Determination of the invertase activity in honey samples as the indicator of the authenticity of honey by UV/VIS spectrophotometric method

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ABSTRACT

Honey is a food consumed by humans of different ages, medical conditions, and professional orientations due to its high health and nutritional properties. Due to other purposes, honey sometimes must be treated, and its values should not be lost. Adulteration of honey is frequently found on the market nowadays, so accurate methods must be used to search for honey-like products. Invertase activity is a good indicator of freshness, thermal treatment, and honey authenticity. A fast UV/VIS spectrophotometric method was modified for the implementation in the food industry for rapid determination of invertase activity of honey samples.

Keywords: invertase, honey, authenticity, etalon.

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Introduction

Honey is a complex mixture produced by honeybees (*Apis mellifera*) from floral nectar containing sugars and water and small quantities of vitamins, minerals, fatty acids, amino acids, and proteins (Hungerford et al., 2020). The purity, floral and geographical origin and authenticity are important factors regarding the quality and the price (Voica et al., 2020). Due to the reason that bees' products have an important place in the chain "toxicant-soil-plant-bee-bee product-man", honey can be used as a not expensive method for monitoring environmental pollution (Nikolov et al., 2019). Regarding the presence of natural radionuclides ^{40}K and ^{137}Cs, the investigation performed on honeys from Turkish cities shows that found activities of ^{137}Cs do not exceed the highest permitted level of the radionuclide for food slightly contributed to the natural radioactivity of honey (Altekin et al., 2015). Honey, one of the oldest sweetening agents known for wound treatment, was evaluated for anti-bacterial activities; it was found that it possesses good activity against Gram-positive pathogens (Bhushanam and Madhusudan, 2019).

The most widespread enzymes in honey are diastase (amylase), invertase, glucose oxidase, catalase, and phosphatase (Pascual-Mate et al., 2018). Invertase is produced in the hypopharyngeal gland of the honeybee (Crane, 1975). The role of invertase is to make a very concentrated solution of sugars resisting fermentation, thus representing a high-energy foodstuff occupying the minimum area in honeycombs (Crane, 1975; 1990). Invertase activity is an important parameter for estimating honey quality and freshness (Julika et al., 2020). It is a better freshness indicator than diastase number because of its easy determination, susceptibility towards heating and storage, and presence in higher quantities than diastase (Bogdanov et al., 1999; Sanchez et al., 2001; White et al., 1964).

The study aimed to evaluate the authenticity of several honeys supplied by individual honey producers to Timomed d.o.o. Knjazevac based on the invertase activity determined by the UV/VIS spectrophotometric method.

Experimental

The harmonized method for the determination of the invertase activity of the International Honey Commission (International Honey Commission, 2009) and the method approved in

Moscow 2017 (Interstate Council for Standardization, Metrology and Certification, 2017) was modified with the aim to decrease the quantity of used chemicals necessary for the determination of the invertase activity in industrial conditions.

To prepare buffer solution (0.1 M; pH = 6.0), it was dissolved 11.66 g of potassium hydrogen phosphate (KH_2PO_4) and 2.56 g of disodium hydrogen phosphate ($Na_2HPO_4 \times 2H_2O$) in water and diluted to 1 L. The substrate was a *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG) solution. It was dissolved 1.2 g of *p*NPG in buffer solution and made up to 200 mL. *p*NPG is sparingly water-soluble, but the solution is not very stable. The buffer solution was dissolved by heating but not above 60 ^oC and cooled immediately after the solution was complete. Reaction-terminating solution (pH = 9.5) was made by dissolving 36.342 g of tris-(hydroxymethyl) aminomethane in water and diluting to 100 mL. A pH-value of 9.5 was adjusted with conc. hydrochloric acid.

Sample preparation and blank preparation are nicely described in Figure 1.

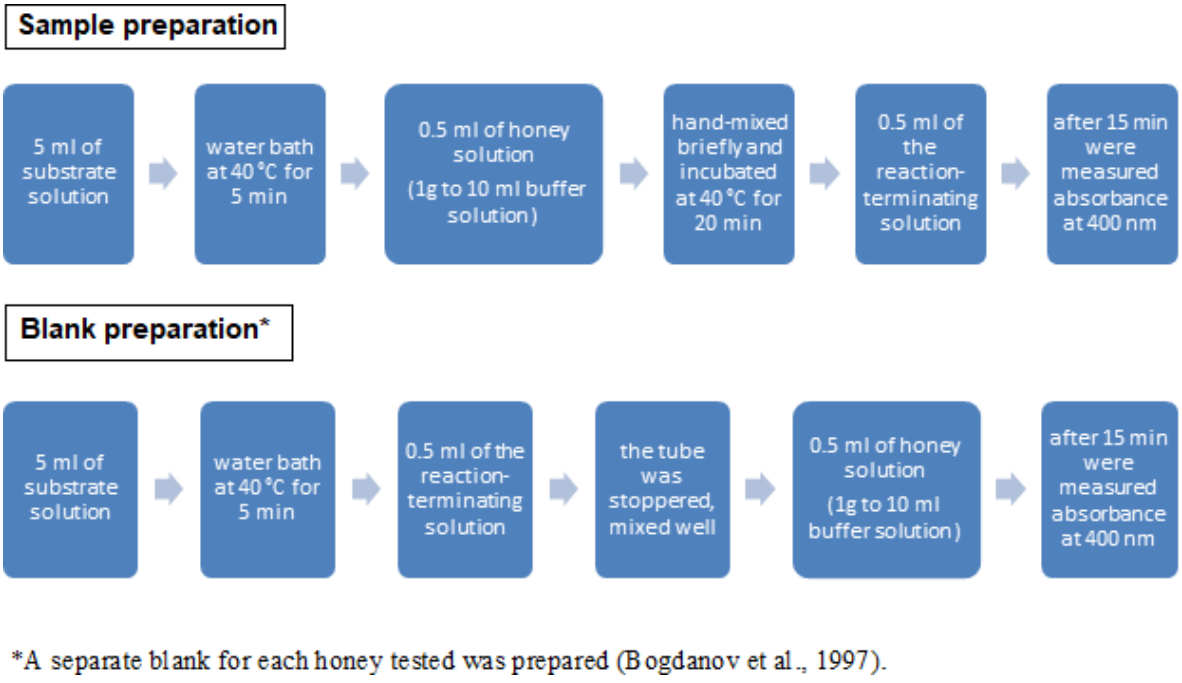

Figure 1. Schematic representation of the sample and blank preparation process

The absorbance of the blank was subtracted from that of the sample solution (ΔA400). The amount of *p*-nitrophenol in µM produced during the test corresponds exactly to the amount of substrate in µM utilized. Therefore, the honey invertase activity (IA) can be calculated from the absorbance measured at 400 nm and is indicated in units/kg (U/kg):

$$IA\ (U/kg) = 2 \times (6 \times 0.05 \times 0.05298 \times 10^4 \times \Delta A) = 317.88 \times \Delta A$$

where 6 = factor for the ml of sample solution used (total volume), 0.05 = converts reaction time from 20 min to 1 min, 10^4 = converts the amount of honey taken to 1 kg, 0.05298 = conversion factor for µg into µM per ml, 2 = conversion factor for honey quantity.

This method (Figure 1) was later again modified, and the modifications include 2.5 ml *p*NPG solution, 0.25 ml honey solution and 0.25 ml reaction-terminating solution. The invertase activity, in that case, was calculated according to:

$$IA\ (U/kg) = 4 \times (3 \times 0.05 \times 0.05298 \times 10^4 \times \Delta A) = 317.88 \times \Delta A$$

where 3 = factor for the ml of sample solution used (total volume), 0.05 = converts reaction time from 20 min to 1 min, 10^4 = converts the amount of honey taken to 1 kg, 0.05298 = conversion factor for µg into µM per ml, 4 = conversion factor for honey quantity.

It is common to express the invertase activity as invertase number (IN). The IN indicates the amount of sucrose per g hydrolyzed in 1 hour by the enzymes contained in 100 g of honey under test conditions (Hadorn and Zürcher, 1966). The relation between U/kg and IN (Oddo et al., 1999) is:

$$IA\ (U/kg) = 7.345 \times IA'\ (IN)$$

Results and Discussion

Nine samples from individual honey producers supplied to Timomed d.o.o. Knjazevac were analyzed in the laboratory at the Department of Chemistry, Faculty of Sciences and Mathematics, University of Nis, Nis (Table 1), where the method for the determination of the invertase activity in honey samples was developed. Except for one sample with the found invertase activity negative (-6.83 U/kg, -0.93 IN), all other eight samples show positive values going from 30.36-167.68 U/kg (4.13-22.83 IN).

Table 1. Invertase activity of selected honey samples analyzed at Department of Chemistry, Faculty of Sciences and Mathematics, University of Nis

Sample	**Total solution volume (ml)**	**A_{blanc} (average) ±SD***	**A_{sample} (average)±SD****	**ΔA**	**Invertase activity (U/kg)**	**Invertase activity (IN)**
1	6	0.482±0.002	1.01±0.05	0.5275	167.68	22.83
2	6	0.41±0.06	0.506±0.009	0.0955	30.36	4.13
3	6	0.477±0.001	0.637±0.006	0.1600	50.86	6.92
4	6	0.43±0.05	0.41±0.04	-0.0215	-6.83	-0.93
5	6	0.392±0.007	0.55±0.06	0.1620	51.50	7.01
6	3	0.6±0.1	0.97±0.06	0.4195	133.35	18.16
7	3	0.54±0.07	0.68±0.08	0.1440	45.77	6.23
8	3	0.4875±0.0007	0.6±0.1	0.1645	52.29	7.12
9	3	0.59±0.03	0.94±0.09	0.3565	113.32	15.43

*n=2, n-number of measurements

**n=3, n-number of measurements

SD-standard deviation

The invertase activity of another five different honey samples supplied from individual honey producers to Timomed d.o.o. Knjazevac was determined in the laboratory at Timomed d.o.o. Knjazevac using the developed method and shows values from 45.62-245.72 U/kg. The invertase number goes from 6.21 to 33.45 IN (Table 2). Due to the reason that industry needs not such precise values to decline/accept the honey from the supplier, one blank probe was used and two probes of the sample.

Table 2. Invertase activity of selected honey samples analyzed at Timomed d.o.o. Knjazevac

Sample	Total solution volume (ml)	A_{blanc}	A_{sample} (average)±SD*	ΔA	Invertase activity (U/kg)	Invertase activity (IN)
10	3	0.449	0.592±0.008	0.1435	45.62	6.21
11	3	0.482	1.26±0.02	0.7730	245.72	33.45
12	3	0.450	0.69±0.02	0.2355	74.86	10.19
13	3	0.444	0.7±0.1	0.2650	84.24	11.47
14	3	0.448	0.6360±0.0000	0.1880	59.76	8.14

*n=2, n-number of measurements

SD-standard deviation

Eleven analyzed samples have invertase activity of more than 4 and less than 20 IN, which is following the research performed by Oddo et al. (1999). One sample with a colour different from others (darker) has an invertase activity of more than 30, which is a characteristic of honeydew honey (Oddo et al., 1999). Honeydew honey and honeydew, which honey bees collect from trees and leaves of conifers and deciduous trees, are a subject of numerous studies (Markwell et al., 1993; Martins-Mansani et al., 2021; Pita-Calvo and Vazquez, 2018; Santas, 1983; Shaaban, 2020; Ülgentürk et al., 2020; Ünal et al., 2017; Utzeri et al., 2018). The higher invertase activity of honeydew honey compared to other honey types can be explained by the high presence of enzymes (particularly invertase) in raw material (honeydew) which has the origin in the secretions of salivary glands and the gut of plant-sucking insects (Oddo et al., 1999). Unfortunately, one analyzed sample showed bizarre behaviour, and that sample is not honey in any sense (-0.93 IN).

The variability in invertase activity can be a result of a series of factors (*e.g.*, nectar collection period, an abundance of nectar flow and its sugar content, age of the bees, pollen consumption, *etc.* (Brouwers, 1982; 1983; Fluri et al., 1982; Huang et al., 1989a, b; Oddo et al., 1999; Simpson et al., 1968).

Nowadays, the adulteration of honey is a common feature, and their producers using the various methods tend to destroy the excess of the enzyme invertase. It was shown that long processing times and high temperatures (> 6h and > 65 ^{0}C) were necessary to start the inactivation of the enzyme invertase (Sahin et al., 2020). Therefore, it is logical that adulterated honey would

have lower IN than natural. We can suspect that samples 2, 3, 5, 7, 8, 10 and 14 are not entirely natural based on the recommendation of the International Honey Commission (IN>10). However, they can be honeys with low enzymatic activity (IN>4) (Bogdanov et al., 1999). On the other hand, various processing operations have been introduced over the last decades to ensure a pleasant and homogeneous presentation of the product in liquid or semisolid form requiring heating (Serra Bonvehı et al., 2000; Vicente Pascual et al., 1987). Various heating methods were proposed decreasing to a small extent the invertase activity but changing the product in the desired direction (Serra Bonvehı et al., 2000).

The absorbance of blank samples for selected honey samples varies from 0.4 to 0.6 (Tables 1 and 2). We suggest the use of blank solutions of true bee honeys (those have IN very close to 4 and 20) as etalons for the comparison with the blank solution of the unknown sample with the satisfactory electrical conductivity (due to the existence of the correlation of minerals (contributing significantly to the electrical conductivity of honeys) and invertase) (Vorlova and Celechovska, 2002) and taste without the use of the equipment, such as UV/VIS, using only visual inspection due to coloration. The color of the blank is not enough, as it was seen in the case of sample 4 where the color was artificial, and no invertase was present there. Therefore, for the rapid determination, some other additional fast measurement must be used as suggested to be complementary method to determine the authenticity of honey sample if we do not have time for a full UV-VIS spectrophotometric determination including sample and blank preparation as described in this work.

Conclusion

Invertase activity has been shown to be a good indicator of honey's freshness, thermal treatment, and authenticity in the food industry. The developed UV/VIS spectrophotometric method is fast and easy to use in academia and particularly in industry. The absorbance of the blank sample can tell a lot about the quality of the honey (together with electrical conductivity and the taste) and sometimes, in a hurry, the blank solutions of the authentic honeys can be etalons for the comparison with a blank solution of the unknown sample (with proper electrical conductivity and the taste) thus without the use of equipment such as UV/VIS.

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Conflict-of-Interest Statement

None.

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