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Changes in concentrations of 5-hydroxymethylfurfural, carbohydrates, and ethanol during the mead production

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This work is focused on monitoring changes in the concentrations of 5-hydroxymethylfurfural, ethanol, and carbohydrates during the mead production using various analytical techniques, namely micellar electrokinetic chromatography with spectrophotometric detection, gas chromatography coupled to flame-ionization detector, and hydrophilic interaction liquid chromatography with refractive-index detector, respectively. In this study, production of three mead samples made by different Czech beekeepers has been monitored. The individual mead fractions were regularly sampled for two months of fermentation and always analysed with the aid of the optimised analytical approach.

Keywords: Mead production; Ethanol; Carbohydrates; 5-hydroxymethylfurfural (5-HMF); Separation techniques

Introduction

Mead is a traditional alcoholic beverage made by fermenting a honey-water solution, usually using the yeast strain *Saccharomyces cerevisiae*. Although simple sugars are fermented to ethanol by yeast during the mead production, the monosaccharides fructose and glucose are still the most abundant constituents of mead. Carbohydrates can be determined by various techniques [1,2], of which HPLC is the most frequently used.

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Since carbohydrates do not contain any chromophore or fluorophore in their structure, conventional spectrophotometric (UV/VIS) or fluorescence detection cannot be used without their prior derivatization. Therefore, refractive-index (RID), evaporative light scattering, and electrochemical detectors or mass spectrometer (MS) are being predominantly employed [3−8].

The content of ethanol anticipated in mead depends on many factors, such as the amount of fermentable carbohydrates available, the content and composition of nutrients, the type of yeast used, and the fermentation temperature, as well as the corresponding ranges between 7−22 % (*v/v*) [5,6,9−14]. Distillation followed by measurement of pycnometric density is the traditional method for the determination of ethanol in alcoholic beverages. However, this technique is laborious, time-consuming, and insufficiently sensitive. Therefore, the precise and sensitive technique of gas chromatography coupled with a flame-ionization detector (GC-FID) is currently the most common approach [5,15,16].

5-hydroxymethylfurfural (5-HMF) is a food contaminant formed by dehydration of reducing sugars. This compound is harmless to humans at low concentrations, but it can cause irritation of eyes, mucous membranes, and skin. Moreover, it is even considered a potential carcinogen and mutagen at higher quantities. 5-HMF is usually absent in fresh mead samples; however, due to the excessive heat treatment, inappropriate storage conditions, or adulteration of mead (especially with invert syrup), it can occur at higher concentrations, decreasing the quality of the final products. Hence, it can serve as an indicator to verify both the mead freshness and the compliance with good manufacturing practice [1,2,8,17−22]. Albeit the Codex Alimentarius Commission, as well as the E.U. have established the maximal 5-HMF level in honey as 40 mg/kg (and even 80 mg/kg for honeys coming from a tropical climates) [23,24]; the standard for Czech honey having been set to only 20 mg/kg [25]. Many analytical methods employing spectrophotometry [17,22], HPLC [7,8,13,17,18,20,22,26], GC-MS [19], nuclear magnetic resonance [2], micellar electrokinetic capillary chromatography (MEKC) [27], and capillary electrophoresis [1], have already been developed for the determination of 5-HMF in honey.

The aim of this study was to reveal how the concentrations of selected analytes may change during the fermentation of honey-water solution. For this purpose, the production of three meads made by different Czech beekeepers has been monitored. Throughout their fermentation process, the corresponding fractions were sampled and analysed using GC-FID, HPLC-RID, and MEKC to determine the amount of ethanol, carbohydrates, and 5-HMF, respectively.

Experimental

Chemicals and reagents

Ethanol (96%), thiourea (p.a.; both Lach-Ner, Neratovice, Czech Republic), 1-propanol (99%; Penta, Chrudim, Czech Republic), 5-HMF (99%; Fluka, Neu-Ulm, Germany), glycerol, fructose, glucose, and sucrose (all ≥99%, Merck, Darmstadt, Germany) were used as standards. Mobile phase was composed of acetonitrile (Sigma-Aldrich, St. Louis, MO, USA) and high-purity water prepared by Milli-Q purification system (Merck Millipore, Billerica, MA, USA). Then, sodium dodecyl sulfate (99%), boric acid (99.5%), and sodium tetraborate (99.5%; all from Fluka) were used for the electrolyte preparation.

Sample pre-treatment

Honey samples intended for studying the mead production were obtained from three traditional Czech beekeepers coming from the regions of Havlíčkův Brod, Potštejn, and Domažlice. At the beginning of the fermentation process of honey-water solutions, the individual fractions were sampled every second day. Towards the end of mead production, the respective fractions were sampled at longer intervals. Prior to analysis, fractions were always two- or five-fold diluted with deionized water and filtered through Nylon filters (0.45 μm, 25 mm; Merck).

Instrumentation and analysis

Gas chromatography

Analysis of ethanol was performed using a GC-2014 system equipped with flame ionization detector (Shimadzu, Kyoto, Japan) tempered at 180 °C. Separation was carried out on a Supelcowax-10 capillary column (15 m × 0.32 mm, 0.5 μm; Sigma-Aldrich, Milwaukee, WI, USA). Nitrogen (Linde Gas, Prague, Czech Republic) was used as a carrier gas at a constant linear velocity of 15.6 cm/s. The column temperature program was set up as follows: the initial temperature was kept at 50 °C for 1 min and then increased up to 130 °C at a rate of 20°C/min. Injection of the sample (1 μL) was performed by a microsyringe (Hamilton Company, Reno, NV, USA) in the split mode (split ratio 1:100) and the injector was maintained at 180 °C.

Calibration solutions of ethanol were prepared in water at six concentration levels (0.5−8 %; *v/v*). Mead samples were diluted five times with water and filtered. As an internal standard (IS), 1-propanol at the concentration of 3 % (*v/v*) was used. All measurements were repeated five times ($n = 5$).

Liquid chromatography

The HPLC system equipped with two LC-20AD binary gradient pumps, a DGU-20A_5 degassing appliance and RID-10AD refractive-index detector (all Shimadzu), a six-port injection valve with 20 μL external loop (Valco-Vici, Schenkon, Switzerland), and a Supelcosil LC-NH_2 column (250 × 4.6 mm, 5 μm particles) with the corresponding guard column (20 × 3 mm; Sigma-Aldrich) was used for analysis of carbohydrates in mead samples. The separations were performed in the isocratic elution mode with 80% aqueous acetonitrile (*v/v*) at a flow rate of 2 mL/min at laboratory temperature.

Calibration solutions of glucose, fructose, and sucrose were prepared in water at nine concentration levels (1.2−42 g/L). Mead samples were diluted two times with water and filtered. All measurements were repeated three times (n = 3).

Micellar electrokinetic capillary chromatography

A set-up for capillary electrophoresis (model Beckman P/ACE 2100, Beckman-Coulter, Brea, CA USA) equipped with diode-array detector and a non-coated fused-silica capillary (ID: 75 μm, total length: 47 cm, effective length to the detector: 40 cm) tempered at 20 °C was used for the determination of the content of 5-HMF. The sample was injected hydrodynamically for 5 seconds and borate buffer (c = 5 mmol/L, pH 9.2–9.3) together with sodium dodecyl sulphate (c = 120 mmol/L) used as electrolyte. Applied voltage was 25 kV and the wavelength of UV detection set at 280 nm. Thiourea (c = 6 mmol/L) was used as a marker of electroosmotic flow (EOF).

Calibration solutions of 5-HMF were prepared in water at seven concentration levels (1−15 mg/L). Mead samples were diluted two times with water and filtered. All measurements were repeated three times (n = 3).

Method validation and data processing

The quantities of the compounds monitored were calculated from the calibration equations of the corresponding standards obtained from the dependence of the peak area of the given standard (carbohydrates and 5-HMF) on its concentration. In case of ethanol, the ratio of the peak areas of the ethanol and the internal standards were used. The regression diagnostic of the studied compounds was performed using QC Expert 2.9 program (TriloByte, Staré Hradiště, Czech Republic). Influential points were identified using graphical diagnostics (Pregibon, Williams, and L-R graphs) with elimination of potential outliers. The linearity of calibration curves was verified by residual plots and the significance of the intercept of the

regression straight-lines was tested using Student's *t*-test. The regression parameters together with their standard deviations and coefficients of determination (R^2) are shown in Table 1.

The method was validated in terms of limits of detection (LOD), limits of quantification (LOQ), and linearity (Table 1). The instrumental LOD and LOQ were calculated as the concentrations corresponding to signal-to-noise ratios S/N = 3 and S/N = 10, respectively. While LODs for carbohydrates, 5-HMF, and ethanol reached values of 15–42 mg/L, 1.3 mg/L, and 0.0015 %, the respective LOQs were determined at the concentrations of 50–140 mg/L, 4.4 mg/L, and 0.005 %. The coefficients of determination were higher than 0.9998 for all standards, demonstrating a high linearity.

Table 1 Limits of detection (LOD) and quantification (LOQ), regression equations (given with corresponding standard deviations of intercept and slope), and coefficients of determination (R^2) of all target compounds

STANDARD	LOD	LOQ	REGRESSION EQUATION	R^2
Ethanol	0.0015 %	0.0050 %	$y = 0.239\ (0.003)x - 0.007\ (0.002)$	0.9999
Fructose	15 mg/L	50 mg/L	$y = 5.8\ (0.2)x - 0.2\ (0.5)$*	0.9998
Glucose	42 mg/L	140 mg/L	$y = 3.85\ (0.01)x - 1.8\ (0.3)$	0.9999
Sucrose	30 mg/L	100 mg/L	$y = 3.37\ (0.01)x - 1.4\ (0.1)$	0.9999
5-HMF	1.3 mg/L	4.4 mg/L	$y = 0.0752\ (0.0003)x - 0.007\ (0.002)$	0.9998

* intercept is not significant at a 95% significance level

The final results are calculated and presented as confidence intervals $\bar{x} \pm s \cdot t_{1-\alpha}$, where $\bar{x}$ is the arithmetic mean, s the standard deviation, and $t_{1-\alpha}$ the critical value of Student's *t*-distribution for three (2.353) or five (2.015) replicates at a significance level α of 0.05 (95% probability).

Results and discussion

Optimization of separation conditions

First, the optimization of selected device parameters was necessary to be carried out. Regardless of the technique used, the aim of all optimizations was to obtain the shortest possible separation with sharp symmetric non-coeluting peaks of the target analytes, showing a minimal resolution of 1.5.

Determination of ethanol using GC

The parameters, such as the initial column temperature (50, 60, and 70 °C), steepness of the temperature gradient (5, 10, 15, and 20 °C/min), and linear velocity of nitrogen (10, 15.6, 20, and 25 cm/s) were optimized. The best separation was achieved by using conditions given in the experimental section and the optimized chromatographic separation of standards of ethanol and 1-propanol (IS) is shown in Fig. 1a.

Determination of carbohydrates using HPLC

Hydrophilic interaction liquid chromatography was selected for the analysis of glucose, fructose, and sucrose present in the mead samples. The chromatographic separation was performed on Supelcosil LC-NH_2 column with acetonitrile/water mobile phase. All experiments were carried out under isocratic conditions due to the refractometric detection chosen. The concentration of acetonitrile in the mobile phase (75 and 80 %; *v/v*) was optimized together with its flow rate (1 and 2 mL/min). The sufficient resolution of separated carbohydrates was achieved by using the conditions given in the experimental section and an example of chromatographic separation of carbohydrate standards is illustrated in Fig. 1b.

Determination of 5-HMF using MEKC

Within the optimization, two types of sample injection modes were tested, namely: hydrodynamic injection for 5 or 10 s and electrokinetic dosing at 5 kV for 5 s) along with different capillary temperatures (20 and 25 °C). The best separation was achieved by using the conditions given in the experimental section and an example of electrophoretic separation of standards of 5-HMF and thiourea (EOF) is shown in Fig. 1c.

Sample analysis

The quantification was performed by means of the calibration curve method with either external standards (for carbohydrates) or internal standards (for 5-HMF and ethanol). The parameters of individual calibration curve are listed in Table 1.

The ethanol content increased during the fermentation of meads up to final concentration of 19.61, 13.41, and 17.05 % in mead samples Nos. 1, 2, and 3, respectively (Fig. 2). While mead No. 2 showed a relatively linear increase in the concentration of ethanol during the whole fermentation process, a very rapid

increase in ethanol at the beginning of fermentation, followed by a slowdown until almost constant value, occurring in meads No. 1 and 3. (Fig. 2).

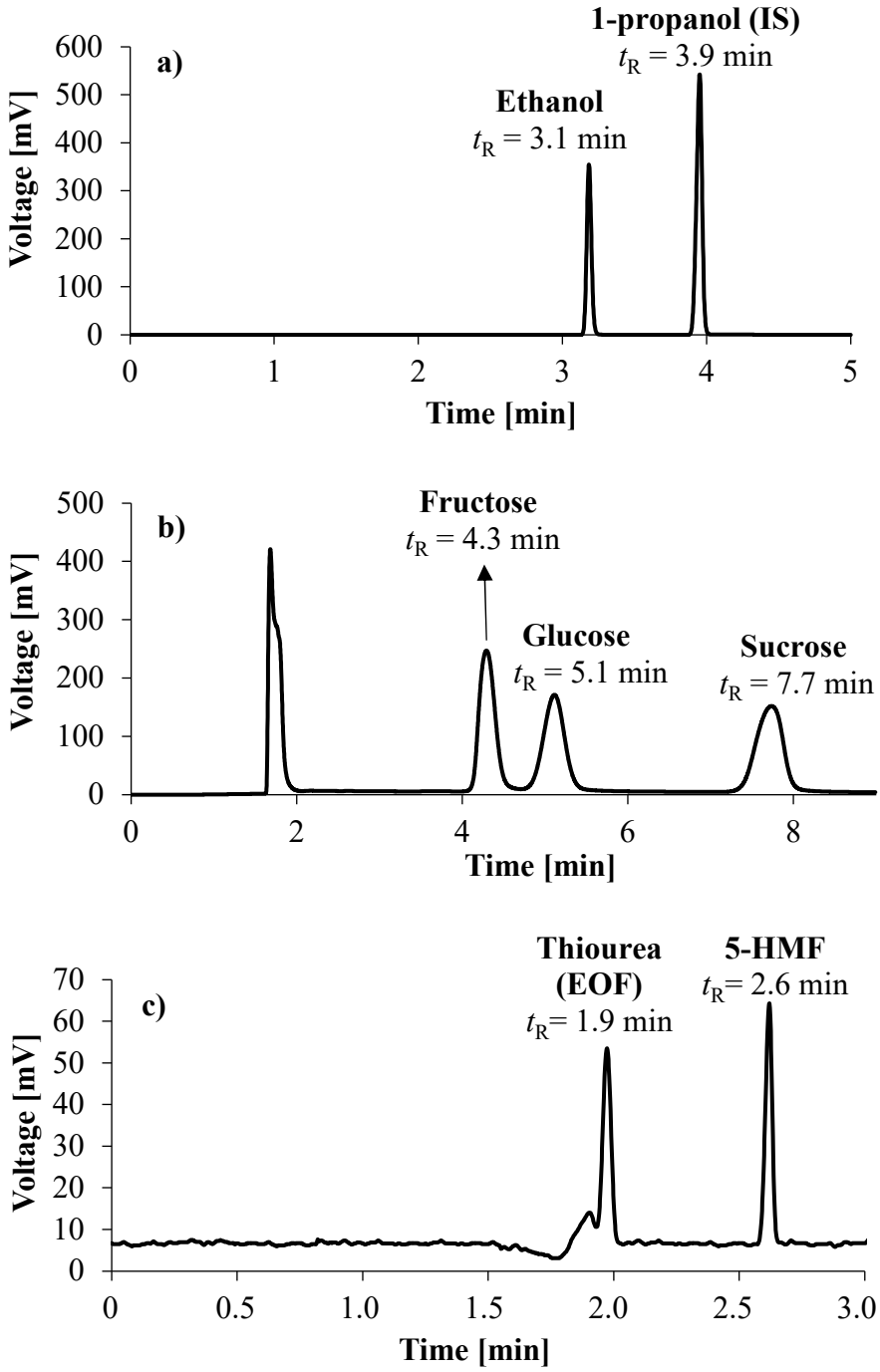

Fig. 1 Demonstration of separations of target compounds using optimized instrumental conditions (for specific instrumental parameters of each measurement, see the Experimental section):

a) GC chromatogram of the standards of ethanol (c = 3 %) and of 1-propanol (internal standard; IS; c = 3 %)
b) HPLC chromatogram of carbohydrates standards (c = 42 g/L)
c) MEKC chromatogram of the standards of 5-HMF (c = 15 mg/L) and thiourea (electroosmotic flow; EOF; c = 6 mmol/L)

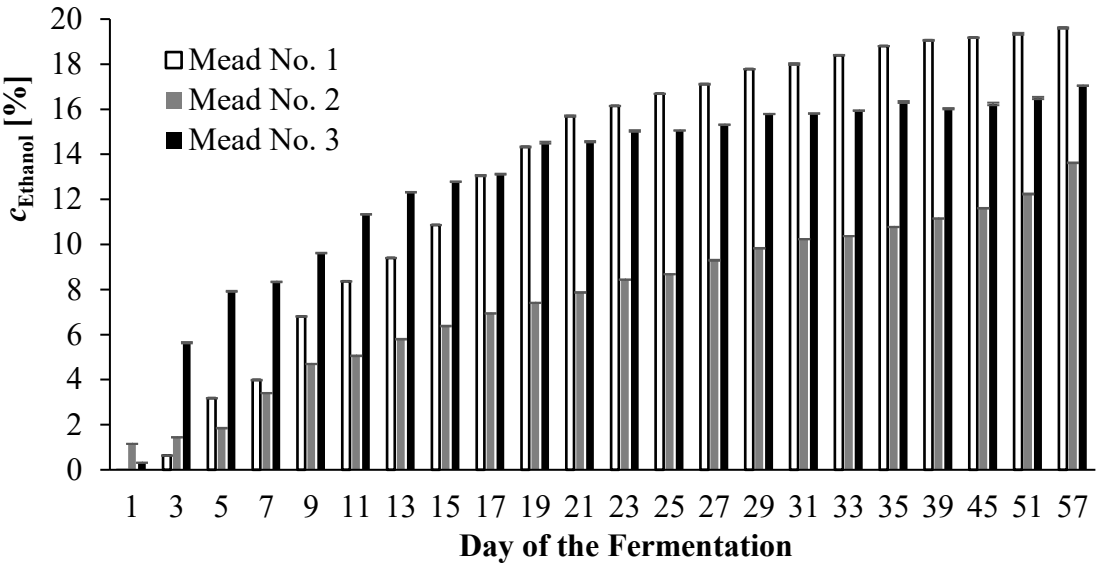

Fig. 2 Increase in the concentration of ethanol during the fermentation process of honey-water solution

Regarding carbohydrates, all mead samples showed a decrease in glucose and fructose in the honey-water solution during fermentation (Fig. 3). However, the content of glucose diminished more significantly because of a preferential consumption by yeast in alcoholic fermentation. Compared to the initial glucose content in honey-water solution of samples Nos. 1, 2, and 3 (147.88 ± 0.13, 175.38 ± 0.14, and 144.19 ± 0.08), its concentration decreased by 99.3, 94.0, and 97.8 % during fermentation, respectively. At the same, fructose was reduced by only 96.6, 75.2, and 83.4 % of the original concentrations (121.33 ± 0.04, 149.79 ± 0.01, and 142.76 ± 0.03) present in samples Nos. 1, 2, and 3, respectively. Changes in the representation of glucose, fructose, and ethanol throughout the fermentation of the individual honey-water solutions are clearly demonstrated in Fig. 4.

Sucrose is one of the frequent illegal additives of honey or meads. For this reason, sucrose was also monitored in all samples. Nonetheless, this was not detected in any of them, which confirms the good manufacturing practice.

During the fermentation process of all three honey-water solutions, a new compound with the retention time of 2.6 min was detected in chromatograms of the real samples (Fig. 5). Based on the literature and subsequent comparison with the relevant standard, the unknown compound was identified as glycerol, representing one of the by-products of the alcoholic fermentation process. Subsequently, the glycerol content in the final drinks was also quantified using the external standardization when the concentration of glycerol standard was 20 g/L. Nevertheless, in the final beverages, its content was below 1 % and thus did not pose a health risk for potential consumers.

Since 5-HMF is a known contaminant of food containing carbohydrates, especially at elevated temperature, its potential occurrence in the meads analysed was monitored. 5-HMF was not detected in any mead throughout the entire production process, demonstrating that the natural increase in temperature during fermentation is not sufficient for initiating the formation of this substance and that the honey had not been dissolved in excessively hot water during the preparation of the honey-water solution, which could subsequently reduce the quality of the resulting beverage.

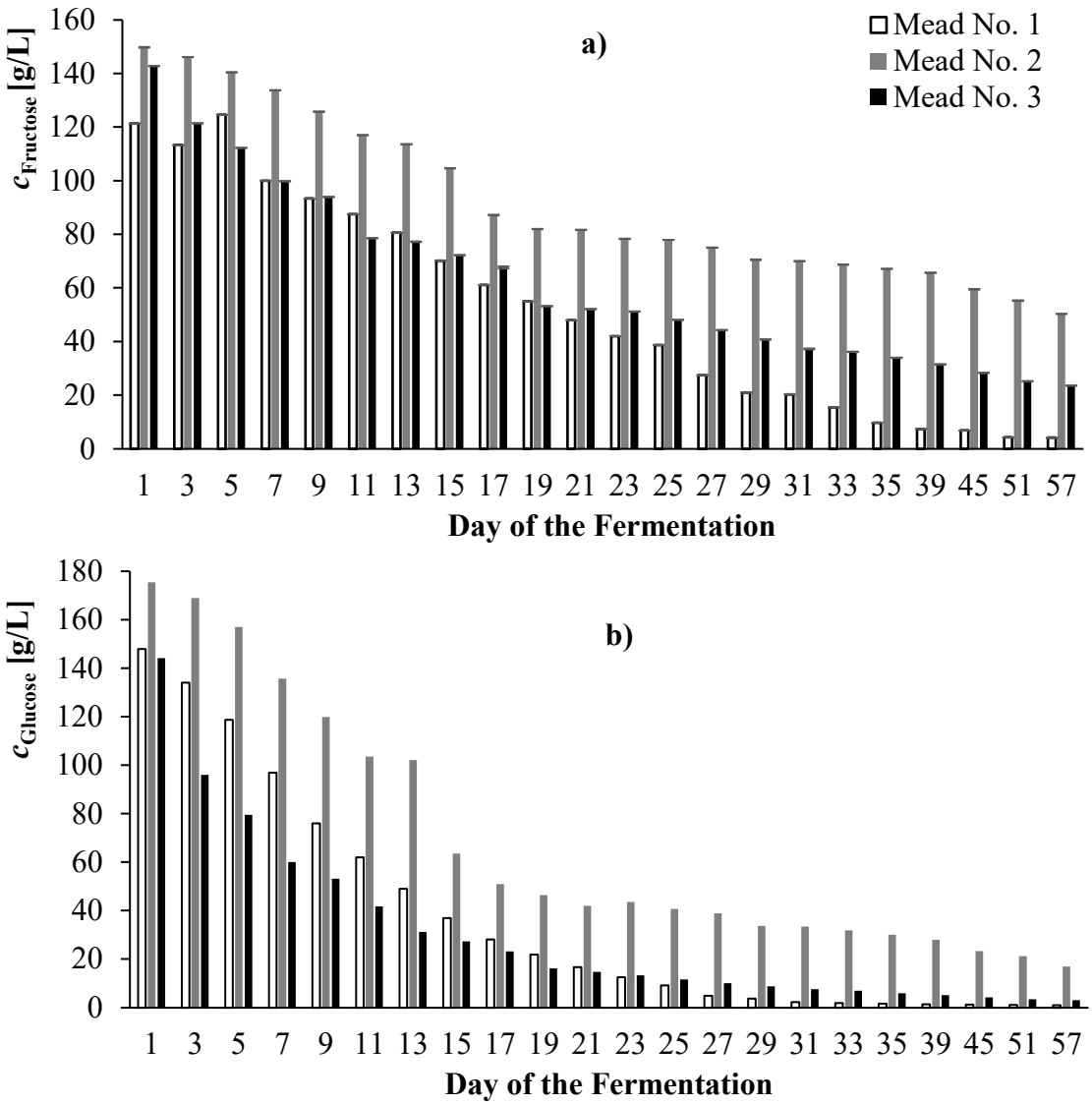

Fig. 3 Consumption of fructose (a) and glucose (b) during the mead fermentation

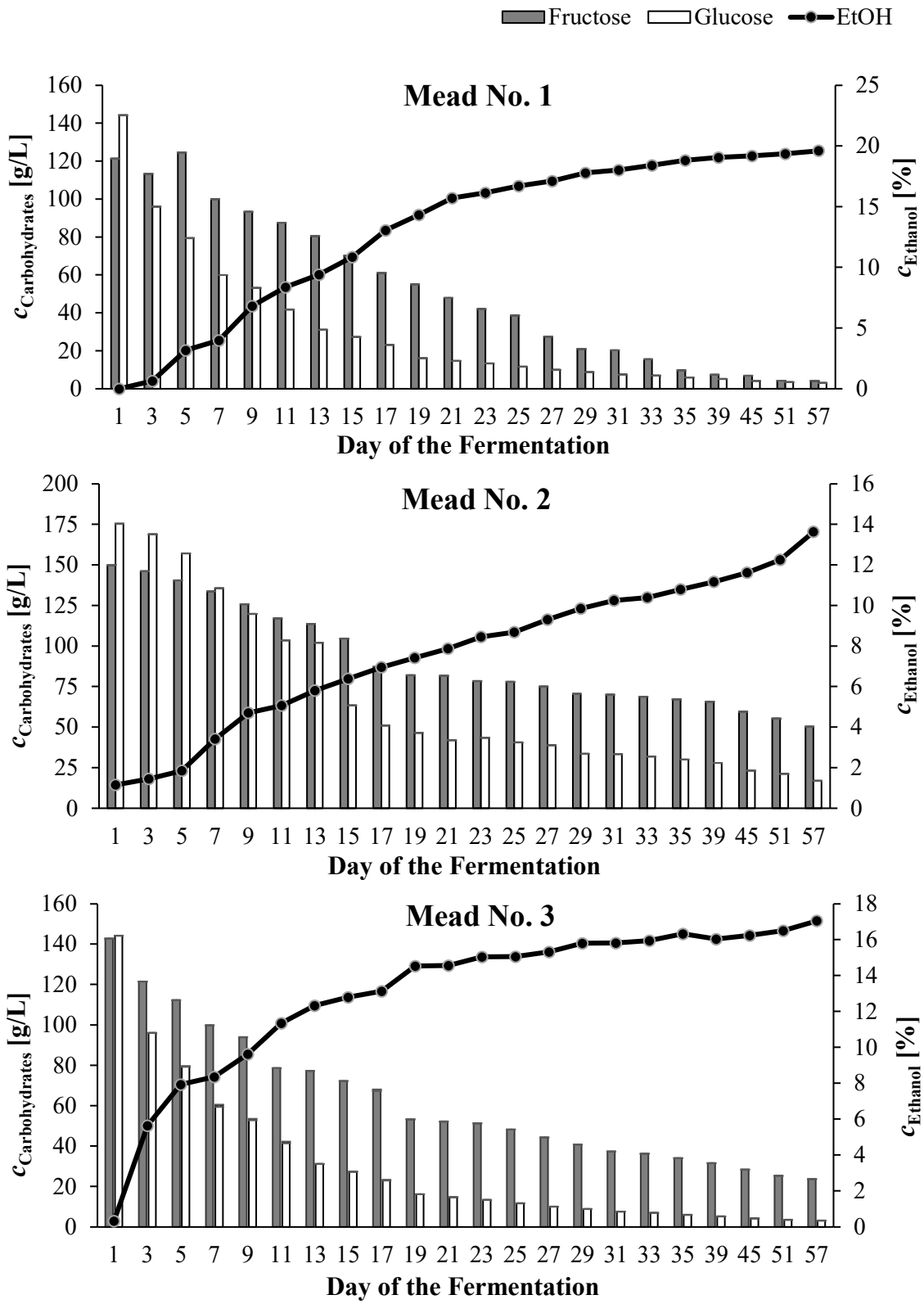

Fig. 4 Consumption of glucose and fructose with simultaneous increase of the content of ethanol during fermentation of honey-water solution

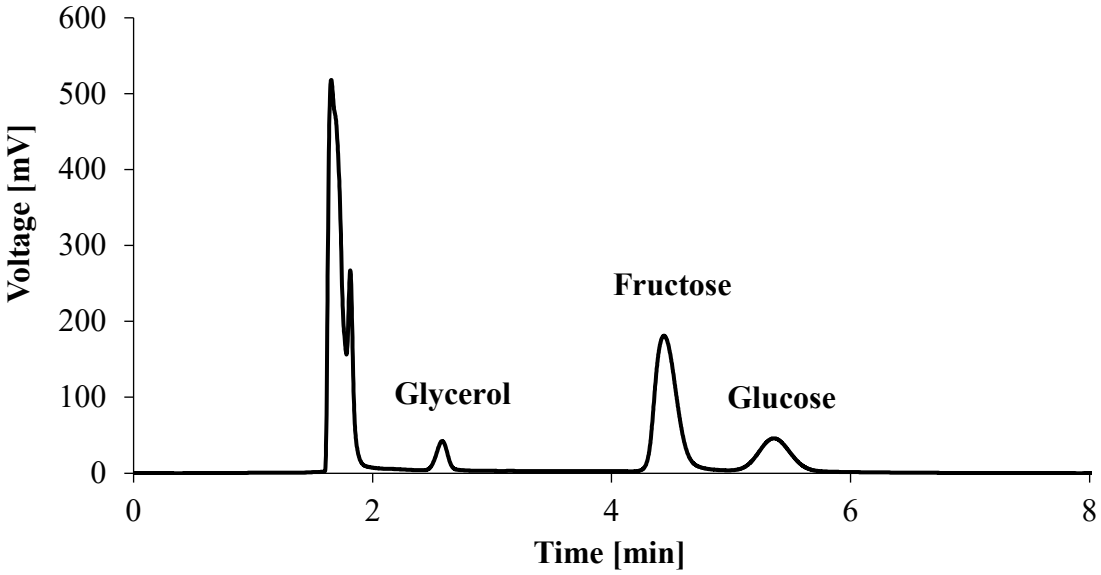

Fig. 5 Example of HPLC chromatogram of mead sample No. 2 (51st fermentation day)

Experimental conditions: Supelcosil LC-NH_2 (250 × 4.6 mm, 5 μm); mobile phase 80% acetonitrile, column temperature 25 °C; flow rate 2 mL/min; injection volume 20 μL; refractive-index detector

Conclusions

Changes in the representation of carbohydrates (glucose and fructose) and ethanol throughout the production of three meads have been monitored using hydrophilic interaction liquid chromatography combined with refractive-index detector and gas chromatography with flame ionization detector, respectively. Glucose was found to be more consumed by yeast than fructose; however, all the mead samples contained negligible amounts of both carbohydrates at the end of the fermentation process. On the contrary, as the carbohydrates are fermented, the amount of ethanol naturally increases. The greatest increment in the content of ethanol was observed at the beginning of the fermentation process, whereas when approaching the end, its concentration was almost constant. Sucrose is an unauthorized mead additive; therefore, monitoring its potential presence was also one of the objectives of this work. Nevertheless, it was not detected in any of the meads studied, thus indicating a good manufacturing practice.

An unknown peak occurred in the HPLC chromatograms of all the mead samples. Later, this peak was identified as a signal belonging to glycerol. However, its concentration had never exceeded even 1 %, meaning no health risk for consumers.

As confirmed, undesirable formation of the contaminant 5-hydroxymethylfurfural may occur during the preparation of honey-water solution (honey dissolves in water at excessive temperature) or alcoholic fermentation. Therefore, the presence of this potential carcinogen was monitored by using the micellar electrokinetic capillary chromatography with spectrophotometric detection. The concentration of 5-HMF was below the limit of detection throughout the production of all the samples studied. Thus, it was verified that gentle heating was used for preparation of honey-water solution, preserving the quality of the product, and that the fermentation of the honey-water solution does not cause such an increase in temperature, which would subsequently initiate the formation of 5-HMF in the sample.

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