Direct Analysis of Dark Honeys for Total Content of Copper and Its Fractionation Forms

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Abstract Suitability of direct analysis of honey solutions by air–acetylene flame atomic absorption spectrometry for reliable measuring total concentrations of Cu was evaluated and compared with determinations completed after open vessel wet oxidative digestion of honeys with a mixture of HNO_3 and H_2O_2 . Total Cu concentrations determined in amber to dark color raw honeys were within 0.95–1.68 mg kg^{-1} . Additionally, solutions of honeys were subjected to fractionation analysis by means of a two-column solid phase extraction procedure with a non-ionic adsorbing resin Amberlite XAD-16 and a strongly acidic cation exchanger Dowex 50W $\times$ 8-200. Another set of solutions was subjected to fractionation analysis using ultrafiltration with membranes of molecular weight cut-off size of 100, 50, 30, 10, and 5 kDa. Both approaches used to partition Cu species enabled to obtain information on chemical and physical fractionation patterns. It seems that predominant classes of species (67–94%) are cationic and residual species which could be the most accessible forms of Cu from honey due to very low molecular size and possible association with certain organic compounds.

Keywords Cu · Honey · Direct analysis · Solid phase extraction · Ultrafiltration · Flame atomic absorption spectrometry

Introduction

Raw honeys demonstrate a great variability in the content for Cu that is dependent on botanical and geographical origins of floral resources available for bees and different kind of impurities that bees carry from surroundings into a hive (Pohl 2009). Another source of contamination could be beekeepers themselves and their equipment and tools used for honey processing. High concentrations of Cu in honey, even up to 35 mg kg^{-1} , can indeed indicate contaminations related to honey processing, shipping, and storage (Baroni et al. 2009; Pohl 2009). This is because honey is acidic and may release Cu and other related elements (Cr, Fe, Ni) by contacting with surfaces of steel and galvanized tools and containers during harvesting, producing, and packing (Paramas et al. 2000).

As in case of other foodstuffs, bio-accessibility of Cu from honey depends on different factors, including physicochemical forms of Cu species and other dietary components present (Gaetke and Chow 2003). Up to 50% of a total Cu intake can be absorbed in a gastrointestinal digestion tract, mostly as simple Cu(II) ions and species of Cu bound to amino acids (histidine, methionine, cysteine) and organic acids (citric, gluconic, lactic, acetic; Turnlund et al. 1997; Wapnir 1998). Possible toxicity of high concentrations of Cu can be manifested in neurodegenerative disorders and propensity of simple Cu ions to participate in formation of reactive oxygen species (ROS; Bremner 1998). The latter species are capable of reacting

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with practically all biological molecules, including lipids, proteins, and DNA, causing their oxidative damage (Gaetke and Chow 2003). An ability of polyphenols to chelate Cu ions can however result in antioxidative effects, i.e., lowering Cu redox activity, inhibiting ROS formation, or scavenging these species (Andrade et al. 2005).

In reference to this, an ability to determine not only total concentrations of Cu in honey but also differentiate and quantify distinct Cu species or their classes differing due to certain physicochemical properties, including for example charge, reactivity, or molecular weight size, seems to be of special significance and importance. Information on Cu speciation would be more appropriate to predict possible physiological or nutritional effects of this element. This however implies special procedures and methods of analysis that enable separating and distinguishing various Cu species. Unfortunately, overwhelming literature reports devoted to honey analysis are merely focused on total concentrations of Cu and other elements.

This work was aimed at direct analysis of honey by means of flame atomic absorption spectrometry (F-AAS) for total concentrations of Cu in several amber to dark color honeys. For that reason, an appropriateness of a more effortless and faster method for determining total quantities of Cu in mentioned honeys was evaluated. In this alternative method of analysis, samples of honey were dissolved and diluted in water and directly analyzed by F-AAS using external calibration with aqueous standard solutions. Although a two-column solid phase extraction (SPE) procedure using a non-ionic macroreticular adsorbing resin Amberlite XAD-16 coupled to a gel strong cation exchange resin Dowex 50W $\times$ 8-200 was already applied to partition Cu, Fe, and Mn species in blossom honeys (Pohl and Sergiel 2010), here both. SPE and ultrafiltration (UF) procedures were used to fractionation analysis of fractionate Cu species in more nutritionally relevant dark honeys. In this way, it was possible to discriminate distinct chemical and physical fractions of Cu species, respectively, and compare results achieved with both approaches in respect to special features of certain groups of compounds binding Cu ions. Accordingly, a chemical partitioning of Cu in analyzed honeys was carried out using a two-column SPE and resulted in distinguishing three operationally defined fractions differ due to hydrophobicity and charge of Cu species. Fractionation due to molecular weight of species with which Cu is associated was attained when honey solutions were ultrafiltered through membranes with molecular weight cut-offs (MWCO) between 5 to 100 kDa. With this complementary data, it was possible to attribute a more defined chemical structure to separated fractions and predict Cu availability from honey.

Materials and Methods

Instrumentation

A Perkin Elmer 1100 B air–acetylene F-AAS was used to determine Cu concentrations. Operating conditions selected for F-AAS followed manufacturer recommendations given for Cu measurements in a lean flame, i.e., flow rates of fuel and oxidant of 1.2 and 8.0 l min⁻¹, respectively, an analytical absorption line wavelength of 324.8 nm, a spectral bandwidth of 0.7 nm, and a HCL lamp current of 15 mA. Absorbance readings were integrated at 0.1 s intervals over a 1-s integration time. Three replicate measurements were made and averaged in each read cycle. Solutions were analyzed versus aqueous standard solutions of Cu at concentrations within a 0.05–1.00 mg l⁻¹ range and suitable procedural reagent blanks. An instrumental detection limit of 0.006 mg l⁻¹ was assessed for Cu under these operating conditions. Precision expressed as relative standard deviation (RSD) for three replicate measurements of 0.05, 0.10, and 0.20 mg l⁻¹ Cu solutions varied from 0.9 (at 0.20 mg l⁻¹) to 5.5% (at 0.05 mg l⁻¹).

Reagents and Materials

Analytical reagent grades of HCl, HNO₃, NaOH, and C₂H₅OH were purchased from POCh (Gliwice, Poland). A 1,000-mg l⁻¹ standard solution of Cu from Merck KGaA (Darmstadt, Germany) was used to prepare standard solutions for external calibration. Ultra-pure water from a Pro-11G reverse osmosis water purification system (Wigo, Wroclaw, Poland) was used in all preparations. Glass columns from Sigma-Aldrich (Saint Louis, MO, USA) with coarse frits and Teflon stopcocks were used for SPE. Sigma-Aldrich non-ionic macroreticular adsorbing Amberlite XAD-16 (mesh size 20–60, surface area 800 m² g⁻¹, dipole moment 0.3 D) and a gel-type strongly acidic cation exchange Dowex 50W $\times$ 8-200 (mesh size 100–200, sorption capacity 1.7 meq ml⁻¹) resins were used. A Cole-Parmer 4-channel MasterFlex L/S peristaltic pump (Vernon Hill, IL, USA) was used to maintain and control flows of solutions through columns.

Sample Preparation

Ripened amber to dark color honeys, including buckwheat (B), buckwheat-goldenrod (BG), heather (H1, H2), and honeydew (HD1, HD2), were kept in plastic containers in a dark place. Total concentrations of Cu were determined by direct analysis of solutions resulted from dissolving samples in water and digesting with oxidizing reagents. In first case, 2.5-g samples were placed in 100-ml beakers and dissolved in about 10 ml of water and then diluted to 50 ml.

Wet acid digestion was carried out in open vessels as following: 2.5-g samples were placed in 250-ml beakers and dissolved with 10 ml of a 1 + 1 HNO₃ solution. Resulting aliquots were brought to boil on a hot plate and refluxed for 2 h under cover glasses. After cooling, 10 ml of a 30% (*m/v*) H₂O₂ solution were added and sample solutions were heated to reduce volume to about 1 ml. Residues left were dissolved with water and made up to 50 ml. In both cases, resulting 5.0% (*m/v*) honey solutions were directly aspirated to F-AAS and analyzed versus external aqueous standard solutions. Three independent analyses were made for each honey, and respective blanks were considered in final results.

Resin Conditioning and Chemical Fractionation

Amberlite XAD-16 was initially dried at 110 °C in an oven for 4 h and then, its 1.0-g portions were wetted with methanol, subsequently with water, and finally, poured into columns. Resin beds formed were washed with 10 ml of a 1-mol l⁻¹ HCl solution and next with 20 ml of water to remove excessive HCl. Portions (1.0 g) of Dowex 50W × 8-200 as received were wetted with water and then poured into columns. Resin beds formed were washed with 10 ml of a 1-mol l⁻¹ HCl solution and then with 20 ml of water. It was followed by rinsing them with 10 ml of a 1 mol l⁻¹ NaOH solution and finally, with 20 ml of water to remove excessive NaOH. Conditioning solutions and water were driven through columns at 2 ml min⁻¹.

A two-column SPE procedure was used to partition Cu species in honeys due to their chemical properties (chemical fractionation). Accordingly, 50-ml 5.0% (*m/v*) honey solutions were passed at 2 ml min⁻¹ through a first column with Amberlite XAD-16 to retain hydrophobic Cu species. Effluents of this column were directly passed through a second column at 2 ml min⁻¹ to retain cationic Cu species on Dowex 50W × 8-200. After passing 40 ml of sample solutions, 10-ml effluents from the first and the second columns were sampled to evaluate contents of Cu in polyphenolic and residual Cu species fractions, respectively. Columns were split up and cationic Cu species retained by the second column were eluted using 10 ml of a 2-mol l⁻¹ HCl solution at 1 ml min⁻¹.

Physical Fractionation

A Millipore (Billerica, MA, USA) 8200 UF stirred cell with a stirring bar and a 200-ml sample reservoir were used. Pressure in the cell was maintained with compressed N₂ and controlled by a pressure adjustment valve fitted with a pressure gauge. At first, 200 ml of 5.0% (*m/v*) honey solutions were filtrated through Nylone 66 0.45 µm membrane filters (Sigma-Aldrich).

Filtrates were collected and successively passed through Millipore Biomax PB polyethersulfone ultrafiltration membranes with MWCOs of 100, 50, 30, 10, and 5 kDa. Each time, permeates that left the cell after filtration through a membrane of a certain MWCO were collected and passed through the membrane with a lower MWCO. Portions (10 ml) of respective permeates were taken to determine concentrations of Cu associated with species below the certain MWCO.

Results and Discussion

Determination of Cu in Honey-Like Matrices

Honey can be treated as an organic sample matrix containing mostly two simple carbohydrates, i.e., glucose and fructose, which contribute to it up to 0.80–0.85 kg kg⁻¹ (Belitz et al. 2004). It can also include relatively high amounts of K and Na that both are recognized to be the most abundant mineral constituents of honey (Pohl 2009). Thus, direct F-AAS analysis of honey solutions resulted from dissolving its samples in water on the content of trace metals, like Cu for example would possibly be prone to matrix effects. To establish if these interferences are related to the presence of mentioned organic and/or mineral constituents, a honey-like matrix was prepared and contained on average 0.45 kg kg⁻¹ of glucose, 0.40 kg kg⁻¹ of fructose, 600 mg kg⁻¹ of K, and 150 mg kg⁻¹ of Na. Then, a set of 100-ml solutions containing 1.0%, 2.5%, 5.0%, 7.5%, 10.0%, 15.0%, and 20.0% (*m/v*) of this matrix in addition to Cu at a concentration of 0.20 mg l⁻¹ were analyzed by F-AAS versus external aqueous standard solutions. Additionally, to check the effect of a real honey matrix on the performance of F-AAS for the determination of Cu, water solutions of honeydew honey (HD1) were also prepared and analyzed on the content of Cu using external aqueous standard solutions.

Results of these analyses are given in Table 1. It can be seen that recovery of Cu gradually decreases when the content of the honey-like matrix is increasing. It reaches only 70% of the initial concentration in case of analysis of a 20.0% (*m/v*) honey-like matrix solution. However, measuring corresponding solutions but without both carbohydrates, it was established that concentrations of Cu determined using external calibration with aqueous standard solutions were on average 0.198±0.006 mg l⁻¹. With respect to this, it was possible to conclude that analytical performance of F-AAS is predominantly affected by glucose and fructose, which change viscosity of solutions, especially when present in elevated quantities (Ioannidou et al. 2005; Lopez-Garcia et al. 1999). Under

Table 1 Concentrations Cu determined in solutions containing different contents of honey-like (Na, K, glucose, and fructose) and honeydew honey matrices using F-AAS external calibration with simple aqueous standard solutions

Content (%; <i>m/v</i>)	Honey-like matrix	Honeydew honey (HD1) matrix	
	Concentration ^a (mg l ⁻¹)	Concentration ^a (mg kg ⁻¹)	<i>t</i> _{calculated} ^b
1.0	0.200±0.003	1.25±0.16	-1.52
2.5	0.199±0.004	1.40±0.06	0.75
5.0	0.201±0.002	1.44±0.07	–
7.5	0.189±0.004	1.22±0.05	-1.77
10.0	0.165±0.004	1.07±0.08	-3.98
15.0	0.154±0.002	NA	–
20.0	0.139±0.002	NA	–

NA not analyzed

^a Average values (*n*=3) ± SDs^b *t* statistic for 4 degrees of freedom and at 95% level of confidence (*t*_{critical}=2.78) was used to compare mean concentrations determined in a 5.0% (*m/v*) honeydew honey solution with those found in remaining honeydew honey solutions

such conditions, external calibration with aqueous standard solutions is not any more possible. Corresponding results were obtained when analyzing solutions with different proportions of honeydew honey (HD1), i.e., 1.0%, 2.5%, 5.0%, 7.5%, and 10.0% (*m/v*). On the basis of *t* test carried out (Table 1), it was found that differences between Cu concentrations obtained for 1.0%, 2.5%, and 5.0% (*m/v*) honeydew honey solutions were statistically insignificant. Nevertheless, uncertainty of results achieved for the lowest concentrated honey solution was quite high (13% as RSD, *n*=3). Analysis of much concentrated honeydew honey solutions, i.e., 7.5% and 10.0% (*m/v*), versus external calibration with aqueous standard solution gave notably lower Cu recoveries as compared to 2.5% and 5.0% (*m/v*) honey solutions.

Direct Analysis of Honeys

Concentrations of Cu and other transition metals in honey are commonly determined by F-AAS after sample digestion aimed at decomposing carbohydrate-rich matrix and releasing mineral constituents. For measurements of very low concentrations of this metal in honey (<0.1 mg kg⁻¹), a more sensitive technique is required, and this is the case of atomic absorption spectrometry with electrothermal atomization (Vinas et al. 1997). Typically dry ashing or wet ash digestions are used for that purpose (Baroni et al. 2009; Nanda et al. 2009; Dag et al. 2006). Curiously, direct analysis of honey, including only simple dissolving of samples in water, is quite occasional (Soares dos Santos et al. 2008; Hernandez et al. 2005; Lopez-Garcia et al. 1999) even though such kind of analysis offers a reduced sample preparation time and a lower risk of losing measured elements or contaminating samples with impurities.

Sample handling and preparation of direct analysis of honey is undoubtedly easier, and hence, after verifying the effect of the honey matrix on the response of Cu in F-AAS, it was decided to analyze 5.0% (*m/v*) solutions of studied dark honeys against external calibration with aqueous standard solutions. Results obtained using this sample treatment were compared with those achieved after analysis of solutions originated from decomposition of samples with concentrated HNO₃ and 30% (*m/v*) H₂O₂ using *t* test (Table 2). It was found that differences between these two sets of Cu concentrations were statistically insignificant at a confidential level of 95.0%. Precision of direct analysis results, evaluated from three independent replicates, was acceptable since it changed from 4.2% to 8.8% (with respect to RSDs). Accuracy was checked by examining recoveries of spiked samples of honeydew honey (HD1). Spiked and original sample solutions were measured under external calibration with aqueous standard solutions and apparent recoveries were determined. Their values achieved, i.e., 97.3±2.9%, 98.6±3.2%, and 99.1±2.1% for spiking samples with 0.020, 0.050, and 0.070 mg l⁻¹,

Table 2 Total concentrations of Cu obtained using direct analysis of honey (A) and after its mineralization in open vessel system (B) in addition to contributions of Cu species fractions distinguished using SPE

Honey	Concentration ^a (mg kg ⁻¹)		Fraction contribution ^b (%)			
	A	B	Hydrophobic	Cationic	Residual	Sum
B	1.25±0.11	1.44±0.13	7.2±1.0	39.2±3.8	54.4±5.8	100.8±7.0
BG	1.06±0.05	1.10±0.06	11.3±1.1	55.7±2.8	35.8±1.9	102.8±3.6
H1	1.08±0.05	1.01±0.05	15.7±1.2	40.7±3.4	42.6±2.7	99.0±4.5
H2	0.95±0.04	1.02±0.07	20.0±2.3	33.7±2.5	47.4±2.9	101.1±4.5
HD1	1.44±0.12	1.54±0.06	30.6±2.9	46.5±3.9	20.1±1.8	97.2±5.2
HD2	1.68±0.09	1.70±0.10	28.9±2.1	42.6±2.8	28.3±2.4	99.8±4.2

^a Average values (*n*=3) ± SDs^b Average values (*n*=3) ± pooled RSDs

respectively, supported that results of direct analysis are reliable and accurate.

Fractionation Pattern for Cu Obtained with Solid Phase Extraction and Ultrafiltration Approaches

It is generally accepted that food products are suitable for consumption if they do not contain minor and trace element contaminants above certain permitted limits. Unfortunately, such limits are commonly expressed as total concentrations, but it is well recognized that bioavailability and metabolic activity of elements are related to physicochemical forms they are present (Dean 2007). Amber to dark color honeys are presumed to contain much higher amounts of flavonoids and phenolic acids as compared to light color honeys (Pohl and Sergiel 2010; Bogdanov et al. 2008); hence, partitioning of Cu due to hydrophobicity, charged and molecular weight of its species in these honeys is worth of studying in view of their food safety and quality. Accordingly, solutions of analyzed honeys were treated with a two-column SPE fractionation scheme. In addition, solutions of three selected honeys (BG, H1, and HD1) were subjected to UF using membrane filters with MWCO within 5–100 kDa in order to compare results obtained for both fractionation approaches.

In reference to results of chemical fractionation (Table 2), it can be seen that predominant fractions for Cu are almost equally abundant cationic and residual species. These Cu species classes account for 34–56% and 20–54%, respectively, regarding total concentrations of Cu in analyzed amber to dark honeys. It seems that these two Cu fractions particularly include species with molecular weight <5 kDa since in case of BG, H1, and HD1 honeys sums of contributions of cationic and residual fractions well corresponds to respective contributions of this low molecular weight fraction (LMW: from 67% to 88%) obtained by UF (Table 3). The first group of species (cationic fraction) could be simple ions of Cu and its labile complexes. In view of biological activity of small charged element species ingested with food in living organisms, it is valid to speculate that this fraction is the most bioavailable (Turnlund et al. 1997) and should be considered when assessing, for example, toxic levels of Cu in case of its elevated concentrations in honeys. Mentioned residual species could be stable and/or neutral complexes with relatively LMW compounds, i.e., some hydroxycarboxylic acids (citric, oxalic, ascorbic, gluconic, lactic) and amino acids (particularly histidine, methionine, and cysteine). These species also make ingested Cu readily absorbable (Turnlund et al. 1997).

The contribution of hydrophobic Cu species was found to be within 7–31% when regarding total concentrations of this element. Apparently, this fraction is likely to be attributed to species of Cu bound to apolar and moderately polar high molecular weight (HMW) organic compounds of

Table 3 Contribution of Cu species fractions distinguished using UF

Fraction	Contribution ^a (%)		
	BG	HD1	H1
>0.45 μm	— ^b	— ^b	— ^b
>100 kDa	— ^b	— ^b	— ^b
50–100 kDa	1.3 $\pm$ 0.1	2.7 $\pm$ 0.9	4.5 $\pm$ 0.3
30–50 kDa	— ^b	— ^b	— ^b
10–30 kDa	10.2 $\pm$ 1.9	26.8 $\pm$ 4.2	18.3 $\pm$ 1.2
5–10 kDa	— ^b	— ^b	— ^b
<5 kDa	88.1 $\pm$ 4.1	67.1 $\pm$ 3.5	80.1 $\pm$ 3.9
Sum	99.9 $\pm$ 4.5	96.6 $\pm$ 5.5	102.9 $\pm$ 4.1

^a Averaged values ($n=3$) $\pm$ SDs

^b Concentrations of Cu in a filtrate is the same or a little higher than in a feeding solution

50–100 kDa and >100 kDa. A notable correlation between sums of contributions of these two HMW fractions and shares of respective hydrophobic fractions distinguished in BG, H1, and HD1 honeys was observed. Considering that complexes of polyphenols with Cu impair its bioavailability from food (Vitali et al. 2008), this fraction can be regarded as highly inaccessible and possibly have a decreasing effect on dietary Cu intake. Information on this class of species is also important in respect to a role that polyphenols play in inhibiting Cu-induced oxidation process and free radical damage by forming stable complexes with Cu ions (Andrade et al. 2005; Hadi et al. 2007).

With reference to accuracy and precision, the SPE fractionation procedure enables recover of Cu from analyzed honeys with relative errors within –2.8% to +2.8%. Precision expressed as RSD and estimated for separated fractions was found to range from 1.0% to 5.8%. Corresponding accuracy for the UF procedure was within –3.4% to +2.9%. Precision of repeated measurements ($n=3$) varied between 0.1% and 4.1%. All these figures verify dependability of both fractionation procedures used for Cu partitioning.

Conclusions

A very simple method of total content analysis honey by means of F-AAS was demonstrated on the example of Cu. It can save time and costs of honey pre-treatment before analysis by F-AAS using direct aspiration of honey solutions and external calibration with aqueous standard solutions.

It seems that this concomitant element is highly bio-accessible from amber to dark honeys since percentage shares of cationic and residual Cu species fractions were

found to change within 67–94% in relation to total concentrations. Both Cu species classes can be presumed to be simply available, and thus, their evaluation is very important in reference to quality, safety, and nutrition values of honey. In this regard, proposed simple and versatile SPE and UF methods to classify and determine relevant groups of Cu species associated with compounds of different MWs appear to be relevant for evaluating bioavailability of Cu. Data obtained with both approaches are equally important and comprehensive as they give the rightest answer about chemical nature of possible group of species of Cu and their availability from honey. These two complementary procedures can also be applied to partition other biologically relevant elements in honey.

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