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Research Article

Rapid Method for the Assessment of Hydroxymethylfurfural (HMF) Levels in Honey by Absorption Spectroscopy

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Abstract: Honey is a complex mixture of compounds and a high added value food with diverse health benefits. The study focuses on Hydroxymethylfurfural (HMF), a compound that can form in honey over time due to various factors such as exposure to heat exposure, storage conditions, and aging. Elevated HMF levels in honey indicate overheating or poor storage. This study presents a rapid classification method for honey samples based on HMF levels using Ultraviolet (UV) absorption spectroscopy and Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA). The study aims to establish a concentration threshold of 15 mg/kg for HMF level, providing a basis for effective classification. Results achieved excellent accuracy in categorizing samples above and below this threshold. This innovative technique, requiring no sample pretreatment, offers a rapid and cost-effective analysis for assessing HMF levels, contributing to quality and safety considerations.

***Keywords*:** honey, HMF, absorption spectroscopy, optical spectroscopy, multivariate statistical analysis

1. Introduction

Honey is the natural sweet substance produced by Apis mellifera bees [1]. Honey is a complex mixture with a wide range of compounds, primarily carbohydrates, with glucose and fructose at approximately 80%. Another major component of honey is water, which is between 13% and 23% of its composition [2, 3]. In addition to the major components, honey consists of various minor substances such as proteins, amino acids, phenolic compounds, minerals, and trace elements [2, 4]. This complex mixture of substances contributes to honey's high nutritional and therapeutic properties with several health benefits [5].

Hydroxymethylfurfural (HMF) (Figure 1) is a compound that occurs in honey as a result of the Maillard reaction [6]. HMF is a heat-induced degradation product, and several factors, such as temperature and time, can influence its formation. Its content in HMF can be used as a quality term for honey as it can appeal to the storage conditions of the honey sample [6, 7]. The Codex Alimentarius of the World Health Organization and the European Union have established a maximum quality level regarding the HMF content in honey, with a maximum limit of 40 mg/kg [1]. HMF is not considered highly toxic, but the maximum limits in honey ensure it meets quality and safety standards. Otherwise,

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the high levels of HMF in honey indicate the degradation of the quality of honey with possible health risks. The analysis method is described in Codex Alimentarius, Association of Official Analytical Chemists (AOAC) [8-10], and requires chemical solvents.

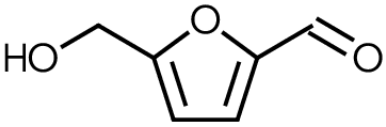

Figure 1. Chemical structure of hydroxymethylfurfural (HMF)

To ensure the safety and quality of honey, it is crucial to monitor the HMF levels with a rapid, easily adaptive method with the capability of on-site application. Optical spectroscopy techniques provide an alternative analytical approach to food analysis [11, 12]. Techniques such as Raman and Fourier Transform Infrared (FT-IR) have been applied to honey products [13-16] for different purposes, such as botanical origin identification and other agricultural products [17-19]. In addition, several reports refer to the application of fluorescence spectroscopy in honey [20, 21] and other food products (e.g. olive oil) [17, 22] and have capabilities for on-site analysis [23]. Among the optical spectroscopic techniques, absorption spectroscopy can also, serve as an appropriate method for the analysis of food products [24] and as well as for honey, either after the sample pretreatment [25, 26] or with a direct application without the sample pretreatment, making it a versatile option for field studies [14].

This study aims to present a rapid method for classifying honey samples based on their HMF content, using Ultraviolet (UV) absorption spectroscopy and statistical analysis. The primary objective is to introduce a practical approach that facilitates a quick determination of HMF levels in honey. The proposed method extends the application of UV absorption spectroscopy in honey, building upon previous work focused on botanical origin determination [14]. By demonstrating the broader potential of rapid food analysis employing UV absorption spectroscopy, this study contributes to the evolving landscape of analytical techniques in the field.

2. Materials and methods

2.1 *Honey samples*

The study was based on the analysis of 149 honey samples collected in 2020 and 2021 belonging to different botanical origins (multi-floral: 55 samples, thyme: 44 samples, and honeydew: 50 samples). The samples were provided and characterized by Chemicotechniki Lab (Rethymno, Greece) in terms of pollen analysis and their physicochemical parameters. The HMF content determination was based on the official AOAC method [8]. The samples were kept in a dark place and at room temperature until their analysis was performed.

2.2 *UV absorption spectroscopy*

UV absorption spectra were recorded using UV-3600i Plus UV-VIS-NIR Spectrophotometer. The recorded spectra were obtained in the range of 200-400 nm. The analysis procedure was according to previous related reported literature [14]. Specifically, a quartz cuvette was constructed for the absorption spectra at this region, as represented in Figure 2. The path length of the cuvette was approximately 0.5 mm, and the analysis required only a drop of the honey sample without pretreatment. All measurements were performed in triplicate.

2.3 *Statistical analysis*

Classification of honey samples with different HMF levels based on absorption spectra was accomplished with multivariate statistical analysis using MATLAB R2011a (MathWorks, MA, USA) with the PLS_Toolbox 8.1

(Eigenvector Research, Manson, WA, USA). Specifically, the employed method was Orthogonal Partial Least Squares regression-Discriminant Analysis (OPLS-DA). Partial Least Squares regression-Discriminant Analysis (PLS-DA) is a supervised statistical method aiming to reduce a large number of initial independent variables (wavelengths) to a smaller number of latent variables. This study has used an extension of PLS-DA, namely OPLS-DA, which incorporates an orthogonal signal correction step and displays the same prediction ability but an improved visualization of model results. The results herein are presented as confusion matrices, score plots, and loading plots. The preprocessing of the UV spectra before statistical analysis was the 1st derivative and mean center.

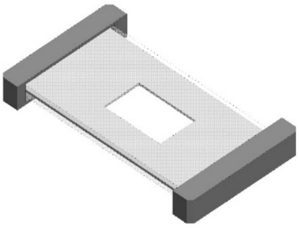

Figure 2. Schematic representation of the custom-made quartz cuvette used

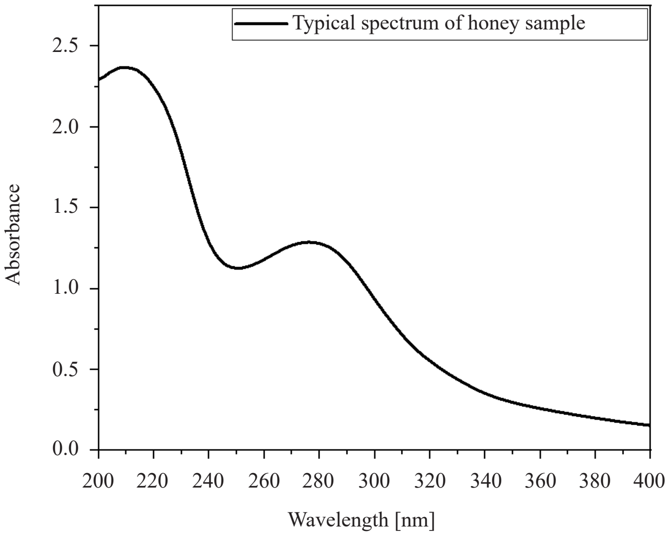

Figure 3. A typical ultraviolet absorption spectrum of honey in the region of 200-400 nm

3. Results and discussion

The absorption spectra of honey samples in the UV region were recorded using the custom-made cuvette, which was necessary for overcoming the high absorption of honey. Figure 3 displays a typical honey spectrum in this region. The UV spectra of honey consist mainly of two bands (located at 210 nm and 275 nm) corresponding to various compounds in honey, such as phenolics and sugars [14]. In addition, HMF depicts a maximum of its absorption in the UV region [27]. Precisely, as shown in the literature where HMF has been extracted and studied by UV absorption spectroscopy, the maximum absorption of HMF is at 284 nm. The advantage of this study arises from the direct analysis of honey samples by UV absorption spectroscopy. The HMF content has not been extracted from the honey samples, so the absorption related to HMF overlaps with the other compounds in honey. This is due to several compounds that absorb in the UV region. The UV spectrum in Figure 3 reflects the chemical fingerprint of honey samples as several compounds (including HMF) absorb at this spectral region.

As mentioned, the maximum absorption of HMF is located at 284 nm, but the corresponding absorption is a broad band located from 250 nm to 320 nm. To enhance the results and provide information from the spectra relevant to the analysis for the discrimination of honey samples based on their HMF content, the spectral region used was restricted from 230 nm to 340 nm. The absorption spectroscopic data from this region have been processed with an OPLS-DA statistical model. Several factors were considered for the selected 15 mg/kg HMF content threshold. The official regulatory limit for HMF level in honey is 40 mg/kg, which is crucial for consumer safety and product quality and was first and foremost acknowledged. In addition, an essential consideration for setting the threshold at 15 mg/kg was that 80% of the samples (120 out of 149) showed HMF levels below 15 mg/kg, while 29 honey samples were above this cutoff. This distribution reinforced the selected threshold, making it a valuable classification parameter.

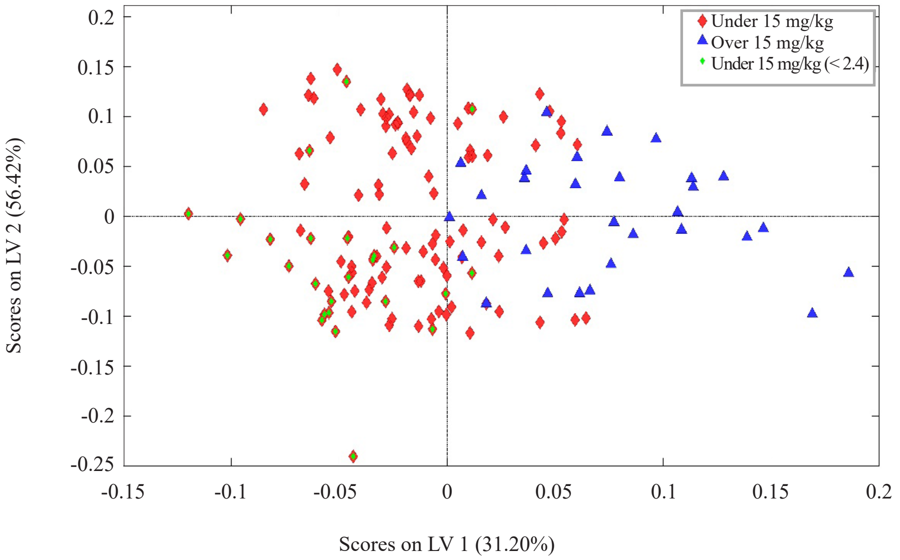

Figure 4. Score plot of first (LV 1) and second (LV 2) latent variables from the OPLS-DA model using ultraviolet (UV) absorption spectroscopic data from 230 to 340 nm for the discrimination of honey samples based on their HMF content. Samples with HMF content over 15 mg/kg (n = 29) are represented by blue triangles, samples with HMF content under 15 mg/kg (n = 95) are represented by red rhombuses and samples with HMF content less than 2.4 mg/kg (n = 25) by green rhombuses with red outline

The application of OPLS-DA on the relevant absorption spectroscopic data brought on a clear separation of the honey samples that exhibit over 15 mg/kg HMF content from the samples with under 15 mg/kg HMF content. In the score plot of Figure 4, the discrimination is accomplished based on the first latent variable (LV 1), as was expected because the OPLS-DA model classification helps to enhance the interpretability of the model by removing unwanted variation and isolating the variation related to the class separation on the first latent variable. It is worth mentioning that, within the group with low HMF levels (under 15 mg/kg), a subcategory of the samples with lower HMF levels (below 2.4 mg/kg) is detected based on the first latent variable. These samples are marked with green color among the other group samples in Figure 4. Table 1 describes the OPLS-DA model performance constructed from the specific absorption spectroscopic data. As displayed in the table, the model successfully classified samples and achieved 82.7% correct classification of samples with HMF content over 15 mg/kg and 87.5% correct classification of the samples with HMF content under 15 mg/kg. Figure 5 displays the corresponding loading plot of the first latent variable (LV 1), which indicates the significant variables (wavelength) for the accomplished discrimination. From the inspection of the loading plot of the first latent variable, the influence of the HMF content on the discrimination of honey samples is observed. The loading plot reveals the regions of HMF absorbance as the corresponding variables for discrimination. Specifically, the two prominent points of positive and negative maximum at 270 nm and 304 nm, with the zero point at 286 nm, and noting that the first derivative has been applied on provided data, indicated the compounds absorbing at around 286 nm to be dominant for the discrimination, which corresponds to HMF as occurs from the literature.

Table 1. Orthogonal partial least squares discriminant analysis classification of honey samples into samples that contained over 15 mg/kg and under 15 mg/kg HMF based on ultraviolet absorption spectroscopic data

		Predicted label	
		Under 15 mg/kg	Over 15 mg/kg
True label	Under 15 mg/kg	**105**	15
	Over 15 mg/kg	5	**24**

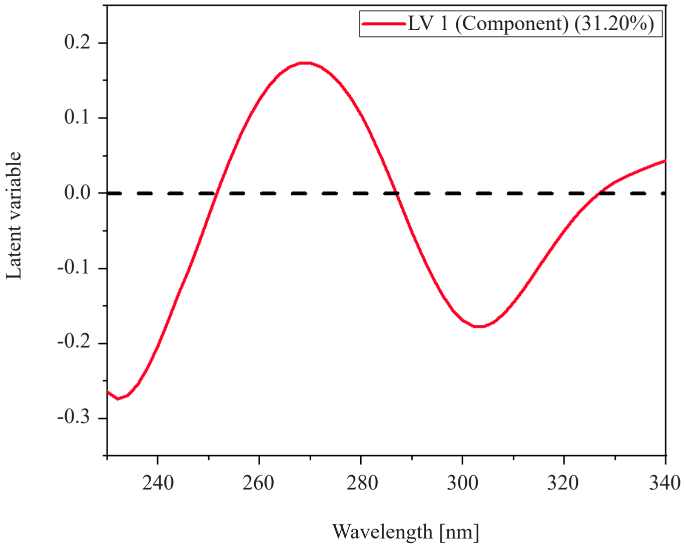

Figure 5. Loading plot of LV 1 for the discrimination of honey samples with HMF content over 15 mg/kg and under 15 mg/kg HMF level from OPLS-DA model based on UV absorption spectroscopic data from 230 nm to 340 nm

From a comprehensive literature review [28] on HMF concentrations in honey from various geographical sources and storage conditions, our study's findings align with established global trends. The variation in HMF levels in honey is well-documented, with factors such as storage time, temperature, and geographical origin influencing the HMF concentration. Categorizing honey samples based on HMF content above and below 15 mg/kg is consistent with the broader spectrum observed in countries like Italy, Turkey, and India, where fresh honey exhibits HMF concentrations ranging from 1.23 to 5.95 mg/kg, 0.00 to 11.50 mg/kg and 0.15 to 1.70 mg/kg, respectively [28]. While all the mentioned concentration ranges for fresh honey from various countries fall below the 15 mg/kg threshold, it is noteworthy that with prolonged storage time, the reported levels of HMF exhibit a notable increase, surpassing the 15 mg/kg limit. For instance, honey from Argentina, stored within two months, shows HMF concentrations ranging from 1.48 to 34.08 mg/kg. Similarly, honey from Turkey, stored for over six months, demonstrates concentrations between 19.20 and 28.6 mg/kg [28]. Furthermore, when considering the different storage temperatures, it becomes evident that significant increases in HMF levels occur, presenting a stark contrast to the concentrations found in fresh honey. For instance, honey from Turkey, stored within one year in storage temperatures at 20 °C ± 2, shows HMF concentrations ranging from 8.60 to 39.00 mg/kg [28]. These findings underscore the impact of storage duration and conditions on HMF levels, emphasizing the need for vigilant monitoring and rapid categorization methods, such as the one proposed in our study, to assess and ensure honey quality.

4. Conclusions

In this work, honey samples were analyzed by UV absorption spectroscopy, and they were classified using OPLS-DA statistical analysis in terms of exceeding a specific concentration threshold for the HMF compound that we set at 15 mg/kg. The performance of the statistical model showed remarkable results in sorting honey samples based on their HMF content with correct classification of 82.7% for the samples that exceed 15 mg/kg and 87.5% for the samples below 15 mg/kg HMF. It is worth mentioning that the analysis of honey samples by UV absorption spectroscopy was accomplished without sample pretreatment. Our findings demonstrate the successful discrimination among honey samples, even though the exhibited HMF concentrations were below the established regulatory limit of 40 mg/kg.

The results showed that UV absorption spectroscopy can rapidly detect honey samples with elevated HMF levels due to the elimination of sample pretreatment and the availability of the technique for on-site analysis. These results complement previously reported literature where absorption spectroscopy was used for the botanical origin identification of honey samples and enhanced its application as an alternative and cost-effective analysis. This technique can serve as an alternative valuable tool for the quality control of honey, ensuring consumers' safety and the compliance of honey with regulations.

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Conflict of interest

The authors declare no conflict of interest.

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