Comparison of Different Methods for Determining 5-hydroxymethylfurfural in Assessing the Quality of Honey from Greater Poland

Joanna Zembrzuska, Łukasz Pakulski, Bożena Karbowska, Jarosław Bartoszewicz, Edyta Janeba-Bartoszewicz, Jarosław Selech, and Przemysław Kurczewski

Abstract: This paper presents the results of testing the content of 5-hydroxymethylfurfural (HMF) in honey from Greater Poland. The production of honey from this area of Poland, its processing, and its consumption are closely related to the sustainable development of this region. It is created in the process of the production, storage, and transport of honey, especially during its thermal processing. Therefore, it can be an indicator in the analysis of sustainable development throughout the life cycle of honey and its products. The aim of this research was to determine the HMF content in Greater Poland’s honey available on the Polish market using three different methods and to compare the obtained results. The methodology used was as follows: two spectrophotometric methods (White and Winkler) and an HMF determination method based on the LC-MS/MS technique developed especially for this study. The determined HMF content in all tested honey samples did not exceed the applicable standard. The determined HMF values in all honey samples ranged from 0 to 28.5 mg HMF/kg honey. All results fell within the scope of European Council Directive 2001/110/EC of 20 December 2001, i.e., they did not exceed the HMF content in honey, which is 40 mg/kg. The results will encourage modifications to the universal honey testing procedures to be developed for the agri-food sector. In the study, seven honeys of different origins and types were selected; three HMF detection methods were selected; a comparative procedure was developed; and the content of the reference ingredient in honey was determined.

Keywords: honey; 5-hydroxymethylfurfural; sustainable honey processing; spectrophotometric methods; LC-MS/MS methods

1. Introduction

One of the goals of sustainable development is responsible consumption and the production of honey and its products. This includes four phases related to the following: honey production, transport, storage, and processing. The quality indicator of honey is often the HMF content in honey, which is determined using various analytical methods. The data analysis in the article covers honey obtained and processed in Greater Poland. Greater Poland is characterized by a strong industrial sector and agriculture based on crops grown on soils requiring high fertilization. Honey coming from inflorescences found in Greater Poland therefore has its own individual characteristics compared to other honeys from...
Poland. It is therefore important to develop research standards that ensure sustainability in the honey processing sector.

Honey is a natural product with high economic, ecological, and social value. There are several aspects that can influence sustainability in the context of honey production and processing. All four aspects of sustainable development are of equal importance. One of many aspects is how to reliably assess honey contamination. The presented research intends to increase the state of knowledge in this area by comparing research methods and indicating the optimal scope of their use.

The chemical composition of honey is very complex [1,2]. Honey, as a whole natural food, is exposed to several pollutants, such as antibiotics, pesticides, heavy metals, and other toxic compounds. Of the above-mentioned impurities/chemical hazards found in honey, which are also very common, one example is 5-hydroxymethylfurfural (HMF). They all reduce the health benefits and quality of honey. 5-hydroxymethylfurfural deserves interest due to its specific properties. On the one hand, it belongs to carcinogenic compounds, has a cytotoxic effect, and hinders or prevents DNA replication/repair [3–5]. On the other hand, it can be used in high-mountain diseases and brain hypoxia and can remove free radicals [5–7]. HMF is a six-carbon heterocyclic organic compound containing both an aldehyde and an alcohol (hydroxymethyl) functional group. 5-Hydroxymethylfurfural is a solid, yellow substance that exhibits a low melting point and high water solubility [8]. It is a natural toxicant that is formed from hexoses by heating and storing foods containing high amounts of sugar. There are two main ways in which 5-hydroxymethylfurfural is formed. The first is the caramelization of sugars at temperatures exceeding 150 °C. The process occurs most easily in sugar reduction reactions, including glucose, fructose, or ribose. Also, it is catalyzed by an acidic environment. The second method of HMF formation is the Maillard reaction, i.e., a non-enzymatic browning reaction. The reaction takes place as a result of the product’s long storage time or its heating. The name “Maillard reaction” comes from the French chemist Louis Maillard, who was the first to describe the reactions between sugars and amino acids in 1912. The aldehyde group of a carbohydrate reacts with the amino group derived from an amino acid or protein to form colorless intermediates, Amadori compounds, which in turn, when heated, form HMF. As a result of further changes taking place, many colored products are created. The products of the Maillard reaction reduce the nutritional value of food by blocking or destroying certain amino acid residues (lysine, cysteine, methionine, and tryptophan) and reducing their bioavailability [9]. Natural bee honey, freshly prepared, contains 2–5 mg/kg HMF. After one year, the quantity of HMF changes, and its content increases to 7–10 mg/kg. After two years, it is 20–25 mg/kg, while longer storage of honey causes even a further increase in HMF content, up to 50–100 mg/kg [10]. Scientists recommend that honey, regardless of its origin, be consumed within a year [11]. The permissible HMF content in honey established by the European Council Directive 2001/110/EC of 20 December 2001 [12] relating to honey is up to 40 mg/kg for products from the European Union. In turn, for honey produced in tropical regions, the limit is higher and amounts to 80 mg/kg. The fact that the quantity of HMF has been exceeded indicates that honey is adulterated or overheated [12]. HMF is easily formed at low temperatures; however, it must be supported by the second condition, which is low pH. Additionally, long-term storage and high temperatures increase its concentration. In addition to temperature and pH, the rate of HMF formation in the product also depends on the moisture content of honey, so there are frequently taken steps to keep moisture content low [13]. Based on the conducted research, numerous scientists have concluded that both temperature and the duration of heating affect the formation of HMF in honey. The work [14] shows the results of honey research collected in Anatolia, Turkey. Honey samples were heated at 135 °C for 100 s, which produced a similar quantity of HMF as when the samples were heated at 150 °C for 40 s. Whereas showed that honey, regardless of its origin, should be consumed within a year [15]. They researched Malaysian honey. In their research, they showed that the HMF content in honey changes accordingly with storage time. In honey samples stored for 3–6 months, the level of HMF ranged from
2.80 to 24.87 mg/kg, which was within the limits set by the European Commission (up to 80 mg/kg for honey from tropical regions) [12]. On the other hand, the extension of the storage time from 12 to 24 months resulted in exceeding the permissible standard for HMF levels, which ranged from 128.19 to 1131.76 mg/kg. HMF has both harmful and positive effects on the human body. Most of the studies and observations in this regard have been carried out primarily on mice and rats under laboratory conditions. HMF and its derivatives are genotoxic, mutagenic, carcinogenic, and destructive to DNA. The compounds also inhibit the work of enzymes in organisms. HMF is an indirect mutagen. It is converted into sulfuric acid ester (VI): 5-sulfoxymethylfurfural (SMF) as a result of activation by sulfotransferases in the liver. SMF is a highly mutagenic compound [3]. Hence, HMF and its derivative, SMF, are strong carcinogens, a fact proven in a great number of studies. HMF is cytotoxic in high concentrations, it irritates mucous membranes, skin, eyes, and upper respiratory tracts [5]. It is a selective inhibitor of a DNA polymerase enzyme, which disturbs or completely impedes DNA synthesis during DNA replication or repair [4]. HMF has been shown to have a positive effect on human health. Zhao et al. showed in their studies that HMF can remove free radicals depending on the dose (0.8–6.4 mM) [16]. HMF also increases survival when oxygen levels are low, resulting from, for example, hypoxia caused by staying at high altitude (mountain climbing), atherosclerosis, or cancer. Therefore, it can be a therapeutic agent used for treating mountain sickness, cerebral edema, or pulmonary edema at high altitudes [6,7]. HMF measurements are used to assess the quality of honey. It is generally present in trace amounts or does not occur in fresh honey. There are many methods used to determine HMF levels in honey. The 5-hydroxymethylfurfural content is determined in a clear, filtered, aqueous solution of honey using a method based on the HPLC technique with a reversed phase and UV detection [6,7,17]. Whereas the International Honey Commission recommends the use of three methods: two spectrophotometric methods, White and Winkler, and the HPLC method [18]. In addition, other methods are used to determine HMF in various types of samples, not necessarily honey [19]: HPLC with refractive index detector (HPLC-RID), HPLC coupled with mass spectrometry (LC-MS), gas chromatography coupled with mass spectrometry (GC-MS), [20] electrochemical biosensors [21], and micellar electro-kinetic capillary chromatography (MECK) [22].

The objective of this study was to determine 5-hydroxymethylfurfural (HMF) levels in honeys available to producers in Greater Poland using the following three methods: two spectrophotometric developed by White and Winkler and one developed for this work based on the LC-MS/MS technique. The indirect aim of the study was to assess the usefulness of all three methods for determining HMF content in honey. This should confirm the thesis that, based on the measurement of the amount of HMF, it is possible to develop a research procedure in the area of environmental protection monitoring for a diversified system of obtaining honey and its products. It is necessary to compare methods with different scopes of applicability in agriculture, transport, storage, and processing, which will enable their standardization. The presented research aims to improve the state of knowledge in this area by comparing research methods and indicating the scope of their use.

2. Materials and Methods
2.1. Field of Research

Wielkopolska (Latin Polonia Maior) is a historical land in Central and Western Poland (Figure 1), the Wielkopolska Lake District and the South-Greater Poland Lowland, in the Central and Lower Warta basins [23]. The fertile land is in black and in the eastern tracts. Contemporary Greater Poland is characterized by a great industrialization in the area of the processing industry. The mining industry is gathered around the brown coal deposits in the eastern part of the region and gas extraction in the north. Numerous production centers with high energy consumption are located in Greater Poland. All of the above
factors mean that this area has its own specificity in agri-industrial production compared to other regions of Poland.

Figure 1. Location of Greater Poland and sources of samples.

According to data from 2022 published by the Food and Agriculture Organization (FAO), honey production in Poland in 2021 amounted to 18.4 thousand tons and was higher than in 2020 by approx. 5.8 thousand tons. In Poland, the most expensive and rarest honeys are heather and coniferous honeydew honeys, and the cheapest and most popular are multifloral and rapeseed honeys. Greater Poland is the second out of sixteen honey producers in Poland, with an amount of 1.97 thousand tons in 2021.

2.2. Samples

Seven honey samples, (1) honeydew; (2) honey from forest areas; (3) nectar phacelia honey; (4) rapeseed honey; (5) honeydew honey; (6) multiflower nectar honey; and (7) multiflower honey collected from local producers (in Poland in the Greater Poland region) and available for general sale in shops and supermarkets, were assessed for their HMF content. Each time, three honey samples were taken from different producers for analysis.

2.3. Apparatus and Reagents

In the work, the following compounds were used for honey determination: potassium hexacyanoferrate (II) trihydrate: purity ≥ 99.5%, POCh, Poland; p-toluidine: high purity grade (99.6%) and barbituric acid: high purity grade (99%) purity, Sigma Aldrich, St. Louis, MO, USA; zinc acetate dihydrate: analytical grade, Merck, Germany, sodium bisulfate: analytical purity, Sigma Aldrich, Shanghai, China, 5-hydroxymethylfurfural: purity ≥ 99%, Sigma Aldrich, China, MS-grade methanol, acetonitrile, and isopropanol were provided by Sigma-Aldrich (St. Louis, MO, USA). The formic acid (90%) used as an addition to the mobile phase was purchased from Baker (UK). Water was prepared by reverse osmosis in a Demiwa system, followed by double distillation from a quartz apparatus. Only freshly distilled water was used. HMF was analyzed using high-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). LC analysis was performed using the chromatographic system UltiMate3000 RSLC from Dionex (Sunnyvale, CA, USA) connected in series with a 4000 QTRAP Hybrid Triple Quadrupole Linear Ion Trap mass spectrometer equipped with a Turbo Ion Spray source (Applied Biosystems-Sciex, Foster City, CA, USA). Spectrophotometric methods for determining HMF contents were performed using a Red Tide USB650UV spectrometer, Ocean Optics, the USA. Measurements were performed using a 10 mm quartz cuvette.
2.4. Procedures

2.4.1. Preparation of Solutions for HMF Determination

HMF levels in the analyzed honey samples were determined with the LC-MS/MS technique and spectrophotometric methods by White and Winkler, as described by the International Honey Commission [18]. Before HMF determinations, the following solutions were prepared for honey samples:

- **Carrez I**: 15 g of potassium hexacyanoferrate (II) trihydrate was dissolved in distilled water in a 100 mL volumetric flask.
- **Carrez II**: 30 g of zinc acetate dihydrate was dissolved in distilled water in a 100 mL volumetric flask.
- A 0.2% sodium bisulfate (IV) solution: 0.1 g sodium bisulfate (IV) was dissolved in distilled water in a 50 mL volumetric flask (prepared immediately before use).
- **A 0.5% barbituric acid solution**: 0.5 g of barbituric acid was dissolved in distilled water in a 100 mL volumetric flask.
- **p-toluidine solution**: 10 g of p-toluidine was dissolved in propan-2-ol in a 100 mL volumetric flask. The preparation of the solution must take place 24 h before use and can be used for up to three days.

2.4.2. The White Spectrophotometric Method

The spectrophotometric method by White, described by the International Honey Commission, was used to determine HMF content [18]. A total of 5 g of honey was dissolved in 25 mL of distilled water and transferred quantitatively into a 50 mL volumetric flask. Then, the Carrez solution I (0.5 mL) and the Carrez solution II (0.5 mL) were added to the honey solution and made up to 50 mL with water. Such a prepared solution was filtered through a filter paper, rejecting the first 10 mL of the filtrate. In the next step, aliquots of 5 mL were put in two flasks. One flask was filled with water up to the mark (sample solution), while the other was filled with 5 mL of 0.2% sodium bisulfate (IV) (reference solution). The absorbance of the solutions at 284 and 336 nm was determined. The HMF content in honey was calculated using the following equation [18,23,24]:

\[
\text{HMF} = (A_{284} - A_{336}) \times 149.7 \times 5 \times D/W \text{ [mg/kg]} \tag{1}
\]

where \(A_{284}\) is the absorbance at 284 nm, \(A_{336}\) is the absorbance at 336 nm, 149.7 = (126 \times 1000 \times 1000)/(16,830 \times 10 \times 5) is constant, 126 is molecular weight of HMF, 16,830 is molar absorptivity \(\varepsilon\) of HMF at \(\lambda = 284\) nm, 1000 is conversion g into mg, 10 is conversion 5 into 50 mL, 1000 is conversion g of honey into kg, 5 is the theoretical nominal sample weight, \(D\) is dilution factor in case dilution is necessary, and \(W\) is the weight in g of the honey sample.

2.4.3. The Winkler Spectrophotometric Method

In Winkler’s method, 10 g of honey was weighed into a beaker, and 20 mL of distilled water was added. The content was mixed using a magnetic stirrer. The mixed solution was transferred into a 50 mL volumetric flask. Then, 1 mL of Carrez solution I and 1 mL of Carrez solution II were added to the honey solution and filled with distilled water up to the mark. Such a prepared solution was filtered through a filter paper, discarding the first 10 mL. The next step was to collect 2 mL of the filtered honey into two 10 mL flasks. A total of 5 mL of p-toluidine solution was added to both of them. Then, 1 mL of distilled water (reference solution) was added to one flask, and to the second, 1 mL of 0.5% barbituric acid solution was added (sample solution). The absorbance of the solutions was determined at 550 nm. HMF content was calculated from the following equation [15,18,24,25]:

\[
\text{HMF} = 192 \times A \times 10/W \text{ [mg/kg]} \tag{2}
\]
where $A$ is absorbance, 192 is the factor for dilution and extinction coefficient, and $W$ is the weight of honey in grams.

2.4.4. The LC-MS/MS Method

The chromatographic separations were obtained at a temperature of 35 °C on a 100 × 2.1 mm id, particle size 1.9 µm, Hypersil Gold C18 analytical column with guard column, both supplied by Thermo Fisher Scientific (168 Third Avenue, Waltham, MA USA). The mobile phase was a mixture of 0.1% aqueous formic acid (A) and methanol (B). The flow rate was 0.2 mL/min, and the injection volume was 2 µL. The following gradients were used: 0 min 70% B, 2 min 100% B, and 2.5 min 100% B. The ESI interface was implemented in a positive ion mode. The analyses were performed in multiple reaction monitoring, monitoring two transitions between the protonated precursor ion and the most abundant fragment ions for the compound. HMF was detected using the following settings for the ion source and mass spectrometer: curtain gas 10 psi, nebulizer gas 40 psi, auxiliary gas 40 psi, temperature of 350 °C, ion spray voltage 5500 V, and collision gas set to medium. The MS/MS parameters used for quantitative HMF determination are shown in Table 1, where DP is the decluttering potential, CE is the collision energy, and CPX is the collision cell exit potential.

<table>
<thead>
<tr>
<th>Pseudo Molecular Ion [M + 1]+</th>
<th>DP [V]</th>
<th>MRM</th>
<th>CE [V]</th>
<th>CPX [V]</th>
</tr>
</thead>
<tbody>
<tr>
<td>127</td>
<td>46</td>
<td>127 → 109 *</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>127</td>
<td>81</td>
<td>127 → 81</td>
<td>23</td>
<td>6</td>
</tr>
<tr>
<td>127</td>
<td>53</td>
<td>127 → 53</td>
<td>31</td>
<td>8</td>
</tr>
</tbody>
</table>

* MRM for quantitative analysis.

2.4.5. Validation of the LC-MS/MS Method

For the LC-MS/MS method, there were determined validation parameters, i.e., limit of detection (LOD), the limit of quantification (LOQ), and the linearity range. Also, the measurement of the expanded uncertainty ($U$) was calculated for the following HMF concentrations: 0.001 µg/mL; 0.0025 µg/mL; 0.005 µg/mL; 0.01 µg/mL; 0.025 µg/mL; 0.05 µg/mL; 0.1 µg/mL; 0.25 µg/mL; and 0.5 µg/mL. The limit of detection (LOD) for HMF, defined as the concentration that yielded S/N ratio greater than or equal to 5, and the limit of quantification (LOQ), defined as the concentration that yielded S/N ratio greater than or equal to 10, were determined for HMF in methanol (the solvent used for the introduction of the compound into the LC-MS/MS system). Determination of the linearity range. Linearity was determined for a series of standard solutions of the analyzed HMF content in the concentration range from 0.000025 µg/mL to 1 µg/mL.

Calculations regarding the measurement of expanded uncertainty were made based on the Ishikawa diagram (Figure 2), which shows the influence of the uncertainty of individual parameters of the analytical process on the final uncertainty of the preparation of the standard solution. The expanded uncertainty was calculated using Formulas (3) and (4) [26], where $U$ denotes the expanded uncertainty, $k$ is the coverage factor (usually 2), $U(c1...c5)$ is the uncertainty of the standard solutions of appropriate concentrations, RSD is the RSD of the results, and the number of independent determinations is as follows:

$$U_{(c5)}^{(c5)} = \sqrt{[U(c1)^2 + U(c2)^2 + U(c3)^2 + U(c4)^2 + U(c5)^2 + \frac{\text{RSD}^2}{n}]}$$

$$U = kU(c5)$$
Figure 2. The Ishikawa diagram for an HMF solution with the concentration of \( c = 0.005 \) µg/mL.

The Statistica program was used to analyze the obtained results.

2.4.6. Preparation of Honey for Determinations by LC-MS/MS

As in the case of spectrophotometric methods, Carrez I and Carrez II solutions were also used during the preparation of honey samples for determinations by LC-MS/MS. The weights of honey samples were determined based on the results obtained from spectrophotometric measurements, so that 300 µL of the honey sample solution contained approximately 0.05 µg of HMF. After weighing, the honey sample was dissolved in 25 mL of distilled water. The dissolution process was accelerated by stirring with a magnetic stirrer. During the next stage, the mixed solution was poured into a 50 mL flask, where 1 mL of Carrez solution I and 1 mL of Carrez solution II were added and then filled with distilled water up to the mark. Such a prepared solution was filtered through a filter paper, discarding the first 10 mL. Furthermore, the honey sample solution was filtered using PTFE syringe filters with a diameter of 13 mm and a pore size of 0.22 µm to remove solid impurities. Then, 300 µL of the filtered honey solution was taken, and 700 µL of methanol was added. The samples thus prepared were subjected to the LC-MS/MS analysis described above.

3. Results and Discussion

3.1. Validation of the LC-MS/MS Method

The equation of the calibration curve as well as the limit of detection (LOD) and the limit of quantification (LOQ) are shown in Table 2. Linearity was tested by analyzing HMF samples at different concentrations ranging from 0.000025 µg/mL to 1 µg/mL. Good linearity was achieved with correlation coefficients of 0.9992. The LOD was 0.0005 µg/mL, while the LOQ was 0.001 µg/mL (White method: LOQ = 4 µg/g; Winkler method: LOQ = 2 µg/g).

Table 2. Linearity range, limits of detection, and quantification for HMF.

<table>
<thead>
<tr>
<th>Linearity Range</th>
<th>Curve Equation</th>
<th>Correlation Coefficient</th>
<th>LOD [µg/mL]</th>
<th>LOQ [µg/mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>From LOQ to 0.5 µg/mL</td>
<td>( y = 2 \times 10^7 \times x + 35,595 )</td>
<td>0.9992</td>
<td>0.0005</td>
<td>0.001</td>
</tr>
</tbody>
</table>

The measurement of the expanded uncertainty for the method based on the LC/MS-MS technique was calculated for the HMF with the following concentrations from 0.001 to
0.1 µg/mL. The calculations were made on the basis of the dependencies described above. The obtained uncertainties are summarized in Table 3. The measurement of the expanded uncertainty increases with the concentration of the standard solution of HMF. The lowest concentration was achieved at 0.001 µg/mL.

Table 3. Measurement of the expanded uncertainty of HMF solution with different concentrations.

<table>
<thead>
<tr>
<th>Concentration [µg/mL]</th>
<th>Measurement Uncertainty [µg/mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.001</td>
<td>$7.6 \times 10^{-5}$</td>
</tr>
<tr>
<td>0.0025</td>
<td>$2.16 \times 10^{-4}$</td>
</tr>
<tr>
<td>0.005</td>
<td>$5.5 \times 10^{-4}$</td>
</tr>
<tr>
<td>0.010</td>
<td>$7.97 \times 10^{-4}$</td>
</tr>
<tr>
<td>0.025</td>
<td>$2.13 \times 10^{-3}$</td>
</tr>
<tr>
<td>0.050</td>
<td>$2.59 \times 10^{-3}$</td>
</tr>
<tr>
<td>0.100</td>
<td>$5.16 \times 10^{-3}$</td>
</tr>
<tr>
<td>0.250</td>
<td>$1.32 \times 10^{-2}$</td>
</tr>
<tr>
<td>0.500</td>
<td>$2.72 \times 10^{-2}$</td>
</tr>
</tbody>
</table>

3.2. Results Analysis

A qualitative analysis was performed on the basis of HMF content determinations by LC-MS/MS and spectrophotometric methods. The results of qualitative analysis are presented in Table 4.

Table 4. Qualitative analysis results.

<table>
<thead>
<tr>
<th>Honey Sample Number</th>
<th>HMF Presence (LC-MS/MS)</th>
<th>HMF Presence (White)</th>
<th>HMF Presence (Winkler)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Honey samples were prepared following descriptions corresponding to the method used. A quantitative analysis of HMF content in individual honey samples was performed. The standard curve technique was used to determine the content of the method based on the LC-MS/MS technique. In the case of spectrophotometric methods, the quantity of HMF in honey samples was determined based on the equations described above. Each sample was analyzed three times, regardless of the method applied. The obtained results are summarized in Table 5 and Figure 3.

Table 5. Qualitative analysis results.

<table>
<thead>
<tr>
<th>Honey Sample Number</th>
<th>HMF Quantity [mg/kg] ± SD (LC-MS/MS)</th>
<th>HMF Quantity [mg/kg] ± SD (White)</th>
<th>HMF Quantity [mg/kg] ± SD (Winkler)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$3.4 \pm 0.2$</td>
<td>$15.0 \pm 0.0$</td>
<td>$0.0 \pm 0.0$</td>
</tr>
<tr>
<td>2</td>
<td>$2.7 \pm 0.4$</td>
<td>$0.0 \pm 0.0$</td>
<td>$3.7 \pm 0.1$</td>
</tr>
<tr>
<td>3</td>
<td>$3.7 \pm 0.2$</td>
<td>$4.5 \pm 0.0$</td>
<td>$8.8 \pm 1.6$</td>
</tr>
<tr>
<td>4</td>
<td>$2.6 \pm 0.1$</td>
<td>$1.5 \pm 0.0$</td>
<td>$3.7 \pm 2.5$</td>
</tr>
<tr>
<td>5</td>
<td>$2.7 \pm 0.2$</td>
<td>$1.5 \pm 0.0$</td>
<td>$5.1 \pm 1.3$</td>
</tr>
<tr>
<td>6</td>
<td>$3.0 \pm 0.2$</td>
<td>$23.5 \pm 1.7$</td>
<td>$23.4 \pm 0.1$</td>
</tr>
<tr>
<td>7</td>
<td>$3.6 \pm 0.2$</td>
<td>$28.5 \pm 1.5$</td>
<td>$20.2 \pm 1.4$</td>
</tr>
</tbody>
</table>
Two spectrophotometric methods, White and Winkler, were negative for the presence of HMF in the two honey samples. In the case of the first method, it was sample no. 2, and in the case of the second, it was sample no. 1. This may have resulted from numerous factors, i.e., the sensitivity of the method, its accuracy, or its repeatability. When comparing the results for honey samples obtained with the method based on the LC-MS/MS technique (Table 5 and Figure 3), it was noticed that the highest content of HMF occurred in honey samples numbers 7 and 3. In none of the tested honey samples, HMF content exceeded the permitted quantity specified in the regulation of the European Commission. The highest HMF content in the tested honey samples determined by the White method was found in honey samples numbers 6 and 7. HMF was not detected in honey sample number 2; therefore, it can be concluded that the value of HMF was below the quantification limit of this method. Similar dependencies were observed for the Winkler method. The highest HMF content was also found in honey samples numbers 6 and 7. However, in honey sample number 1, HMF was not marked; thus, it can be concluded that the value of HMF was below the detection limit of this method. When analyzing Table 5 and Figure 3, it was noticed that the quantity of HMF differed depending on the method applied. This may result from numerous factors, i.e., the place where the honey sample was taken for testing (external or internal part of the container), the sensitivity of the method, its accuracy, or its repeatability. It could also be affected by the presence of HMF derivatives (selectivity), which are quantified when testing honey samples using spectrophotometric methods, or by the presence of compounds that facilitate the ionization of HMF in the ion source of the mass spectrometer and overestimate the final result or hinder ionization and underestimate the results obtained by a method based on the LC-MS/MS technique. The obtained results are characterized by high repeatability, which is exhibited through low values of standard deviations.

It is impossible to explain exactly why these methods are not compatible, which has been confirmed by previous work [17]. They suggested that this could be due to the formation of hydroxymethylfurfural precursors. They also looked at the overestimation of the Winkler method compared to the White method and HPLC. Previous studies have shown that none of the previously used methods for determining HMF in honey reflect its actual content [27]. Other authors recommend a method based on the HPLC technique with UV detection [11]. Spectrophotometric methods (White and Winkler) are fast but have low specificity and sensitivity. HPLC is more accurate and precise, but time-consuming.
Contrary to other studies, based on our research, it is not possible to unequivocally indicate the best method of determining this compound in honey samples. The method based on the LC-MS/MS technique is supported by its very high sensitivity and selectivity. The latter parameter results from the detection method of fragmentation reaction monitoring. This is a specific example of targeted analysis.

Nevertheless, further research on HMF content in honey should be carried out on a larger sample pool. In addition, it is desirable to develop an LC-MS/MS or other methods to not only determine HMF but also its derivatives, which would give a more complete picture concerning spectrophotometric methods. Furthermore, honey samples should be homogenized before testing by thorough mixing, which will certainly increase the reliability of HMF content determination. Additionally, it is recommended to take into account the effect of the matrix on the final results of HMF content (overestimation/underestimation) obtained through a method based on the HPLC-MS/MS technique. HMF content in honey samples detected with this method should be determined using a multiple standard addition technique. According to the European Council standard, the obtained results of HMF content range up to 40 mg/kg (Figure 3). This means that they meet the normative requirements, but not all methods give consistent results.

4. Conclusions

The purpose of the research presented in the article was to confirm the assumptions that HMF may have a honey quality indicator and that it is possible to use three methods of its determination as equal. This has allowed the development of industry guidelines to assess the balance in the development of the acquisition and production of honey and its preserves. The research issue is commissioned as part of a regional project implemented at the Poznan University of Technology in cooperation with the industrial sector.

The validation of the 5-hydroxymethylfurfural qualitative and quantitative method based on the HPLC-MS/MS technique was developed and carried out. The developed method was used to determine HMF content in honey samples. The method is distinguished by the linearity range in concentrations from 0.001 µg/mL to 0.5 µg/mL and low values of detection and quantification limits. Both spectrophotometric methods are distinguished by low sensitivity but high repeatability. The HMF contents determined by these three methods are divergent. This may result from numerous factors, i.e., the sensitivity of the method, its accuracy or repeatability, the presence of HMF derivatives, or the presence of compounds that facilitate the ionization of HMF in the ion source and thereby overestimate or lower the results obtained by a method based on the LC-MS/MS technique. The determined HMF content in the tested honey samples does not exceed the applicable European Council standard, i.e., they do not exceed the value of HMF content in honey, which is 40 mg/kg. This means that the kinds of honey present on the Polish market are of high quality. All research methods should be considered equal in HMF analysis. Their selection should be dictated by the advanced and technical capabilities of their use and the availability of injection of field trials at producers, producers, and carriers.

Author Contributions: J.Z.: methodology, formal analysis, investigation, project administration, supervision, writing—original draft; Ł.P.: investigation, formal analysis, methodology, validation visualization, writing—original draft; B.K.: data curation, formal analysis, software, validation, visualization; J.B.: formal analysis, validation, investigation; E.J.-B.: formal analysis, funding acquisition, methodology writing—review and editing; J.S.: formal analysis, supervision, validation, writing—original draft; P.K.: formal analysis, funding acquisition, project administration writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This work has been financed by the Statutory Activities Fund of the Institute of Machines and Motor Vehicles, Poznan University of Technology (PL) 0414/SBAD/3622, and the Ministry of Science and Higher Education (no. 911/SBAD/2404).

Institutional Review Board Statement: Not applicable.
Informed Consent Statement: Not applicable.

Data Availability Statement: The data are available on request.

Conflicts of Interest: The authors declare no conflicts of interest.

References

5. Morales, F.J. Hydroxymethylfurufural (HMF) and Related Compounds; Wiley: Hoboken, NJ, USA, 2008; Volume 135. [CrossRef]
11. Khalil, M.I.; Sulaiman, S.A.; Gan, S.H. High 5-hydroxymethylfurufural concentrations are found in Malaysian honey samples stored for more than one year. Food Chem. Toxicol. 2010, 48, 2388. [CrossRef]
23. Stankowski, W. Wielkopolska; OCLC 830198134; WisP: Maxwell, CA, USA, 1999; ISBN 83-02-07148-X.
25. Tomczyk, M.; Zagula, G.; Puchalski, C.; Dzukan, M. Transfer of some toxic metals from soil to honey depending on bee habitat conditions. Acta Univ. Cibiniensis 2020, 24, 49. [CrossRef]


27. Anklam, E. A review of the analytical methods to determine the geographical and botanical origin of honey. *Food Chem.* 1998, 63, 549. [CrossRef]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.