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Article

Determination of the Hydrophobic Fraction of Ca, Fe, Mg and Zn in Dark Color Honeys Using Solid Phase Extraction and Flame Atomic Absorption Spectrometry

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Neste estudo, avaliou-se a fração hidrofóbica de Ca, Fe, Mg e Zn em méis escuros por meio de extração em fase sólida utilizando material à base de sílica gel com grupos ativos fenil ligados monomericamente. Verificou-se que Fe está associado com esta fração, provavelmente compostos polifenólicos, no mais alto grau. A contribuição da fração hidrofóbica no conteúdo total deste elemento nos méis analisados representou 7-43%. A confiabilidade destes resultados foi verificada através de extração em fase sólida utilizando material à base de sílica gel com grupos ativos octadecil ligados polimericamente (Superclean™ ENVI DSC-18). Além disso, estabeleceu-se uma forte correlação positiva entre a concentração de Fe na fração hidrofóbica avaliada e a densidade óptica dos méis escuros, indicando uma possível contribuição dos complexos azuis deste metal com substâncias fenólicas para a cor global do mel.

In this study, the hydrophobic fraction of Ca, Fe, Mg and Zn in dark honeys was assessed by means of solid phase extraction using silica gel based material with monomerically bonded phenyl active groups (Discovery® DSC-Ph tubes). It was found that Fe is associated with this fraction, likely polyphenolic compounds, to the highest degree. The contribution of the hydrophobic fraction to the total content of this element in analyzed honeys accounted for 7-43%. The reliability of these results was verified using for solid phase extraction silica gel based material with polymerically bonded octadecyl active groups (Superclean™ ENVI DSC-18). Additionally, a strong positive correlation was established between the concentration of Fe in the hydrophobic fraction assessed and the optical density of dark honeys, indicating a possible contribution of blue complexes of this metal with phenolic substances to the overall color of honey.

Keywords: metals, dark honey, flame atomic absorption spectrometry, solid phase extraction, hydrophobic fraction

Introduction

The elemental analysis of honeys reported in the literature is primarily devoted to determinations of total concentrations of various metals. It is mostly intended to verify their geographical and/or botanical provenience due to recognized elemental patterns and to evaluate their wholesomeness and quality according to certain food safety regulations.[1-4] Interestingly, this kind of the analysis is also capable of providing an informative value about the environmental pollution of the area from which honey is derived.[5-7]

From the data of recent studies, in which the functional speciation approach was used to analyze samples of dark honeys,[8,9] it transpires that such metals as Ca, Fe, Mg and Zn are not exclusively present in this matrix in the form of simple ions but are bound to some extant with different endogenous bioligands that can modulate their mobility and availability. Accordingly, using a non-ionic adsorbing resin Amberlite XAD-16 and a strong acidic cation exchange resin Dowex 50W×8 connected in a series, it was possible in quoted works to extract three operationally defined fractions of metals differing in the hydrophobicity and charge of their species, i.e., hydrophobic, cationic and residual. Considering that the two last fractions are likely to include simple ions of metals, their labile species and stable charged and/or neutral complexes with low molecular mass compounds, it is valid to suppose that these two classes of species have the highest mobility and could be the most bioavailabile

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fractions of metals from honeys.[10,11] In contrast to this, non-ionic and hydrophobic species, which fractions were found to contribute 1-18% (Ca), 17-25% (Fe), 3-18% (Mg) and 7-16% (Zn) to the total concentrations of these metals, are likely to be associated with very high molecular mass organic compounds, e.g., polyphenols and/or their conjugates with the polysaccharides.[8] In consequence of the presence of this group of species, the bioaccessibility of Ca, Fe, Mg and Zn from honey could be impaired[12] and this points that total concentrations of metals are rather inappropriate to evaluate the safety and wholesomeness of honey.

For reasons stated above, the piece information about the share of the hydrophobic fraction in honey seems to be quite important in terms of reflections on the uptake of metals from honey and eventual nutritional effects related to the ingestion of this food product. The possibility to evaluate the contribution of this class of species could also be important due to the role that metals play in the inhibition of the metal-induced oxidation processes and demonstrate the antioxidant properties of honey, case of Fe bound to polyphenolic compounds.[13-15]

In connection with this problem, the present work is an extension of earlier works and aims at verifying the suitability of fast solid phase extraction (SPE) via commercial reversed-phase (RP) cartridges for the determination of the hydrophobic fraction of selected nutritionally important elements (Ca, Fe, Mg and Zn) in dark honeys, for which sums of concentrations of these elements are the highest.[16] The elements selected for the study are components of enzymes essential for a variety of metabolic reactions in the body (Fe, Mg and Zn) or play an important role in bodily functions (Ca and Mg) and should be supplied from the diet. At the outset, conditions of the retention of complexes of metals with hydrophobic phenolic compounds possessing aromatic hydrocarbon groups and condensed rings in their structures by SPE tubes were selected. Their sorption behavior toward simple ions of Ca, Fe, Mg and Zn and their charged or neutral complexes with low molecular mass compounds were verified to ensure that the hydrophobic fraction contributions would not be overestimated. Two SPE tubes, i.e., Discovery® DSC-Ph and Superclean™ ENVI DSC-18, were selected and applied to determine and compare results related to shares of the hydrophobic fraction of Ca, Fe, Mg and Zn in Polish dark honeys. Additionally, to establish the direction and the strength of the relationship between total concentrations of Ca, Fe, Mg and Zn, concentrations of these metals in the hydrophobic fraction assessed and the optical density of honey Spearman's rank correlation coefficients were calculated.

Experimental

Reagents

Ultra-pure water from a PRO-11G reverse osmosis water purification station (Wigo, Wroclaw, Poland) was used throughout. ACS grade solutions of concentrated reagents, i.e., 30% (m/m) H_2O_2 and 65% (m/m) HNO_3, were supplied by J. T. Baker (Deventer, Netherlands). Merck KGaA AAS Titrisol (Darmstadt, Germany) standards containing 1000 mg of Ca (as $CaCl_2$ in water), Fe (as $FeCl_3$ in 15% HCl), Mg (as $MgCl_2$ in 6% HCl) and Zn (as $ZnCl_2$ in 0.06% HCl) were used to prepare single-element stock standard solutions. Mixed standard solutions for the calibration were prepared by subsequent dilutions of stock solutions.

For SPE of metal species from solutions of honey samples via the RP retention mechanism, the following SPE tubes (bed weight of 500 mg and volume of 6 mL) purchased from Sigma-Aldrich (St. Louis, MO, USA) were used: Discovery® DSC-Ph, Supelclean™ ENVI-18, Supelclean™ ENVI-8, and Supelclean™ ENVI-Carb™. Before the sample loading, SPE tubes were washed with 10 mL of methanol, followed by 10 mL of water.

Instrumentation

A Perkin Elmer 1100B single-beam flame atomic absorption spectrometer (FAAS) with a deuterium arc lamp background corrector was used for the determination of concentrations of Ca, Fe, Mg and Zn in all sample solutions and effluents. The instrument was equipped with a single-slot 10 cm Ti burner head for the air-acetylene flame. A stainless steel nebulizer fitted into the end cup of a wettable plastic coated mixing chamber with a drain interlock was applied for the aspiration of solutions. Absorbance readings were carried out using a time-average integration mode, i.e., three readings were integrated at intervals of 0.1 s over an integration time of 1 s and averaged. Concentrations of Ca, Fe, Mg and Zn were measured using recommended operating conditions.

A Thermo Scientific (Bremen, Germany) single-beam Spectronic 20D+ digital visible spectrophotometer was used to measure the color of honey as the optical density at 420 nm of the undiluted samples.[17]

Honey samples and their analysis

Nine commercially available samples of dark honeys were analyzed, i.e., buckwheat, heather and honeydew from coniferous tree honeys distributed by Sadecki Bartnik

(SB-B, SB-H and SB-HDC), buckwheat and honeydew from coniferous and deciduous tree honeys distributed by Huzar Ltd. (H-B, H-HDC and H-HDD) and buckwheat, heather and honeydew from coniferous tree honeys distributed by CD Inc. (CD-B, CD-H and CD-HDC). Total concentrations of Ca, Fe, Mg and Zn in these honeys were determined as described in detail elsewhere.[8,9] In brief, samples of 2.5 g were dissolved in water and diluted to 50 mL. The resulting 5.0% (m/v) honey solutions were analyzed by FAAS against simple standard solutions. The results achieved for three parallel samples were averaged.

To determine hydrophobic fraction contributions of Ca, Fe, Mg and Zn in the analyzed honeys, 10 mL of 5.0% (m/v) solutions of dark honey samples were passed at the flow rate of 2 mL min^{-1} through Dicovery™ DSC-Ph or Superclean™ ENVI DSC-18 SPE tubes to retain non-ionic and hydrophobic species of these metals likely to be bound to polyphenolics. Column effluents (portions of 7 mL after discarding the first 3 mL) were collected and directly analyzed by FAAS against simple standard solutions. Concentrations of Ca, Fe, Mg and Zn in the hydrophobic fraction retained by sorbents were assessed by subtracting concentrations of these metals found in the effluents from their total concentrations determined in sample solutions loaded. By rationing these values to total concentrations of Ca, Fe, Mg and Zn in sample solutions, respective contributions (in %) of the mentioned fraction, were established.

Results and Discussion

Sorption behavior of SPE tubes

To assure that the hydrophobic fraction contributions of Ca, Fe, Mg and Zn would not be overestimate due to the sorption of simple ions of these metals and their stable neutral and/or anionic complexes, the sorption behavior of SPE tubes toward these metal species was examined. For that purpose, 10 mL of working standard solutions (pH 3.5, 4.0 and 4.5) containing simple ions of Ca, Fe, Mg and Zn (at 5.0, 0.2, 1.0 and 0.1 mg L^{-1}, respectively) only or with added disodium salt of ethylenediaminetetraacetic acid (EDTA) at the concentration of 0.01 mol L^{-1} were passed at the flow rate of 2.0 mL min^{-1} through SPE tubes. The pH and concentrations of Ca, Fe, Mg and Zn in working standard solutions were selected with respect to the pH and average concentrations of these metals in 5.0% (m/v) solutions of Polish dark honeys.[1,2] The effluents of SPE tubes were collected and the concentrations of Ca, Fe, Mg and Zn were measured by FAAS to evaluate the contributions of the eventually retained fraction of these metals. The retention efficiencies (in %) were assessed by relating concentrations of the retained metals to their original concentrations in loaded working standard solutions.

All SPE tubes applied were established not to retain simple ions of Ca, Fe, Mg and Zn in the whole pH range examined. It was found that these metals are entirely, i.e., with efficiencies higher than 98, 91, 97 and 95%, respectively for Ca, Fe, Mg and Zn, present in respective effluents. When working standard solutions contained EDTA (added to complex Fe and Zn ions in the pH range of 3.5-4.5) were passed through SPE tubes, it was established that, except for Superclean™ Envi-Carb™, their sorption behavior is quite similar as in case of the sorption behavior toward simple ions. Accordingly, Ca, Fe, Mg and Zn were not retained by Discovery® DSC-Ph, Superclean™ NVI DSC-18 and Superclean™ ENVI DSC-8 SPE tubes in the presence of EDTA in solutions. Respective effluents contained over 97, 94, 98 and 95%, respectively for Ca, Fe, Mg and Zn, of initial quantities of these metals present in working standard solutions. In case of Superclean™ ENVI-Carb™ SPE tubes, from 17 to 22% of the total Fe and from 20 to 27% of the total Zn present in loaded solutions were retained likely due to the formation of complexes with EDTA and a high affinity of this sorptive material toward polar organic species. Such sorption behavior of Superclean™ ENVI-Carb™ SPE tubes precluded their use in further experiments.

Determination of total concentrations of Ca, Fe, Mg and Zn

It is widely accepted that a non-ionic macroreticular adsorbing resin Amberlite XAD-2 is used for the extraction of total hydrophobic and weakly polar phenolic substances from honey after the acidification of sample solutions to pH 2 what results in the appearance of non-dissociated forms of these substances in analyzed sample solutions.[18,19] Here, to retain this fraction of metals under consideration, the pH of sample solutions of analyzed honeys remained unchanged not to alter the existing equilibrium. Discovery® DSC-Ph SPE tubes were used to determine the hydrophobic fraction of Ca, Fe, Mg and Zn because they are recognized to exhibit the improved sorption of substances with ring structures.

As can be seen in Table 1, the highest total concentrations among all determined metals were established for Ca (the average value of 64.3 ± 31.2 μg g^{-1}) followed by Mg (the average value of 47.8 ± 53.4 μg g^{-1}). The average concentrations of Fe and Zn were quite corresponding, i.e., 5.9 ± 7.4 and 4.1 ± 5.0 μg g^{-1}, respectively. Since results of the analysis of honeys sold in a general trade market of Poland have not been reported so far, concentrations of Ca, Fe, Mg and Zn in commercial honeys studied here were

compared with those for honeys donated by individual beekeepers or their associations and locally marked.[1,2]

Table 1. Total concentrations (in μg g^{-1}) of Ca, Fe, Mg and Zn in dark honeys

Sample	Ca	Fe	Mg	Zn
SB-B	32.4 ± 0.6	3.1 ± 0.3	13.1 ± 0.2	16.8 ± 0.4
SB-H	55.6 ± 3.0	0.5 ± 0.1	16.5 ± 1.4	0.7 ± 0.1
SD-HDC	40.7 ± 1.2	2.6 ± 0.3	40.5 ± 0.2	1.6 ± 0.1
CD-B	29.8 ± 0.9	24.3 ± 1.7	7.2 ± 0.2	5.1 ± 0.2
CD-H	63.1 ± 1.0	2.4 ± 0.2	18.0 ± 0.1	4.2 ± 0.6
CD-HDC	118.8 ± 1.1	9.5 ± 0.6	46.4 ± 1.6	4.7 ± 0.1
H-B	49.8 ± 0.8	5.9 ± 1.0	12.6 ± 0.2	0.7 ± 0.1
H-HDC	91.1 ± 1.4	2.8 ± 0.7	120.0 ± 2.7	1.9 ± 0.4
H-HDD	97.0 ± 0.5	2.2 ± 0.4	155.5 ± 2.6	1.5 ± 0.1
Min.-Max.	29.8-118.8	0.5-24.3	7.2-120.0	0.7-16.8

Average value ± standard deviation (n = 3).

The reliability of the results of the total concentrations of Ca, Fe, Mg and Zn obtained through the direct analysis of 5.0% (m/v) water sample solutions was verified by the recovery of these metals added (in the form of simple ions) to sample solutions of buckwheat (SB-B) and coniferous tree honeydew (CD-HDC) honeys. The respective recoveries of the added metals were found to be within 96-100% Ca, 92-93% Fe, 93-95% Mg and 97-99% Zn, proving the dependability of the method applied. It was also established that total concentrations of Ca, Fe, Mg and Zn determined in both selected honeys were comparable to those measured in sample solutions resulted from the wet digestion of respective samples in the mixture of concentrated HNO_3 and 30% H_2O_2 solutions. Apparently, using the *t*-test at the 95% significance level,[20] differences between concentrations of Ca, Fe, Mg and Zn achieved with both sample preparation methods were found to be statistically insignificant. Limits of detection for Ca, Fe, Mg and Zn obtained with the direct analysis of 5.0% (m/v) water sample solutions were 2, 8, 0.5 and 5 μg L^{-1}, respectively, and they were assessed on the basis of the 3σ criterion and using 40 g L^{-1} solutions of glucose as blanks (honey-like matrix at the level of 5.0%). The precision (as the relative standard deviation, RSD) for repeated (n = 3) measurements of solutions containing 40 g L^{-1} of glucose and 0.05, 0.10 and 0.20 mg L^{-1} of Ca, Fe and Zn were within 2.2-6.5% (Ca), 4.3-8.2% (Fe) and 1.8-5.7% (Zn). In case of Mg (at 0.01, 0.02 and 0.05 mg L^{-1}), RSD values were in the range of 3.2-6.1%.

Determination of the hydrophobic fraction of Ca, Fe, Mg and Zn

The hydrophobic fraction contributions of Ca, Fe, Mg and Zn to their total concentrations are given in Table 2. It was found that among studied metals, Fe has the most non-ionic character and the highest affinity to form complexes with hydrophobic organic substances, particularly phenolics that are present in dark honeys in relatively large quantities.[16] The concentration of Fe in the fraction separated in the analyzed dark honeys was establish to vary from 0.2 to 3.7 μg g^{-1}, what corresponds to the share of this fraction within the range of 7-43% in reference to the total content of this metal. These results well conform with outcomes reported in a previous study in which the donation of the hydrophobic fraction of Fe, presumed to be associated with the presence of complexes of this element with polyphenolics and assessed using a more complex and laborious tandem SPE approach, was in the range from 17 to 25% of its total content in the analyzed honeys.[8]

Table 2. Contributions (in %) of the hydrophobic fraction of Ca, Fe, Mg and Zn to total concentrations of these metals in dark honeys obtained after solid phase extraction with Discovery® DSC-Ph tubes. Results achieved using Superclean® ENVI DSC-18 tubes are given in brackets

Sample	Ca	Fe	Mg	Zn
SB-B	3.1 ± 0.2 (6.0 ± 2.0)	32.0 ± 0.4 (30.0 ± 3.7)	3.0 ± 2.0 (3.4 ± 1.7)	6.1 ± 4.4 (5.9 ± 2.6)
SB-H	7.7 ± 0.6 (6.6 ± 4.3)	42.6 ± 8.3 (51.1 ± 8.0)	3.5 ± 0.1 (2.4 ± 0.1)	8.8 ± 4.2 (10.0 ± 4.7)
SD-HDC	13.8 ± 0.6 (14.1 ± 0.9)	7.4 ± 1.0 (9.4 ± 3.5)	1.7 ± 0.5 (0.8 ± 0.1)	[a] (1.2 ± 1.0)
CD-B	4.0 ± 0.6 (1.7 ± 0.9)	15.8 ± 4.7 (18.7 ± 1.1)	[a] [a]	6.7±1.7 (6.5± 2.1)
CD-H	2.2 ± 0.4 (4.3 ± 2.5)	10.3 ± 0.6 (12.4 ± 2.0)	3.1 ± 0.9 (4.1± 2.2)	9.4 ± 1.0 (11.7 ± 2.9)
CD-HDC	7.5 ± 0.5 (6.3 ± 0.7)	29.8 ± 9.8 (35.7 ± 1.0)	1.0 ± 0.4 (2.8 ± 1.5)	[a] (2.8 ± 1.7)
H-B	4.6 ± 2.6 (6.5 ± 1.8)	35.7 ± 3.5 (34.0 ± 4.4)	[a] [a]	[a] (6.0 ± 5.7)
H-HDC	8.0 ± 3.7 (8.4 ± 0.7)	25.5 ± 4.1 (28.3 ± 3.9)	4.3 ± 0.6 (4.5 ± 0.5)	6.4 ± 1.5 (5.9 ± 2.5)
H-HDD	5.7 ± 2.0 (8.2 ± 3.1)	26.5 ± 6.3 (34.8 ± 4.9)	1.7 ± 1.0 (3.1 ± 2.2)	10.5 ± 3.7 (9.5 ± 2.1)
Min.-Max.	2.2-13.8	7.4-42.6	[a]-4.3	[a]-10.5

Average value ± standard deviation (n = 3); [a]the same or slightly higher concentrations in the effluent as in the loaded solution.

In case of Ca, Mg and Zn, the contributions of the separated fraction to total concentrations of these metals were much lower as compared to Fe, i.e., below 14, 4 and 10%, correspondingly. This indicates that the contribution of the bioaccessible fraction of Ca, Mg and Zn to the total concentration of these metals in the dark honeys is reasonably high and that these elements may be easily converted into soluble forms in honey during its ingestion and intaken by the body.[21] The situation is

Table 3. The Spearman's rank correlation analysis ($p < 0.05$) among the total concentration (T) of metals, their concentrations in the hydrophobic fraction (HF) and the optical density (OD)

	T Ca	T Fe	T Mg	T Zn	HF Ca	HF Fe	HF Mg	HF Zn	OD
T Ca	1.000								
T Fe	–0.300	1.000							
T Mg	0.817	–0.450	1.000						
T Zn	–0.267	0.617	–0.200	1.000					
HF Ca	–0.117	0.150	0.083	0.050	1.000				
HF Fe	–0.100	0.883	–0.383	0.533	–0.150	1.000			
HF Mg	0.690	0.647	0.724	–0.204	0.017	–0.443	1.000		
HF Zn	–0.100	–0.085	–0.186	0.559	–0.322	0.085	0.199	1.000	
OD	0.017	0.883	–0.167	0.383	0.100	0.900	–0.434	–0.271	1.000

different for Fe, because a much larger contribution of the hydrophobic fraction of this element may result in its lower bioaccessibility.

The validity of the evaluation results of the hydrophobic fraction contributions was confirmed using Superclean™ ENVI DSC-18 SPE tubes since C_{18} cartridges were previously used to extract phenolic substances in honey.[19,22-24] As can bee seen from Table 2, very comparable results were obtained using both SPE tubes. The differences between contributions assessed using Discovery® DSC-Ph and Superclean™ ENVI DSC-18 SPE tubes were established to be statistically insignificant according to the *t*-test applied at the 95% significance level.[20] This proves the reliability of the analytical procedure used for the determination of the hydrophobic fraction contributions of Ca, Fe, Mg and Zn in honey.

Rank correlation analysis

To indicate any dependence between the total concentrations of metals (TC), the concentrations of metals in the determined hydrophobic fraction (HFC) and the optical density (OD), and to estimate its significance, coefficients of the Spearman's rank correlation were calculated for the set of variables achieved for analyzed dark honeys (Table 3). As can be seen, the strong positive correlations were found between total concentrations of Fe, Mg and Zn and the concentrations of these metals in the fraction assessed using Discovery DSC-Ph SPE tubes. The respective *r* values were equal to 0.883, 0.724 and 0.559 and proved that the metals are bound to hydrophobic compounds like phenolic acids and flavonoids, which may alter their mobility and bioaccessibility or have other implications to the honey properties. No such relation has been reported so far.

Inter-correlations between total concentrations of studied metals, i.e., for couples Ca-Fe, Ca-Zn, Fe-Mg and Mg-Zn, were established to be negative and very weak ($r < 0.500$). Quite similar observations for these metals were reported before.[25-27] The only exception was found for couples Fe-Zn ($r = 0.883$) and Ca-Mg ($r = 0.690$), for which strong positive correlations were found between their total concentrations, likely to high chemical similarities of these metals.

The strong positive correlation was found between the total concentration of Fe and the optical density of honey representing its color ($r = 0.883$). A corresponding relation in case of dark honeys was lately ascertained.[28,29]. Even a much stronger positive correlation was found in the present study between the optical density and the concentration of Fe in the hydrophobic fraction ($r = 0.900$). The significance of this dependence points out that the contribution of complexes of Fe with polyphenols to the color of dark honeys could be quite high. By similarity to the formation of blue complexes of Fe with polyphenols in wine,[30] this effect, especially evident in dark honeys, could be related to the batochrome displacement to the blue region and an increase of the intensity of the red increment, however, a further elucidation is required. No such effect has been described so far. Interesting, in case of Zn, which is capable of forming complexes with polyphenols of honey as well, the correlation between the optical density and the concentration of this metal in the distinguished fraction was weak ($r = –0.271$), showing that such complexes are probably colorless.

Conclusions

A simple and fast method of the determination of hydrophobic fraction of Ca, Fe, Mg and Zn in dark honeys was proposed and included the extraction of respective species by a silica gel based material with phenyl active groups (Discovery® DSC-Ph), offering the improved retention of such large hydrophobic molecules containing ring structures. To simplify the procedure and overcome the inconvenience associated with a too strong retention

of some phenolic-metal species, a non-elution approach was used.

It was established that among studied metals, Fe showed the greatest degree of the complexation by polyphenolic substances. The contribution of the polyhenolic fraction of this metal to its total concentration in analyzed honeys was within 7-43%. In case of Ca, Mg and Zn, respective contributions of this fraction were lower, indicating a higher mobility and bioaccessibility of these metals in dark honeys relative to Fe. The occurrence of a strong correlation between the optical density of honey and the content of Fe in the hydrophobic fraction indicates a significant influence of colored complexes of Fe with polyphenols on the appearance and the color of dark honeys likely due to the batochrome effect.

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