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DETERMINATION OF HMF, GLUCOSE AND FRUCTOSE CONTENTS IN VARIOUS HONEY SAMPLES

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The paper deals with estimation of the concentration changes of 5-hydroxymethyl-2-furfuraldehyde (HMF), fructose and glucose in various honey samples after their heating. The content of HMF in honey was analysed by liquid chromatography (HPLC) method coupled with ultraviolet (UV) detection. The content of glucose and fructose in honey was analysed by HPLC method using evaporative light scattering detection (ELSD). Chromatographic conditions were optimized for HMF, fructose and glucose analyses. The obtained limits of detection (LOD) in honey were 0.25 mg kg^{-1} for HMF, 0.18 g kg^{-1} for fructose and 0.26 g kg^{-1} for glucose. Samples of honey were exposed to the temperatures of 50, 60, 70, and 80 °C and the concentrations of target analytes were monitored. The highest increase in HMF was found in liquid honey heated up to 80 °C, where the acceptable limit (40 mg kg^{-1} HMF in honey by EU) was exceeded after 5 hours of

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heating. The creamed honey did not exceed the limit of HMF even after 8 hours. The amount of HMF was significantly higher in honey samples purchased from retailers compared to samples purchased from beekeepers.

Introduction

5-hydroxymethyl-2-furfuraldehyde (HMF) is a mutagenic compound that promotes the rise of toxic substances. Its derivates incurred as by-products of Maillard reaction are cytotoxic, genotoxic and carcinogenic [1,2]. HMF is a typical product of honey degradation. The occurrence and accumulation of HMF in honey depends on the type of honey, storage conditions, temperature and pH [3].

The creamed honey is honey adapted into cream consistence using controlled crystallization, without any heating or addition of other compounds. This honey retains the substances and does not undergo further crystallization.

The liquid honey is honey that has been heated to melt the honey crystal and filtered to remove foreign material and unmelted crystals. It is unstable and tends to revert to the crystalline state [4].

The limit of the HMF content in honey determined by the Czech legislation is 40 mg kg^{-1} honey [5]. In the international legislation the acceptable limit is defined in the European Honey Directive [6] and Codex Alimentarius [7]. These limits are 40 mg kg^{-1} of honey and 80 mg kg^{-1} of honey, respectively.

There are three different methods for HMF determination in honey cited in Harmonized Methods of the European Honey Commission. The first two methods are spectrophotometric and the last one is HPLC method with UV detection [8]. Moreover, a lot of various methods for determination of HMF in honey using HPLC-UV methods were published in various journals [9-12]

The main honey sugars (fructose and glucose) could be determined by various methods based on the utilisation of their physical characteristics [13]. The content of saccharides are usually measured by chromatographic methods. HPLC with silica-based amino columns and refractometric detection [9,14,15] or with ion-exchange columns and pulsed amperometric detection [16,17] is simple and more suitable for routine analysis. Another method of saccharides determination in honey can be the HPLC with evaporative light scattering detector (ELSD) [18]. ELSD detects whichever substances less volatile then is mobile phase and removes common problems connected with refractive or UV detection [19].

The first aim of this work was to adjust and validate the method for glucose and fructose determination in honey using HPLC with ELSD detection, validate the method for HMF determination in honey using HPLC. The next objective was to determine the HMF, glucose and fructose contents in samples of liquid honey compared with samples of creamed honey and their dependence on the various heating temperatures and time. Finally, the last part of this study was focused on

the comparison of honey from beekeepers, shops and bio-farms.

Materials and Methods

Honey Samples

Altogether 21 honey samples of mixed floral sources were analyzed: 6 samples of fresh honey (No. 1-4 liquid honey, 5 and 6 creamed honey) and 4 samples of 1-year-old honey (No. 7-10) were obtained from the beekeepers from Pardubice (The Czech Republic). The next 6 samples (No. 11-16) of honey were purchased in the local shops, 3 samples (No. 17-19) of honey were bought in the bio-farm (No. 17 liquid honey, No. 18 creamed honey, No. 19 honeydew honey) and 2 foreign samples were obtained from Austria local shop (No. 20 creamed honey) and Malta local shop (No. 21). All the honey samples have been stored at 4 °C until to analysis.

Standards and Solvents

The standards of glucose and fructose were purchased from Merck (Darmstadt, Germany). Their stock solutions were prepared by dissolving 0.4381 g fructose or 0.6826 g glucose in redistilled water and filled up to the 50 ml in a volumetric flask. The HMF standard was bought from Sigma-Aldrich (Steinheim, Germany). Its stock solution was prepared by dissolving 10 mg standard in redistilled water and filling up to 25 ml in a volumetric flask. All the standards were analytical grade.

Acetonitril and methanol (both HPLC grade) were purchased from Merck (Darmstadt, Germany). For the evaporation of sample the nitrogen gas (purity 5.0, Linde Gas a.s., Prague, The Czech Republic) was used.

Methods

The saccharides in honey were separated and quantified on an HPLC chromatograph equipped with SP 8800 pump (Spectra-Physic, San Jose, USA), Shodex Pegas KT-35M degasser (Showa Denko KK, Tokyo, Japan), and evaporative light scattering detector (ELSD) Chromachem® 6100 (ESA Inc., Chelmsford, USA). The saccharides were separated on Purospher Star® NH_2 column (4 mm × 250 mm, Merck, Darmstadt, Germany) at 35 °C with LCO 101 column heater (ECOM, Prague, The Czech Republic). A flow rate of 1.0 ml min^{-1} was used with mobile phase acetonitrile/water (80 : 20, v/v). The Chromachem®

6100 detector was set at 45 °C (evaporation temp.) and 30 °C (nebulisation temp.) with 1.8 $\times 10^5$ Pa of nitrogen gas. The mobile phase was filtered through a membrane filter (0.45 μm, 47 mm, Merck, Darmstadt, Germany) and degassed in ultrasonic bath SONOREX RK 52 H (Bandelin electronic GmbH & Co. KG, Berlin, Germany). The injection volume of sample was 10 μl.

The HMF was analyzed with a HP-1090M (Hewlett-Packard, Avondale, USA) liquid chromatograph equipped with diode array detector (DAD). Separation was achieved on the LiChrospher®60 RP-select B, 5 μm, 4 mm × 250 mm column (Merck, Darmstadt, Germany) and LiChrospher® 100 RP-18e, 5 μm guard column (Merck, Darmstadt, Germany). A flow rate of 1.0 ml min^{-1} was used with mobile phase methanol/water (10 : 90, v/v) and the detection of the HMF was performed at the wavelength 285 nm. The mobile phase was filtered through membrane filter (0.45 μm, 47 mm) and degassed with helium at the overpressure of 0.1 MPa. The injection volume of sample was 10 μl.

For the evaluation of the effect of the heating time and temperature on the analyte contents the aliquot of 50 g of honey samples were warmed up in water bath using the thermostat (JULABO F 12 Seelbach, Germany).

Sample Preparation

A sample of 5 g honey was used for the determination of HMF. The sample was dissolved in small amount of redistilled water, quantitatively transferred into 25 ml volumetric flask and made up to mark with redistilled water.

The amount of 0.1 g was taken from the honey sample for the determination of saccharides. The honey was dissolved in small volume of redistilled water, quantitatively transferred into a 10 ml volumetric flask and made up to mark with redistilled water.

All these samples were filtered through the membrane filter (PTFE, 0.45 mm, 13 μm, Merck, Darmstadt, Germany) prior to chromatographic analysis.

Results and Discussion

Optimization of the HPLC method for the determination of saccharides: The chromatographic conditions were taken from literature [18] with some minor modification. It was necessary to perform these modifications because of the different sample matrix analysed in comparison to that in publication.

The mobile phase composition was set up at the ratio 80 : 20 (v/v) in the isocratic mode at the flow rate of 1.0 ml min^{-1}. These conditions were found to be fully suitable for separation of the target saccharides (fructose and glucose) within an acceptable time period. The appropriate retention times were 6.1 min for

fructose and 6.8 min for glucose, respectively.

To maintain the constant temperature, it was necessary to use the column thermostat. According to this, the various temperatures ranging from 25 to 40 °C (increments of 5 °C) were tested. The temperature of 35 °C was found to be the best choice for analysis of the target saccharides.

The next part of this optimisation was to find appropriate conditions for ELSD detector (e.g. evaporation and nebulisation temperatures, pressure of N_2 and detector sensitivity). Based on the mobile phase composition, its flow rate and sample volatility and according to the manufacturer's instruction manual we selected the nebulisation temperature of 30 °C and the evaporation temperature of 45 °C. For the evaporation of sample the nitrogen gas at the pressure of 26 PSI (1.8×10^5 Pa) was used.

Validation of the HPLC method for the determination of saccharides: For the validation of HPLC-ELSD method for determination of the saccharide contents in honey samples the linearity was evaluated based on the creation of calibration curves and is expressed as the correlation coefficients. Eight calibration solutions were prepared for each calibration dependency in the concentration range of 0.35-6.0 g l^{-1} for fructose and 0.4-6.0 g l^{-1} for glucose. Calibration equations and appropriate coefficients of correlation are presented in Table I.

Table I Calibration characteristics of the target compounds

	Retention time, min	Calibration range	Calibration equation	R^2	LOD***
Fructose*	6.1	0.35-6.0 g l^{-1}	$y = 1266.7\,x - 309.3$	0.9994	17 mg l^{-1}
Glucose*	6.8	0.4-6.0 g l^{-1}	$y = 1100.4\,x - 444.1$	0.9991	25 mg l^{-1}
HMF**	7.8	0.5-18.0 mg l^{-1}	$y = 50.246\,x - 3.461$	0.9992	50 µg l^{-1}

* HPLC-ELSD method; ** HPLC-UV method; *** Calculated as three times noise level at the appropriate retention time

The detection limits were calculated as three times the noise level at the retention time of individual target analytes and are shown in Table I.

Optimization of the HPLC method for the determination of HMF: For the selection of appropriate chromatographic conditions for analysis of HMF in honey we used several publications [1,3,8-12]. From the obtained results and according to analysed sample matrix we found that the suitable mobile phase composition is methanol/water (10 : 90, v/v) at a flow rate of 1.0 ml min^{-1} in the isocratic mode. The appropriate retention time for HMF was 7.8 min.

Validation of the HPLC method for determination of HMF: For the validation of

HPLC-UV method for determination of the HMF contents in honey samples, the linearity was evaluated based on the generation of calibration dependence and is expressed as the correlation coefficient. Nine calibration solutions were prepared for the calibration curve in the concentration range of 0.5-18.0 mg l^{-1}. The obtained calibration equation and appropriate coefficient of correlation are shown again in Table I.

The detection limit was calculated as three times the noise level at the retention time of HMF and is presented in Table I.

The dependences of target analyte contents on the heating temperature and time: Before the analysis of the real samples of honey it was necessary to select a suitable quantification method. For this case the method of calibration curve and the standard addition methods were tested and compared. Because no significant differences in the results obtained by these two methods were found, the method of calibration curve was selected for the quantification of all the target analytes in honey samples.

The dependences of the concentration changes of HMF, glucose and fructose on the heating temperature and time were evaluated using the samples of liquid honey (sample No. 1) and creamed honey (sample No. 5). The samples were heated up to the temperatures of 50, 60, 70 and 80 °C. These temperatures were used with regards to the literature [3,20,21], where the concentration changes of components are evident for temperatures above 50 °C. For this study the samples were collected every hour during the time period of 8 hours. No distinct changes were observed for the temperature 50 °C, therefore the samples were collected also after 12, 24, 36, 48, 60, 72 and 84 hours. The most significant concentration changes were observed at the temperature 80 °C. The liquid honey exceeded allowed limit of HMF content after 5 hours at this temperature. The creamed honey did not exceed the limit of HMF even after 8 hours. The obtained results are presented in Figs 1 and 2 where the EU acceptable limit of HMF is symbolised by dot line.

In Figs 3 and 4 are shown the dependences of the HMF, fructose and glucose contents in honey on the heating time at the temperature of 80 °C. On these charts it can be seen that the concentration of HMF increased with decreasing saccharides concentrations.

Comparison of HMF, fructose and glucose content in different honey samples: The honey samples No. 1-21 were analysed using the optimised chromatographic conditions mentioned above. For this comparison study the fresh honey and 1-year old honey samples from beekeepers together with those purchased in the Czech local shops, bio-farm and two foreign honeys were used. Concentration of HMF was observed within the concentration range of 0.159-71.523 mg kg^{-1} honey with

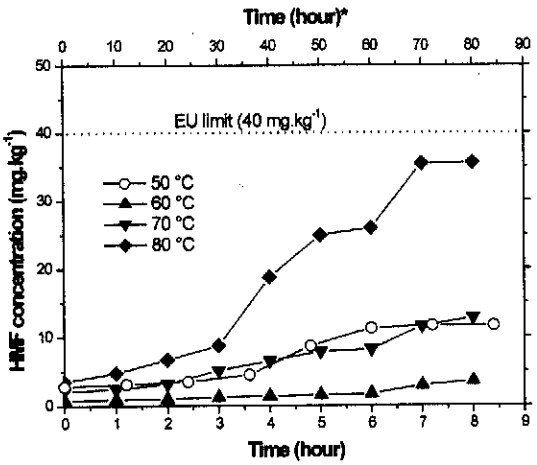

Fig. 1 The dependence of the HMF concentration in creamed honey on the heating temperature and time (* – values for 50 °C)

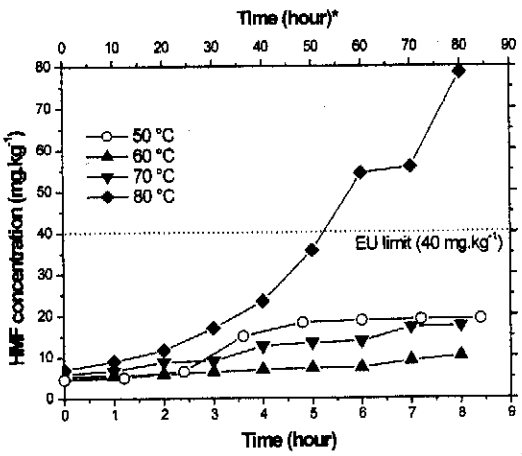

Fig. 2 The dependence of the HMF concentration in liquid honey on the heating temperature and time (* – values for 50 °C)

the average RSD 1.66 % (calculated from 5 replications). As mentioned above, the acceptable limit of HMF in EU is 40 mg kg^{-1} honey. It was found that in some of the honey samples bought in the local shops the EU acceptable limit were exceeded (samples No. 11, 13 and 14). The same can be stated in the case of the honey from Malta (sample No. 21).

The obtained results are summarised in Figs 5 and 6. On these charts it can be seen that the content of HMF depends on the age and the form of the honey.

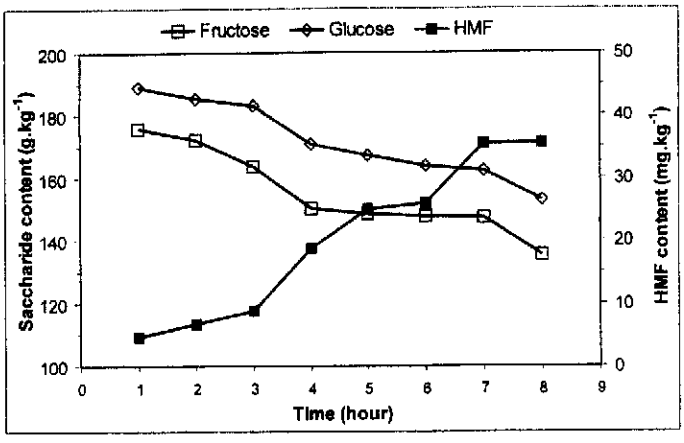

Fig. 3 The dependence of the HMF, glucose and fructose contents in creamed honey on the heating time at 80 °C

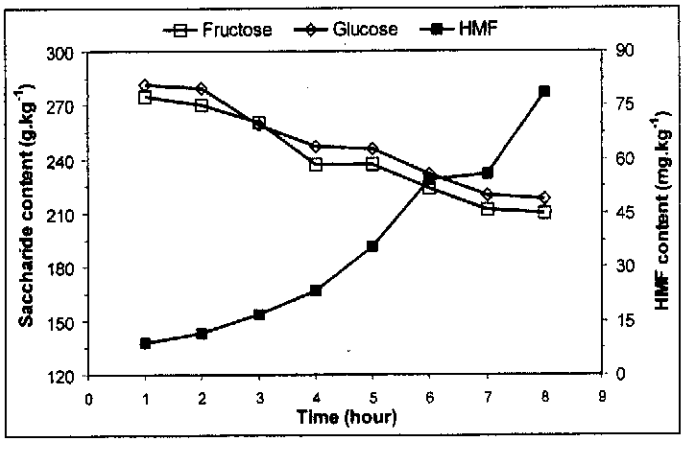

Fig. 4 The dependence of the HMF, glucose and fructose contents in liquid honey on the heating time at 80 °C

The fresh and creamed honeys contain the lowest amount of HMF. The amount of fructose was observed within the range of 183.24- 502.70 g kg^{-1} honey with the average RSD 2.61 % and the amount of glucose was obtained in the range of 194.69-512.56 g kg^{-1} honey with the average RSD 2.32 % for all the measured samples. The RSD values were calculated from the 5 replications of analysis. The obtained concentrations are presented in Figs 5 and 6.

According to the results obtained for HMF the contents of fructose in honey samples exceeded the glucose contents for the fresh samples. For the rest of analysed samples the fructose content was lower than the content of glucose (see Figs 5 and 6).

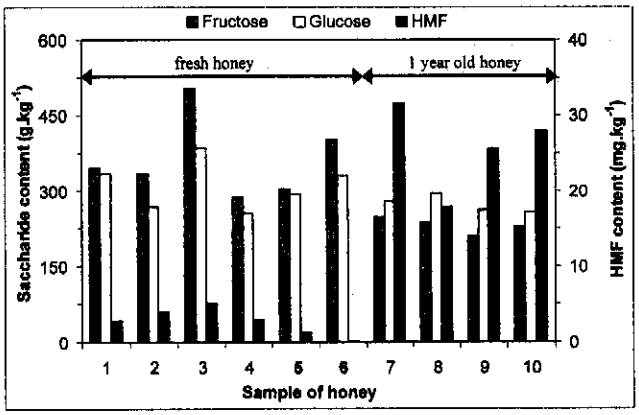

Fig. 5 Comparison of HMF, glucose and fructose contents of various honey samples from beekeepers; bold labels = creamed honey

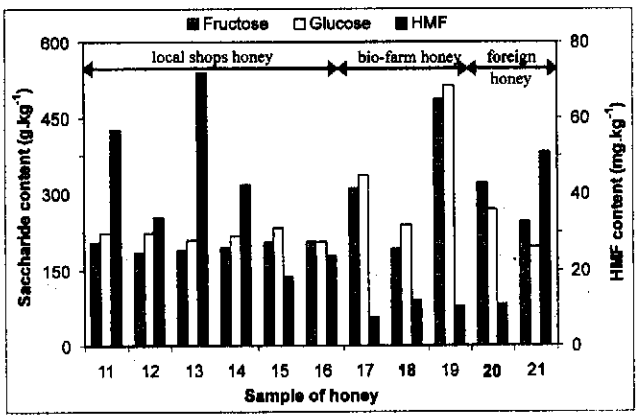

Fig. 6 Comparison of HMF, glucose and fructose contents of various honey samples from other sources; bold labels = creamed honey

Conclusion

The simple HPLC methods for the determination of HMF, fructose and glucose contents in honey were described and evaluated. The method HPLC-UV for analysis of HMF and the HPLC-ELSD method for analysis of saccharides were used. Both methods were optimised and a simple validation was performed. These methods were finally used for the evaluation of effects of heating temperature and time on the content of target analytes and for the comparison of individual honey samples composition.

On the basis of the results obtained it can be claimed that the increase in HMF content is much slower in the case of the creamed honey compared to that in the liquid honey. According to this finding, the creamed honey seems to be

more suitable for human consumption.

The higher amount of HMF in the honeys could be caused by the wrong storing conditions or by the undesirable heating of honey. Moreover, the fructose content is decreasing during the shelf ageing of honey.

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