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Research Article

A STUDY OF PHYSICOCHEMICAL PROPERTIES, VOLATILE COMPONENT ANALYSIS AND ANTIOXIDATIVE PROPERTIES OF HONEY

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ABSTRACT

Honey samples from five different floral origins were analysed through solid phase microextraction (SPME) with objective to identify and compare their volatile organic compound profile. In addition, the levels of water, HMF, free proline, total acidity, diastase activity and sugar content have also been reported. The samples showed adequate water and HMF content. Total phenolics varied from 75.6 to 98.5mg/g, while total flavonoids were comprised between 1.86 and 4.93mg/g, expressed as quercetin equivalents (the lowest and highest values were also found for Eucalyptus honey and neem honey, respectively). The IC50 value for DPPH has been found to be ranged from 4.97 to 9.45mg/ml. The highest DPPH RSA was found in Eucalyptus honey, followed by mustard honey and neem honey.

Keywords: Solid Phase Micro-Extraction, DPPH, Proline, Flavonoid, Antioxidant.

INTRODUCTION

Flavonoids are a large class of phytochemicals which are omnipresent in human diets. They can be found in fruits, vegetables, tea, chocolate and wine and have a number of beneficial effects on human health, being antioxidant, anti-inflammatory, antiallergic, antiviral and anticarcinogenic agents [1, 2]. The evaluation of phase 2 detoxification and antioxidant enzymes by isothiocyanates, carotenoids, flavonoids and other phytochemicals is now recognized as one of the mechanisms by which fruits and vegetables, in particular, cruciferous vegetables, exert their chemoprotective effects. Some of these phytochemicals are also found in dietary ingredients that are produced either commercially or from plants or plant parts. Thus, the presence in honey of similarly acting phytochemicals, such as the flavonoids pinocembrin, pinostrobin, pinobanksin and chrysin makes this natural sweetner a logical source of chemoprotective activity [3-9].

The main goal of this work was to assess the floral origins of different unifloral honey by evaluating the volatile organic compound profiles through solid phase micro-extraction and gas chromatography coupled to mass spectrometry. In addition, the physicochemical and antioxidative properties have been investigated.

MATERIALS AND METHOD

Sample collection

Samples of raw honey from six different sources, viz. Trifolium aleaxandrium L. (berseem clover), Brassica camprestris (mustard), Helianthus annus (Sun flower), Eucalyptus globulus and Azadirachta indica (Neem) were used. A commercial sample of honey was procured from the local market. Honey samples were collected in central Uttar

Pradesh. Sampling area covered the most important production zones. Samples were collected stored at 00C until analysis, which occurred no longer than one month after extraction from the hives by beekeepers.

Pollen analysis

The botanical origin of the samples were determined using techniques described before [10]. For floral identification, 5 g of the diluted honey sample was centrifuged at 10, 000 rpm for 15 min, to separate the pollens. Samples of separated pollen grains were spread with the help of a brush on a slide containing a drop of lactophenol. The slides were examined microscopically at 45x, using a bright field microscope.

Physicochemical Characteristics

Honey was analysed according to methods previously reported for pH, moisture, Brix, ash content, electrical conductivity, free lactone and total acidity, diastase activity, hydroxymethyl furfural determination [11]. Two replicate analyses were performed for each sample.

pH

The pH was measured by pH-meter model Systronics, with a precision of ±0.002pH units. The pH of honey was measured for a solution of 10 g honey in 75mL of CO2 free distilled water.

Moisture content

Moisture was determined by refractometry, using an EI model Abbe's refractometer with direct reading display, and results were expressed as 0Brix.

Ash

Ash content was measured by calcinations, overnight, in furnace at 5500C, until constant mass.

Electrical conductivity

Electrical conductivity of a 20% (dry matter basis) honey solution in CO2-free deionised distilled water, was measured at 200C in a Systronics model conductivitimeter, and results were expressed as µS cm-1.

Free lactones and total acidity

Free lactones and total acidity were determined by titrimetric methods: the addition of 0.05M NaOH was stopped at pH 8.50 (free acidity), immediately a volume of 10mL 0.05 M NaOH was added and, without delay, back titrated with 0.05 M HCl to pH 8.30 (lactone acidity). Total acidity results were obtained by adding free and lactone acidities.

Diastase activity

Diastase activity was measured using buffered soluble starch solution and honey, which was incubated in a thermostatic bath at 400C. Absorption was followed by using EI spectrophotometer and a chronometer. Using regression (without using the data point at 0 min) lines were fitted to the absorption data and the diastase number was calculated from the time taken for the absorbance to reach 0.235. For samples of low diastase activity, the regression was made on the basis of the last three data points to improve the linear correlation. In samples of high diastase activity the time taken for the absorbance to reach 0.235 was determined with absorbance at 5 and 10, or 5, 15 and 20 min, depending on the activity. Results were expressed (as Gothe degrees) as ml of 1% starch hydrolysed by enzyme in 1g of honey, in 1 h.

Hydroxymethyl furfural content (HMF)

The Winkler method was used to determine the HMF content of honey samples, treating 5g of each sample with a clarifying agent (Carrez). The volume was adjusted to 50ml and the solution was filtered. The absorbance of the filtered solution was measured at 284 and 336 nm against an aliquot treated with NaHSO3.

Estimation of total phenolic and flavonoids contents

The Folin-Ciocalteu method [12] was used to determine total phenolic content. Each honey sample (5g) was diluted to 50 ml with distilled water and filtered through Whatman No. 1 paper. To this solution, 0.5mL of 0.2 N Folin-Ciocalteu reagent for 5 min and 2 mL of 75g/l Na2CO3 were then added. After incubation at room temperature for 2h, the absorbance of the reaction mixture was measured at 760nm. The mean of three readings was used as a standard to produce a calibration curve. Total phenolic content was expressed in mg of gallic acid equivalents (GAE)/100g of honey (mean of three readings).

The total flavonoid content was determined using the Dowd method as adopted by Arvouet-Grand et al.[13]. Briefly, 5ml of 2% aluminium trichloride in methanol was mixed with the same volume of a honey solution (0.01 or 0.02 mg/ml). Absorption readings at 415 nm (EI spectrophotometer) were taken after 10 min against a blank sample consisting of a 5ml honey solution with 5ml methanol, without addition of

AlCl3. The total flavonoid content was determined using a standard curve prepared using quecetine (0-50 mg/l) as standard. The mean of three readings was used and expressed as mg of quercetin equivalents (QE)/100 g of honey.

Estimation of Proline content

The proline content was determined using the method of Ough as adapted by Bogdonov, [14]. A 0.5 ml solution of honey (0.05 mg) mixed with 1 ml formic acid (make), 1 ml ninhydrin solution (3% ethyleneglycol monomethyl ether) and shaken vigorously for 15 min and transferred to a 700C bath for 10 min. A 5 ml solution of 50% 2-propanol in water was then added and the mix was left to cool and the absorbance determined (510 nm) 45 min after removal from the 700C water bath. Water was used as blank and a 0.032 mg/ml solution of proline was used as standard solution. Proline concentration in mg/kg of honey was calculated as follows:

Proline (mg/kg) = (Es/Ea)x(E1/E2)x80, where Es is the absorbance of the sample solution; Ea is the absorbance of the proline standard solution (average of three readings); E1 is the mg of proline used for standard solution; E2 is the weight of honey in grams; 80 is the dilution factor. The mean of three readings was used.

Radical scavenging Activity and antioxidant content

The DPPH assay constitutes a quick and low cost method. Because of its odd electron, DPPH gives a strong absorption band at 517 nm (deep violet colour). In the presence of a free radical scavenger, this electron becomes paired, resulting in an absorption loss and consecutive stoichiometric decolourization. The absorption change produced by this reaction was used to evaluate the antioxidant potential of the samples. Honey was diluted with ethanol (5 µL, 300 µgmL-1) to give a final extract concentration of 1 μgmL^{-1}. The mix was shaken vigorously and left for 5 min.

The absorbance of the resulting solution was measured at 517 nm. All tests were executed in triplicate and averaged. The radical scavenging activity was calculated using the formula:

Percentage inhibition= [1-(absorbance of DPPH+Sample-Absorbance of Sample)]x100/ Absorbance of DPPH

The mean of three IC50 (Concentration Causing 50% inhibition) values of each honey samples was determined graphically. The antioxidant content was evaluated with some modifications. Honey samples were dissolved in methanol (0.02 0.04 g/ml), and 0.75 ml of each solution was than mixed with 1.5 ml of a 0.02 mg/ml solution of DPPH in methanol.

Volatile compounds Analysis

The solid phase microextraction (SPME) was carried out with Supelco SPME devices coated with polydimethylsiloxane (PDMS, 100 lm), used for sampling the honey samples placed into a 10 ml glass septum vial and allowed to equilibrate for 30 min. After equilibration time, the fibre was exposed to the headspace for 30 min at room temperature. At the end of sampling, the fibre was withdrawn into the needle and transferred to the injection port of the GC and GC/MS system, operating as follows.

The GC analyses were accomplished with a HP-5890 Series II instrument equipped with HP-WAX and HP-5 capillary columns (30 m X 0.25 mm, 0.25 µm film thickness), and with the following conditions: temperature programme 600C for 10 min, followed by an increase of 50C/min to 2200C; injector and detector temperatures at 2500C; carrier gas helium (2 mL/min); splitless injection; detector dual FID.

The identification of the chemicals was performed for both the columns through comparison of their retention times with those of pure authentic samples and by means of their Linear Retention Indices (LRI) relative to the series of n-hydrocarbons. GC/EIMS analyses were performed with a Varian CP-3800 gas chromatograph equipped with a HP-5 ms capillary column (30 mX 0.25 mm; coating thickness 0.25 µm) and a Varian Saturn 2000 ion trap mass detector. The analytical conditions were the following: injector and transfer line temperatures 2500C and 2400C respectively; oven temperature from 600C to 2400C at

30C/min; carrier gas helium at 1 mL/min; splitless injection. The identification of the constituents was based on a comparison of their retention times with those of authentic samples, comparing their linear retention indices relative to the series of n-hydrocarbons, and on computer matching against commercial mass spectra (NIST 98 and ADAMS 95) and those of our library, built up from pure substances and components of known essential oils and MS literature data [15-19]. Moreover, the molecular weights of all the

Table 1: Distribution data for physicochemical parameters in honey samples

Sample	pH	Moisture (%)	^{0}Brix (%)	Ash (%)	Electrical conductivity (μScm^{-1})	Free Acidity (meq/Kg)	Lactone acidity (meq/Kg)	Total acidity (meq/Kg)	HMF (mg/Kg)	Diastase Activity (^{0}Gothe)
Eucalytus globules	3.48±0.03	17.5±0.16	79.5±0.3	0.120±0.003	0.45±0.01	26.5±0.93	4.7±0.17	31.0±0.17	12.7±0.45	12.0±0.93
Azadirachta indica	3.92±0.08	15.8±0.01	80.4±0.4	0.687±0.004	0.22±0.003	29.3±0.50	4.2±0.38	34.1±1.08	11.75±0.32	18.2±0.65
Helianthus annus	4.67±0.05	14.3±0.02	78.7±0.3	0.235±0.006	0.34±0.008	17.3±0.60	3.9±0.82	33.5±0.82	14.7±0.34	23.2±0.94
Bressica comprestris	4.98±0.07	16.5±0.07	81.4±0.7	0.929±0.007	1.59±0.002	27.3±0.70	6.2±0.53	38.6±1.03	9.10±0.23	20.6±0.49
Trifolium alexadrium	4.38±0.07	17.9±0.06	79.2±0.1	0.558±0.002	1.22±0.007	32.6±0.41	6.2±0.98	37.9±1.02	15.54±0.63	28.2±0.55

Table 2: A Comparison of data from honey samples obtained in north India

S. No	Floral Origin	Harvest date	Total Phenolic Content (mg GAE/100g ±SD)	Total Flavonoid Content (mg QE /100g ±SD)	Proline Content (mg/kg± SD)	RSA IC_{50} (mg/ml±SD)	AEAC (mg/100g±SD)	QEAC (mg/100g±SD)
1	*Trifolium aleaxandrium* L. (berseem clover)	Aug, 2010	75.64±0.90	3.92±0.67	723.4±13.2	4.97±0.57	23.47±0.51	12.49±0.05
2	*Brassica camprestris* (mustard)	Sep, 2010	83.96±0.63	2.61±0.05	823.4±17.3	7.63±0.23	29.27±0.26	9.67±0.71
3	*Helianthus annus* (Sun flower)	Oct, 2010	85.6±0.48	4.53±0.34	687.3±19.7	5.27±0.07	17.59±0.07	11.06±0.24
4	*Eucalyptus globules*	Nov, 2010	78.85±1.83	1.86±0.03	969.4±19.8	9.45±0.28	35.63±0.53	7.47±0.31
5	*Azadirachta indica* (Neem)	Jan, 2011	98.5±0.04	4.93±0.02	764.1±11.5	6.49±0.37	37.48±1.23	8.39±0.07

AEAC: Ascorbic acid equivalent antioxidant content
GAE: Gallic acid equivalent
I_{50} : 50% inhibitory Concentration
QEAC: Quercetin equivalent antioxidant content
RSA: Radical Scavenger activity
SD: Standard Deviation

Table 3. Volatiles of honey from different regions sampled by SPME

S. No.	Constituents	L.R.I.	Sample 1	Sample 2	Sample 3	Sample 4
1.	Ethyl acetate	609	-	3.7	-	29.1
2.	3-hydroxy-3-butanone	707	2.5	-	-	-
3.	2-hexanone	799	-	13.3	5.8	-
4.	furfural	835	1.6	15.0	9.9	16.6
5.	2,3-dihydro-5-methyl-2-furanone	868	-	1.6	0.5	-
6.	2-acetyl furan [1-(2-furanyl)-ethanone]	912	0.1	1.6	3.2	1.1
7.	6-methyl-2-heptanone	954	1.6	-	-	-
8.	benzaldehyde	963	-	-	-	3.0
9.	5-methyl furfural	964	-	3.2	2.0	-
10.	1-heptanol	970	1.0	-	-	-
11.	methyl 2-furoate	974	-	23.5	33.9	2.1
12.	1-octen-3-ol	981	1.2	-	-	-
13.	6-methyl-5-hepten-2-one	986	-	1.7	-	2.4
14.	octanal	1002	-	-	-	1.1
15.	2-ethyl-1-hexanol	1031	-	-	0.5	-
16.	benzyl alcohol	1034	0.2	-	-	2.7
17.	Phenyl acetaldehyde	1045	0.6			2.0
18.	*(E,E)*-3,5-octadien-2-one	1074		0.3	0.6	
19.	*cis*-linalool oxide (furanoid)	1077	55.1		0.6	1.9
20.	2,5-furandicarboxaldehyde	1078		13.4	21.9	
21.	*trans*-linalool oxide (furanoid)	1089	17.7		0.1	
22.	linalool	1101	0.4			
23.	nonanal	1103		1.3	0.6	4.6
24.	isopentyl isovalerate	1104	1.0			
25.	phenylethyl alcohol	1112	0.2	0.1		1.7
26.	2-ethylhexanoic acid	1121				3.8
27.	isophorone	1119	0.2			
28.	4-ketoisophorone	1145	0.8			1.1
29.	lilac aldehyde A	1155	0.3			
30.	*cis*-linalool oxide (pyranoid)	1174	4.0			
31.	*trans*-linalool oxide (pyranoid)	1176	5.3			
32.	a-terpineol	1192	0.4			
33.	safranal	1199	0.5			
34.	decanal	1205	0.1	2.5	1.1	4.5
35.	5-hydroxymethyl furfural	1238		3.2	6.4	
36.	nonanoic acid	1276			0.6	2.0
37.	ethyl nonanoate	1298				1.4
38.	*n*-tetradecane	1400	0.1	0.8	0.6	1.3
39.	*(E)*-geranylacetone	1455		4.2	1.0	4.9
40.	*n*-pentadecane	1500	0.2	1.4	1.0	2.5
41.	*n*-hexadecane	1600		0.8	1.1	3.4
42.	*n*-heptadecane	1700			0.6	0.9
	Oxygenated monoterpenes		**83.2**	**–**	**0.7**	**1.9**
	Apocarotenoids		**1.5**	**–**	**–**	**1.1**
	Non-terpene derivatives		**10.4**	**91.6**	**91.3**	**91.1**
	Total identified		**95.1**	**91.6**	**92.0**	**94.1**

identified substances were confirmed by GC/ClMS, using MeOH as Cl ionizing gas.

RESULTS AND DISCUSSION:

Physico-chemical parameters:

The results of the physicochemical analyses of honey samples from different sources are summarised in Table 1. Honey pH is affected by conditions during extraction and storage which also influences texture, stability and shelf life. Indeed, pH is a useful indicator of possible microbial growth, since most bacteria grow in a neutral and mildy alkaline environment, while yeast and molds can develop in an acidic environment (pH= 4.0-4.5) and do not grow well in alkaline media. The pH values of the analysed honey samples ranged from 3.4 to 4.9. These values are in an acceptable range for honey Adams, 1995 [20].

Percent moisture in the analysed honeys ranged from 14.3 to 17.9. The water content of honey depends on various factors, like harvesting season, the degree of maturity reached in the hive and climate factors. All samples contained less than 20% water.

Moisture and sugar content are strictly correlated, and anomalous values of Brix degrees (directly related with the sugar content) may be a reliable index for adulteration [21]. The analysed samples presented Brix degrees ranging from 79.2 to 81.4.

Ash content is a parameter used for the determination of the botanical origin .The results found (0.1-0.9%) are within the values allowed for floral honeys, indicating clearness of honey samples and possibly lack of adulterations.

The electrical conductivity of honey is closely related to the concentration of mineral salts, organic acids and proteins. This parameter shows great variability according to the floral origin and it is important for the differentiation of honeys of different floral origins. The results obtained for the honey samples varied between 0.22-1.59 µScm-1.

Honey due its acidity to the presence of organic acids, mainly gluconic acid, in equilibrium with the corresponding lactones, and to inorganic ions such as phosphate, sulphate and chloride .[22-24] Lactone acidity is considered as the acidity reserve when the honey becomes alkaline, while the total acidity is the sum of free and lactone acidity. Lactone acidity ranged from 3.9-6.2 meq/Kg and the total acidity varied from 31.0 to 38.6 meq/Kg.

HMF content is a widely used parameter for measuring the freshness of honey samples. Several factors influence the formation of HMF, such as storage condition and floral sources. It is well known that the heating of honey results in the formation of HMF, which is produced during acid catalysed dehydration of hexoses, such as fructose and glucose. All samples presented HMF level below 20mg/Kg of honey, ranging from 9.1 to 15.5 mg/Kg.

Diastase activity is a parameter used to determine if honey has been extensively heated during processing, because the enzyme is susceptible to heating and storage factors. Values ranged between 12-28° Gothe.

Phenolic, flavonoid and proline content

The total content of phenolic compounds in the honey samples varied from 75.6 to 98.5 mg/g (Table 2). Neem honey was found to possess the highest phenolic content, followed by sunflower, mustard, eucalyptus and berseen clover ones. The total content of flavonoids ranged from 1.86 to 4.93 mg/g, expressed as quercetin equivalents (the lowest and highest values were found for Eucalyptus and neem honey, respectively). The ratio of total content of phenolics and flavonoids was calculated in order to evaluate the distribution of flavonoids and non-flavonoid compounds in honey. It resulted about 20. This value suggests that the composition in phenolics may have strong correlation with the botanical source of honey.

The proline content (mg/Kg) varied from 687 to 969 mg/kg. The highest proline content was observed for eucalyptus and lowest one for berseem clover honey.

Radical scavenging activity and antioxidant content

The results of the DPPH radical scavenging activity (RSA) and the antioxidant content of the five different honey samples are summarised in Table 2. The IC50 value for DPPH ranged from 4.97 to 9.45 mg/ml. The highest DPPH RSA was found for Eucalyptus honey, followed by mustard and neem ones. Using the standard curves of ascorbic acid and quercetin, it was shown that the highest antioxidant content was observed for neem and eucalyptus honeys: 37.48 and 35.63 mgAEAC/100g and 12.49 and 11.06 mg QEAC/100g, respectively.

Volatile compounds analysis: The results are reported in Table 3. Altogether 42 compounds were identified, accounting from 91.6% to 95.1% of the whole volatiles.

Honey samples were found to emit numerous volatile organic compounds belonging to different chemical classes, such as terpenes, apocarotenoids and various non-terpene aliphatic, oxygenated and aromatic derivatives. Aromatic aldehydes such as benzaldehyde and phenyl acetaldehyde have been previously reported as common components of various unifloral honey. Sample 1 was found to be rich in cis-linalool oxide (furanoid) (55.1%), trans-linalool oxide (furanoid) (17.7%), trans-linalool oxide (pyranoid) (5.3%), and cis-linalool oxide (pyranoid) (4.0%). Sample 2 contained as major components methyl-2-furoate (23.5%), furfural (15.0%), 2,5-furan dicarboxaldehyde (13.4%) and 2-hexanone (13.3%). The major volatiles detected in sample 3 were methyl-2-furoate (33.9%), 2,5-furandicarboxylaldehyde (21.9%), furfural (9.9%) and 2-hexanone (5.8%). Ethyl acetate was found as major component in sample 4 (29.1%), together with furfural (16.6%), nonanal (4.6%) and decanal 4.5%. It is interesting to note that the different chemical classes of volatiles are not homogeneously distributed among the various samples. In particular, sample 1 was found to be very rich in oxygenated monoterpenes (83.2%). In the same honey sample apocarotenoids reached 1.5% and non-terpene derivatives 10.4%. On the contrary, oxygenated monoterpenes were not very represented in samples 3 and 4 (0.7 and 1.9%, respectively) and were not detected at all in sample 2, which emitted only non-terpene derivatives (91.6%). This latter chemical class was also the main one detected in samples 3 and 4 (91.3 and 91.1%, respectively). In sample 4, also a small amount of apocarotenoids (1.1%) was characterized among its volatiles.

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