Sugar Spectrum, Hydroxymethylfurfural and Diastase Activity in Honey: A Validated Approach as Indicator of Possible Adulteration

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Abstract

Honey is used for centuries both as nutrient and in medicine, being an exhaustless source of benefic factors for health and also for different biotechnological processes. This is a complex matrix which possesses antioxidant and antimicrobial properties due to its peroxidic activity and bioactive compounds. A fully validated approach for the determination of sugars, hydroxymethylfurfural content and diastase activity in honey samples were presented in accordance with the official methods from the International Honey Commission. Spectrophotometric method was used for diastatic activity determination and chromatographic determinations for HMF content (photodiode array detection) and sugars (refractive index detection). The limits of detection and quantification were calculated. Accuracy, precision and uncertainty were determined in the spectrophotometric and chromatographic techniques using the certified reference material. Diastase activity, sucrose content and hydroxymethylfurfural (HMF) content were easily differentiated and these parameters were used for indication of a possible adulteration of the honey. The present study confirms that, in order to have an authentic honey sample, diastatic activity must be above 8 DN (diastatic number) and HMF content less than 40 mg/kg. Sugar spectrum is different for every honey type. Consequently, physico-chemical analysis should be combined for the determination of authentic honey and proper labeling.

Keywords: authenticity, diastase activity, honey, hydroxymethylfurfural, sugar spectrum.

Introduction

Honey is "a natural sweet substance produced by the honey bees *Apis mellifera* from the nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances

of their own, deposit, dehydrate, store and leave in the honey comb to ripen and mature", as stated by Directive 2014/63/EU. In fact, honey is a very complex mixture of sugars and water (as major components) and a multitude of substances (vitamins, minerals, proteins, amino acids, organic acids, hydroxymethylfurfural, antioxidants from

the class of phenolic acids and flavonoids and different enzymes) as minor components.

Beside its nutritional value, honey possess a wide range of bioactive properties, due to its complex chemical composition: antioxidant, antibacterial, hypoglycemic, antifungal, gastroprotective, anti-inflammatory effects (Alvarez-Suarez *et al.*, 2013; Vallianou *et al.*, 2014; Machado De-Melo *et al.*, 2017; Scephankova *et al.*, 2017; Soares *et al.*, 2017; Bobiş *et al.* 2018; Fazalda *et al.*, 2018). The importance of honey in human nutrition is obvious, and studied since early times, nowadays the modern methods used for chemical composition determination and also high resolution equipments, responded to the questions raised over the time (Oroian & Ropciuc, 2019; Bobiş *et al.*, 2020; Geana & Ciucure, 2020; Pauliuc *et al.*, 2020). Beside this, it is demonstrated that different types of honey possess many healing properties, first against reactive oxygen species, which are the principal responsible for different diseases, many of them being considered deadly of with low chances of curing (Alvarez-Suarez *et al.* 2016; Afrin *et al.*, 2019; Kocyigit *et al.*, 2019). The apoptotic effect of honey or it's extracts on different carcinoma cells is also demonstrated in a series of scientific studies (Samarghandian *et al.*, 2011; Morales & Haza, 2013; Rady *et al.*, 2018).

Similar to other food products, honey may be subjected to possible adulteration. Two aspects should be considered when talking about honey authenticity: authenticity related to production and processing and authenticity related to description (Mădaş *et al.*, 2019a; Geana *et al.*, 2020). Honey chemical composition determination, comprises physico-chemical determinations, spectrophotometric and chromatographic methods, sensory analysis and palynological determinations of uniflorality (Mădaş *et al.*, 2019b).

Sugar profile in honey vary due to its botanical origin. Although glucose and fructose are the most important sugars in honey, their quantity and ratio, may give informations about botanical origin of honey (Mărghitaş *et al.*, 2010; Kamal and Klein, 2011; Aronne *et al.*, 2014; Mădaş *et al.*, 2019b; Vranic *et al.*, 2019). Also, the presence of different minor sugars are used as potential markers for different types of honey (Sanz *et al.*, 2005; Pereira *et al.*, 2014). Honey is different from other natural sweeteners, by the presence of enzymes. Naturally, honey contain small amounts of enzymes. They are

added by the bees, but may came from the nectar of the floral source. The predominant enzymes in honey are diastase, invertase and glucose oxidase (Bogdanov *et al.*, 2008). Diastase (or amylase) is an important indicator of honey freshness and authenticity (Persano-Oddo *et al.*, 1990; Alvarez-Suarez *et al.*, 2013). It came from the bees in the process of transforming the nectar into honey. The nectar type and flow, and the age of the bees are the main factors for the amount of diastase found in honey (Persano-Oddo *et al.*, 1990). Invertase degrades sucrose in more simple constituents, the reason why in honey different minor sugars are present, although they are not present in nectar (Donner, 1977). Invertase is present in ripen honey, and may be determined spectrophotometrically (Persano-Oddo *et al.*, 1999). Glucose oxidase (GOx) is an important enzyme of honey, directly related to the antibacterial activity of honey. This enzyme is degrading glucose to a gluconolactone and further to gluconic acid (the main organic acid in honey) and hydrogen peroxide, the main substance responsible for the antibacterial activity. The enzyme was found in the hypopharyngeal gland of the bees, from where it reaches honey (Bucekova *et al.*, 2014). Hydroxymethylfurfural (HMF) is a furane compound, generally formed in the caramelisation process (thermal treatment applied on carbohydrate containing products). The presence of simple sugars and different acids in honey, combined with high temperature storage or procession, lead to the appearance of high amounts of HMF, which may be found in very small amounts even in fresh honey. Codex Alimentarius Standard Commision have stated a limit of 40 mg/kg (80 mg/kg for honeys from Tropical regions), which permit moderate heating of honey to have a lower amount of HMF present in commercial honey properly processed (Codex Alimentarius, 2001).

In order to have a guarantee that such methods may be used as indicators for honey authenticity, their validation is required. Laboratory for Quality Control of Bee Products have validated internally the above mentioned methods according to Eurachem CITAC Guide (2012; 2019) and with proficiency testing participations.

Materials and methods

The research has been carried out in Laboratory for Quality Control of Bee Products and Bee

Diseases from University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania.

Honey samples. Twenty-four honey samples of different botanical and geographical origins (5 samples of acacia honey, 3 polifloral, 4 rape (canola), 3 honeydews, 2 buckwheat, 2 sunflowers, one of linden, raspberry and heather from Romania and also one sample of eucalyptus and lavender from the Proficiency Testing Trial). All honeys were melisopalynologically characterized, and their botanical origin was established according to the validated method used in our laboratory (Louvreaux *et al.*, 1978).

Sugar spectrum determination. Sugar content determination in the laboratory is made according to European Honey Commission (1997) Method, using high performance liquid chromatography and refractive index detection for principal sugars present in honey (Bonta *et al.* 2008). The HPLC analysis of the carbohydrates is carried out on a modified Alltima Amino 100Å stainless steel column (4.6 mm diameter, 250 mm length, particle size 5 µm). The SHIMADZU instrument (LC-10AD VP model, Shimadzu, Kyoto, Japan) is equipped with degasser, two pumps, auto sampler, thermostat oven, controller and refractive index detector. The injection volume was 10 µl and the flow rate was 1.3 ml/min. The mobile phase is a solution of HPLC purity acetonitrile and ultrapure water (80/20 v/v). Five g of honey are dissolved in water (40 ml) and transferred into a 50 ml volumetric flask, containing 12 ml HPLC grade purity methanol and filled up to the mark with water. The solution is filtered through a 0.45 µm membrane filter, collected in sample vials and placed in autosampler for analysis. For the quantification of main sugars, different calibration curves in the range 50–0.25 g/100 g (fructose 50–20 g/100 g; glucose 10–40 g/100 g; sucrose 0.3–15 g/100 g; turanose, maltose, isomaltose, erlose 0.25–5 g/100 g), with regression coefficients (R^2) higher of 0.998 were obtained. The results are expressed in g/100 g honey.

Diastase activity. Diastase activity is determined following the protocol of Megazyme International Ireland (2014) and validated in the lab. Two g of honey are weighted and dissolved in 40 ml maleat buffer 100mM (pH 5.6) in a beaker and transferred quantitatively in a 50 ml volumetric flask. 1 ml of the diluted honey is placed in a glass tube and is pre-incubated at 40°C for 5 min.

An Amylzyme tablet is added to the tube, without removing from the water bath and without stirring. The incubation is continued for another 10 min. 10 ml of Trizma base solution is added to terminate the reaction and the tube is stirred on a vortex for about 30 sec. After 5 min at room temperature, the solution is filtered through filter paper and the absorbance is read against a reaction blank (prepared in the same way, except adding the sample). For a lot of Amylzyme tablets (200 pcs) a calibration curve is provided by the beneficiary. For the analysed honey samples presented in this study, the diastase activity was calculated following the equation:

$$\text{Diastazic activity} = 20 \times \text{DO}_{590}$$

HMF analysis. HMF determination was based on the method described by the European Honey Commission (1997) and validated in the lab. HMF is determined from a clear, filtered aqueous solution of honey, using high performance liquid chromatography with photodiode array detection, where the signal is compared with a standard of known concentration. The separation is made on a Discovery HS C18 (5µm; 4.6x250 mm) column, equipped with a C18 pre-column, mobile phases: ultrapure water (solvent A) and HPLC grade methanol (solvent B), injection volume 20µl and 0.8 ml/min flow rate in gradient mode. The gradient started at 10% B until 13 min, increase to 90% B at 15 min and run isocratically (90% B) up to 20 min. The percent B decreased to 10% B and runs 10 min at the same concentration. The detection is made at 283 nm. Ten grams of honey is weighted in a glass beaker and approx 25 ml water is added for dissolution. The honey solution is transferred to a 50 ml volumetric flask, and 0.5 ml Carrez I solution is added. After proper mixing, 0.5 ml Carrez II solution is added and filled to the sign with water. After mixing all reagents, the solution is filtered through filter paper and the first 10 ml are discharged. The solution is again filtered through Millipore filter (0.45 µm) and injected into HPLC system. A calibration curve is constructed with known concentration of HMF standard, subjected to the same preparation protocol as the sample and the unknown concentration of HMF from honey samples are determined using the calibration curve values. The result is expressed as mg HMF/kg honey.

Method validations. Honey is a complex matrix, different from one sample to another. Because the validation of an analytical method need a reference material, an *in house* reference material was used, a honey with all parameters determined, divided into small amounts and kept in the freezer. The reference material was used to determine the limit of detection (LOD), limit of quantification (LOQ). *In house* reference material was used for accuracy, precision and uncertainty of the proposed methods, following Eurachem/CITAC Guidelines (2012, 2019). Limit of detection is the lowest concentration of analyte that can be determined with confidence. For the determination of LOD, 7 independent standard solutions of fructose, glucose and sucrose in mixture, were measured (repeatability level 0.1%). The average concentration for each sugar and standard deviation of the 7 determinations was calculated. LOD was expressed as " $c+3STDEV$ ". The limit of quantification (LOQ) is strictly, the lowest analyte concentration that can be determined with an acceptance level of fidelity or repeatability and accuracy. 7 independent standard solutions of fructose, glucose and sucrose in mixture were measure under repeatability conditions, each measured once (concentration level 0.1%). The average concentrations and standard deviation of the 7 determinations were calculated. The LOQ was expressed as having the value of " $c+10STDEV$ ". Precision was studied by repeatability (the results of independent tests were obtained by the same method, from the same honey sample, in the same laboratory, by the same operator using the same equipment, in short intervals) and reproducibility (the results of independent tests were obtained with the same method, from the same honey sample, in the same laboratory, but by different analysts under different conditions, on different days). Established performance criteria: the relative standard deviation of the value of determinant, measured under repeatability conditions should not exceed 5%. Accuracy expresses the degree of agreement (approximation) between the average value obtained in a series of test results and a reference value (true value). It is ideal to compare the analytical result with a certified value. Established performance criteria: accuracy expressed as the relative difference between the average of 6 determinations and the actual (reference)

value should not exceed 5%. Uncertainty is a parameter associated with the result of a measurement, which characterizes the dispersion of values that can reasonably be assigned to the measurement. Uncertainty was calculated for a 95% confidence interval and a coverage factor of $k = 2$. Established performance criteria: extended uncertainty should not exceed 20% of the measurement value.

Same validation parameters were made for HMF determination: LOD, LOQ, accuracy, precision and uncertainty, following Eurachem/CITAC Guidelines (2012, 2019). Diastase validation followed uncertainty, accuracy and precision determinations, similar to sugars and hydroxymethylfurfural determination.

Results and discussions

Method validation results. Detection and quantitation limits (LOD and LOQ) were determined on reference materials (standards of HMF and sugars). Average value of the seven measurements was determined (0.117 mg/L HMF) and relative standard deviation (0.0095). LOD was expressed using the formula: $c+3STDEV$. Obtained value was 0.15 mg/L (the lowest concentration which may be determined with confidence). For LOQ determination, the same 7 determinations were used, with the same values of the mean and standard deviation. The value obtained for LOQ was 0.21 mg/L. Established performance criteria: $LOD < LOQ < \text{the lowest value from the work concentrations domain}$. LOD and LOQ determination for individual sugars (fructose, glucose and sucrose), a mixture of the three sugars was made, 0.1% each, following the described method. The LOD values were: fructose 0.13%; glucose 0.13%; sucrose 0.15%, and LOQ values were: fructose 0.22%; glucose 0.23%; sucrose 0.22%.

Precision was studied by repeatability and reproducibility determinations, taking into analysis three *in house* reference materials with different levels of HMF concentration. The average value of the 6 measurements was: 3.38, 15.99 and 42.43 mg/kg. Relative standard deviation (%RSD) was calculated for every concentration level, and 2.11, 1.80 and 1.85% was obtained. Under reproducibility conditions, values of 8.51, 7.79 and 4.94 % were obtained, which must be lower than 10% (Eurachem Citac

Table 1. Uncertainty calculated for the three parameters (sugars, diastase and HMF) in APHIS Laboratory

Level of concentration	U	Calculated result	U·100/c (%)
Sugars (g/100g)			
Fructose: 41.20	1.98	$C_{\text{fructose}} = 41.20 \pm 1.98$ g/100g honey	4.81
Glucose: 25.82	1.66	$C_{\text{glucose}} = 25.82 \pm 1.66$ g/100g honey	6.43
Sucrose: 1.37	0.25	$C_{\text{sucrose}} = 1.37 \pm 0.25$ g/100g honey	18.25
Diastase (SU)			
Level : 7.17	0.48	7.17 ± 0.48 Shade units/g	6.69
Level : 12.42	2.19	12.42 ± 2.19 Shade units/g	17.63
Level : 22.49	1.73	22.49 ± 1.73 Shade units/g	7.69
HMF (mg/kg)			
Level : 3.38	0.63	$C_{\text{HMF}} = 3.38 \pm 0.63$ mg/kg	18.67
Level : 15.9	2.71	$C_{\text{HMF}} = 15.9 \pm 2.71$ mg/kg	17.10
Level : 42.07	5.10	$C_{\text{HMF}} = 42.07 \pm 5.10$ mg/kg	12.15

U-extended uncertainty.

Table 2. The chemical composition of analysed honey samples

Honey type	HMF (mg/kg)	Diastazic index	Palynological analysis
Acacia 1	1.5	2.8	< 20% acacia pollen
Acacia 2	3.1	2.5	30.6% acacia pollen
Acacia 3	5.9	8.9	rape and <i>Graminee</i> pollen
Acacia 4	7.0	8.4	multifloral pollen
Acacia 5	0.6	6.0	18.4% acacia pollen
Multifloral 1	3.1	4.6	-
Multifloral 2	1.2	10.8	-
Multifloral 3	6.7	2.8	-
Rape 1	4.4	8.1	>30% rape pollen
Rape 2	4.8	7.4	>30% rape pollen
Rape 3	10.2	9.4	>30% rape pollen
Rape 4	1.7	9.7	>30% rape pollen
Honeydew 1	17.7	10.4	honeydew elements present
Honeydew 2	4.1	9.2	honeydew elements present
Honeydew 3	0.8	10.7	honeydew elements present
Linden	7.8	9.0	>30% linden pollen
Sunflower 1	2.4	7.5	>40% sunflower pollen
Sunflower 2	14.0	6.1	>40%
Raspberry	13.9	9.5	>25% raspberry pollen
Buckwheat	22.1	5.2	buckwheat pollen present
Buckwheat	4.7	10.0	buckwheat pollen present
Heather	1.3	57.4	heather pollen present
Eucalyptus	45.8	4.7	>60% eucalyptus pollen
Lavander	40.4	7.7	lavender pollen present

Guide, 2000). For sugars and diastase method validation, the same protocol was followed, and lower %RSD values that 5% were obtained in the case of repeatability and lower that 10% for reproducibility.

Due to the lack of certified material (honey), we took an *in house* reference material with known HMF value (3.38 mg/kg) and a fortification with another 3.3 mg/kg and 20 mg/kg HMF standard were made. Average value concentration, following 6 time determinations was 6.65 mg/kg and 23.32 mg/kg. Recovery percent was determined and values of 97.42% for the first fortification level and 99.70% for the second fortification level. The established performance criteria (recovery percent 90-100% and %RSD<5%) was obtained. Recovery values for sugars were determined, and 101.8% (fructose), 99.7% (glucose) and 100.6% sucrose were obtained.

The uncertainty was determined on *in house* reference material, having the concentrations of sugars, HMF and diastase shown in Table 1 (Level of concentration), following Eurachem Citac Guide (2000).

Extended uncertainty was calculated for a confidence interval of 95% and covering factor $k=2$. Established performance criteria was accomplished, extended uncertainty (U), did not exceed 20% from the measured value.

Analysed samples results. Twenty-four honey samples (5 samples of acacia honey, 3 polifloral,

4 rape (canola), 3 honeydews, 2 buckwheat, 2 sunflowers, one of linden, raspberry and heather from Romania and also one sample of eucalyptus and lavender from the Proficiency Testing Trial) were subjected to chemical analysis of sugars, diastase activity and hydroxymethylfurfural content. Before chemical composition analysis, palynological determinations were made to confirm or not the declared botanical origin (Table 2) for the unifloral declared honey samples.

HMF and diastase were determined following the accredited methods presented in previous section. Romanian honey samples presented HMF content within the national and international regulations (<40 mg/kg), with values ranging from 0.6 to 22.1 mg/kg, depending on the botanical origin of each honey. Regarding eucalyptus and lavender honeys, the background is unknown, samples being shipped from France in the Proficiency Test Trial of the laboratory, but from the other physico-chemical and sensorial analysis, resulting a probable heating of the sample (Figure 1).

Diastase index is a parameter showing the "involvement" of the bee in the process of transforming the nectar into honey. National standard regulations state a minimum diastatic index value of 6.5 in the case of Acacia honey, 10.9-13.9 for honeydew and other honey types a minimum of 10.9 units, in order to declare a sample as authentic honey. The analyzed samples presented a wide range of values, and for this reason other differ-

Figure 1. Overlapped chromatogram of HMF standard (2 mg/L), honeydew honey (17.7 mg/kg) and eucalyptus honey (45.8 mg/kg)

ent methods are needed to determine the authenticity. Some of Acacia honey samples, have lower diastatic values than the minimum admitted by the standard, but if Council Directive 2001/110 or Codex Standard for Honey (2001) is taking into account, minimum limit of 3 units is accepted for low natural diastase content honeys, and HMF content not higher than 15 mg/kg.

Sugar spectrum is registered as a chromatogram by the HPLC system (Figure 2). The results obtained for all honeys are presented in Table 3. The first two acacia samples, have indeed the F/G ratio higher than 1.2, and correlated with palynological origin, show that the samples are monofloral acacia samples. The other two,

have higher amounts of glucose, and also the palynological analysis did not reveal acacia pollen in the sediment, so they can't be classified as acacia. The last declared acacia sample have a very high amount of sucrose, much more than the maximum limit. Here we can suppose a possible falsification. One of the multifloral samples have also high sucrose content. Regarding honeydew honeys, the presence of melezitose was investigated (data not shown). The presence of this tri- saccharide, in two of three samples, correlated with the presence of honeydew elements and high electrical conductivity (data not shown), were enough to classify the samples as honeydew honeys.

Table 3. HPLC determination of sugar spectrum for the analysed honey samples

Honey type	Fructose (%)	Glucose (%)	Sucrose (%)	F/G ratio
Acacia 1	41.65	24.84	3.38	1.68
Acacia 2	44.16	26.76	0.78	1.65
Acacia 3	41.97	32.36	0.60	1.30
Acacia 4	40.19	37.73	0.03	1.07
Acacia 5	30.74	27.11	14.84	1.13
Multifloral 1	39.02	26.58	1.15	1.47
Multifloral 2	36.7	26.08	6.59	1.41
Multifloral 3	33.47	34.46	11.44	0.97
Rape 1	36.71	41.86	Nd	0.88
Rape 2	36.98	42.30	Nd	0.87
Rape 3	36.15	38.96	Nd	0.93
Rape 4	39.1	39.9	Nd	0.98
Honeydew 1	38.40	27.13	0.06	1.42
Honeydew 2	39.08	30.76	0.98	1.27
Honeydew 3	33.98	26.16	Nd	1.30
Linden	36.21	33.01	0.06	1.10
Sunflower 1	38.74	40.42	Nd	0.96
Sunflower 2	38.69	35.87	0.05	1.08
Raspberry	42.84	27.74	0.07	1.54
Buckwheat	36.93	36.90	Nd	1.00
Buckwheat	37.51	30.11	0.41	1.25
Heather	39.65	31.37	0.03	1.26
Eucalyptus	38.77	33.44	Nd	1.16
Lavander	37.96	32.11	1.81	1.18

Figure 2. Sugar HPLC chromatogram of Acacia (A) and Honeydew honey samples (B)

Conclusion

The present study describes a validated approach of sugar content, diastase activity and HMF determinations in order to be used in honey authenticity determination. These are considered important methods to obtain accurate results in the authenticity process determination. The fact that low LOD and LOQ were established, give very good credentials for the methods, and also obtaining accurate results in the determinations. Overall conclusion is that several laboratory determinations need to be used in order to obtain data regarding honey authenticity.

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