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ARTICLE

Determination and Classification of Jordanian Propolis Samples According to Their Elemental Profile

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Abstract: It is possible to classify various propolis samples based on their botanical or geographic origins, since environmental conditions and the harvest season have an impact on the chemical ingredients that make up propolis. In the current research, the determination and geographical classification of propolis samples according to their mineral/metal content are addressed. Acid digestion was employed for mineral/metal recovery and inductively-coupled plasma-optical emission spectrometry was used for accurate quantification. Multivariate techniques, PCA and PLS-DA, were applied for unsupervised and supervised classification for different propolis samples. The measured analytical data can be simply manipulated using chemometric tools, like PCA and PLS-DA. Adequate results were reported by PLS-DA to separate propolis samples that were obtained from industrial and agricultural, but not desert regions. The variations in mineral/metal contents present in propolis were helpful for discrimination purposes. Minerals, like Ca, Na, and K, were the most significant variables for propolis discrimination according to their geographical origins. Based on mineral and metal content, PLS-DA model was effective to classify propolis samples with 92% accuracy as estimated from the confusion matrix.

Keywords: ICP-OES, Jordanian propolis, Elemental determination, Regional classification.

Introduction

Consumed as food, honey is a vital ingredient in many processed foods. It could be a significant source of vitamins and other essential macro- and micronutrients for human health[1]. Aside from honey, other bee products, including pollen and propolis, are commonly utilized in medicine to treat cancer, diabetes and heart diseases. Propolis and bee pollen have anti-inflammatory and antioxidant properties because of their high flavonoid and other phenolic compounds' concentrations[2,3]. In addition, propolis is also a good source of useful minerals[4–6]. Flavonoids, terpenes, phenylpropanoids, lignans and coumarins were the principal organic chemicals found in various types of propolis[7]. Propolis has also been suggested as a chemical preservative in certain food products or as a germicide for food packaging[2]. Due to the presence of many active ingredients, propolis can be utilized in endodontic treatments as an intracanal medication and as a canal-irrigation solution[8]. Recent studies have revealed that using propolis as a storage medium can keep the periodontal ligament cells healthy and can also prevent osteoclastic activity thanks to one of the active components that it contains[8]. Propolis can both stimulate the synthesis of tubular dentin and lessen pulp inflammation during critical pulp therapy[8,9].

On the other hand, mineral content in propolis is often utilized to identify the regions of origin as a sign of environmental pollution[10]

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and to provide trustworthy traceability techniques[5,6,10]. The occurrence of harmful substances in propolis is linked to anthropogenic environmental contamination in the area surrounding the apiaries, which comes from a variety of sources, including air, water, plants and soil. Industrial sources are some likely sources of emissions of cadmium and lead[10]. Actually, the ability of some plant species to collect significant amounts of heavy metals in their biomass is well known and they are classified as hyperaccumulators[10]. In regions where beekeeping is practiced for commercial purposes, it is crucial to recognize bee plants with this characteristic in order to ensure that the product fully complies with the technical and sanitary specifications imposed by regulatory agencies and the demanding consumer market in modern times[6]. Furthermore, it is well-known that flavonoids frequently combine with metals, including iron, chromium, nickel, copper or lead, to form stable complexes[10]. These metals become thus, as a result of this behavior, one of the main propolis pollutants.

The determination of minerals and other heavy metals is often accomplished by atomic absorption spectrometry and inductively-coupled plasma–optical emission spectrometry[11,12]. Analysis of a large number of propolis from different countries indicated that minerals' contents, like Ca, Fe, K, Mg and P, were much higher than thse of heavy metals, like Cr, Cu, Ni and Zn[6,11]. There are many published research articles that correlate the presence of heavy or toxic metals in propolis with industrial or agricultural activities. For instance, Gong *et al.* investigated the relationship between geographical origin and the contents of Ca, Al, Mg, K, Fe, Na, Zn, Mn, Sr, Cu, Cr, Ni and other metals, like Ar, Cd and Pb[13]. Moreover, the authors have applied advanced statistical tools for sample classification according to their geographical sources[13].

Several methods of data analysis can be carried out for the classification of profiles of diverse backgrounds employing sets of features provided by various analytical instruments, such as NMR spectroscopy, UV spectroscopy, IR spectroscopy and ICP[14,15]. For the aim of sample classification, for example, certain features from various methodologies are chosen in order to remove redundant and irrelevant data that could potentially cause noise. Unsupervised categorization techniques may be employed to find propolis groups, if sample class labels are not available or if the existence of novel classes is suspected. Principal-component analysis (PCA), for example, is a technique for transforming data that may be adequate to show class structure in the samples. On the other hand, supervised classification can be supported by class labels, if they are present. Then, predictive models or classifiers can be created to categorize fresh, unlabeled data. For instance, support vector machines, artificial neural networks and partial least squares-discriminant analysis (PLS-DA) have all been used to create classification models for propolis[14,15].

The current study is significant, because there are few studies locally that examine the mineral content of propolis. Large amounts of propolis are produced and commercialized by a sizable number of beehives in Jordan[6]. Given the widespread use of honeybee products, including propolis, in human nutrition and the potential for hazardous metal contamination, the authors chose to investigate the levels of Ca, K, Na, Mg, Al, Zn, Fe, Mn and V in multi-floral propolis harvested from different regions throughout Jordan. Our research also sought to identify the best propolis products for environmental monitoring while taking into account the correlations between the metal levels in different propolis. Moreover, multivariate analysis by principal-component analysis PCA and partial least squares-discriminant analysis PLS-DA is proposed for sample classification based on their geographical sources.

Materials and Methods

Collection of Propolis Samples

Propolis samples were collected in ten apiaries located in Amman Salt Road **ASR**, Sahab **SAH**, Al-Hashimiyah **ALH**, Al-Aghwar Middle **ALGM**, Kurayma Al-Aghwar **KUR**, Al-Subahi **ALS**, Kherbat as-Souk **KHE**, Abu Alanda **ABU**, Amman City Centre **ACC** and Al Jabal Al Abyad **ALJ**. The regional classification of the selected locations was indusial, agricultural and desert as reported in reference [6]. Moreover, the locations of the apiaries were also provided in a previous study[6]. Five hives in each apiary were chosen at random. One propolis sample was obtained from each selected hive to end up with 50 samples of different geographical origins. The data was gathered between March and October of 2017. The

samples were taken using special traps, transported to a sterile plastic container after collection and then correctly labeled with an appropriate procedure. The collected samples were preserved in plastic containers and kept at (5 ^{o}C). Each sample measured 3.0 g.

Preparation of Propolis for Inductively coupled Plasma Atomic Emission Spectrometry (ICP-OES)

Each sample was accurately weighed and then put into a clean, 25-mL Erlenmeyer flask. After adding 5.0 mL of HNO_3 acid, the flask was covered and heated for 15–20 minutes or until the fluid began to boil and turned rose in color. After allowing the liquid to cool to room temperature, 3.0 mL of $HClO_4$ acid was added gradually over the course of three steps (1.0 mL per step) and the mixture was heated until the color emerged. With distilled water, the capacity was finally finished up to 20.0 mL. Under slightly-modified conditions from those used for the digested samples, a blank sample was created[16]. Using an ICP-OES (Inductively Coupled Plasma-Optical Emission Spectrometer), minerals and metals were quantified (Model: VISTA-PROCCD Simultaneous ICP-OES, Varian, Australia). Distilled water was used as a blank and relative humidity was maintained at 21.0%. The outlined sample preparation procedure and analytical measurement were adopted from the works of Formicki[5] and Aldgini[6] with slight modifications. In the earlier procedures, complete dissolution of samples is achieved under the action of acid at 90–100 ^{o}C. A five-point calibration graph was created for each element. Table 1 gives the operating information.

Table 1. Operational parameters of ICP-OES.

Element	Detection wavelength (nm) - mode	LoD (µg/mL)
Al	167.079 - Axial	0.0008
Sn	189.989 - Axial	0.0004
Cu	217.894 - Axial	0.0005
Zn	481.053 - Axial	0.0002
Cr	359.349 - Axial	0.0012
Fe	238.204 - Axial	0.0006
Pb	220.353 - Axial	0.0015
Cd	228.802 - Axial	0.0001
Mn	257.610 - Axial	0.0002
As	189.042 - Axial	0.0008
V	309.311 - Axial	0.0012
Ni	221.647 - Axial	0.0003
Co	237.862 - Axial	0.0017
Ca	393.366 - Radial	0.0004
K	769.896 - Radial	0.0170
Na	588.995 - Radial	0.2636
Mg	279.553 - Radial	0.0001
Ba	493.409 - Radial	0.0005
Power/kW	1.15	
Sample flow rate (L.min^{-1})	1.0	
Auxiliary gas flow rate (L.min^{-1})	0.5	
Nebulizer gas flow rate (L.min^{-1})	0.5	
Plasma gas flow rate (L.min^{-1})	12	

Multivariate Data Analysis

Classification of propolis samples was made using unsupervised and supervised methods. All statistical analysis was carried out using Chemoface 1.61 software under Matlab (R) Mathworks, 8.6, USA)[17].

Multivariate data is arranged as a data matrix $\mathbf{X}_{n\times i}$ of *n* propolis samples measured at *i* variables (metals). Herein, two matrices **X** were created from analytical data: (50 samples × 9 variables). In addition to **X**, a classification vector $\boldsymbol{y}_{n\times 1}$ is created which contains the assigned classes of propolis samples. The samples were assigned to three different geographical classes (Table 2). It is important to mention that the data provided in Table 2 presented the average concentration of minerals/metals in the samples taken for each area. The data was processed using a normalizing methodology which allowed for better PCA outputs and classification outputs.

Principal-component Analysis PCA

Application of PCA is necessary for natural classification of propolis. In PCA, matrices $\mathbf{X}_{50\times 9}$ are decomposed into two matrices; **T** (score matrix) and **L** (loading matrix) using singular value decomposition or nonlinear iterative partial least-squares[18,19]. $\mathbf{X}_{50\times 9}$ is decomposed into $\mathbf{T}_{50\times h}$ and $\mathbf{L}_{h\times 9,}$ where *h* is the number of optimum factors needed for optimum matrix decomposition. The value of *h* is estimated by leave-one-out cross-validation mythology[19,20]. Once *h* is computed, scores and loading vectors are viewed to assess the natural clusters of samples and the significance of variables for propolis clustering.

Partial Least Squares - Discriminant Analysis PLS-DA

PLS-DA proposes to find latent variables in order to represent the maximum covariance between a dependent variables' matrix X (which contains mineral/metal contents) and a given characteristic ***y*** (i.e., different classes) of propolis described by the dependent variables[21]. In the training step of PLS-DA classifier, the analytical data of samples was assigned with 1, 2 and 3 (**y** vector) representing the propolis classes and the main classification vectors were estimated by the model[21,22]. The estimated discriminant functions DF were used to view sample classification based on their geographical origins.

Results and Discussion

The detection of the mineral or heavy metals in propolis is often carried out by ICP-OES, ICP-MS, electrothermal-AAS, UV-Vis spectrophotometry, neutron activation, electroanalytical methods and graphite furnace-AAS[10]. Moreover, sample preparation is carried out by direct digestion with HNO_3. Among the examined analytical methods, ICP-OES was the most applied one due to its high sensitivity, reasonable cost and short analysis time. As indicated in Table 1, the adopted ICP procedure can handle more than 15 metals in a single run. Both axial and radial modes were operated during ions' detection. The resolving power of isolated wavelengths reached 0.001 nm, which enables very sensitive detection of metals. A separate calibration curve was created for each ion over the range 0.1–5.0 μg/mL for Ca, Co, Mg, Sn and Na and from 0.1–2.0 μg/mL for the rest of ions. From calibration lines, the limit of detection LoD was estimated (3 × SD blank/calibration sensitivity) and is provided in Table 1. As indicated in Table 1, all ions can be detected at levels < 0.001 μg/L except K and Na which were detected at 0.0170 and 0.2636 μg/mL, respectively. The high detection level of K and Na would be attributed to their serious ionization at extremely high temperatures. The measured LoD values of different ions were low enough to permit accurate quantification of metals in propolis samples. It should be mentioned that the level of each metal in propolis is expressed in μg/g by the following appropriate mass balance equation:

Metal conc. = metal conc. in extract × volume of extract / mass of propolis

Both accuracy and precision of the proposed analytical method ICO-OES were adequate. The average mineral/metal contents in different propolis, estimated according to their geographical origins, are presented in Table 2 and Figure1.

In small amounts, propolis samples contain a range of mineral compositions. The mineral components of honey, propolis and bee pollen depend on the source plant and region[5]. Metals that are derived from soil, carried to plants that produce nectar through their roots, conveyed through the nectar, and then into the honey that bees produce remain stable in honey and propolis for a very long period[4]. At the bees' foraging area, where they may bring pollutants from the plants, air, water and soil into the hives, heavy metals

Table 2. Average contents of mineral and heavy metals (µg/g) in collected propolis samples from different regions.[a]

Element	Amman Salt Road (In)[b]	Sahab (In)	Kherbat as-Souk (In)	Abu Alanda (In)	Amman City Centre (In)	Al-Aghwar Middle (Ag)	Kurayma Al-Aghwar (Ag)	Al-Subahi (Ag)	Al-Hashimiyah (De)	Al Jabal Al Abyad (De)
Ca	3.2	7.0	1.0	7.5	5.0	2.5	1.0	2.5	7.0	4.5
K	2.2	1.2	0.7	0.9	1.0	1.0	0.7	1.8	11	1.0
Na	2.5	3.0	2.5	1.5	2.0	2.5	0	4.0	3.5	2.0
Mg	0.4	0.5	0.4	0.9	0.3	0.4	0	0.1	1.0	0.2
Al	1.8	1.0	1.0	1.0	1.0	0.4	0.1	0.3	0.8	0.3
Zn	0.05	0.05	0.05	0.2	0.05	0.05	0.05	0.05	0.05	0.05
Fe	0.2	1.1	0.6	0.9	0.3	0.2	0.08	0.5	0.7	0.1
Mn	0.05	0	0	0	0	0	0	0	0	0
V	0.05	0	0.05	0.05	0.05	0	0.05	0.05	0	0.05

[a] The results provided in the table represent the average contents of the analysis of five propolis samples that were taken from the same apiary or geographical area (RSD 1.9–10.7%).
[b] Regional classification of areas is provided between brackets (In: Industrial; Ag: Agricultural; De: Desert).

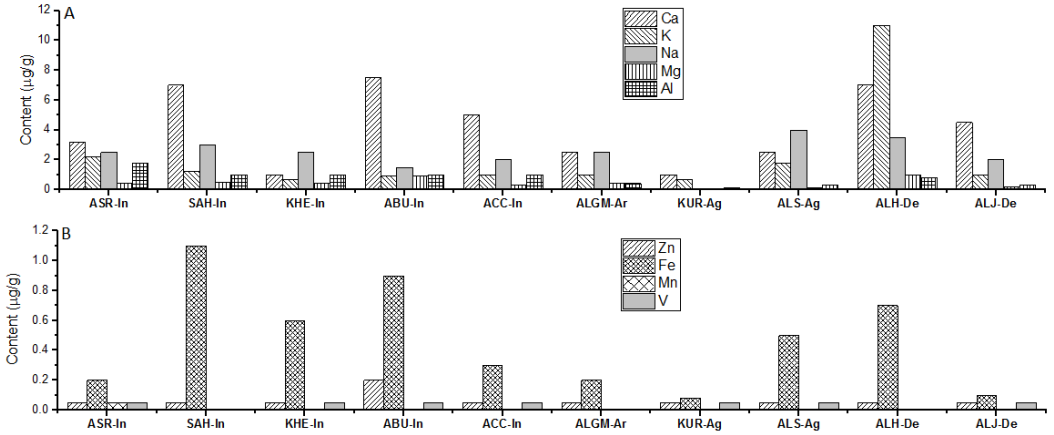

Figure 1. Detected minerals/heavy metals in propolis samples collected from different regions. ASR: Amman Salt Road; SAH :Sahab; ALH: Al-Hashimiyah; ALGM: Al-Aghwar Middle; KUR: Kurayma Al-Aghwar; ALS: Al-Subahi; KHE: Kherbat as-Souk; ABU: Abu Alanda; ACC: Amman City Centre; ALJ: Al Jabal Al Abyad.

can also serve as a biomarker of environmental pollution[23]. To determine the originality or authenticity of honey, propolis and bee pollen, variations in their mineral contents are attributed to their botanical or geographic origin. Table 2 lists the concentrations of three heavy metals (Al, Mn and V), as well as those of the six mineral elements K, Na, Ca, Mg, Fe and Zn that were found in propolis samples. Ca was found in significant excess among minerals (1–7.5 mg/kg). Propolis from Abu Alanda, Sahab and Al-Hashimiyah had the highest concentration of this mineral (7–7.5 µg/g), which can be related to the physical location of these regions. At the same time, the level of Fe was similar to that found in propolis of Polish ancestry[5]. The current findings are notable in that the mineral content of the propolis is significantly lower than that of the Spanish type[10] and somewhat comparable to that of Jordanian samples that have been published[6]. As shown in Figure 1, the detected ions can be grouped into two levels: a high level (around 1.0 µg/kg and higher) including Ca, K, Na, Mg and Al as depicted in Figure 1A and a low level (< 1.0 µg/kg) including Zn, Fe, Mn and V as shown in Figure 1B. As indicated in Figure 1A, Ca, K and Na were available in higher excess compared to Mg and Al. The high level of Ca and K in ALH samples can be attributed to the intense desert environment compared to other regions. In general, samples obtained from KUR have low contents of minerals, which may be attributed to the agricultural nature of this region. The detection of the earlier minerals is expected and also well reported in the literature[10]. On the other side, detection of heavy metals, like Zn and Fe, would indicate industrial activity around the apiaries. For instance, detection of Fe in large excess in SAH samples compared to the other metals is attributed to the high industrial activity in this region. As mentioned earlier, the reported levels of minerals/heavy metals are much lower than reported in the literature[10].

Unsupervised Classification of Propolis Samples by Principal-component Analysis

As indicated in Figure 1, the contents of some mineral and metal ions were variable and this can permit the application of PCA as an unsupervised classification method. Moreover, PCA can detect the main variables that control sample classification. The PCA results are shown in Fig 2.

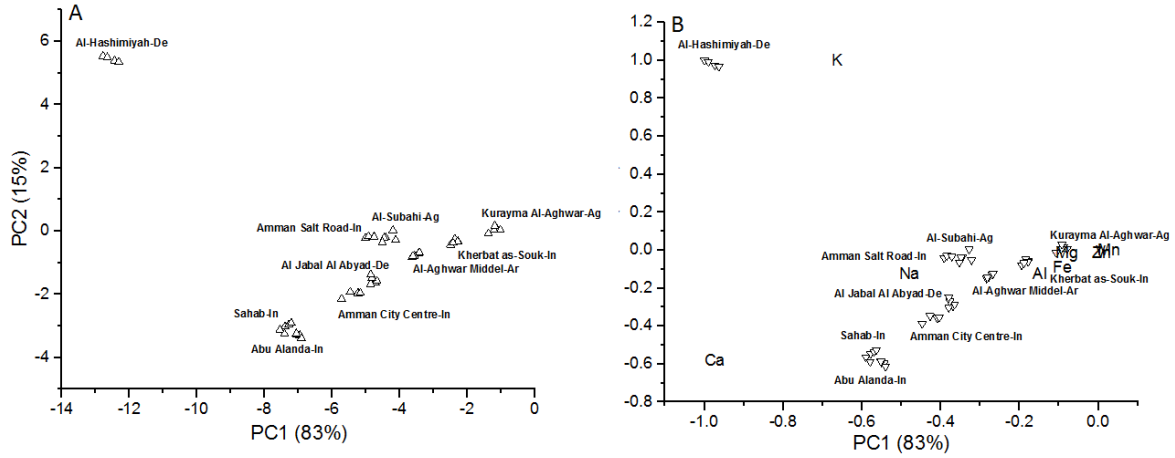

Figure 2. PCA of ICP results for propolis: A) score plot and B) bi-plot.

As indicated in Figure 2A, the data was fairly presented by two PC factors as they captured 98% of the variability in the original data: PC1 and PC2 were making 83% and 15% of the original data. Hence, both PC1 and PC2 were used to cluster propolis samples. As shown in Figure 2A, samples from SAH and ABU regions are isolated together. The earlier PCA analysis seems to be reasonable as the earlier areas are industrial. Although samples from KHE, KUR and ALGM were isolated together (Figure 1A), the propolis of (ASR & ALS) and (ACC & ALJ) were not properly isolated. The interesting point in Figure 2A was the excellent separation of propolis harvested from the ALH region. The significance of variables for propolis separation is shown in the bi-plot as indicated in Figure 2B. As shown, K played a significant role in the isolation of ALH samples, while Ca has a significant role in the separation of ABU and SAH samples from the rest of samples. As indicated in Table 2, samples from ABU & SAH

were rich in Ca with values of 7.0 and 7.5 mg/kg, respectively. On the other hand, the maximum content of K (11.0 mg/kg) was reported in ALH samples and this can explain the unique separation of these samples. In the following section, classification of propolis samples is fairly presented according to their geographical regions using PLS-DA.

Geographical Classification of Propolis Samples Using PLS-DA

One method for processing data is principal-component analysis (PCA), which may be sufficient to reveal class structure in the samples. On the other hand, when class labels are present, supervised classification can be supported. Then, classifiers or prediction models can be developed to categorize brand-new, unlabeled data. Moreover, PLS-DA can generate a better classification of the samples compared to PCA[15]. For instance, classification models for propolis have been developed using support vector machines, artificial neural networks, and partial least squares-discriminant analysis (PLS-DA)[14,15]. In fact, the collected samples could be assigned to their geographical origins. As indicated in Table 2, the collected samples were assigned to industrial, agricultural, and desert backgrounds. Hence, regional classification of propolis based on their mineral/metal content is applicable for the local samples. The graphical representation of PLS-DL classification is displayed in Figure 3.

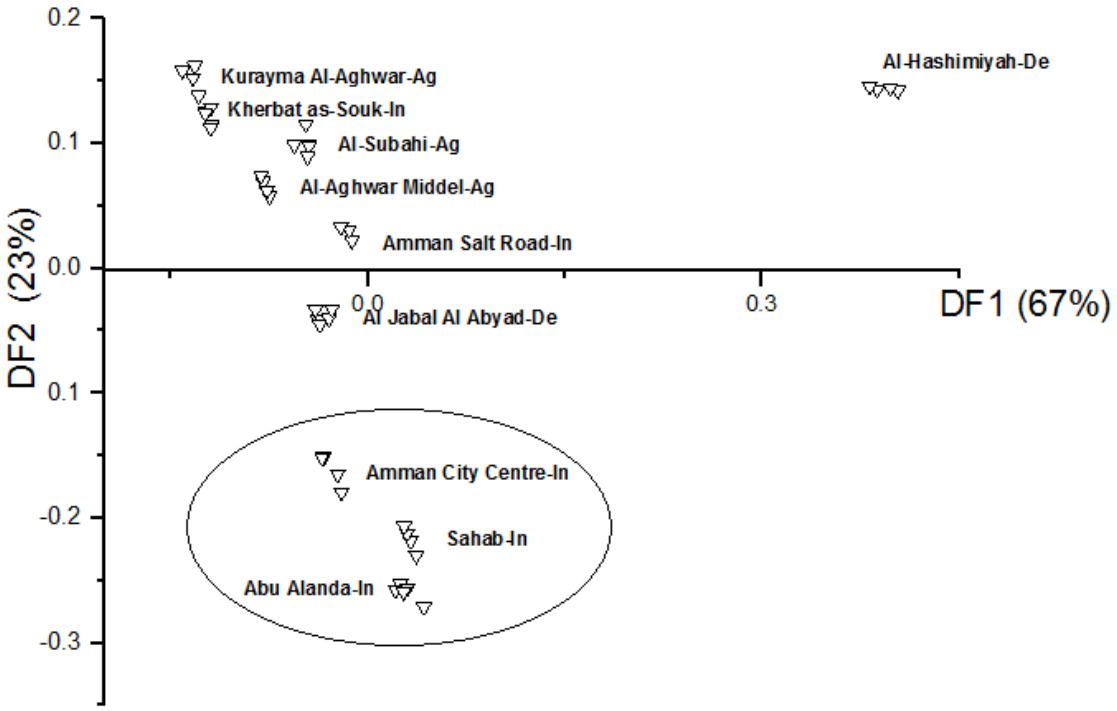

Figure 3. Score plot of the first two discriminant functions (DF1 and DF2) for classification of propolis samples according to their geographical origins.

Indeed, PLS-DA outperformed PCA by its capability to assign samples according to their origin. As indicated in Figure 3, PLS-DA has separated samples from industrial regions (which include AAC, SAH and ABU). As was the case in PCA, samples from ALH region were separated alone due to their distinguished chemical composition. PLS-DA outperformed PCA by better isolation of ACC and ALJ samples. Moreover, PLS-DA has also separated ASR samples from the rest of samples. The unique isolation of AAC, SAH and ABU samples is mainly attributed to the high Ca content in these samples (5.0–7.5 μg/kg), as indicated in Table 2. Unfortunately, the model was not workable to cluster samples from desert origin alone (like the case of samples from industrial regions). As shown in Fig 3, samples collected from ALH and ALJ were not classified close to each other, and this may be attributed to the high K content in the former samples (11 μg/kg, Table 2). To assess the overall

performance of PLS-DA for propolis classification, the accuracy was estimated from the confusion matrix[22,24]. The accuracy of classification is estimated as:

$$Accuracy = \frac{\sum_{g=1}^{G} c_{gg}}{n} \times 100 \quad (1)$$

where c_{qq} is the number of samples correctly assigned to class *g*, *G* is the total number of classes, *n* is the total number of samples. The results of sample classification are provided in Table 3.

Table 3. Confusion matrix of regional classification of propolis using PLS-DA.

Propolis	Sample	**Predicted Samples**									
		ASR -In	SAH -In	ALH -De	ALGM -Ag	KUR -Ag	ALS –Ag	KHE -In	ABU –In	ACC -In	ALJ -De
ASR–In	5	**5**	0	0	0	0	0	0	0	0	0
SAH-In	5	0	**5**	0	0	0	0	0	0	0	0
ALH-De	5	0	0	**5**	0	0	0	0	0	0	0
ALGM-Ag	5	0	0	0	**5**	0	0	0	0	0	0
KUR-Ag	5	0	0	0	0	**4**	0	**1**	0	0	0
ALS–Ag	5	0	0	0	0	0	**4**	**1**	0	0	0
KHE-In	5	0	0	0	0	**1**	**1**	**3**	0	0	0
ABU–In	5	0	0	0	0	0	0	0	**5**	0	0
ACC-In	5	0	0	0	0	0	0	0	0	**5**	0
ALJ-De	5	0	0	0	0	0	0	0	0	0	**5**

As indicated in Table 3, most samples of industrial origin (except KHE samples) were accurately classified. For KHE, three samples were correctly assigned to their origin, while two samples were misclassified to KUR and ALS origin. One sample from KUR region was misclassified to KHE. At the same time, only one sample from ALS was misclassified to KHE. The final accuracy of the developed model to classify propolis samples was 92% as estimated from Equation 1.

Conclusions

ICP-OES enables quick and easy multi-element quantification in propolis samples. Chemometric tools, like PCA and PLS-DA, can be used to quickly understand the obtained data. Promising results were obtained using PLS-DA to differentiate propolis samples obtained from agricultural and industrial regions. However, the model was not workable to isolate samples from desert regions. Based on mineral and metal contents, PLS-DA model was effective to classify propolis samples with 92% accuracy as estimated from the confusion matrix. The variations in mineral/metal contents present in propolis were helpful for discrimination purposes. Minerals, like Ca, Na and K, were the most important variables for propolis discrimination according to their geographical origins.

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