Bull. Chem. Soc. Ethiop. **2024**, 38(1), 1-10.
DOI: https://dx.doi.org/10.4314/bcse.v38i1.1

ISSN 1011-3924
Printed in Ethiopia
Online ISSN 1726-801X

# INFLUENCE OF HEAVY METAL IONS AND STORAGE TIME ON HYDROXYMETHYLFURFURAL FORMATION IN HONEY COLLECTED FROM ILU WOREDA, OROMIA REGIONAL STATE, ETHIOPIA

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(Received May 15, 2023; Revised October 14, 2023; Accepted October 17, 2023)

**ABSTRACT**. The effect of heavy metals on the formation of hydroxymethyl furfural (HMF) from degradation of reducing sugars in honey collected from Ilu Woreda, West Shewa Zone of Oromia Regional State, Ethiopia were studied. The concentration level of Pb, Cd, Cr and Ni in honey sample was determined using AOAC Official Method. The concentration of HMF in honey was determined using international honey commission standards. The concentrations of heavy metals in fresh honey were 11 ± 0.001, 454 ± 0.002, 630 ± 0.001 and 1 ± 0.001 μg $kg^{-1}$ for Pb, Cr, Ni and Cd, respectively. The influence of heavy metals on the formation of HMF in honey samples was studied on 5, 30 and 60 days after spiking at concentration level of 10,000 μg $kg^{-1}$ of each heavy metal. The concentration of HMF for untreated honey samples was ranged 1380-2200 μg $kg^{-1}$ for the storage time of 5, 30 and 60 days. The concentration of HMF in honey samples spiked with Pb, Ni, Cr and Cd were found to be in the range of 2100-2150, 1900-2430, 2100-2270 and 2050-2330 μg $kg^{-1}$, respectively. The results of this study indicate that contamination of honey with heavy metal facilitate the formation of HMF.

**KEY WORDS**: Fresh honey, HMF, Heavy metal ions, HPLC-DAD and Honey quality

## INTRODUCTION

Honey is a natural, sugary and viscous fluid produced by honeybee from the nectar of vegetation and/or plants [1]. It is one of the most widely consumed food products due to its unique nutritional and medicinal properties [2, 3]. Honey compositions are mainly water (15-20%), sugars (80–85%, w/w) including the two main sugars (dextrose and levulose) and 22 other more complex sugars. In addition, honey contains nitrogenous compounds, proteins, lactone, phenol antioxidants, antibiotic-rich inhibine, fragrance compounds, enzymes, amino and organic acids, phenolic acids, gluconic acid, minerals, flavonoids, vitamins, hydroxymethyl furfural (HMF), and other phytochemicals [4].

Even though honey differs in appearance, sensory perception and composition due to the variation of botanical origin, the main nutritional and health relevant components are carbohydrates, mainly fructose and glucose [5, 6]. The presence of certain ingredients like some alkaloids, heavy metals, and HMF and its derivations may contribute to honey's toxin [7].

As shown in Figure 1, HMF is a cyclic aldehyde produced during food processing or long storehouse of honey from sugar degradation through a non-enzymatic browning response [4, 8]. HMF is not found in honey naturally and it is potential cancerous, mutagenic and cytotoxic which is also toxic to honey bees [7]. The amount of HMF is influenced by a variety of factors, including honey quality, climate, honey kinds, geographic location, preservation, method of processing, and other factors [4]. Heating or preserving honey may produce HMF which is a toxic substance [7]. Additionally, the presence of heavy metals like lead, cadmium, mercury and arsenic in honey can catalyze the production HMF from sugar [7, 8] and heavy metals in honey is also more of a health danger to plants, animals and human life [9] than altering physicochemical characteristic of honey [10].

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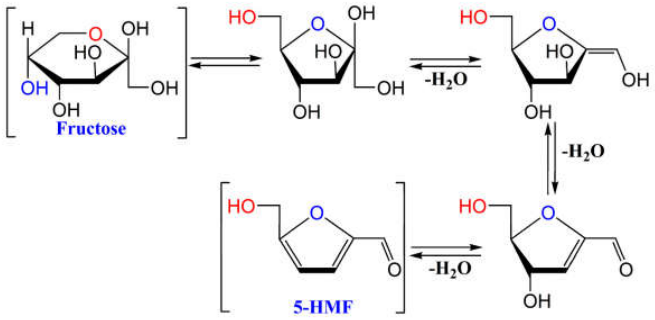


Figure 1. Reaction pathway of fructose to form HMF.

The death of honey bees by HMF after 15 to 30 days of eating on it has been reported by Gregorc *et al.* [11]. A large-scale experiment at relatively low concentration of HMF (150,000 g $kg^{-1}$) detected in acid-hydrolyzed inverted sugar syrup also cause 50% bee mortality after 16 days commencement of feeding. The inverted syrup for feeding bees should not contain HMF more than 20,000 µg $kg^{-1}$ [11, 12]. HMF can cause mutagenic, carcinogenic, and other dangerous consequences (genotoxic, cytotoxic and enzyme inhibitory) in humans. For this reason the maximum residues level (MRL) for HMF in honey is 40,000 µg $kg^{-1}$ for honey from the continental area and 80,000 µg $kg^{-1}$ for honey from the tropical area [13].

In general, the amount of HMF in honey is not only an indicator of food storage conditions and quality, but it could also be used as an indicator of the potential occurrence of heavy metal contamination [11, 13]. It is therefore important to examine and understand the potential effects of heavy metals on formation of HMF from degradation of sugar in honey. Thus, the objective of this study was to evaluate effect of heavy metals on the degradation of sugar of honey to form HMF. The influence of heavy metal ions on the formation of HMF in controlled fresh honey and contaminated honey with certified standard heavy metals solution (Pb, Cr, Ni, and Cd) samples was studied in different time intervals.

## EXPERIMENTAL

*Description of study area*

The study was conducted on honey samples collected from Teji, which is located in the Oromia regional state, Ethiopia. It is located at latitude 8°38' N and longitude 38°17' E with elevation of 2068 m above sea level. It is approximately 64 km far from Addis Ababa in South West direction. The map of sampling area is given in Figure 2.

*Sampling and sample preparation*

Freshly harvested honey was sampled randomly from three modern hives (1 kg from each hives) from Teji, Oromia, in the month of April 2022. The three samples were mixed and stored in a clean plastic bottle. Then, the sample was transported to Ethiopian Conformity Assessment Enterprise (ECAE) Biochemical Analytical Laboratory and kept at room temperature of 22 ± 3 °C. Before analysis of HMF of the honey samples, it was filtered using plastic sieve against gravity at room temperature and shaken to homogenize and solubilize crystallized sugar [14, 15]. The determination of heavy metals from honey samples were analyzed using standard procedure of

AOAC Official Method: 2015.01 as described below. A test sample was digested with $HNO_3$ (70%) and $H_2O_2$ (30%) under pressure in a closed vessel microwave digester. Then the resulting solution was diluted with 50 ml de-ionized water and the concentration level of Pb, Cd, Cr, and Ni were determined by ICP-MS. The experimental procedure used to determine HMF was the International Honey Commission method [14]. All analyses were carried out in triplicate [16].

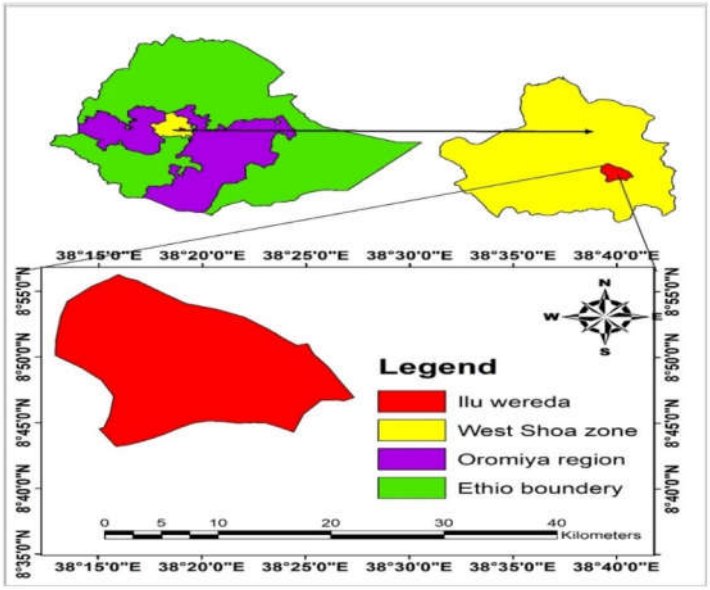


Figure 2. Map of sampling site.

*Apparatus and equipment*

In this study, digital analytical balance (Mettler Toledo, OH United States) with ± 0.0001 g precision, pH/ORP and conductivity meter/TDS/NaCl (HI 2550 pH/Cond meter, HANNA Instruments) were used to determine electrical conductivity, pH and free acidity. Digital refractometer (Scientific Laboratory Supplies, RFM960) was used for refractive index and moisture content determination. Furthermore, spectrocolorimeter (Lovibond PFR-i series) for honey color determination, ultra wave sonicator (Scientific Laboratory Supplies) for degassing air bubbles from deionized water solutions, fume cupboard (ENVAIR Ltd., England), magnetic stirrer hotplate 2400 rpm,150 W (78-1Magnetic stirrer, China), high performance microwave digestion system (ETHOS UP) for honey samples digestion were used throughout the experiment. The heavy metal analysis was performed using a PerkinElmer inductively coupled plasma mass spectrometer (NexION1000) instrument (ICP-MS). Waters-e 2695 UHPLC (alliance 2998 PDA Detector) was used for HMF analysis of honey samples.

*Reagents and chemicals*

All reagents and chemicals used were of analytical grade. Deionized water produced by a water purification system (Thermo fisher, Germany) was used for all solution preparations. Different chemicals such as methanol (99.9%), hydroxymethyl furfural, kosher ≥ 99%, nitric acid ($HNO_3$,

70%), acetonitrile (99.9%) and acetic acid (35%), were purchased from Sigma-Aldrich Chemie GmbH, Germany. Hydrogen peroxide ($H_2O_2$ 30%), and reference standard solution of lead, cadmium, chromium and nickel 10,000 (g $kg^{-1}$, were obtained from Romil pure chemistry, UK. Deionized water was used throughout the experiment to prepare diluted reference and working standard solutions.

*Preparation of standards*

A 1000 µg $kg^{-1}$ standard solution of each metal studied was prepared by diluting 10,000 µg $kg^{-1}$ certified reference standard solution of lead (Pb), chromium (Cr), nickel (Ni), and cadmium (Cd) standard solutions. A working standard solution was prepared from 100 g $kg^{-1}$ secondary standard for each heavy metal analysis. The concentration of each metal in the honey samples were obtained from the calibration graph for each metal. The calibration curve was prepared with series concentration (0.1, 0.5, 1, 5, 10, 50, and 100 g $kg^{-1}$).

Reference standard solution of 100 g $kg^{-1}$ HMF was prepared in deionized water by dilution from certified reference standard material of 1000 g $kg^{-1}$ HMF. A 100 g $kg^{-1}$ reference standard was used for preparing working standard solutions in the concentration range of 0.1-10 g $kg^{-1}$. The HMF standard solutions were freshly prepared in every analysis.

*Physicochemical analysis methods*

The parameters of honey samples such as metal contents, and HMF content, were analyzed following the standard methods and procedures of European Union Directive (EU), International honey commission (IHC), Ethiopian standards agency standard method and codex alimentarius commission methods [17, 18].

*Honey sample preparation*

The determination of heavy metals from honey samples were analyzed with the standard procedure of AOAC Official Method: 2015.01. A 0.2 g of honey samples (spiked and unspiked sample) were weighed into the digestion vessels. Then, 7.0 ml of concentrated $HNO_3$ and 1.0 mL of ($H_2O_2$, 30%) were added to the spiked and unspiked samples. Samples were digested for 120 min at temperature of 200 $^oC$ in microwave digester. After cooling the entire digested samples were transferred through filtration into acid rinsed 50 mL volumetric flask and diluted to its final volume with deionized water. Then, the concentration of Pb, Cd, Cr, and Ni were determined by ICP-MS instrument. Similarly, blank samples were also prepared and analysed for the calculation of LOD and LOQ. Furthermore, a 10 g of honey samples were also diluted in 50 ml deionized water, filtered through 0.22 µm syringe filter and immediately injected in a HPLC instrument for HMF analysis.

*Instrumentation*

The four elements were simultaneously determined using AOAC 2015.01-ICP-MS method after digested by the Microwave digestion method in a mixture of nitric acid and hydrogen peroxide with the closed-vessel microwave digestion system [19]. The metal analysis was carried out using a PerkinElmer inductively coupled plasma mass spectrometer (NexION1000) method (ICP-MS). UHPLC (Waters, e 2695) equipped with a Photo 2.6.2 Diode Array Detector (Alliance -2998 PDAD) and SB C18 (3 x 250 mm, 5 µm) column was used during analysis of HMF. The UHPLC conditions for separation were the following: isocratic mobile phase, 90% water at 1% of acetic acid and 10% methanol; flow rate, 0.7 mL $min^{-1}$; injection volume, 10 µL. All the solvents were HPLC grade (Sigma-Aldrich, Milan). The wavelength of the chromatograms was monitored at

285 nm. The peak of HMF was identified by comparing the spectrum of HMF standard with that of honey samples. The amount of HMF was determined using an external calibration curve [14, 20].

*Method validation*

The precision was evaluated as the relative standard deviation of 10 repeated determinations for one sample. The accuracy of the analytical quality control was also verified by the recovery experiments for the 4 elements [21, 22]. The numerical values of the calibration curves regression parameters were the basis for estimating the value of the limit of detection and quantification of the analytical method. The limit of detection (LOD) and limit of quantification (LOQ) was calculated from standard deviation (SD) of seven replicate of blank analyses and slope of calibration curve (S) using the following equation [23]:

$$LOD = 3.3*SD/S \quad (1)$$

$$LOQ = 10*SD/S \quad (2)$$

## RESULTS AND DISCUSSION

*Methods validation*

The method used for heavy metals analysis has wide linear ranges (LR) with satisfactory coefficient of determinations ($R^2$) ranging from 0.9998–0.9999. The LOD and LOQ of the method were in the range of 0.0003–0.089 and 0.0009–0.27 µg $kg^{-1}$, respectively. The coefficient of variation (CV %) for all the elements analyzed ranged between 0.0009–2.28%. Percent recovery were also evaluated and found to be in the range of 94–111%. The analytical performance characteristic of method used for analysis of HMF was also evaluated. The experimental values for LOD, LOQ, CVs and recovery were found to be 22 µg $kg^{-1}$, 68 µg $kg^{-1}$, 0.007 and 94.5%, respectively. All validation parameters (Table 1) were in the acceptable ranges indicating the method is efficient for the analysis of target heavy metals and HMF in honey samples [24, 25].

Table 1. LOD, LOQ, precision and recovery of heavy metals.

| Analyte | LR (µg $kg^{-1}$) | $R^2$ | LOD (µg $kg^{-1}$) | LOQ (µg $kg^{-1}$) | Precision (CV %) | Recovery (%) |
|---|---|---|---|---|---|---|
| Pb | 0.1-100 | 0.9998 | 0.002 | 0.006 | 2.28 | 100 |
| Cd | 0.1-100 | 0.9998 | 0.0003 | 0.001 | 0.001 | 94 |
| Cr | 0.1-100 | 0.9999 | 0.089 | 0.27 | 0.006 | 111 |
| Ni | 0.1-100 | 0.9999 | 0.029 | 0.089 | 0.007 | 105 |
| HMF | 100-10000 | 0.9997 | 22 | 68 | 0.001 | 94.5 |

Peak identification was done by comparing the chromatogram of extract of freshly harvested honey and HMF standard. As can be seen from Figure 3, the retention time is almost the same.

*Concentration of heavy metals in honey sample*

The ICP-MS (NexION 1000 STD*)* system was calibrated by the method of external standards [19, 23]. The reagent blank solution was spiked with Pb, Cr, Ni and Cd standard solutions. The background interferences from the plasma gases, air entrainment and solvent were corrected by subtraction of reagent blank signals. Good accuracy was assured by the analysis of standard reference materials and % of recovery was calculated. The metal concentrations vary among different places due to a number of factors, according to a research that examined heavy metals determination in honey using an inductively coupled plasma optical emission spectrometer (ICP-OES) [26].

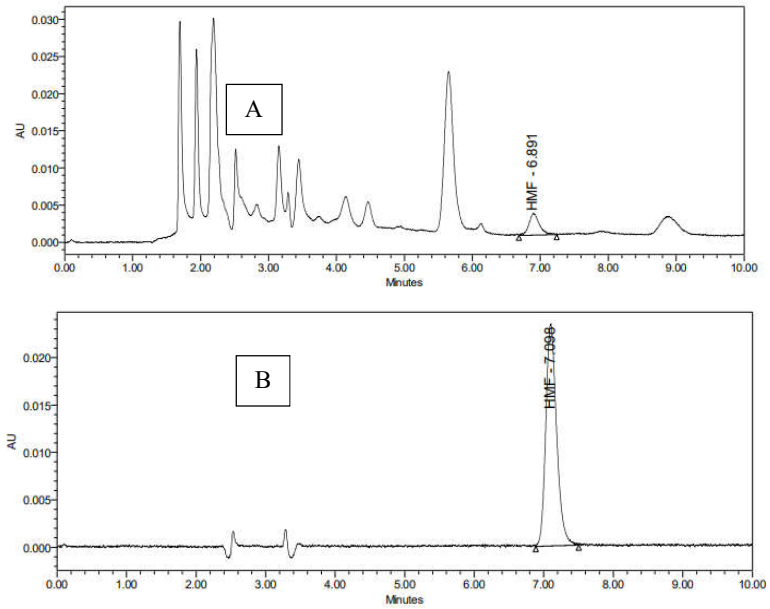


Figure 3. Chromatogram of extract of freshly harvested honey (A) and HMF standard (B).

Table 2. Mean concentration of heavy metals (n = 3) in freshly harvested honey determined by inductively coupled plasma mass spectrometer (ICP-MS).

| Metal | Concentration (µg $kg^{-1}$), Mean ± SD | Ethiopian standard (µg $kg^{-1}$) | General Codex standard (µg $kg^{-1}$) |
|---|---|---|---|
| Pb | 11 ± 0.001 | 50 | 50 |
| Cr | 454 ± 0.002 | - | 300 |
| Ni | 630 ± 0.001 | - | 500 |
| Cd | 1 ± 0.001 | 50 | 50 |

The concentration of target heavy metals in freshly harvested honey were determined by inductively coupled plasma mass spectrometer (ICP-MS). As indicated in Table 2 above, the mean concentration of Pb, Cr, Ni and Cd, in freshly harvested honey was 11 ± 0.001, 454 ± 0.002, 630 ± 0.001 and 1 ± 0.001 µg $kg^{-1}$, respectively. The mean concentration of lead and cadmium in freshly harvested honey were below the maximum residual limit, 50 µg $kg^{-1}$, set by Ethiopian standard and general codex standards [27]. Even though, there is no maximum residual limit set by Ethiopian standard and general codex standards the mean concentration of chromium and nickel in honey were remarkable. The source of metals in honey sample is linked to factors such as application of fertilizers for agricultural practices, nature of soil and environment [28] in the area or the growing plant diversity.

*The effect of heavy metals and storage time on formation of HMF in honey sample*

The effect of heavy metals (Pb, Ni, Cr and Cd) on formation of HMF in honey samples was studied and the results are presented in Table 3. The initial concentration of HMF in freshly harvested honey sample was determined prior to addition of target heavy metals and it was found to be 1380 µg $kg^{-1}$. The concentration of HMF for untreated honey samples was found to be in

the range of 1380-2200 μg $kg^{-1}$ for the storage time of 5, 30 and 60 days. The concentration of HMF in honey samples spiked with Pb, Ni, Cr and Cd were ranged 2100-2150, 1900-2430, 2100-2270 and 2050-2330 μg $kg^{-1}$, respectively.

Although, the concentration of HMF in freshly harvested and spiked honey were below the allowed limit (40,000 μg $kg^{-1}$) [15], the concentration of HMF in honey samples increases with storage and presence of heavy metals. Ni is the major influencing factor for the formations of HMF for prolonged storage of honey sample (Table 3). This may be due to the higher catalytic reaction rate and stronger interaction between fructose and metal cations due to the surface charge increases and the ionic radius decreases [29]. HMF formation is correlated with chemical characteristics such as pH, free acid content, total acidity, lactone content and mineral content, which in turn are related to the floral source of collected honey samples [4]. The prolonged storage conditions can be also the major factor for the HMF production in honey samples [30].

Table 3. Determination of HMF from fresh and contaminated honey samples stored at room temperature after 5, 30 and 60 days.

| Added metal ion concentration | Concentration of HMF in honey μg $kg^{-1}$ (Mean ± SD | | | |
|---|---|---|---|---|
| | 0 Day | 5 Days | 30 Days | 60 Days |
| Unspiked honey | 1380 ± 9.3 | 1800 ± 8.7 | 2050 ± 7.6 | 2200 ± 6.2 |
| Lead (Pb), 10,000 μg $kg^{-1}$ | 1380 ± 9.3 | 2100 ± 9.5 | 2150 ± 8.3 | 2400 ± 7.1 |
| Nickel (Ni), 10,000 μg $kg^{-1}$ | 1380 ± 9.3 | 1900 ± 8.5 | 2100 ± 6.8 | 2430 ± 5.3 |
| Chromium (Cr), 10,000 μg $kg^{-1}$ | 1380 ± 9.3 | 2100 ± 6.2 | 2150 ± 4.8 | 2270 ± 5.7 |
| Cadmium (Cd), 10,000 μg $kg^{-1}$ | 1380 ± 9.3 | 2050 ± 7.4 | 2200 ± 8.6 | 2330 ± 4.9 |

In addition, the concentrations of metallic ions present in honey have also been reported to have a positive effect on HMF formation [31]. There is statistically significance differences between the concentrations of HMF at 95% confidence level ($p < 0.05$) with respect to storage time. The concentration of HMF in unspiked and spiked honey sample is stastitistically different at 95% confidence level ($p < 0.05$).

*Comparison of the results with reported literature*

HMF concentration is one of potential parameter affecting honey freshness or quality. Table 4 shows its concentration as a function of storage time of honey from different geographical locations. The concentration of HMF in real honey samples in this study is less than concentration of honey from Bangladesh [32], Turkey [33] and Kenya [34]. The concentration of spiked honey with metal is comparable with honey from Bangladesh [32], Kenya [34] and Argentinean Patagonia [35] and Ethiopia [36].

Table 4. Variation in HMF concentration in honey samples with storage time and geographical locations.

| Country of origin | Storage time | Storage temperature (°C) | Concentration of HMF (μg $kg^{-1}$) | References |
|---|---|---|---|---|
| Bangladesh | > 1.5 years | 20 - 25 | 3180–703100 | [32] |
| Turkey | 1 year | 20 ± 5 | 8600–39000 | [33] |
| Kenya | < 1 year | 25 ± 2 | 3070–389036 | [34] |
| Argentinean Patagonia | > 3 years | - | 0.0–14070 | [35] |
| Ethiopia | < 6 months | - | 680–6560 | [36] |
| Ethiopia | 0 - two months (unspiked with metals) | 22 ± 3 | 1380-2200 | This study |
| | 0 - two months (spiked with metals) | 22 ± 3 | 1900-2430 | This study |

## CONCLUSION

The natural concentration of heavy metals (Pb, Ni, Cr and Cd) in honey samples was determined and their levels of concentrations were comparable with the international standards. From the metals analyzed the most concentrated was nickel, followed by chromium and lead, while cadmium was in low concentration. All metal ions studied enhanced the rate of production of HMF by catalyzing the formation of HMF from simple sugars in honey. The metal ions added to the honey performed the catalytic effects due to their positive charge which can coordinate to several donor atoms. The catalytic effect of heavy metals in current study is time dependent and increases with time. The additions of Pb, Ni, Cr and Cd heavy metal ions in honey have more influence than the fresh honey stored in these storage time. Therefore, it is very crucial to control any source of heavy metals during honey processing and storage.

## ACKNOWLEDGMENTS

Kotebe University of Education, Department of Chemistry is acknowledged for the laboratory facilities and consumables provided. Mr. Tsedeke Birhanu extended his acknowledgment to Ethiopian Conformity Assessment Enterprise for the sponsorship.

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