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ORIGIN OF HONEY PROTEINS AND METHOD FOR ITS QUALITY CONTROL

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Abstract

As one of the best suppliers of energy and the necessary substances in human's nutrition, honey has numerous consumers all over the world. There are some protein compounds in honey in addition to sugars, lipids and mineral compounds. Relative quantity of proteins in honey compound is considered as a quality index. Determination of the quantity of plant origin (pollen) and animal origin (honey bee) of the proteins of honey is an important but unknown issue. Knowing this ratio can be an index for quality control of honey. In this research, 6 honey "unifloral" samples were collected from "Sirachal" region located in Karaj-Chaloos Road, 40 km from North of Karaj in different months. After a quantity of each honey sample was diluted with water, pollens were counted in 10 gram of each sample using optical microscope. Total quantity of proteins in each sample was specified through method of "Bradford". Electrophorus profile of pollen proteins used by bees, pollens in honey and honey proteins were prepared and compared through electrophorus method of Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. Statistical study of tests results and repeating them was done by *T* test. The results showed that honey collected in June-July has the most quantity of pollen (680000 in each 10 *g* honey). Average of the whole pollen proteins that were mostly used by honey bees is 35.91±5.56 of the dry weight of the pollens. Honey collected in June-July with the most amounts of pollens has the most quantity of total protein means 0.99% of the total weight of the pollen and honey collected in Aug.-Sep. with the least amount of pollen (147000 in each 10 *g*) has the least quantity of total protein means 0.37% of the total weight of the pollen. Average protein of the whole honey during hive establishment is 0.64±0.26% and average quantity of pollen proteins of honey in this period is 0.24±0.14%. Since proteins are among very valuable compounds in human's nutrition and plays vital role on growth and health of cells, quantity of the whole protein of honey is one of the quality indices and since based on the tests carried out in this research, about 1/3 of proteins of honey penetrate it through pollens, ratio of pollen proteins to total proteins of honey can be considered as a new index for examination of honey quality.

Introduction

Numerous methods have been introduced for quality control and standardization of honey. For example, measuring *HMF* (Hydroxy Methyl Furfural), ratio of fructose to glucose, measure of moisture, pH, quantity of protein, quantity of unbound amino acid, electric conduction, however, quantity of pollen proteins of honey have been less considered as an index of quality control of honey. Knowing what quantity of total protein of honey has plant origin (pollen origin) is still questionable.

Honey is a semi liquid product (water: 15-18% approx.) which contains a complex mixture of carbohydrates, mainly glucose and fructose; other sugars are present as traces, depending on the floral origin. Moreover, organic acid, Lactones, aminoacids, minerals, vitamins, enzymes, pollen, wax and pigments are present. Honey is produced either from many flowers or from single flower pollens. The single flower origin should assure a better quality of the product, when it guarantees a specific and well-defined flavour and aroma (Fallico *et al*., 2003).

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Melissopalynological and sensory characterization have been performed in order to check the reliability of botanical origin of the samples (Lušić *et al*., 2007). Honeys were considered to be *monofloral* whenever the dominant pollen was found to be over 45% of total pollen (Andrade *et al*., 1999).

The chemical properties of honey, which are related to the floral source from the honey has been extracted, such as *Eucalyptus lanceolatus* honey showed higher protein, diastase and catalase activity than *Helianthus annuus* honey, while proline, conductivity, total acidity, free acids and lactone content were higher in *Helianthus annuus* honey(Bath & Singh, 1999).

Pollen pellets collected from honey bees foraging at 62 floral species were analysed for protein and amino acid content and their value for honey bee nutrition was determined. The crude protein levels of all pollen pellets analysed ranged from 9.2% for *Hypochoeris radicata* (flatweed) to 37.4% for *Echium plantagineum* (Paterson's curse) with a mean of 25.9% (Somerville & Nicol, 2006).

The protein contents in honey samples of different floral origins, commercialized in several states of Brazil, were determined using the method of Bradford. The spectra of pollen of the honeys collected in those areas were studied, in order to establish the correlation between the different botanical species and the protein contents. The physicochemical properties of the honeys (colour, moisture, pH and acidity) were also determined. These were shown to be efficient and it allowed the detection of elevated protein in honey samples of *Borreria verticillata*, known in Brazil as vassourinha, from Piauí State (Azeredo *et al*., 2003). Also Baroni *et al*., (2002) used honey proteins as chemical markers of the floral origin of honey.

Nutritionally, simple carbohydrates make up 82.4% of honey, being primarily fructose (38.5%) and glucose (31%), with some maltose, sucrose and other sugars. Honey also contains some 17.1% water, with protein and micronutrients making up the balance (0.5%) (Boylan, 2000). Krell, 1996, has reported that pollen proteins are 7.5-35% by weight to the total weight of pollens.

In this research, total quantity of proteins of 6 honey samples collected from bee hives of "Sirachal" in different months have been measured and compared. Quantity of proteins of the pollens used more by honey bees and also quantity of pollen proteins in these honey samples as well as the ratio of pollen proteins (plant originated) to total proteins of honey were studied and compared in order to determine what quantity of honey proteins has plant origin and what quantity has animal origin. This ratio can be considered as one of the quality indices of honeys.

Materials and Methods

Six honey samples were collected from "Sirachal" in Tehran in different months. This region is located in northern latitude between 35°, 59′ to 36°, 3′ and eastern longitude between 51°, 8′ to 51°, 13′. Pollen traps have been installed on a number of bee hives of this region and pollen loads were collected in 3-day intervals. Pollens of all regional plants were also collected in order to be used for comparison and identification of pollen loads obtained from pollen traps and pollens extracted from honey.

Honeys were collected in 15-day intervals as well. Ten grams of each honey were analyzed per month to study pollens. Melissopalynology were used by Sawyer Method (Sawyer, 1988).

Chemical and biochemical analyses including fructose, glucose, sucrose tests, ratio of fructose to glucose, pH by pH meter HORIBA, model of M-12 and moisture were measured by refractometer ATAGO, model of NAR- 3T made in Japan. Quantity of total pollen less proteins and separated-pollen proteins of honey was determined by Bradford Method (Bradford, 1976). Of 150*g* of each sample, upon dilution and centrifuging for three times, deposited pollens were collected by pastor pipette and dried in a dry oven. Fat removal was done by oil either for 10 minutes and then Bradford stages were performed.

Bradford method: Pollen extracts with alkali phosphate buffer with weight ratio of 15% (*w/v*) were prepared and then they were shaken on the shaker at 4^{o}C for 24 hours, floating extraction and storage were performed at – 20^{o}C up to the usage time, Standard protein solution was prepared, 9 compounds with different volumes of pollen extract with extract buffer (5:95 …45:55) were prepared such that the volume of each compound does not exceed 100 micro liter, Similar compounds were prepared from standard protein solution and extract buffer, each compound was soaked in 5 micro liter of reagent "Comasie Blue" G 250 (Bradford reagent) and finally absorption spectrum of all the compounds was read after 5 minutes in absorption spectrum of 595 nanometer by spectrophotometer. Protein concentration in each pollen extract (extracted from honey or pollen trap) as well as pollen less honey was determined by standard curve.

Measuring through electrophoresis: Using *SDS-PAGE* Method (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis), electrophoresis profile of the pollen proteins that were mostly used by honey bees such as pollens separated from pollen trap and also pollens separated from honey were specified and compared (Fig. 1). Measurement was repeated at least three times and the results were evaluated and compared by calculation of the results mean (*T*-test).

Results

The palynological studies showed that the honeys tested are unifloral honey with pollen domination of *Berberis vulgaris* L. (88%). (Table 1, Figs. 2, 3). Percentage of glucose, fructose, sucrose and ratio of fructose to glucose is shown in Table 2. As these results show, the least quantity of fructose, glucose and the lowest ratio of fructose to glucose are in the honey collected in June-July.

Measuring honey pH in different months did not show considerable difference and June-July honey is somehow less acidic (Table 3). Moisture of honeys tested is shown in Table 4. The least moisture is for July-Aug. (14.85%) and the most moisture is for May-June (19.4%). Results of studies of measuring the purified honey proteins (pollen less) and pollens separated from honey in different months were obtained by Bradford method (Table 5). These studies show that the quantity of total honey proteins was the most (0.99%) in June-July, 2004 and the least in Aug.-Sept., 2005 (0.37%).

Discussion

Measuring total honey proteins: Comparing natural honey proteins obtained in this study, even in July-Aug. that has the least percentage of protein (0.57%) and pollen protein is 0.11%, with protein of another honey (23%) that is sold in the market as a natural honey under a well-known mark and its pollen quantity is 0.01% draws our attention to considerable quantity of pollen protein of natural honey with good quality and can be regarded as a good criteria for specifying a natural honey.

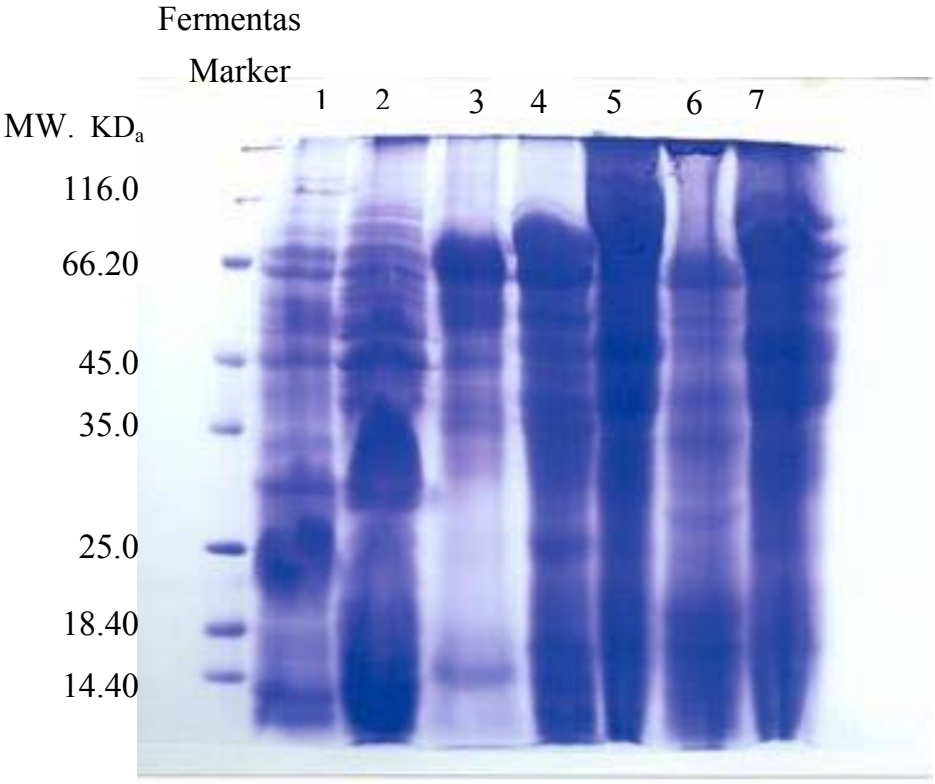

Fig. 1. Electrophoresis profile of pollens (Dominant with middle attractiveness) which used by honeybee and extracted pollen from honey.1- *Cousinia nekarmanica* Rech.f, 2- *Crepis sancta* (L.) Bobcock, 3- Extracted pollen from honey, 4- *Berberis vulgaris* L., 5- *Buffonia macrocarpa* Ser., 6- *Pterocephalus canus* Coult. ex DC., 7- *Acanthophyllum microcephalum* Boiss.

Looking at Table 5, we see that the more the number of pollens, the more the pollen protein will become. For example, June-July honey with over 6,000,000 pollens in each 10 g honey (Table 1) has the most pollen protein (0.21%) and total protein (0.99%) (Table 5). Also in July-August. pollen protein decreases as the quantity of pollen decreases.

Total protein of honey is between 0.1% to 0.65% (Hashemi, 2001). Average protein of honey is 0.64% in this study that is almost the highest quantity in this range and its pollen protein is 0.12. This result is considerably different from that of a commercial honey with total protein of 0.23 and pollen protein of 0.01.

SDS-PAGE electrophoresis study of pollen and honey proteins: Although Azeredo *et al*., (2003) determined the total protein content honey but in addition we could separate the plant and zoo protein contents in honey.

Through electrophoresis on pollens, a great number of blue bands (after gel being painted with Comasie blue) indicated a great amount of protein in the pollens used by honey bees (Fig. 1, pollens 1-7) which are expanded from low (14.40 KD_a) to high (116 KD_a) molecular weight.

Comparison of columns 3 and 4 in the bands with molecular weight of high (about 91 KD_a)to medium(about 30 KD_a) indicates the remarkable similarity of these bands. Comparing column 4 that allocates to *Berberis vulgaris* with column 3 which belongs to the pollens extracted from honey shows that the main plant proteins of honey belongs to pollens of *Berberis vulgaris*. We have already achieved such result in accounting honey pollens (Table 1).

Table 1. Average of number and percent of pollen in each 10 grams honey, collected in June-July.

Percent	Number	Plant species
88.37	6072245	*Berberis vulgaris* L.
5.91	406170	*Isatis kotschyana* Boiss. & Hohen.
1.28	88003.51	*Acanthophyllum microcephalum* Boiss.
0.78	54146	*Eupatorium cannabinum* L.
0.59	40616.836	*Cerasus microcarpa* (C. A. Mey.) Boiss.
0.39	27078	*Epilobium hirsutum* L.
0.39	27078	*Salvia virgata* Jacq.
0.39	27078	*Reseda lutea* L.
0.29	20308.5	*Chearophyllum macropodum* Boiss.
0.29	20308.5	*Cardaria draba* (L.) Desv.
0.29	20308.5	*Phlomis olivieri* Benth.
0.098	6769.5	*Achillea vermicularis* Trin.
0.098	6769.5	*Rosa iberica* Stev.
0.098	6769.5	*Crepis sancta* (L.) Babcock
0.098	6769.5	*Dianthus orientalis* Adams subsp. *orientalis*
0.098	6769.5	*Astragalus aureus* Willd.
0.098	6769.5	*Astragalus effusus* Bunge
0.098	6769.5	*Lonicera iberica* M. B.
0.098	6769.5	*Bromus danthoniae* Trin.
0.098	6769.5	*Melilotus officinalis* (L.) Desr.
0.098	6769.5	Unknown
100	6871045.846	Sum

Table 2. Measure of glucose, fructose and sucrose and ratio of fructose to glucose in different months honey

Sugar / Honey	Glucose (%)	Fructose (%)	Sucrose (%)	Fructose / Glucose (%)
June-July 2004	31.99	31.49	5.8	0.948
July-Aug. 2004	40	39.5	5.4	0.98
Agu.- Sep. & Sep.- Oct. 2004	35.139	34.685	8.24	0.987

Table 3. Measurement of pH of honey in different months.

Collected date	pH
June-July 2004	4.96
July-Aug. 2004	4.45
Aug.-Sept. & Sept.-Oct.	4.77
Average of pH of honey = 0.11 ± 4.72	

Table 4. Measurement of moisture, brix, refractive index of honey in different months.

Collection date of honey	Refractive index	Brix	Moisture (%)
May-June 2004	1.49395	81.2	18.8
June-July 2004	1.49942	83.5	16.5
July-Aug 2004	1.50185	85.15	14.85
Aug.-Sep. & Sep.-Oct. 2004	1.50056	83.7	16.3
May-June 2005	1.49223	80.6	19.4
June-July 2005	1.4980	82.7	17.3

Table 5. Determining average of protein weight percent of honey pollen, honey and total protein in distinct weight of honey.

Average of total protein weight percent	Average of honey pollen and honey protein weight percent	Collected date of honey
0.99	honey pollen=0.21 honey=0.78	June-July 2004
0.57	honey pollen=0.11 honey= 0.46	July-Aug 2004
0.37	honey pollen= 0.05 honey= 0.32	June 2005
0.57	honey pollen= 0.11 honey= 0.46	July 2005

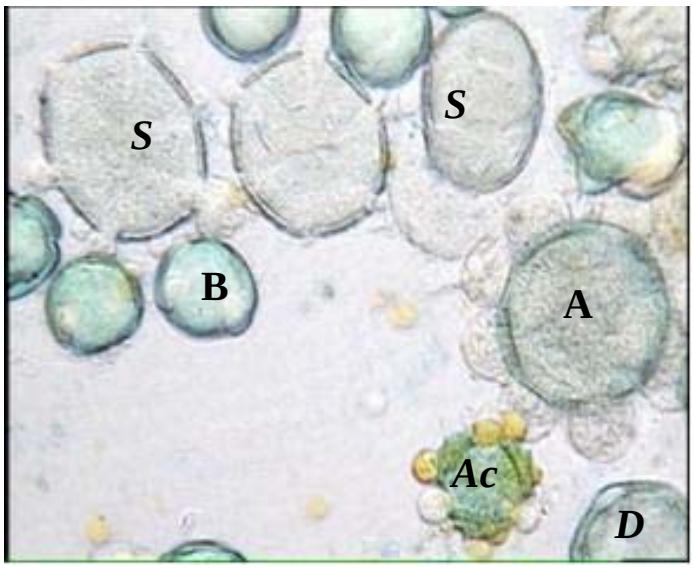

Fig. 2. Pollen of honey collected in June. (×720) *A=Acanthophyllum microcephalum* Boiss., *Ac=Achillea vermicularis* Trin. *B=Berberis vulgaris* L., *D=Dianthus orientalis* Adams subsp. *orientalis, S=Salvia virgata* Jacq.

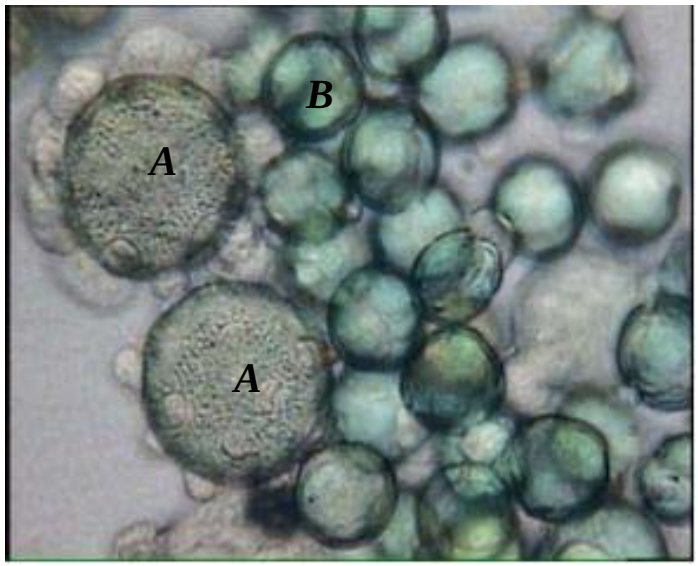

Fig. 3. Pollens of honey collected in 2004. (×720), *B=Berberis vulgaris* Boiss. *A=Acanthophyllum microcephalum* L.

The bandless part of the protein shows the entry of its protein to honey. Thus light extraction and centrifuge due to separation of wax from honey cannot lead to decreasing pollen protein of honey since the main part of pollen protein can even leave the pollen through sticking of the pollen to extractor or to wax before separation and then enter the honey.

Electrophoresis shows that almost half of the pollen protein (with molecular weight of low to medium) can penetrate honey (Fig. 1). Therefore, in addition to the pollen protein (0.12) that was already reported and is consumed by eating honey, about half of honey protein (purified pollenless honey) is pollen type. In general, of the total honey protein, about 1/3 relates to pollen and has plant origin and the remaining 2/3 includes enzymes and proteins with insect origin (honey bee).

Using one of the following two methods, it is possible to present a criterion for quality control of honey:

$$\frac{\text{Pollen Proteins}}{\text{Honey Proteins}} \geq 0.2$$

Thus the higher the ratio of pollen protein to honey protein including plant and animal proteins, the higher the quality of honey.

$$\frac{\text{Pollen Proteins x 2}}{\text{Honey Proteins - Pollen Proteins}} \geq 0.6$$

The ratio of pollen proteins to animal protein is actually obtained from the above formula and thus it multiples by two since pollen protein is about 1/3 and animal protein is about 2/3 of the total protein. The greater this ratio, the higher the quality of honey. For example, for the honey produced in this research (the figures are related to the collection months):

$$\frac{0.12}{0.52} = 0.23$$

$$\frac{0.12 \times 2}{0.52 - 0.12} = 0.6$$

Doing the above operations for the check samples of honey showed that:

$$\frac{0.01}{0.22} = 0.045$$

$$\frac{0.01 \times 2}{0.22 - 0.01} = 0.095$$

In the tests carried out in this research, the ratio of pollen proteins to honey proteins is at its highest i.e., about 0.23. Since total protein of honey in our research is at its highest for a natural honey i.e., 0.64, thus maximum range of the ratio of pollen protein to honey proteins is about 0.23 for all the natural honeys.

Moreover, by knowing the ratio of pollen proteins to honey proteins, it is possible to determine the number of pollens in each 10 *g* honey that the number of its pollen is unknown through the following formula:

$$\frac{\text{Ratio of pollen proteins to honey proteins x } 2563115.067}{0.23} = \text{average of number of pollens in each 10 } g \text{ natural honey}$$

25631150.067= average of number of pollens in honey in this research (from June-July to Sept.-Oct.)

0.23=Ratio of pollen protein to honey (related to average of honeys of June-July to Sept-Oct.)

Therefore this finding has application in honey origin quality control.

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