

Analysis of Hydroxymethylfurfural in Honey-Flavored Syrups by HPLC

Ünal Savsar ^{1,a}, Bilal Yılmaz ^{1,b,*}¹ Department of Analytical Chemistry, Faculty of Pharmacy, Ataturk University, Erzurum, Türkiye.

*Corresponding author e-mail address: yilmazb@atauni.edu.tr

Research Article

History

Received: 15.12.2025

Accepted: 12.04.2026

ABSTRACT

The aim of this study was to develop and validate a rapid, simple and reliable high-performance liquid chromatography (HPLC) method for the accurate determination of hydroxymethylfurfural (HMF) in commercially available honey-flavored syrups. Analyses were performed using a reverse-phase HPLC system equipped with a 250 × 4.6 mm, 5 µm C₁₈ column and a UV detector set at 284 nm. The mobile phase consisted of methanol and acetonitrile (10:90, v/v), delivered at a flow rate of 1.0 mL min⁻¹. Under these conditions, HMF was quantified in less than 6 minutes, demonstrating the method's efficiency and suitability for routine analysis. Method validation followed International Council for Harmonisation guidelines. The calibration curve showed linearity across 0.10-100 µg mL⁻¹ with a correlation coefficient above 0.99. Precision values for both intra-day and inter-day measurements were below 2.37%, while accuracy showed relative error values under 0.87%. Recovery tests, conducted using the standard addition method on three honey-flavored syrups (GrinTuss, Biakaf, Bisolnatur), confirmed the method's robustness. Overall, the developed HPLC procedure provides a straightforward, sensitive and highly reproducible approach for determining HMF levels, supporting quality control and safety assessment of honey-flavored pharmaceutical syrups. This ensures its applicability for routine monitoring in diverse quality control settings today.

Keywords: Honey-Flavored Syrup, HPLC, Hydroxymethylfurfural, Validation

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a  0009-0008-1972-3360b  0000-0002-8574-7570

1. Introduction

Hydroxymethylfurfural (HMF) (Figure 1) is a compound formed through the dehydration of hexoses under acidic conditions or via the Maillard reaction, a non enzymatic browning process [1]. Its presence is closely linked to the freshness and quality of carbohydrate rich foods and serves as an important indicator in evaluating honey quality [2-4].

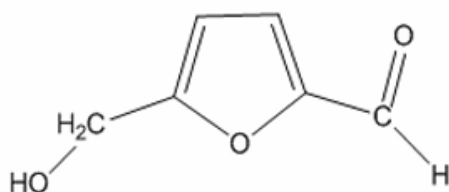


Figure 1. Chemical structure of hydroxymethylfurfural

Acid catalyzed reactions at elevated temperatures cause the dehydration of monosaccharides producing furfural from pentoses and HMF from hexoses [5]. Keto pentoses and hexoses are particularly susceptible to this process [6]. Reaction rates are influenced by factors such as pH water activity reducing sugar and amino acid content and temperature with studies reporting a fourfold increase for every 10°C rise [7]. Honey's chemical composition including sugar content pH total acidity and

mineral content along with processing and storage conditions strongly affects HMF formation [8]. Heat treatments improve honey stability by preventing fermentation and crystallization but prolonged high temperature exposure markedly increases HMF levels. Improper storage further accelerates HMF accumulation reducing shelf life [9,10]. Regulatory limits exist for HMF content with the Turkish Food Codex setting a maximum of 40 mg/kg for blossom and honeydew honeys [11].

Thermal processing extends the shelf life of many foods but also promotes HMF formation. Appropriate heat treatments improve sensory qualities, whereas excessive heating or prolonged treatments accelerate HMF production and degrade nutritional value [12]. HMF is widely accepted as a chemical indicator to monitor food processing and storage conditions [13]. Studies on commercial jams, baby foods, and molasses have shown that HMF levels increase proportionally with storage time and temperature, confirming its reliability as a quality marker [14]. Analyses of various food products, including dried foods, fruit juices, honey, cereals, vinegar, and halva, have reported HMF concentrations ranging from 0 to 3500 ppm [15]. Rapid cooling after pasteurization has been shown to reduce HMF formation in fruit juices, with uncooled samples exhibiting 4.2 to 21.4 percent higher HMF levels [16]. Many heat processed sugar rich foods, including dried fruits, fruit juices, marmalades, jams,

molasses, tomato paste, vinegar, biscuits, and caramelized products, contain significant amounts of HMF. Elevated HMF can negatively affect food quality by causing browning, altering taste and aroma, and reducing nutritional value. Additionally, high HMF levels raise health concerns, prompting the establishment of regulatory limits for some food products [17].

The Turkish Food Codex establishes specific maximum limits for HMF content in various food products. According to these regulations, the maximum allowable concentration of HMF in honey is 40 milligrams per kilogram (2012/58). For molasses, the limits differ depending on its physical state: liquid molasses is permitted to contain up to 75 milligrams per kilogram, while solid molasses can contain up to 100 milligrams per kilogram (2007/27) [11]. Although HMF formation is a natural consequence of thermal processing, it is particularly observed in heat treated products such as milk, dairy based beverages, fruit flavored powders, and whey. Despite the potential for HMF accumulation in these products, monitoring and control measures are often insufficient, which can result in elevated HMF levels if appropriate processing and storage practices are not followed [18].

The potential health risks of HMF and related furan compounds have been well documented [19]. Formed from pentoses and hexoses under acidic conditions, HMF exhibits mutagenic activity and can irritate the respiratory tract eyes skin and mucous membranes [20]. High dietary intake may cause genetic damage, and studies indicate that HMF activated by sulfotransferases can harm DNA [21,22]. Animal experiments have shown cytotoxic effects at high doses, with an oral lethal dose in mice reported as 3.1 grams per kilogram body weight [20]. Given the carcinogenic potential of such compounds and the rising global cancer incidence, controlling HMF formation during food processing is essential, with legal limits and strict monitoring necessary to ensure safe and high quality products. Honey, a nutrient rich natural sweetener valued for its safety and health benefits, requires careful production storage and quality assessment, especially for vulnerable populations such as children the elderly and patients [21,22].

Today, HMF levels are widely regarded as indicators of honey exposure to high temperatures or improper storage. Historically, HMF was used to detect adulteration of honey with glucose and fructose syrups. Monitoring chemical quality indicators such as HMF is particularly important for sensitive populations [23-25]. HMF analysis is used as a chemical marker to assess whether foods such as fruit juices, milk, honey, molasses, cereals, and jams have been properly processed and stored (3,4,15,16).

However, in Türkiye, no HPLC-based studies have reported HMF levels in commercially available honey-flavored syrups, emphasizing the novelty and significance of this research. Accordingly, the present study aims to develop a simple, reliable, and rapid HPLC method for

determining HMF concentrations in honey-flavored syrups sold in pharmacies.

2. Materials and Methods

2.1. Chemicals

5-Hydroxymethylfurfural ($\geq 99\%$), methanol and acetonitrile were provided Sigma-Aldrich (St. Louis, MO, USA). The pharmacy in Erzurum, Türkiye, supplied the HMF-containing GrinTuss, Biakaf and Bisolnatur medicines. All of the chemicals were analytically pure.

2.2. HPLC System and Chromatographic Conditions

Agilent HPLC equipment from the 1260 series was used for the method development and validation investigations. This chromatographic system was equipped with an auto injector (G7129A), quaternary pump (G7111A), and UV detector (G71144A). The separations were performed at 25 °C using the Ace C₁₈ (250×4.60 mm ID, 5µm) analytical column. Methanol-acetonitrile (10:90, v/v) was used as the mobile phase in the process. The method's UV detection and mobile phase flow rate were 284 nm and 1.0 mL min⁻¹, respectively.

2.3. Preparation of Standard and Quality Control Solutions

A stock solution of HMF was prepared in methanol at a concentration of 100 µg mL⁻¹ and stored at -20 °C. This stock solution was then diluted with methanol to obtain standard solutions of HMF ranging from 0.10 to 100 µg mL⁻¹. In addition, quality control (QC) samples were prepared at concentrations of 2.50, 37.5 and 75 µg mL⁻¹.

2.4. Procedure for Pharmaceutical Preparations

Three commercially available honey-flavored syrup products (GrinTuss, Biakaf, and Bisolnatur) were first homogenized and finely ground, and a portion of the resulting powder was accurately weighed. The powder was then diluted with the appropriate volume of methanol in a 100 mL amber volumetric flask. After sonication for at least fifteen minutes to facilitate dissolution, the mixture was filtered through Whatman No. 42 filter paper. To ensure that the final HMF concentration of the solution (50 µg mL⁻¹) fell within the working range of 0.10-100 µg mL⁻¹, an appropriate volume of the filtrate was further diluted with methanol before analysis.

3. Results and Discussion

3.1. Development and Optimization of the Method

The significance of the HPLC method for drug analysis in regular quality control has received a lot of attention lately. A suitable method for figuring out HMF in pharmaceutical dose forms was suggested. For the experiment to be successful, the chromatographic conditions were improved. The suitability of the mobile phase used was evaluated in terms of optimization. Parameters such as pump pressure, HMF separation, peak

shape, retention time, capacity factor, and symmetry ratio were optimized, and it was determined that the most appropriate mobile phase composition was a methanol-acetonitrile mixture (10:90, v/v). Therefore, the study was conducted using this mobile phase.

It was found that the retention time was 5.85 minutes. The assay took about ten minutes to complete. Following multiple trials using various solvent mixes, the mobile

phase was selected. Peak characteristics (symmetry, tailing), run duration, cost, and ease of preparation were all carefully taken into account while choosing the mobile phase. Figure 2 displays a representative chromatogram that was produced by using the suggested method to analyze a typical HMF sample. The medicine may be quickly determined thanks to the retention time measurements.

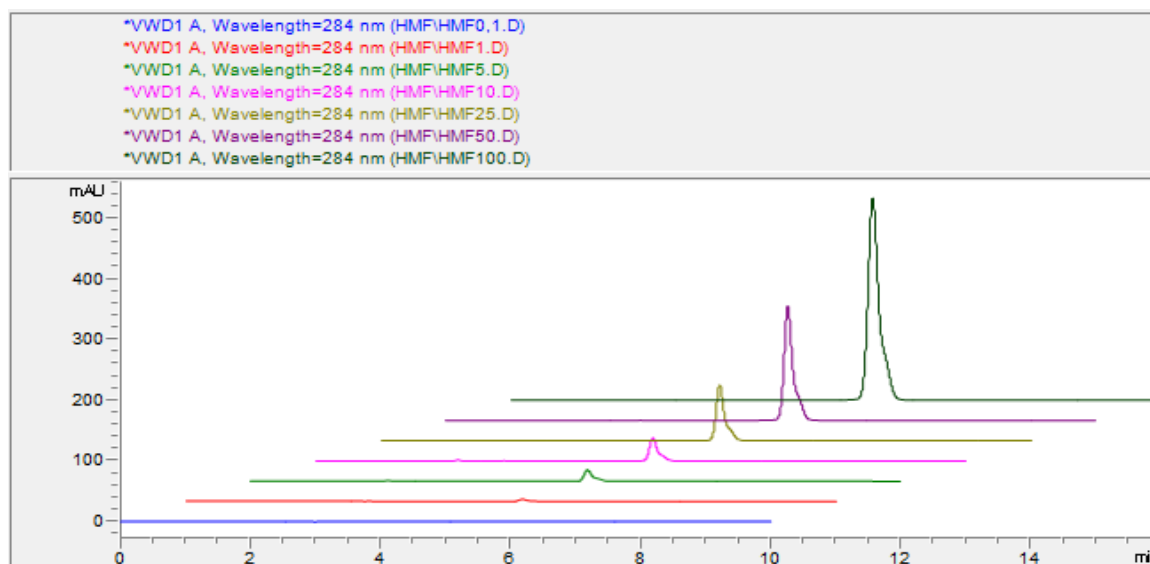


Figure 2. The chromatograms of HMF (0.10, 1.0, 5.0, 10, 25, 50 and 100 $\mu\text{g mL}^{-1}$, Rt:5.85 min)

3.2. Validation of the Method

The purpose of method validation is to confirm that the analytical procedure is appropriate for its intended application in accordance with ICH guidelines. Validation of the method included assessments of linearity, accuracy, precision, limits of detection and quantification, recovery, stability, selectivity and system suitability [26,27].

3.2.1. Linearity

If an analytical technique yields test results that are exactly proportional to the analyte concentration in the sample within a given range, either directly or via a clearly stated mathematical transformation, it is said to be linear. First, a plot of signal versus analyte concentration can be used to visually evaluate this. The test results should be verified using the proper statistical techniques if the association seems linear (e.g., by generating a regression line using the least squares approach). In certain situations, achieving linearity between the analyte's reaction and its concentration may necessitate a mathematical modification of the test results.

The data obtained from the regression line itself can be useful for analytically evaluating the degree of linearity. The regression line's slope, residual sum of squares, y-intercept, and correlation coefficient must all be reported. Plotting the HMF concentration on the X-axis and the HMF peak area on the Y-axis produced the standard curve (Figure 3).

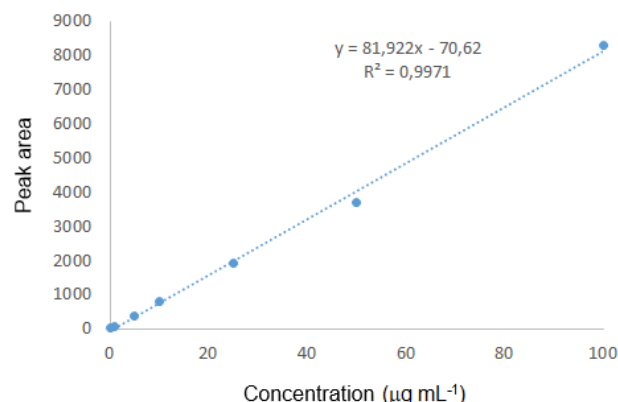


Figure 3. The standard curve of HMF (0.1, 1.0, 5.0, 10, 25, 50 and 100 $\mu\text{g mL}^{-1}$)

Using the least squares regression approach to construct the linear regression analysis, the linearity was assessed. The regression equation was computed from the calibration graphs (Table 1).

Table 1. Linearity of HMF (n=6)

Range ($\mu\text{g mL}^{-1}$)	Linear Regression	R ²
0.10-100	$y = 81.922 \times -70.62$	0.9971

3.2.2. Accuracy and precision

The degree to which the procedure's test findings closely match the real value is known as accuracy. It is commonly expressed as the percentage recovery when known amounts of analyte are added to the experiment. The analytical process's precision is measured by

accuracy. The variances of the acquired findings were computed using the true values, and the results were then reported as percentage accuracy.

Precision refers to the consistency of an analytical procedure when applied repeatedly to multiple aliquots of a homogeneous sample. It is commonly expressed using the standard deviation (SD) or relative standard deviation (RSD) of a set of measurements. Precision reflects the

method's repeatability or its reproducibility under normal operating conditions. The accuracy of the assay, considering both intra-day and inter-day variations, was evaluated by analyzing the QC samples six times. As summarized in Table 2, the precision of the QC samples ranged from 0.78% to 2.37%, while the accuracy values fell between 0.40% and 0.87%.

Table 2. Precision and Accuracy of HMF

Added ($\mu\text{g mL}^{-1}$)	Found \pm SD	Intra-day		Found \pm SD	Inter-day	
		Accuracy (relative error %)	Precision RSD%		Accuracy (relative error %)	Precision RSD%
2.50	2.48 \pm 0.020	-0.80	0.81	2.49 \pm 0.031	-0.40	1.24
37.5	37.79 \pm 0.759	0.77	2.00	37.72 \pm 0.894	0.59	2.37
75	75.61 \pm 0.590	0.81	0.78	75.65 \pm 1.709	0.87	1.46

3.2.3. LOD and LOQ

The LOD and LOQ of HMF were determined by analyzing different HMF solutions and assessing the signal-to-noise ratio for each concentration. A signal-to-noise ratio of approximately 3:1 was used to define the LOD, whereas the LOQ was defined as the concentration yielding a signal-to-noise ratio of around 10:1 with a relative standard deviation below 10% based on triplicate measurements. Using the HPLC method, the LOD and LOQ were established as 0.03 $\mu\text{g mL}^{-1}$ and 0.10 $\mu\text{g mL}^{-1}$, respectively.

3.2.4. Recovery

Recovery was evaluated by spiking pre-analyzed tablet samples with known amounts of the pure reference

compound at different concentration levels within the analytical range of the proposed method (e.g., low, medium, and high levels). Each spiked sample was prepared and analyzed in triplicate using the established chromatographic procedure to ensure reliability and reproducibility. The percentage recovery was calculated by comparing the measured concentration with the added amount of the standard. The results demonstrated high accuracy of the method, with recovery values falling within acceptable limits, indicating that common excipients present in the tablet matrix did not interfere with the determination of the analyte. Additionally, the low %RSD values confirmed the precision of the recovery measurements. Overall, these findings verify that the proposed method is accurate, reliable, and suitable for quantitative analysis. The detailed recovery data are summarized in Table 3.

Table 3. Recovery of HMF in Pharmaceutical Preparations (n=6)

Pharmaceutical preparation	Added ($\mu\text{g mL}^{-1}$)	Intra-day			Inter-day		
		Found \pm SD	Recovery%	RSD%	Found \pm SD	Recovery%	RSD%
GrinTuss (10 $\mu\text{g mL}^{-1}$)	15	14.79 \pm 0.231	98.6	1.56	14.81 \pm 0.24	98.7	1.62
	40	40.23 \pm 0.134	100.6	0.33	40.33 \pm 0.152	100.8	0.38
	90	90.49 \pm 0.191	100.5	0.21	91.07 \pm 0.369	101.2	0.41
Biakaf (10 $\mu\text{g mL}^{-1}$)	15	15.19 \pm 0.393	101.3	2.58	14.89 \pm 0.434	98.2	2.91
	40	41.08 \pm 1.070	102.7	2.60	41.07 \pm 1.096	102.7	2.67
	90	90.47 \pm 1.786	100.5	1.97	89.51 \pm 1.786	99.4	1.99
Bisolnatur (10 $\mu\text{g mL}^{-1}$)	15	15.09 \pm 0.306	100.6	2.03	14.91 \pm 0.497	98.5	3.33
	40	40.12 \pm 1.367	100.3	3.40	41.18 \pm 1.402	102.9	3.40
	90	89.74 \pm 1.746	99.7	1.94	90.86 \pm 1.927	100.9	2.12

3.2.5. Stability

Stability studies demonstrated that the samples remained stable for up to 48 hours under all tested storage conditions, including room temperature, refrigeration at 4 $^{\circ}\text{C}$, and freezing at -20 $^{\circ}\text{C}$. Throughout the study period, no significant changes were observed in key analytical parameters such as peak area, retention time, or overall chromatographic profile. The results

indicated that there was no measurable degradation or loss of analyte under these conditions. Furthermore, the consistency of the obtained data confirmed the reliability of the method during short-term storage. Detailed stability study results are presented in Table 4, supporting the conclusion that the samples can be safely stored and handled under the tested conditions without compromising analytical accuracy.

Table 4. Stability of HMF in Solutions

Added ($\mu\text{g mL}^{-1}$)	+25 °C stability (Recovery% \pm RSD%)		+4 °C stability (Recovery% \pm RSD%)		- 20 °C stability (Recovery% \pm RSD%)	
	24 h	48 h	24 h	48 h	24 h	48 h
15	99.6 \pm 2.12	99.5 \pm 2.24	99.7 \pm 2.17	100.2 \pm 2.09	99.8 \pm 2.47	99.4 \pm 2.07
50	100.8 \pm 2.59	99.7 \pm 2.13	100.3 \pm 2.08	100.7 \pm 2.09	100.4 \pm 1.56	99.6 \pm 2.67
80	100.4 \pm 1.29	100.6 \pm 1.95	99.5 \pm 3.05	100.7 \pm 2.72	100.6 \pm 2.74	99.9 \pm 2.79

3.2.6. Selectivity

In this study, the possible interference from common excipients and additives was examined. Control samples were prepared and analyzed to assess their impact. At the concentrations typically present in dosage forms, no interference from these substances was detected. The excipients included are among the most frequently used in the pharmaceutical industry. The method's selectivity was further evaluated by testing common tablet ingredients such as talc, lactose, sodium chloride, titanium dioxide, and magnesium stearate, none of which affected the performance of the proposed method. The results indicate that the procedure can be considered selective.

3.2.7. System suitability

The chromatographic system was put through a system appropriateness test before each validation. As a result, standard solutions with 50 $\mu\text{g mL}^{-1}$ of HMF were

used. For each of the five appropriate injections, the efficiency, tailing factor, and area relative standard deviation were computed. The check standard was calculated using the average of the five appropriate injections. For all sample analysis, the tailing factor was < 1.12, the efficiency was > 3917, and the RSD% was \leq 1.27%.

3.3. Procedure for Pharmaceutical Preparations

For this study, 0.5 grams of samples were weighed from honey-flavored syrups obtained from a pharmacy (GrinTuss, Biakaf, Bisolnatur). The samples were dissolved in 10 mL of methanol and vortexed for 1 minute. After filtration, the sample solutions were analyzed by HPLC, and the peak areas were recorded (Figure 4). The concentrations of HMF in the samples were determined by comparing the chromatogram peak areas with those on the standard calibration curve.

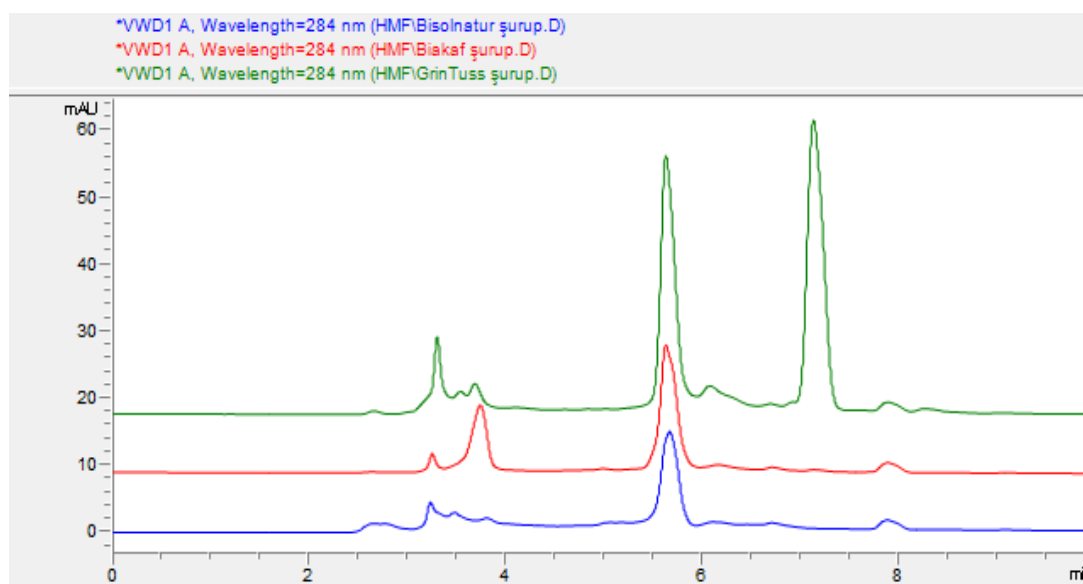


Figure 4. HPLC chromatogram of honey-flavored syrups (GrinTuss, Biakaf, Bisolnatur, 50 $\mu\text{g mL}^{-1}$)

3.4. Comparison of the Methods

The findings of the present study clearly indicate that the proposed HPLC method provides several significant advantages over previously reported analytical procedures for the determination of HMF. As a well established indicator of quality in thermally processed foods particularly honey and honey derived products HMF has become an increasingly important parameter in routine quality control analyses [1-4]. Many conventional HPLC methods described in the literature utilize buffer containing mobile phases or involve complex multi step sample preparation processes which tend to increase

both analysis time and operational costs [12,15]. In contrast the method developed in this study employs a simple buffer free mobile phase consisting of methanol and acetonitrile thereby simplifying the analytical procedure while reducing potential variability associated with pH fluctuations. This streamlined approach enhances both practicality and reproducibility under routine laboratory conditions [10].

From a performance standpoint the reduction in analysis time represents a notable improvement. Earlier HPLC methods generally require between 12 and 20 minutes to achieve complete separation of HMF [6,8].

However in the present study the analyte was eluted at approximately 5.85 minutes allowing the total chromatographic run to be completed in less than 10 minutes. This shorter run time is particularly advantageous for high throughput laboratories and contributes to more efficient analytical workflows.

The method also demonstrates superior linearity compared to previously published studies. While reported calibration ranges are typically limited to 1-50 $\mu\text{g mL}^{-1}$ [14,16] the current method exhibits excellent linearity over a considerably wider range of 0.10–100 $\mu\text{g mL}^{-1}$. This broad calibration interval enables accurate quantification of HMF in both low level and highly concentrated samples without requiring additional dilution steps. In terms of sensitivity previously reported limits of detection and quantification are generally around 0.1 $\mu\text{g mL}^{-1}$ and 0.3–1.0 $\mu\text{g mL}^{-1}$ respectively [9,13]. The lower limit of detection 0.03 $\mu\text{g mL}^{-1}$ and limit of quantification 0.10 $\mu\text{g mL}^{-1}$ achieved in this study indicate improved sensitivity allowing for the detection of trace levels of HMF.

The accuracy and precision of the method were evaluated by assessing both intra-day and inter-day variations. Solutions at three different concentrations within the HMF calibration range were prepared, and chromatograms were recorded on the same day and on different days. The peak areas of each sample were measured in triplicate. The mean values and standard deviations of the obtained results were calculated. Precision was expressed as RSD% and accuracy was expressed as relative error [26,27]. The accuracy obtained here (98.0-102.7%) and the low RSD% values (0.78-2.40%) fall well within, and in several cases exceed, the typical performance criteria cited in the literature [9,13].

Although numerous studies have investigated HMF levels in various food matrices such as honey fruit juices molasses and dairy products [1,3,4,15,16,23] there is a lack of research focusing on pharmaceutical type honey flavored syrups particularly in Türkiye. In this context the present study provides a novel contribution by evaluating HMF levels in commercially available syrup formulations obtained from pharmacies. The analyzed samples prepared at a concentration of 50 mg mL^{-1} exhibited HMF contents ranging from 3.113 to 6.023 $\mu\text{g mL}^{-1}$. The successful application of the proposed method to products such as GrinTuss Biakaf and Bisolnatur further highlights its practical utility and expands current knowledge regarding HMF occurrence in such preparations.

In conclusion the developed HPLC method represents a rapid sensitive and reliable approach for the determination of HMF in honey flavored syrups. Its use of a buffer free mobile phase combined with a short analysis time makes it particularly suitable for routine applications. Moreover the wide linear range low detection limits and high accuracy and precision demonstrate that the method is both efficient and practical when compared to many existing procedures reported in the literature.

Conflict of Interest

There are no conflicts of interest in this work.

Acknowledgments

The research reported in this study was funded by the Atatürk University Scientific Research Projects Coordination Unit under project number TYL-2024-14871.

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