum*), 13% Asphodelaceae (*Asphodelus tenuifolius*), 9% Fabaceae (*Cajanus cajan*) and 2% Malvaceae (*Gossypium arboretum*). Thus, the evaluation of botanical taxa exhibited the true unifloral nature of rapeseed pollen only. However, the coconut and coriander pollen samples would be considered as uni-floral due to the presence of single predominant plant families without any secondary pollen in each case [27].

The microscopic examination showed (Fig. 1) notable differences among the bee pollen samples. The coconut pollen was oval in shape, monad, monoporate and heteropolar with annulus aperture. Our results are in agreement with the previous work on palynology by Ibrahim et al. [28]. The coriander pollen was stick-shaped, monad, isopolar and perprolate with a distinct annulus. The bee pollen from *B. napus* was typically monad and oblate, comprising a colpus aperture. The results for rapeseed pollen were similar to those available in the online palynological database PalDat for the respective family [29]. The multi-floral pollen comprised diverse pollen species with entirely different morphological features. The dominant maize pollen was monoporate and oblate in shape, containing an operculate and psilate aperture (labelled as (i) in Fig. 1D). The pearl millet pollen, label (ii), appeared to be circular, echinate and porate, while onion weed pollen, label (iii), exhibited a sub-triangular to oval shape. Further, the pigeon pea pollen, label (iv), was prolate in shape and monocolpate. The minor pollen, cotton pollen labelled as (v), was spherical and monoporate.

Physico-chemical characteristics and energy value

The results for physic-ochemical characteristics of bee pollen showed (Tab. 2) that pollen samples had lower moisture values (expressed on dry basis) than those established by Brazilian legislations, according to which it should be less than 300 $g \cdot kg^{-1}$ in natural or fresh bee pollen [13]. However, the moisture values determined in our study were higher than those published by Kostić et al. [30] (43.5–143.5 $g \cdot kg^{-1}$). This may be explained on the basis of partial shade drying of the studied pollen at the farm level only, rather than the mechanical dehydration.

Content of carbohydrates, the major component present in pollen, ranged from 423.26 $g \cdot kg^{-1}$ in the coriander bee pollen to 461.58 $g \cdot kg^{-1}$ in coconut pollen. These results fall within the range presented by Campos et al. [3] (130–550 $g \cdot kg^{-1}$) and Swiss legislation [14].

The protein content varied from 196.27 $g \cdot kg^{-1}$ in the rapeseed pollen to 253.92 $g \cdot kg^{-1}$ in coconut pollen sample and was higher than the minimum values established by Brazilian, Argentinean and Swiss regulations [12–14]. The protein content of studied rapeseed pollen was found to be lower than the Chinese rapeseed pollen (272.7 $g \cdot kg^{-1}$) [11].

The lipids content was slightly higher in our study than the maximum value of Swiss legislation (100 $g \cdot kg^{-1}$) [14]. This inconsistency may be due to the plant environment and genetics. The present

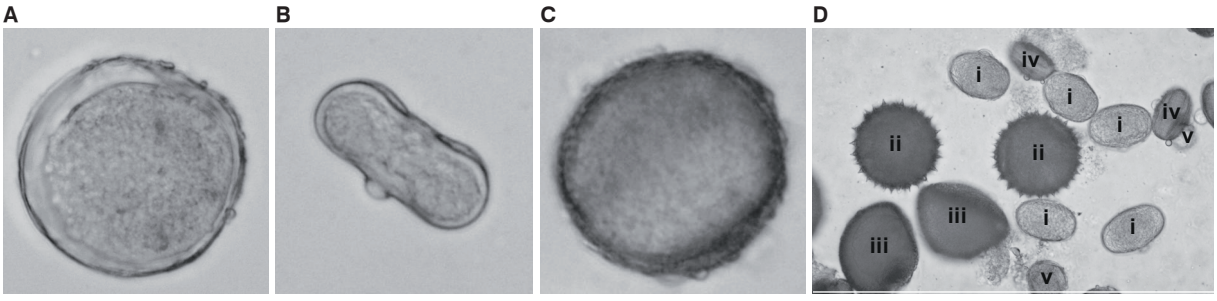

Fig. 1. Compound microscopic view of Indian bee pollen of four botanical origins.

A – coconut bee pollen, B – coriander bee pollen, C – rapeseed bee pollen, D – multi-floral bee pollen.
(i) – maize bee pollen, (ii) – pearl millet bee pollen, (iii) – onion weed bee pollen, (iv) – pigeon pea bee pollen, (v) – cotton bee pollen.

Tab. 3. Fatty acid composition of Indian bee pollen of four botanical origins.

Fatty acids [g·kg^{-1}]		Bee pollen			
		Coconut (n = 6)	Coriander (n = 9)	Rapeseed (n = 10)	Multi-floral (n = 10)
C4:0	Butyric acid	1.4 ± 0.1 ^{a}	ND	ND	ND
C6:0	Caproic acid	2.0 ± 0.2 ^{a}	ND	ND	ND
C8:0	Caprylic acid	5.1 ± 0.1 ^{a}	ND	ND	ND
C10:0	Capric acid	3.5 ± 0.1 ^{a}	ND	3.5 ± 0.1 ^{a}	5.2 ± 0.2 ^{b}
C11:0	Undecanoic acid	ND	ND	1.5 ± 0.2 ^{a}	ND
C12:0	Lauric acid	3.5 ± 0.2 ^{a}	ND	1.3 ± 0.1 ^{b}	ND
C14:0	Myristic acid	3.9 ± 0.1 ^{c}	5.5 ± 0.2 ^{c}	3.0 ± 0.1 ^{b}	16.5 ± 0.3 ^{a}
C15:0	Pentadecanoic acid	ND	0.7 ± 0.1 ^{a}	ND	ND
C16:0	Palmitic acid	86.0 ± 0.1 ^{c}	90.8 ± 0.2 ^{c}	136.8 ± 0.4 ^{b}	146.1 ± 0.3 ^{a}
C16:1, *cis*-Δ^{7} (ω-7)	Palmitoleic acid	3.0 ± 0.1 ^{a}	1.0 ± 0.1 ^{b}	ND	ND
C17:0	Heptadecanoic acid	32.4 ± 0.4 ^{a}	2.5 ± 0.1 ^{b}	ND	ND
C17:1, *cis*-Δ^{7} (ω-7)	Heptadecenoic acid	2.6 ± 0.1 ^{d}	16.6 ± 0.3 ^{ab}	8.7 ± 0.1 ^{c}	17.9 ± 0.3 ^{a}
C18:0	Stearic acid	12.0 ± 0.1 ^{d}	19.0 ± 0.2 ^{c}	24.1 ± 0.1 ^{ab}	27.5 ± 0.2 ^{a}
C18:1, *trans*-Δ^{9} (ω-9)	Elaidic acid	4.4 ± 0.1 ^{ab}	1.1 ± 0.2 ^{c}	2.8 ± 0.1 ^{ab}	4.8 ± 0.1 ^{a}
C18:1, *cis*-Δ^{9} (ω-9)	Oleic acid	104.2 ± 0.2 ^{b}	64.1 ± 0.4 ^{d}	76.1 ± 0.3 ^{c}	126.2 ± 0.3 ^{a}
C18:1, *cis*-Δ^{6} (ω-12)	Petroselinic acid	ND	11.0 ± 0.2 ^{a}	ND	ND
C18:2, *trans,trans*-$\Delta^{9,12}$ (ω-6)	Linoelaidic acid	ND	5.5 ± 0.2 ^{a}	ND	ND
C18:2, *cis,cis*-$\Delta^{9,12}$ (ω-6)	Linoleic acid	113.2 ± 0.2 ^{a}	32.5 ± 0.1 ^{d}	60.8 ± 0.2 ^{c}	80.2 ± 0.4 ^{b}
C18:3, *cis,cis,cis*-$\Delta^{9,12,15}$ (ω-3)	α-Linolenic acid	144.0 ± 0.3 ^{b}	162.8 ± 0.3 ^{a}	87.9 ± 0.2 ^{c}	5.0 ± 0.1 ^{d}
C18:3, *cis,cis,cis*-$\Delta^{6,9,12}$ (ω-6)	γ-Linolenic acid	55.4 ± 0.3 ^{c}	5.5 ± 0.2 ^{d}	290.8 ± 0.2 ^{a}	282.2 ± 0.3 ^{b}
C20:0	Arachidic acid	11.9 ± 0.3 ^{abc}	6.0 ± 0.2 ^{d}	13.2 ± 0.1 ^{ab}	15.0 ± 0.1 ^{a}
C20:1, *cis*-Δ^{11} (ω-9)	Eicosenoic acid	13.2 ± 0.1 ^{a}	10.1 ± 0.1 ^{b}	2.9 ± 0.3 ^{c}	ND
C21:1, *cis*-Δ^{9} (ω-9)	Heneicosenoic acid	4.8 ± 0.1 ^{a}	1.8 ± 0.2 ^{b}	3.7 ± 0.2 ^{a}	ND
C20:2, *cis,cis*-$\Delta^{11,14}$ (ω-6)	Eicosadienoic acid	ND	3.2 ± 0.1 ^{b}	1.7 ± 0.1 ^{bc}	7.2 ± 0.2 ^{a}
C20:3, *cis,cis,cis*-$\Delta^{8,11,14}$ (ω-6)	Dihomo-γ-linolenic acid	ND	0.9 ± 0.1 ^{c}	12.3 ± 0.3 ^{b}	30.1 ± 0.2 ^{a}
C20:3, *cis,cis,cis*-$\Delta^{11,14,17}$ (ω-3)	Eicosatrienoic acid	157.6 ± 0.4 ^{b}	540.1 ± 0.3 ^{a}	138.3 ± 0.3 ^{c}	118.9 ± 0.2 ^{d}
C20:4, *cis,cis,cis,cis*-$\Delta^{5,8,11,14}$ (ω-6)	Arachidonic acid	183.6 ± 0.2 ^{a}	ND	ND	5.8 ± 0.1 ^{b}
C22:0	Behenic acid	9.8 ± 0.1 ^{b}	ND	2.1 ± 0.1 ^{c}	19.7 ± 0.1 ^{a}
C22:1, *cis*-Δ^{13} (ω-9)	Erucic acid	ND	3.2 ± 0.2 ^{a}	4.6 ± 0.2 ^{a}	ND
C22:6, *cis,cis,cis,cis,cis,cis*-$\Delta^{4,7,10,13,16,19}$ (ω-3)	Docosahexaenoic acid	ND	1.5 ± 0.1 ^{a}	ND	ND
C23:0	Tricosanoic acid	ND	1.4 ± 0.1 ^{c}	79.7 ± 0.1 ^{a}	66.5 ± 0.4 ^{b}
C20:5, *cis,cis,cis,cis,cis*-$\Delta^{5,8,11,14,17}$ (ω-3)	Eicosapentaenoic acid	33.6 ± 0.2 ^{a}	5.1 ± 0.2 ^{bc}	3.5 ± 0.1 ^{cd}	7.2 ± 0.1 ^{b}
C24:0	Lignoceric acid	6.6 ± 0.2 ^{bc}	4.3 ± 0.1 ^{bcd}	7 ± 0.2 ^{b}	14.9 ± 0.1 ^{a}
C24:1, *cis*-Δ^{15} (ω-9)	Nervonic acid	ND	1.6 ± 0.1 ^{a}	2.1 ± 0.1 ^{a}	2.1 ± 0.1 ^{a}
Saturated fatty acids (SFA) [g·kg^{-1}]		178.1	130.2	289.0	311.4
Monounsaturated fatty acids (MUFA) [g·kg^{-1}]		127.8	109.4	98.1	146.2
Polyunsaturated fatty acids (PUFA) [g·kg^{-1}]		687.4	757.1	595.3	536.6
Total unsaturated fatty acids (TUSFA) [g·kg^{-1}]		815.2	866.5	693.4	687.6
Ratio TUSFA/SFA		4.6	6.7	2.4	2.2

Results are expressed as mean values ± standard deviations per kilogram of dry matter. Means in a row with same letter in superscript are not significantly different ($p > 0.05$) based on the Duncan's multiple range test.
n – number of samples, ND – not detected.

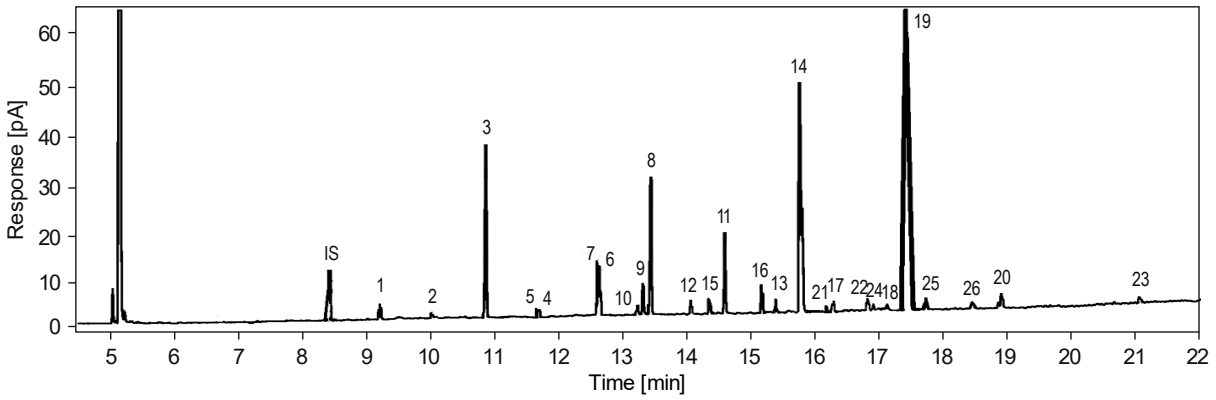

Fig. 2. Chromatogram of fatty acid methyl esters in coriander bee pollen.

IS – internal standard (tridecanoic acid), 1 – myristic acid, 2 – pentadecanoic acid, 3 – palmitic acid, 4 – palmitoleic acid, 5 – heptadecanoic acid, 6 – heptadecenoic acid, 7 – stearic acid, 8 – oleic acid, 9 – petroselinic acid, 10 – elaidic acid, 11 – linoleic acid, 12 – linoelaidic acid, 13 – γ-linolenic acid, 14 – α-linolenic acid, 15 – arachidic acid, 16 – eicosenoic acid, 17 – eicosadienoic acid, 18 – dihomo-γ-linolenic acid, 19 – eicosatrienoic acid, 20 – eicosapentaenoic acid, 21 – heneicosenoic acid, 22 – erucic acid, 23 – docosahexaenoic acid, 24 – tricosanoic acid, 25 – lignoceric acid, 26 – nervonic acid.

data are in accordance with the limit in literature summarized by CAMPOS et al. [3] (10–130 g·kg^{-1}).

In case of ash and crude fibre contents, the values were 22.70–34.59 g·kg^{-1} and 30.54–43.13 g·kg^{-1}, respectively, showing a non-significant relation among the pollen derived from the floral origin of Northern and North-Western regions of India, where the alluvial soil is spread throughout the regions. The similar plant-growing conditions (soil) led to the non-significant difference between the coriander, rapeseed and mixed pollen. Ash content was in accordance with the Brazilian, Argentinean and Swiss legislation [12–14] and crude fibre levels in Indian pollen were found to meet the criteria of Swiss legislation [14].

The low energy value (from 13 888.78 kJ·kg^{-1} to 15 894.59 kJ·kg^{-1}) calculated for Indian pollen qualified them as a low-energy food. These results fall in the range published by KOSTIĆ et al. (14 671.19–16 551.90 kJ·kg^{-1}) [30] and YANG et al. (15 899.20–20 334.20 kJ·kg^{-1}) [11].

The low pH (4.74–5.48) and a_w (0.39–0.47) of bee pollen samples (Tab. 2) may be beneficial in inhibiting the growth of moulds, which might produce the mycotoxins [5]. The obtained values were similar to the data (pH 4.3–5.2, a_w 0.21–0.54) given by FEÁS et al. [5].

Fatty acid profile

The fatty acid composition (Tab. 3) exhibited the significant contents of essential fatty acids such as α-linolenic acid and linoleic acid, which can be useful to prevent diseases like the cardiovascular ones, inflammation and diabetes. Eicosatrienoic acid was found at highest contents (118.9–540.1 g·kg^{-1}), followed by γ-linolenic acid (5.5–290.8 g·kg^{-1}), α-linolenic acid (5.0–162.8 g·kg^{-1}), oleic acid (64.1–126.2 g·kg^{-1}), palmitic acid (86.0–146.1 g·kg^{-1}) and linoleic acid (32.5–113.2 g·kg^{-1}). Several rare fatty acids were present in minute amounts, like eicosapentaenoic acid, which is hardly found in plant foods, dihomo-γ-linolenic, petroselinic, heptadecenoic and lignoceric acids. Moreover, docosahexaenoic acid was also found in coriander bee pollen. This compound is otherwise present in fish oil. It can be claimed that eicosatrienoic, γ-linolenic, eicosapentaenoic, heptadecenoic, dihomo-γ-linolenic and petroselinic acids were detected for the first time in bee pollen and, hence, could be considered as markers of bee pollen collected from Northern, North-Western, and South-Western regions of India (Fig. 2).

Polyunsaturated fatty acids (PUFA) was the dominating fatty acids (536.6–757.1 g·kg^{-1}) category followed by saturated fatty acids (SFA, 130.2–311.4 g·kg^{-1}) and monounsaturated fatty acids (MUFA, 98.1–146.2 g·kg^{-1}). The ratio of total unsaturated to saturated fatty acids varied from 2.2 to 6.7 (i.e. > 1), which supports the novel approach of using bee pollen as a functional food or dietary supplement. Further, ratio ω-6/ω-3, an important criterion to evaluate the health-promoting properties of food, should be 5 : 1 or less. In "western" diets, this criterion is 10 : 1 to 25 : 1, which may lead to the development of various chronic diseases. In this study, the bee pollen had

the ω-6/ω-3 ratio from 0.06 to 3.09, due to the high content of eicosatrienoic acid and α-linolenic acid, which is equivalent to fish. Therefore, the pollen analysed in this research could be considered as an essential source of ω-3 fatty acids for the human diet. The obtained results are more or less in agreement with data on Portuguese [5] and Chinese [11] bee pollen, where α-linolenic, palmitic, oleic and linoleic acids were the major fatty acids detected.

Amino acid profile

The amino acid composition (Tab. 4) revealed that all pollen cultivars contained essential amino acids and the total amino acid amount varied from 188.57 $g \cdot kg^{-1}$ for rapeseed pollen to 246.86 $g \cdot kg^{-1}$ for coconut pollen. Glutamic acid (23.55–30.27 $g \cdot kg^{-1}$), aspartic acid (23.17–28.13 $g \cdot kg^{-1}$), proline (20.81–25.19 $g \cdot kg^{-1}$), leucine (17.61–24.66 $g \cdot kg^{-1}$), alanine (15.87–23.25 $g \cdot kg^{-1}$) and lysine (13.48–20.14 $g \cdot kg^{-1}$) were the major amino acids present in all pollen varieties (Fig. 3). The content of essential amino acids varied from 84.90 $g \cdot kg^{-1}$ in rapeseed bee pollen to 109.38 $g \cdot kg^{-1}$ in coconut pollen. The percentage of total essential amino acids to total amino acids was found highest in the rapeseed pollen sample (45%) and the lowest in coriander pollen (44.2%). The determined values are sufficiently above that of FAO reference protein (33.9%) [31]. These data ensure that Indian origin bee pollen may be a "healthy" source of high-quality protein. The obtained results are similar to Chinese bee pollen [11] where proline, glutamic acid and aspartic acid were the predominant amino acids, along with 39.4% essential amino acid proportion.

Mineral profile

The mineral composition of bee pollen (Tab. 5) exhibited that phosphorus (3 200–4 600 $mg \cdot kg^{-1}$) and potassium (3 600–4 100 $mg \cdot kg^{-1}$) were the dominating elements, and bee pollen from all floral sources except coriander contained a significantly higher amount of iron than its minimum intake value as recommended by FAO [32]. Iron is an essential element needed for red blood cells

Tab. 4. Amino acid composition of Indian bee pollen of four botanical origins.

Amino acids [$g \cdot kg^{-1}$]	Coconut bee pollen (n = 6)	Coriander bee pollen (n = 9)	Rapeseed bee pollen (n = 10)	Multi-floral bee pollen (n = 10)
Essential amino acids				
Arginine	9.76 ± 0.06^{d}	10.32 ± 0.02^{c}	10.84 ± 0.01^{b}	11.98 ± 0.04^{a}
Histidine	12.31 ± 0.01^{a}	5.84 ± 0.03^{c}	7.65 ± 0.02^{b}	3.46 ± 0.07^{d}
Isoleucine	7.27 ± 0.04^{c}	10.09 ± 0.02^{a}	3.86 ± 0.06^{d}	9.55 ± 0.03^{b}
Leucine	24.66 ± 0.05^{a}	19.95 ± 0.06^{c}	17.61 ± 0.01^{d}	22.59 ± 0.04^{b}
Lysine	20.14 ± 0.03^{a}	18.68 ± 0.04^{b}	13.48 ± 0.07^{d}	17.67 ± 0.02^{c}
Methionine	4.68 ± 0.02^{a}	1.23 ± 0.05^{d}	3.45 ± 0.03^{b}	2.71 ± 0.08^{c}
Phenylalanine	6.54 ± 0.06^{d}	9.17 ± 0.01^{a}	8.26 ± 0.05^{c}	8.80 ± 0.03^{b}
Threonine	10.42 ± 0.04^{a}	8.41 ± 0.02^{b}	7.37 ± 0.04^{c}	5.26 ± 0.01^{d}
Tryptophan	3.71 ± 0.05^{b}	2.57 ± 0.03^{d}	2.91 ± 0.02^{c}	4.59 ± 0.08^{a}
Valine	9.89 ± 0.03^{b}	10.26 ± 0.04^{a}	9.47 ± 0.06^{c}	7.14 ± 0.05^{d}
Non-essential amino acids				
Alanine	23.25 ± 0.07^{a}	17.15 ± 0.05^{c}	18.24 ± 0.01^{b}	15.87 ± 0.02^{d}
Aspartic acid	28.13 ± 0.02^{a}	26.49 ± 0.01^{b}	23.17 ± 0.03^{d}	24.32 ± 0.04^{c}
Cysteine	2.54 ± 0.08^{a}	2.31 ± 0.03^{b}	2.09 ± 0.05^{c}	1.86 ± 0.03^{d}
Glutamic acid	30.27 ± 0.04^{a}	27.82 ± 0.02^{b}	23.55 ± 0.09^{d}	25.55 ± 0.05^{c}
Glycine	18.36 ± 0.06^{a}	12.39 ± 0.03^{c}	2.16 ± 0.02^{d}	15.45 ± 0.06^{b}
Proline	21.42 ± 0.05^{c}	23.74 ± 0.04^{b}	25.19 ± 0.01^{a}	20.81 ± 0.02^{d}
Serine	9.08 ± 0.03^{a}	8.26 ± 0.05^{c}	5.24 ± 0.04^{d}	8.46 ± 0.01^{b}
Tyrosine	4.43 ± 0.02^{a}	3.87 ± 0.04^{c}	4.03 ± 0.03^{b}	3.63 ± 0.05^{d}
Total amino acids [$g \cdot kg^{-1}$]	246.86 ± 0.01^{a}	218.55 ± 0.03^{b}	188.57 ± 0.02^{d}	209.70 ± 0.01^{c}
Total essential amino acids [$g \cdot kg^{-1}$]	109.38 ± 0.03^{a}	96.52 ± 0.04^{b}	84.90 ± 0.06^{d}	93.75 ± 0.02^{c}

Results are expressed as mean values ± standard deviations per kilogram of dry matter. Means in a row with same letter in superscript are not significantly different ($p > 0.05$) based on the Duncan's multiple range test.
n – number of samples.

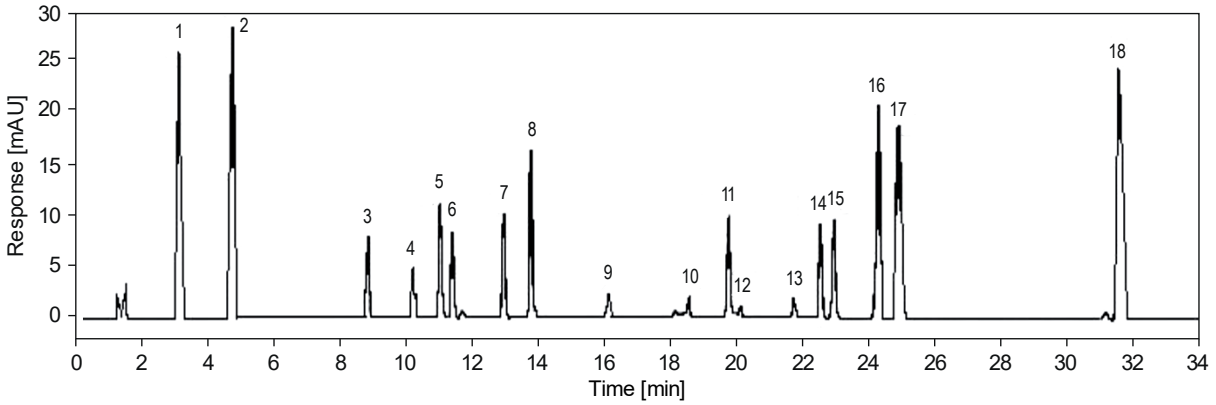

Fig. 3. Chromatogram of amino acids in coriander bee pollen.

1 – aspartic acid, 2 – glutamic acid, 3 – serine, 4 – histidine, 5 – glycine, 6 – threonine, 7 – arginine, 8 – alanine, 9 – tyrosine, 10 – cysteine, 11 – valine, 12 – methionine, 13 –tryptophan, 14 – phenylalanine, 15 – isoleucine, 16 – leucine, 17 – lysine, 18 – proline.

production and may be helpful in preventing anemia in India, where 51 % women are anemic [33]. The content of copper varied from 7.82 mg·kg^{-1} in mixed bee pollen to 12.81 mg·kg^{-1} in rapeseed bee pollen. Chromium was present in a minute amount (0.65–1.79 mg·kg^{-1}), while cobalt was not detected in any pollen sample. The variations in mineral composition of pollen are typically associated with the growing conditions of plants, such as chemical composition irrigation water, soil, plant source and geographic origin [34].

The contents of magnesium, calcium, phosphorus, copper and zinc were found to be within standard limits, i.e. 200–3 000 mg·kg^{-1}, 200–3 000 mg·kg^{-1}, 800–6 000 mg·kg^{-1}, 2–16 mg·kg^{-1} and 30–250 mg·kg^{-1}, respectively, as suggested by Campos et al. [3] who evaluated the composition of bee pollen from Bulgaria, Brazil, Switzerland and Poland.

Correlation among the physico-chemical parameters

The Pearson's correlation matrix revealed a very strong positive correlation between the ω-3 fatty acids and PUFA (r = 0.96). This established the dependence of ω-3 fatty acids on PUFA, while there existed a nearly perfect negative association between ω-6 fatty acids and ω-3 fatty acids (r = –0.98), demonstrating the decrease in ω-6

Tab. 5. Mineral composition of Indian bee pollen from four botanical origins.

Minerals [mg·kg^{-1}]	Coconut bee pollen (n = 6)	Coriander bee pollen (n = 9)	Rapeseed bee pollen (n = 10)	Multi-floral bee pollen (n = 10)
Major minerals				
Ca	1 800 ± 6^{ab}	1 900 ± 11^{a}	1 600 ± 9^{c}	2 000 ± 13^{a}
K	3 600 ± 18^{b}	4 100 ± 23^{a}	3 600 ± 30^{b}	4 000 ± 16^{a}
Mg	920 ± 7^{a}	970 ± 10^{a}	1 050 ± 5^{a}	840 ± 6^{ab}
P	3 300 ± 6^{c}	4 600 ± 13^{a}	3 200 ± 11^{cd}	3 600 ± 8^{b}
Na	284.00 ± 0.22^{a}	150.00 ± 1.3^{d}	171.00 ± 0.85^{c}	204.00 ± 0.50^{b}
Trace minerals				
Fe	133.00 ± 0.51^{c}	82.40 ± 0.20^{d}	204.00 ± 0.64^{b}	243.00 ± 0.18^{a}
Cu	10.95 ± 0.13^{a}	10.42 ± 0.25^{a}	12.81 ± 0.09^{a}	7.82 ± 0.17^{b}
Mn	70.81 ± 0.54^{a}	60.43 ± 0.18^{b}	44.55 ± 0.11^{c}	36.30 ± 0.24^{d}
Zn	53.82 ± 0.51^{a}	40.95 ± 0.64^{b}	39.82 ± 0.38^{bc}	25.27 ± 0.41^{d}
Cr	0.65 ± 0.01^{bc}	1.15 ± 0.04^{a}	0.81 ± 0.02^{b}	1.79 ± 0.084^{a}
Co	ND	ND	ND	ND

Results are expressed as mean values ± standard deviations per kilogram of dry matter. Means in a row with same letter in superscript are not significantly different (p > 0.05) based on the Duncan's multiple range test.
n – number of samples, ND – not detected.

fatty acids with an increase in ω-3 fatty acids. Our results suggested that bee pollen with high ω-3 fatty acids possessed low values of ω-6 fatty acids, which are also beneficial for human health. These results are in agreement with Čeksterytė et al. [35] that revealed the higher level of ω-3 fatty acids (31.6%) in Lithuanian bee pollen than ω-6 fatty acids (9.4%). PUFA and ω-3 fatty acids represented a high negative association with SFA ($r = –0.96$ and $r = –0.91$, respectively) showing the high levels of PUFA and ω-3 fatty acids with respect to lower levels of SFA.

Multivariate analysis

The main aim of multivariate analysis was the demonstration of similarities or differences to differentiate the position of pollen samples from four botanical sources. According to the Kaiser criterion, the major four principal components (PCs) were derived that accounted for 86.9% of the variation in the 35 bee pollen samples. PC1 explained 33.4%, PC2 explained 30.2%, PC3 explained 18.9% and PC4 showed 4.5% of the variation.

Factor loadings for the first four PCs showed that PC1 was positively correlated with iron, SFA and ω-6 fatty acids, revealing a negative association with PUFA and ω-3 fatty acids (Fig. 4A). Likewise, PC2 differentiated the bee pollen samples on the basis of contents of calcium, potassium, lipids, copper, phosphorus and magnesium. PC3 characterized the pollen samples according to sodium content, water activity and fibre content. PC4 with less contribution (4.5%) differentiated the bee pollen samples based on levels of carbohydrates (Fig. 4A).

Fig. 4B shows the two-dimensional bi-plots of studied data where samples from all sources were observed very close forming clusters thus exhibiting a high degree of similarity. All coriander pollen samples positioned on the upper-left of PC1 were strongly associated with "healthy" ω-3 fatty acids and PUFA, while all multi-floral samples on the uppper-right of PC1 were linked to MUFA and minerals, chromium and calcium (Fig. 4B). Thus, ω-3 fatty acids, PUFA and MUFA could be employed to differentiate coriander pollen from multi-floral bee pollen.

For the classification of bee pollen samples, HCA was applied to the data and four clusters were obtained (Fig. 5). The sample similarities were calculated using Euclidean distances. The results of HCA as a dendrogram showing four clusters corresponded to each botanical origin of the bee pollen samples. From the top to bottom, the first cluster consisted of coconut bee pollen samples and second cluster comprised rapeseed pollen samples. In case of the third cluster, all multi-

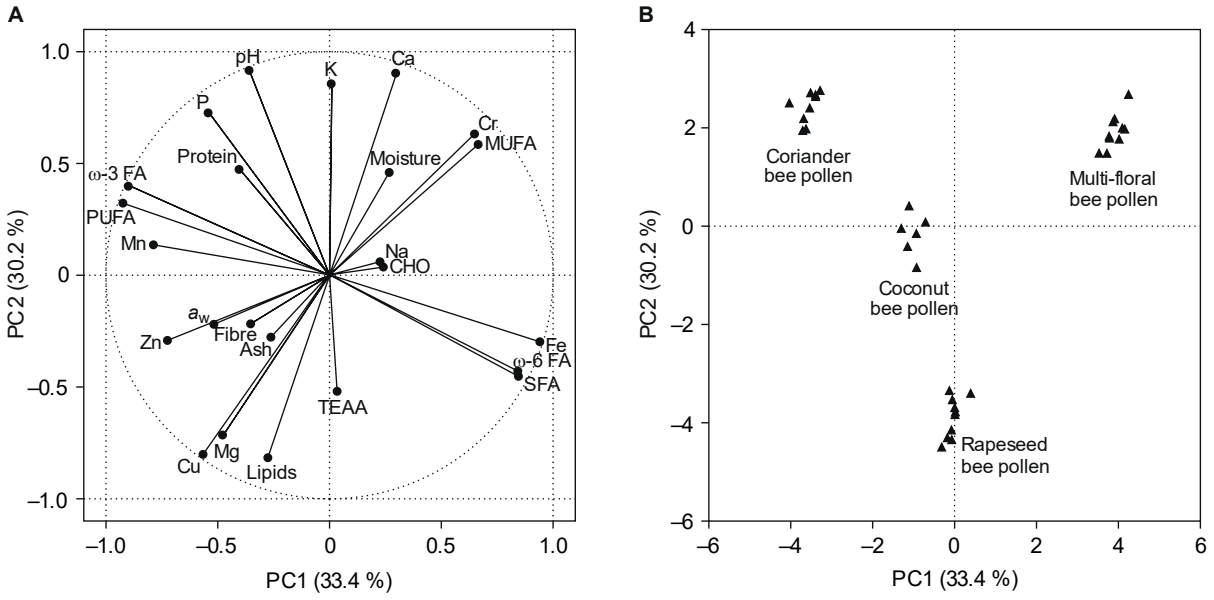

Fig. 4. Principal component analysis of Indian bee pollen of four botanical origins.

A – Projection of variables on factor-plane (1 x 2), B – Projection of botanical origins on factor-plane (1 x 2).
CHO – carbohydrates, a_w – water activity, SFA – saturated fatty acids, MUFA – monosaturated fatty acids, PUFA – polyunsaturated fatty acids, ω-3 FA – omega-3 fatty acids, ω-6 FA – omega-6 fatty acids; TEAA – total essential amino acids.

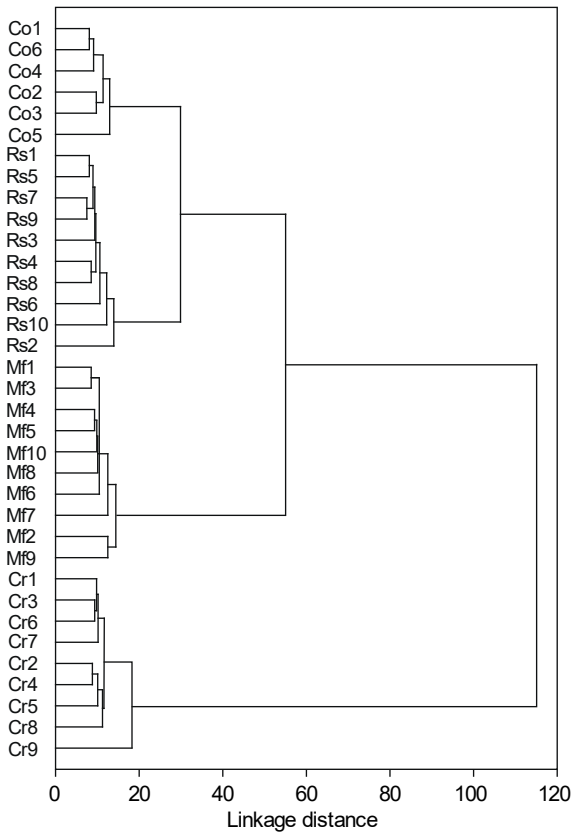

Fig. 5. Hierarchical cluster analysis of Indian bee pollen of four botanical origins.

Co – coconut bee pollen, Cr – coriander bee pollen, Rs – rapeseed bee pollen, Mf – multi-floral bee pollen.

floral samples were aggregated, while the fourth cluster was composed of coriander pollen samples. LDA was also employed to classify the bee pollen samples based on their floral origin and results demonstrated that 100 % of the samples were well classified into each bee pollen origin.

CONCLUSIONS

The present investigation demonstrated significant differences among the pollen samples from different floral sources and acknowledged that physico-chemical properties of Indian bee pollen were similar to other countries such as Brazil, Switzerland or Argentina. The low energy value, high content of essential amino acids and fatty acids, proteins, iron and other minerals suggest that bee pollen is a "healthy" food supplement and potentially functional food. Currently, it is an under-explored commodity in India and this study identifies a strong need to characterize the bee pollen from different parts of India and to establish bee pollen quality standards, providing directions to the Government of India.

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Supplementary data

Supplementary data related to this article can be found at http://www.vup.sk/en/download.php?bulID=1998.

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