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Evaluation of HMF levels in unbranded flower honeys in terms of food safety

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Abstract: 5-hydroxymethylfurfural (HMF) is formed by reducing sugars in honey in acidic environments by the Maillard reaction and is known as a carcinogenic, mutagenic and genotoxic compound. The aim of this study is to investigate the toxic HMF content of unbranded flower honey samples sold under the sun under inappropriate conditions and unsupervised on the highway sides and to make an evaluation in terms of food safety. The quality of the analysis results was guaranteed by participating in an international proficiency test. Analysis was performed on a High-Performance Liquid Chromatography (HPLC) device with Diode Array Detector (DAD) and Refractive Index (RI) detectors- The HMF levels of 5 honey samples were determined to exceed the legal limit (40 mg kg^{-1}). It is revealed by the current study that the honey sold on the roadside is not safe in terms of toxic HMF. In addition, 5 samples were not found to comply with the regulation in terms of glucose + fructose content and 2 samples in terms of fructose/glucose ratio. Thus, it has also been determined that these honeys are not reliable in terms of purity.
Keywords: Bee product, food safety, HMF, honey, toxicity.

Markasız çiçek ballarındaki HMF düzeylerinin gıda güvenliği açısından değerlendirilmesi

Özet: 5-hydroxymethylfurfural (HMF), Maillard reaksiyonu ile baldaki şekerlerin asidik ortamlarda indirgenmesiyle oluşur ve karsinojenik, mutajenik ve genotoksik bir bileşik olarak bilinir. Bu çalışmanın amacı, karayolu kenarlarında güneşin altında uygunsuz koşullarda, denetimsiz satılan markasız çiçek balı örneklerinde toksik HMF içeriğini araştırmak ve gıda güvenliği açısından bir değerlendirme yapmaktır. Analiz sonuçlarının kalitesi, uluslararası bir yeterlilik testine katılarak garanti altına alınmıştır. HMF ve şeker seviyelerinin belirlenmesi için sırasıyla, Diyot Dizisi Dedektör (DAD) ve Refraktif İndeks (RI) dedektöre sahip bir HPLC cihazı kullanılmıştır. Beş bal numunesinin HMF seviyelerinin yasal sınırı (40 mg kg^{-1}) aştığı belirlenmiştir. Bu çalışma, yol kenarlarında satışa sunulan balların toksik HMF bakımından güvenli olmadığını ortaya koymaktadır. Ayrıca, 5 numune glukoz + fruktoz içeriği bakımından, 2 numune de fruktoz/glukoz oranı bakımından yönetmeliğe uygun bulunmamıştır. Böylece, bu balların saflık bakımından da güvenilir olmadığı belirlenmiştir.
Anahtar sözcükler: Arı ürünü, bal, gıda güvenliği, HMF, toksisite.

Introduction

Honey is a natural food containing around 80% carbohydrates (most importantly glucose, fructose, and sucrose) and 20% water. It also contains more than 180 bioactive components, including vitamins, minerals, amino acids, organic acids, enzymes, and phenolic compounds (8, 11, 14). Honey is classified as flower and secretion honey. Flower honey is obtained from plant nectars, and honeydew honey is obtained from the secretions of plant-sucking insects living on plants or from the secretions of living parts in plants according to Turkish Food Codex Honey Communique (21). Heat treatment can be applied to the honey in order to provide sufficient fluidity during filling, to delay or prevent crystallization and to stop microorganism development (9, 11). Acid catalyzed dehydration of hexoses (1, 3) and the Maillard reaction (1, 22) during heat treatment are the main routes for spontaneous formation of 5-hydroxymethylfurfural (HMF). Long-term storage is another factor that contributes to the increase of HMF amounts. Therefore, the determination of the amount of HMF is an indicator for excessive heat treatment or improper storage of honey (6, 11). HMF, which induces reactive oxygen species, has toxic effects (7). Current studies show that HMF can be considered as a potential carcinogen for humans or an agent that may be metabolized to carcinogenic compounds (1, 13). 5-sulfoxymethyfurfural, a conversion product of HMF, is a cytotoxic and mutagenic compound (5). If the literature is taken into account, HMF is a compound that should not only be considered as a quality criterion in

honey, but also be considered in terms of health risks as it is a compound with mutagenic and genotoxic metabolite. Commercial honeys are controlled for HMF levels, purity and other quality criteria by regular sampling of the authorities. However, the honey that is put up for sale on the side of the roads under the sun on makeshift benches by producers and/or people claiming to be producers without legal sales permission, is not followed by the state's control mechanisms. On the other hand, consumers passing through the national parks such as Ilgaz and Küre, buy these honeys considering that they are pure, natural, even organic and very healthy. Examining the HMF levels and compositions of these honeys - under the above-mentioned sales conditions is also extremely important in terms of consumer health and food safety. Therefore, the aim of this study was to examine toxic HMF content and composition of the unbranded flower honey samples sold in Kastamonu province in order to reveal whether consumers are under risk related to food safety and health.

Materials and Methods

***Collection of honey samples*:** A total of 22 unbranded flower honey samples were collected from Kastamonu province in the summer season. The samples (about 1 kilogram each) were obtained from small-scale traditional beekeepers selling on the sides of highways in the Ilgaz and Küre mountain regions. Flower honey samples were stored at room temperature (22 ± 2 °C) in a dark place until analysis.

Chemicals: HMF (≥ 99%), glucose, fructose, and sucrose were provided by Sigma Aldrich (Saint-Louis, MO, USA), AFG Bioscience LLC (Northbrook, USA) and Merck (Darmstadt, Germany). Methanol and acetonitrile (HPLC grade) were obtained from Sigma Aldrich. Ultrapure water was produced using Human Power 2 system (Seoul, Republic of Korea). All the other reagents used were of analytical purity and were obtained from Merck and Sigma Aldrich.

Methods: All analyses were carried out in Kastamonu University Central Research Laboratory Application and Research Center. The quantities of HMF and sugars were determined according to International Honey Commission (IHC) Methods (4) using a High-Performance Liquid Chromatography (HPLC) device (Shimadzu LC-20A Prominence series, Kyoto, Japan) with Diode Array Detector (DAD) and Refractive Index (RI) detector, respectively. Conditions for chromatographic analyses are given in Table 1. In addition, the free acidity and moisture analyses of the samples were completed according to IHC methods (2009).

Sample extraction for chromatographic analyses: A sample of 2.5 g of homogenized honey was accurately weighed into a 50 mL beaker. Then, the sample was dissolved in approx. 25 mL of water and transferred quantitatively to a 50 mL volumetric flask. The volumetric flask content was diluted to 50 mL with water and filtered through a 0.45 μm membrane filter to provide a sample solution ready for chromatography.

Method performance characteristics: Linearity was assessed with a calibration curve that was constructed using standard solutions of HMF and sugars in the ranges of 0.8, 4.0, 8.0, 12.0, 20.0, 40.0, 80.0 μg mL^{-1} and 15, 20, 40, 60, 80%, respectively. Ten replicates of HMF solution (in ultra-pure water spiked at the 0.8 μg mL^{-1} concentration) and sugar solution (glucose, fructose, and sucrose in ultra-pure water spiked at 0.5%; 1%, and 1% concentrations) analyses were performed. The limit of detection (LOD) and limit of quantification (LOQ) of the methods were expressed as 3 × standard deviation (SD) and 10 × SD, respectively, according to Eurachem Guide (2014) (17). To establish repeatability (intra-day precision) and reproducibility (inter-day precision), ten replicates of a homogenized flower honey sample analyses were performed on two different days, separately. Intra-day and inter-day precisions were expressed as the percentage relative standard deviation (RSD %). To establish the accuracy, recovery assays were carried out. For this purpose, homogeneous flower honey was diluted 4 times with ultrapure water. Standard HMF was spiked into this dilute solution with a concentration of 24 and 48 μg mL^{-1}, respectively. Likewise, standard sugars were added so that the in order to reach 5% and 30% dilutions, respectively. Six replicates of these samples were prepared and analyses were performed. Recovery is expressed in percent and calculated using the formula below.

Table 1. Conditions for chromatographic analyses.

Analysis Parameters	Column	Wavelength	Column and Detector Temperature	Mobile Phase	Flow Rate	Sample Volume
HMF	Inertsil C18; (5 μm, 4.6 × 150 mm)	285 nm	Room temperature	Water:Methanol (90:10, v/v)	1.0 (mL min^{-1})	20 μL
Sugars (Glucose, Fructose, Sucrose)	Inertsil NH2 ; (5 μm, 4.6 × 250 mm)	-	40 °C	Acetonitrile:water (80:20, v/v)	1.3 (mL min^{-1})	10 μL

Table 2. LOD, LOQ, intra-day, inter-day precision and recoveries for chromatographic analysis of flower honey.

Analyte	LOD/LOQ	Intra-day precision, %RSD (n=10)	Inter-day precision, %RSD (n=20)	Spiked Levels for recovery assays	Recovery of spiked samples, % ± SD (n=6)
HMF	0.036/0.121 (mg kg^{-1})	1.40	1.69	24 mg kg^{-1} 48 mg kg^{-1}	99.83 ± 1.65 100.65 ± 0.34
Fructose	0.278/0.927 (%)	1.54	0.94	5 % 30 %	91.47 ± 14.24 90.37 ± 1.26
Glucose	0.238/0.732 (%)	1.23	1.03	5 % 30 %	107.40 ± 11.52 99.99 ± 2.33
Sucrose	0.189/0.639 (%)	1.03	3.31	5 % 30 %	96.80 ± 2.56 99.40 ± 1.20

SD: Standard deviation
RSD: Relative standard deviation.

Table 3. Analysis results for flower honey samples.

No	HMF (mg kg^{-1} ± SD)	Free acidity (meq kg^{-1} ± SD)	Moisture% (w / w ± SD)	Fructose% (w / w ± SD)	Glucose% (w / w ± SD)	Sucrose% (w / w ± SD)	Total sugar% (w/w)	Fructose + glucose%	Fructose / glucose
1	**42 ± 1**	10.8 ± 0.1	9.8 ± 0.1	40.8 ± 0.2	26.4 ± 0.1	0.3 ± 0.0	67.5	67.2	**1.55**
2	10 ± 0.3	13.6 ± 0.1	13.9 ± 0.0	43.0 ± 0.3	36.0 ± 0.0	1.2 ± 0.1	80.2	79.0	1.19
3	2 ± 0.1	5.1 ± 0.0	15.4 ± 0.0	38.3 ± 0.15	27.3 ± 0.1	0.4 ± 0.05	66.0	65.6	1.40
4	<LOD	20.4 ± 0.15	15.8 ± 0.0	32.1 ± 0.2	25.4 ± 0.15	0.1 ± 0.0	57.6	**57.5**	1.26
5	7 ± 0.1	30.7 ± 0.1	15.8 ± 0.1	30.4 ± 0.3	22.3 ± 0.2	<LOD	52.7	**52.7**	1.37
6	2 ± 0.0	20.4 ± 0.2	14.4 ± 0.1	40.1 ± 0.1	32.3 ± 0.1	<LOD	72.4	72.3	1.24
7	26 ± 0.5	15.3 ± 0.1	16.6 ± 0.0	36.5 ± 0.1	34.6 ± 0.2	0.1 ± 0.0	71.2	71.1	1.05
8	8 ± 0.2	20.4 ± 0.1	15.8 ± 0.1	36.7 ± 0.2	32.0 ± 0.2	0.6 ± 0.0	69.3	68.7	1.15
9	**60 ± 1.3**	35.7 ± 0.05	17.8 ± 0.0	38.8 ± 0.4	32.6 ± 0.1	0.3 ± 0.0	71.7	71.3	1.19
10	10 ± 0.3	20.4 ± 0.1	15 ± 0.0	42.7 ± 0.2	38.9 ± 0.3	<LOD	81.6	81.6	1.10
11	11 ± 0.1	20.4 ± 0.0	13.1 ± 0.1	42.4 ± 0.2	34.7 ± 0.25	<LOD	77.2	77.2	1.22
12	**55 ± 3.2**	30.7 ± 0.2	14.0 ± 0.1	36.2 ± 0.3	38.3 ± 0.2	<LOD	74.5	74.5	0.95
13	1 ± 0.0	37.5 ± 0.1	16.0 ± 0.0	37.6 ± 0.25	33.4 ± 0.0	0.2 ± 0.0	71.1	71.0	1.13
14	7 ± 0.1	30.7 ± 0.3	15.0 ± 0.0	42.1 ± 0.3	32.7 ± 0.1	0.3 ± 0.05	75.1	74.8	1.29
15	**43 ± 2.4**	30.7 ± 0.1	15.8 ± 0.0	39.8 ± 0.1	32.1 ± 0.1	0.2 ± 0.0	72.1	71.9	1.24
16	**44 ± 1.9**	13.6 ± 0.0	17.0 ± 0.0	32.5 ± 0.1	25.4 ± 0.2	<LOD	57.9	**57.9**	1.28
17	7 ± 0.2	27.9 ± 0.1	15.8 ± 0.0	39.7 ± 0.35	34.0 ± 0.4	<LOD	73.7	73.7	1.17
18	10 ± 0.2	20.4 ± 0.1	13.3 ± 0.1	41.1 ± 0.15	29.8 ± 0.15	<LOD	70.9	70.9	1.38
19	<LOD	37.7 ± 0.05	10.8 ± 0.2	28.7 ± 0.1	17.5 ± 0.1	<LOD	46.2	**46.2**	**1.64**
20	37 ± 1.7	29.8 ± 0.1	13.7 ± 0.1	39.1 ± 0.25	32.1 ± 0.1	<LOD	71.2	71.2	1.22
21	16 ± 0.8	6.8 ± 0.1	13.7 ± 0.0	21.5 ± 0.15	24.6 ± 0.2	0.1 ± 0.0	46.2	**46.1**	0.90
22	5 ± 0.1	17.0 ± 0.2	13.4 ± 0.1	36.5 ± 0.3	30.3 ± 0.1	0.1 ± 0.0	66.8	66.8	1.20
Mean	18.32	22.55	14.63	37.12	30.58	0.18	67.87	67.69	1.23

SD: Standard deviation
Note: Results shown in bold are incompatible with the Turkish Food Codex Honey Communique.

$$\text{Recovery (\%)} = [(C1-C2) / C3] \times 100$$

C1 = Measured concentration of sample and added standard
C2 = Concentration of the sample
C3 = Concentration of the added standard

Analytical quality assurance: The laboratory where this study was carried out participated in "FAPAS® (accredited by UKAS as complying with the requirement of EN ISO/IEC 17043:2010) Proficiency Test No. 2839" for the evaluation of analytical quality assurance.

Statistical analysis: Statistical analysis was performed using IBM SPSS 22.0 software. The results of the analysis parameters were not normally distributed; the non-parametric Spearman correlation test was performed to evaluate the possible association between HMF levels and other analysis parameters.

Results

Method performance characteristics: The calibration curves were linear in the working range of the standards with coefficient of determination (R^2) values of 0.99997, 0.99995, 0.99999, and 0.99993 for HMF, glucose, fructose, and sucrose, respectively. The LOD and LOQ values, and the results of repeatability, reproducibility, and recovery, were shown in Table 2. The percentages of RSDs were < 20% in this study and were below the maximum acceptable RSD values calculated by Horwitz equation (12). The recoveries obtained for all concentration levels were acceptable.

Analytical quality assurance: Assigned values of the FAPAS test sample and z-scores for each analysis parameter of the laboratories participating in the international proficiency tests were determined by FAPAS. The z-score values of Kastamonu University Central Research Laboratory Application and Research Center ranged from 0.5 to 0.9 for all the parameters analysed in this study. Considering that the acceptable range of z-scores must be $-2 \leq z \leq 2$, the quality and reliability of our analytical method was proved.

Analysis Results for Flower Honey Samples: The analysis results of the flower honeys examined in our study and their comparison with the limits in the Turkish Food Codex, Honey Communique were given in Table 3.

Discussion and Conclusion

Turkish Food Codex Honey Communique (21) and European Union Directive (110/2001/ CE) (8) recommend a limit of 40 mg kg^{-1} for HMF in honey. The level of HMF was detected in the range from undetectable to 60.2 mg kg^{-1} (see Table 3) in this study and HMF levels of 5 honey samples were determined to exceed the legal limit given in Turkish Food Codex, Honey Communique. Similar to the results of our study, in a study examining 8 flower honey samples obtained from traditional producers in Bingöl province, HMF value (42.22 mg kg^{-1}) of a Honey sample - exceeded 40 mg kg^{-1} (24). In a study conducted with honey samples obtained from 46 members registered to the beekeepers' association in Gaziantep province, the average HMF value was determined as 27.690 mg kg^{-1}, but the HMF levels of three samples were out of the standard (16). If the measured value of HMF is above legal limit, it indicates that the honey has been stored in an unsuitable hot environment or may have been exposed to heat treatment (6, 9, 11). In contrast, in a study with honeys supplied directly from the producer in the eastern Black Sea Region, HMF was detected in the range from undetectable to 11 mg kg^{-1} (10) and the range of HMF values were 2.06-3.43 mg kg^{-1} for multifloral honeys from the Central Anatolia and 2.03-3.29 mg kg^{-1} for sunflower honeys from the Trakya Region (25). Unlike these studies, the reason for HMF values of some honey samples of this study being above the legal limits, may be exposure to the sun because they are sold on the sides of highways in the Ilgaz and Küre mountains.

In this study, some quality criteria and components of honey were also analyzed. In our study, mean fructose + glucose content was determined to be and 67.69% (see Table 3). But the fructose + glucose% (inverted sugar) content of 5 flower honeys was less than the minimum limit value (60%) determined by the relevant legal regulations. The reason for this situation may be that such honeys sold under the name of flower honey are actually mixed with honeydew honeys (the minimum limit value 45%) or produced with sugar syrups. In the literature, there are other studies in which some of the honeys purchased directly from the producers do not meet the requirements of the regulation in terms of glucose + fructose (10, 11). The mean fructose/glucose value was determined to be 1.23 (Table 3) in this study. While the fructose/glucose ratio should be 0.9-1.4 in flower honey according to Turkish Food Codex Honey Communique, this ratio was significantly higher than 1.4 in 2 samples in our study. This shows that the amount of fructose in the samples is higher than the amount required in these honeys. This suggests the possibility that fructose syrup was added to these honeys. The average sucrose amount was detected to be 0.18% in this study. The sucrose content did not exceed the limit value of 5% in any of the samples. Similarly, in the studies of Batu, Küçük and Çimen (2) and Güzel and Bahçeci (11), the results were found to be within the limits. In contrast, the content of sucrose in 14 of 20 flower honey samples from Erzurum Province exceeded the limit of Turkish Food Codex Honey Communique (20). The free acidity and moisture content of all flower honeys investigated in this study were below the maximum limit values given in the Turkish Food Codex, Honey Communique. The acidity and moisture content of flower honeys examined in other

studies (2, 11, 16, 20, 25) mostly abided by the limits of the relevant communique, in line with our study.

The samples examined in this study were obtained from traditional manufacturers that selling in makeshift huts without legal permission at different points on the highways in Ilgaz and Küre mountains. Thus, it is obvious that these samples were exposed to significant levels of daylight and summer heat. Some of these honeys may probably be produced in previous seasons. High temperatures and long-term storage are known to significantly increase the HMF levels (6, 9, 11). Korkmaz and Küplülü stored flower and honeydew honey samples produced by different companies at different temperatures and examined the effect of this situation on HMF levels (15). In their study, while the average HMF values of the samples kept at 10 ± 2 °C and 22 ± 2 °C for a year did not exceed 40 mg kg^{-1} that is the limit value in the Turkish Food Codex Honey Communique, the HMF level increased rapidly and was above 40 mg kg^{-1} (from 53.1 to 83.7 mg kg^{-1}) starting from the 6th month in samples stored at 35 ± 2 °C. These temperatures are common in the summer months in Kastamonu province, and it is possible that honeys sold on the sides of the highway might be left at this temperature and in the sun for weeks or even months. It is highly probable that the samples with high HMF levels in this study were stored for a long time or exposed to sunlight and/or heat treatment or produced in previous years. Although, moisture and free acidity contents which are important for the Maillard reaction are other parameters affecting HMF levels -(1, 18, 19), no significant relationship between these parameters and HMF levels was determined ($P > 0.05$) in the current study. The reasons for this may be that these parameters do not show much variation in honey samples, and most importantly, the heat and sunlight parameters are more effective on HMF levels under the conditions in which these samples are sold. Honey offered for sale by traditional beekeepers is not inspected and the conditions under which they are stored and sold are unknown, and HMF levels are questionable. The results of our study revealed that unbranded flower honeys sold by beekeepers on the roadside may be problematic in terms of HMF level and also revealed that their purity is questionable. Although the Ministry of Agriculture and Forestry of the Republic of Türkiye increases its legal inspections day by day, it is still not possible to examine all of the honey offered for sale in large number of commercial shops. For example, the average HMF value of honeys obtained from the markets was 56.70 ± 3.83 mg kg^{-1}, in the province of Aydın (23). Even the honeys sold in commercial shops cannot be fully inspected, and for this reason, the sale of honey that does not comply with the criteria in legal regulations cannot be prevented. Therefore, it does not seem possible for the authorities to inspect the honey produced by all traditional producers. That's why it is important that the authorities take the necessary precautions, increase the awareness of producers and consumers and ensure that they are informed about the health risks caused by consuming these products.

Consequently, it is thought that brand-free honeys sold on the sides of the highway by traditional beekeepers are not reliable in terms of HMF levels and purity. Unfortunately, these producers and their products are not inspected by the authorities responsible for monitoring compliance with the provisions of the Turkish Food Codex, Honey Communique. Authorities should make consumers aware of the safety and health risks of unbranded honey sold by the roadside, and producers should be trained on appropriate production, storage and sale conditions. Consumption of packaged and branded honeys is recommended, because these honeys are subject to inspections by authorities in terms of food safety and reliability.

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Ethical Statement

Since the study contains only food analyzes, ethics committee approval is not required.

Conflict of Interest

The author declared that there is no conflict of interest.

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