BRAZILIAN JOURNAL OF FOOD TECHNOLOGY

ISSN 1981-6723
Online version

ORIGINAL ARTICLE

Spectrophotometry of Winkler and White's official methods for the determination of hydroxymethylfurfural in bee honey

Isabel Cabral Maeda[1], Aryele Nunes da Cruz Encide Sampaio[1*], Evelyn Fernanda Flores Caron[1], José Fernando Nardy[1], Sarah Cristina de Oliveira[1], Juliano Gonçalves Pereira[1], Otávio Augusto Martins[1]

[1]Universidade Estadual Paulista (UNESP), Universidade Estadual Paulista (UNESP), Faculdade de Medicina Veterinária e Zootecnia, Botucatu, Departamento de Produção Animal e Medicina Veterinária Preventiva, Laboratório de Inspeção de Alimentos, Botucatu/SP, Brasil

***Corresponding author:** Aryele Nunes da Cruz Encide Sampaio, Universidade Estadual Paulista (UNESP), Faculdade de Medicina Veterinária e Zootecnia, Botucatu, Departamento de Produção Animal e Medicina Veterinária Preventiva, Laboratório de Inspeção de Alimentos, Rua Prof. Walter Mauricio Correa, SN, CEP: 18618-681, Botucatu/SP, Brasil, e-mail: aryele.sampaio@unesp.br

Cite as: Maeda, I. C., Sampaio, A. N. C. E., Flores Canon, E. F., Nardy, J. F., Oliveira, S. C., Pereira, J. G., & Martins, O. A. (2023). Spectrophotometry of Winkler and White's official methods for the determination of hydroxymethylfurfural in bee honey. *Brazilian Journal of Food Technology*, *26*, e2022133. https://doi.org/10.1590/1981-6723.13322

Abstract

The determination of hydroxymethylfurfural (HMF) in bee honey is performed by the spectrophotometric methods Winkler's and White's. The Winkler method reads the absorbances in the visible region of the spectrum, and the White method measures in the ultraviolet (UV) and visible regions of the spectrum. This study aims to compare the spectrophotometric methodologies of White and Winkler to the determination of HMF in bee honey. For the study, it was used a UV-visible spectrophotometer, 10 mm optical path quartz cuvettes, and ten bee honey samples of different flowering. Absorbances at 550 nm were analyzed by the Winkler method; absorbances at 284 nm and 336 nm in the White method; and a comparison of HMF concentration (mg/kg) was carried out between the methods. The results showed significant differences ($p<0.05$) in HMF concentration determined by both methods. The main conclusion is that the Winkler method presents higher HMF values than the White method.

Keywords: Absorbance; Bees; Comparison; HMF; Physicochemical; Spectrophotometer.

Highlights

- There are no significant differences in the absorbance readings in the evaluated methods
- HMF values were different between the White and Winkler methods
- The Winkler method presents higher HMF values than the White method

1 Introduction

Hydroxymethylfurfural (HMF) is a cyclic aldehyde formed from the breakdown of sugars present in various heat-processed foods such as breakfast cereals, dairy products, and juices. In addition, its concentration is a parameter to measure the quality and freshness of the honey, as the maximum acceptable amount is 60 mg/kg. HMF can be present in honey for three reasons: (a) sugar addiction fraud, (b) overheating, and (c) aging (Shapla et al., 2018). Brasil (1981) defines HMF in bee honey as a fructose dehydration product that occurs when sucrose is reversed in an acid medium.

HMF can be detrimental to health by showing effects such as cytotoxicity to mucous membranes, skin, and upper respiratory tract, mutations, chromosomal aberrations, and carcinogenicity. In contrast, HMF has been shown in some studies with positive effects such as antioxidants, antiallergics, and anti-inflammatories (Shapla et al., 2018).

Evaluating the concentration of HMF in honey is important and verifiable by using the official White, Winkler, and high-performance liquid chromatography (HPLC) methods. The White and Winkler methods use UV-visible spectrophotometer equipment that is cheaper than high-value HPLC.

The White method uses potassium ferrocyanide (Carrez I), zinc acetate (Carrez II), and sodium bisulfite solutions to determine HMF in bee honey samples. The main equipment used in the method is the spectrophotometer. While ABNT (Associação Brasileira de Normas Técnicas, 2016) does not mention the use of an ultrasound bath, IAL (Instituto Adolfo Lutz, 2005) cites the use of this equipment for the determination of HMF. Spectrophotometer absorbance readings are taken at two wavelength regions (λ): (a) visible region - 336 nm and (b) ultraviolet region - 284 nm (Association of Official Analytical Chemists, 1995; Bogdanov et al., 1997; Instituto Adolfo Lutz, 2005).

The Winkler method uses barbituric acid and p-toluidine solutions. This method uses a spectrophotometer and the visible region of the spectrum with absorbance readings at 550 nm (Brasil, 1981). The concern in terms of biosafety for this method is that p-toluidine is a substance that has toxic and carcinogenic compounds, and this is the main reason that many routine laboratories for honey analysis do not perform this analysis (Zappalà et al., 2005; Pascual-Maté et al., 2018).

The HPLC method consists of the use of specific chromatography equipment which is expensive and is often not compatible with the reality of many laboratories that analyze the quality of honey in Brazil. However, the spectrophotometric methods use a device easily acquired because it does not depend on its price, which may exceed the economic reality of beekeepers. And spectrophotometric methodologies (White and Winkler) can be more easily applied and economically viable to determine HMF concentration. This study aims to compare the spectrophotometric methodologies of White and Winkler to the determination of HMF in bee honey.

2 Material and methods

2.1 Samples

Ten samples of bee honey from supermarkets and apiaries were used. The samples were sent to the Food Physicochemical Laboratory of the Public Food Guidance Service of the Department of Animal Production and Preventive Veterinary Medicine, São Paulo State University (Unesp), School of Veterinary Medicine and Animal Science, Botucatu City, São Paulo, Brazil and stored in thermal boxes to avoid interference from elevated temperatures.

2.2 Reagents

For the spectrophotometric determination of HMF by the White method, 15% potassium ferrocyanide solutions (Carrez I solution), 30% zinc acetate (Carrez II solution), and 0.2% sodium bisulfite were used. In the Winkler method, the solutions used were 0.5% aqueous barbituric acid solution and 10% para-toluidine isopropyl solution.

Pure water from a reverse osmosis system was used in the analytical assays. All solutions obtained in the present work were made from high-quality analytical reagents of the brands Merck® and Sigma-Aldrich®.

2.3 Equipment

Tecnal® reverse osmosis equipment model TE 4007-10 was used to obtain pure water. The Spectrum® model SP1105 UV-visible spectrophotometer was used to analyze the methods. In the HMF absorbance readings in the spectrophotometer, 10 mm optical path quartz cuvettes and 3.5 mL volume were used.

2.4 Determination of HMF by the Winkler method

The samples were submitted to White and Winkler spectrophotometric methods for the determination of HMF. The assays were made in triplicate totaling thirty assays for each method.

Five grams of honey were weighed into a 50 mL glass vial. It dissolved with the aid of pure water and transferred to a 25 mL volumetric flask. It completed the volume of the volumetric flask with pure water and homogenized it. The solution was used immediately.

Two milliliters of the honey solution prepared above were transferred into 4 clean and dry test tubes. 5 mL of the para-toluidine solution was added to each test tube. In the tube considered white, 1 mL of pure water was added and in the other tubes, 1 mL of barbituric acid solution was added and homogenized. The sequence of addition of reagents did not exceed 2 min.

Waited 4 min. Transferred to a 10 mm optical path quartz cuvette and read the absorbance spectrophotometer at 550 nm. The absorbance value obtained in the reading was applied in the following official formula of the method. Formula: HMF (mg/kg) = 192 x absorbance/cuvette thickness. Where: HMF = hydroxymethylfurfural; 10 mm cuvette thickness of optical path = consider 1 cm; and the value of 192 = dilution factor and extinction coefficient.

2.5 Determination of HMF by the White method

Five grams of honey were weighed into a 50 mL glass and transferred to a 50 mL volumetric flask with the aid of a stick and 25 mL of pure water. Added 0.5 mL of Carrez I solution and 0.5 mL of Carrez II solution. It completed the 50 mL volumetric flask with pure water and homogenized it.

The solution was filtered with filter paper and the first 10 mL were discarded. 5 mL of the filtrate was pipetted into 4 clean test tubes. In the tube considered white (reference) 5 mL of sodium bisulfite solution was added and in the other tubes, 5 mL of pure water was added. It mixed well (Associação Brasileira de Normas Técnicas, 2016).

The absorbance reading was taken on the spectrophotometer with 10 mm optical path quartz cuvettes at 284 nm and then at 336 nm. The results were placed in the official formula of the method: HMF (mg/kg) = $(A_{284} - A_{336})$ x 149.7 x 5 / m. Where: A_{284} = absorbance reading at 284 nm; A_{336} = absorbance reading at 336 nm; m = sample mass (g); 5 = nominal mass of sample; and 149.7 = (126/16830) x (1000/10) x (1000/5).

2.6 Statistical analysis

Statistical analyzes of the triplicate absorbance readings in the spectrum regions (UV and visible) were performed. Statistical analysis of HMF concentrations was also performed using the Winkler and White spectrometric methods. The statistical method was based on an entirely randomized experiment or randomized essay. Performed analysis of variance (ANOVA) supplemented with Tukey test for comparison of means. Statistical analysis considered the significance level of 5%.

2.7 Treatment of chemical residues

All chemical residues generated at the Animal Food Physicochemical Laboratory during this work were sent to the Conservation and Maintenance Section of the General Administration (UNESP, Botucatu, São

Paulo, Brazil). This section manages all chemical residues generated at the Botucatu *Campus* and makes their analytical destination decisions.

3 Results

3.1 Absorbance study in the visible region of the spectrum (550 nm) in the Winkler method.

Table 1 shows the analysis of variance (ANOVA) of the absorbance values obtained at 550 nm by the Winkler method. Statistical analysis of the three repeats of absorbance obtained a value of $p > 0.05$. The mean of the three absorbance repetitions did not show significant differences ($p = 0.9884$) complemented with the Tukey test (Table 2).

Table 1. Analysis of variance (ANOVA) of the absorbance values of the three replicates (triplicate) of each honey sample analyzed and read at 550 nm in the spectrophotometer for the determination of 5-hydroxymethylfurfural using quartz cuvettes with a 10 mm optical path (Winkler method).

Source of variation	Degrees of freedom	Sum of squares	Medium Square
Treatments	2	0.0008441	0.0004220
Waste	36	1.300	0.03611
Total	38	1.301	
F = 0.01169 = ($MS_{treatment}/MS_{residual}$)			

Table 2. Mean ± standard deviation of absorbance values [-Log10 (I/I0)] read at 550 nm in the spectrophotometer of the three replicates (triplicate) of each honey sample for the determination of 5-hydroxymethylfurfural by the Winkler Method. Statistical analysis and Tukey-Kramer multiple comparison test (5% significance level).

Absorbance [- Log_{10} (I/I_0)] ± standard deviation		
1st repetition	2nd repetition	3rd repetition
0,1668 ± 0,0519a[(1)]	0.1639 ± 0.0523a	0.1749 ± 0.0539a

[(1)]Tukey-Kramer test and identical lowercase letters between repetitions indicate that there are no significant differences ($p = 0.9884$).

3.2 Absorbance study in the ultraviolet region of the spectrum (284 nm) in the White method

Table 3 shows the analysis of variance (ANOVA) of the three absorbance repeats at 284 nm. The statistical analysis of absorbance obtained a value of $p > 0.05$ demonstrating that there are no significant differences in repetitions. Tukey's test, which compared the mean absorbance, obtained $p = 0.9998$ (Table 4).

Table 3. Analysis of variance (ANOVA) of the absorbance values of the three replicates (triplicate) of each sample of honey analyzed and read at 284 nm in the ultraviolet (UV) region of the spectrophotometer for the determination of 5-hydroxymethylfurfural using quartz cuvettes of 10 mm of the optical path (White's method).

Source of variation	Degrees of freedom	Sum of squares	Medium Square
Treatments	2	0.0002016	0.0001008
Waste	36	16.336	0.4538
Total	38	16.336	
F = 0.0002221 = ($MS_{treatment}/MS_{residual}$)			

Table 4. Mean ± standard deviation of absorbance values [-Log10 (I/I0)] read at 284 nm in the spectrophotometer of the three replicates (triplicate) of each honey sample for the determination of 5-hydroxymethylfurfural by the White method. Statistical analysis and Tukey-Kramer multiple comparison test (5% significance level).

Absorbance [- Log_{10} (I/I_0)] ± Standard Deviation		
1st repetition	2nd repetition	3rd repetition
0.2788 ± 0.1886a[(1)]	0.2827 ± 0.1874a	0.2773 ± 0.1845a

[(1)]Tukey-Kramer test and identical lowercase letters between repetitions indicate that there are no significant differences ($p = 0.9998$).

3.3 Absorbance study in the visible region of the spectrum (336 nm) in the White method

Like the previous results, Table 5 shows the analysis of variance (ANOVA) of these three absorbance repetitions at 336 nm. The Anova result showed that there are no significant differences ($p > 0.05$) in the absorbance repetitions at 336 nm (visible region of the spectrum). Table 6 shows the comparison of the mean absorbance supplemented with the Tukey test ($p = 0.8991$).

Table 5. Analysis of variance (ANOVA) of the absorbance values of the three replicates (triplicate) of each honey sample analyzed and read at 336 nm in the visible region of the spectrophotometer for the determination of 5-hydroxymethylfurfural using quartz cuvettes with a 10 mm optical path (White's method).

Source of variation	Degrees of freedom	Sum of squares	Medium Square
Treatments	2	0.0002378	0.0001189
Waste	36	0.04014	0.001115
Total	38	0.04038	
$F = 0.1067 = (MS_{treatment}/MS_{residual})$			

Table 6. Mean ± standard deviation of absorbance values [- Log10 (I/I0)] read at 336 nm in the spectrophotometer of the three replicates (triplicate) of each honey sample for the determination of 5-hydroxymethylfurfural by the White method. Statistical analysis and Tukey-Kramer multiple comparison test (5% significance level).

Absorbance [- Log_{10} (I/I_0)] ± Standard Deviation		
1st repetition	2nd repetition	3rd repetition
0.0148 ± 0.0107a(1)	0.0148 ± 0.0076a	0.0201 ± 0.0093a

(1)Tukey-Kramer test identical lowercase letters between repetitions indicate that there are no significant differences ($p = 0.8991$).

3.4 Comparison of HMF concentrations (mg/kg) by Winkler and White methods

The analysis of variance (ANOVA) complemented with the Tukey test showed that there are extremely significant differences between the Winkler and White methods ($p = 0.0042$) evaluated. Statistical analysis in the present study showed that HMF values (mg/kg) in the honey of bees of different flowering were higher in the Winkler method compared to the White method (Table 7).

Table 7. Mean ± standard deviation of 5-hydroxymethylfurfural values (mg/kg) in honey from *Apis mellifera* bees by the official spectrophotometric methods of Winkler and White. Statistical analysis (ANOVA) and Tukey-Kramer multiple comparison test (5% significance level).

Official spectrophotometric methods for 5-hydroxymethylfurfural (HMF)	
Winkler	White
32.52 ± 10.07b(1)	0.61 ± 0.61a

(1)Tukey-Kramer test different lowercase letters indicate that there are significant differences ($p = 0.0042$).

4 Discussion

Our results demonstrated a high variability of HMF values (mg/kg) in the Winkler and White methods, observed in their respective values of standard deviations and coefficient of variation (CV). But we found numerically that the standard deviations of HMF values in the White method were greater than in the Winkler method. This variability of HMF values in the White method is because of technical problems due to equipment manufacturing issues in the automatic switching of the two lamps (tungsten and deuterium). We believe that the technology of many low-cost spectrophotometers is problematic in the changing region of the two lamps (tungsten and deuterium). This problem that exists in this spectral region (UV and visible boundary region) of the low-cost spectrophotometer may be clarified with future methodological research. The absorbance reading at 336 nm in the White method is within the transient limit established by Harris (2005) to ± 400 nm between visible and ultraviolet regions of the spectrum. Harris (2005) mentions that the tungsten lamp is used in the visible region (400 nm to

700 nm) and the deuterium lamp is used in the ultraviolet region (200 nm to 400 nm). Another methodological point, which is an interference with the White method, is the use or not of an ultrasound bath. ABNT (Associação Brasileira de Normas Técnicas, 2016) does not mention the use of an ultrasound bath by the technical standard ABNT NBR 15714-9 (Associação Brasileira de Normas Técnicas, 2016) and IAL (Instituto Adolfo Lutz, 2005) cites the use of an ultrasound bath for the determination of HMF by the White method. Based on this discrepancy, we believe that future studies should verify the efficiency of the use of ultrasound baths to determine HMF in this method to elucidate this question. Therefore, we believe that further methodological studies are needed to clarify the interferences of the Winkler and White methods which may be Chinese low-cost spectrophotometer technology or analytical gears. This work did not evaluate the spectrophotometer equipment of several world manufacturers nor the method steps because our objective in the present work was to analyze if there are differences in the determination of HMF of honeys in the Brazilian reality.

Martysiak-Żurowska & Borowicz (2009) and Kukurová et al. (2006) compared the Winkler and HPLC methods and described that the spectrophotometric method was less effective. Martysiak-Żurowska & Borowicz (2009) and Kukurová et al. (2006) observed higher sensitivity and repeatability on HPLC and found a significantly higher statistical value for HMF in the Winkler method. Martysiak-Żurowska & Borowicz (2009) justified that the Winkler method addresses all furan-derived aldehydes and not just the HMF molecule. This is the possible reason why we get higher HMF values in the Winkler method than in White's method.

Kukurová et al. (2006) reports honey samples with HMF values of less than 1.0 mg/kg are not properly detected by the Winkler method and the HPLC method is the most suitable for the determination. In addition, Martysiak-Żurowska & Borowicz (2009) and Kukurová et al. (2006) commented on the carcinogenicity of the p-toluidine solution used in the Winkler method.

Truzzi et al. (2014) compared the HPLC and White methods and concluded that the HPLC method is the most suitable because it is more accurate for samples with HMF concentrations from 1 mg/kg to 4 mg/kg. Finally, Zappalà et al. (2005) studied the three official methods (Winkler, White, and HPLC) and found that the White and HPLC methods showed comparable results and the Winkler method overestimated them.

Although HPLC is the method with the best ratings, it requires the use of specific equipment that is expensive for the reality of routine laboratories for Brazilian beekeepers. Therefore, the present study evaluated the two official methods (White and Winkler) that are most cost-effective for small honey producers' laboratories.

In our experiment we found that the Winkler method presented higher and more accurate values than the White method, and in both methods, the analyzed samples were in accordance with the European Union Directive 2001/110/EC (European Union, 2001). In the scientific literature they generally compare spectrophotometric methods with the HPLC method and there are virtually no comparisons only between spectrophotometric methods (Martysiak-Żurowska & Borowicz, 2009; Kukurová et al., 2006; Truzzi et al., 2014; Zappalà et al., 2005). In addition, the International Conference on Harmonisation (1995) recommends the use of HPLC, White or Winkler methods for the determination of HMF in bee honey and states that the Winkler method should not be used if other methods are available in the laboratory because of the danger of the use of p-toluidine can provide.

Regardless of the choice of the spectrophotometric method (Winkler or White) for the determination of HMF in bee honeys, in this study, we found statistically no significant differences ($p < 0.05$) when analyzing the ultraviolet and visible regions of the spectrum of each method. However, we observed high HMF values in the Winkler method.

5 Conclusion

The absorbance study in the ultraviolet and visible regions of the spectrum demonstrated stability in the White method. The absorbance study in the visible region of the spectrum demonstrated stability in the Winkler method. Hydroxymethylfurfural (HMF) values were different between the White and Winkler methods. HMF values in bee honey were higher in the Winkler method.

References

Associação Brasileira de Normas Técnicas – ABNT. (2016). *Apicultura – mel. 9: determinação do conteúdo de hidroximetilfurfural por espectrofotometria no UV-Vis* (ABNT-NBR 15714-9). Rio de Janeiro: ABNT.

Association of Official Analytical Chemists – AOAC. (1995). *Official methods of analysis of the Association of Official Analytical Chemists* (method 980.23). Arlington: AOAC.

Bogdanov, S., Martin, P., & Lullman, C. (1997). *Harmonised methods of European Honey Commission. Apidologie*. Paris: International Honey Commission.

Brasil. Ministério da Agricultura, Pecuária e Abastecimento. (1981). *Métodos oficiais para análise de produtos de origem animal* Brasília: MAPA.

European Union. (2001).Council Directive 2001/110/EC of 20 December 2001 relating to honey. *Official Journal of the European Communities*, Bruxelas, 47-52.

Harris, D. C. (2005). *Análise química quantitativa* (6. ed.). Rio de Janeiro: LTC.

Instituto Adolfo Lutz – IAL. (2005). *Métodos físico-químicos para análise de alimentos*. São Paulo: IAL.

International Conference on Harmonisation – ICH. (1995). *Validation of analytical procedures: methodology. Q2B* (CPMP/ICH/281/95). Europe: ICH.

Kukurová, K., Karovičová, J., Greif, G., Kohajdová, Z., & Lehkoživová, J. (2006). Determination of 5-hydroxymethylfurfural after Winkler and by the HPLC method for authentication of honey. *Chemical Papers*, *60*(3), 186-191. http://dx.doi.org/10.2478/s11696-006-0034-8

Martysiak-Żurowska, D., & Borowicz, A. (2009). A comparison of spectrophotometric winkler method and hplc technique for determination of 5-hydroxymethylfurfural in natural honey. *Chemia Analityczna*, *54*, 939-947.

Pascual-Maté, A., Osés, S. M., Fernández-Muiño, M. A., & Sancho, M. T. (2018). Methods of analysis of honey. *Journal of Apicultural Research*, *57*(1), 38-74. http://dx.doi.org/10.1080/00218839.2017.1411178

Shapla, U. M., Solayman, M., Alam, N., Khalil, M. I., & Gan, S. H. (2018). 5-Hydroxymethylfurfural (HMF) levels in honey and other food products: effects on bees and human health. *Chemistry Central Journal*, *12*(1), 35. PMid:29619623. http://dx.doi.org/10.1186/s13065-018-0408-3

Truzzi, C., Illuminati, S., Annibaldi, A., Finale, C., Rossetti, M., & Scarponi, G. (2014). Physicochemical properties of honey from marche, central italy: classification of unifloral and multifloral honeys by multivariate analysis. *Natural Product Communications*, *9*(11), 1595-1602. PMid:25532290. http://dx.doi.org/10.1177/1934578X1400901117

Zappalà, M., Fallico, B., Arena, E., & Verzera, A. (2005). Methods for the determination of HMF in honey: a comparison. *Food Control*, *16*(3), 273-277. http://dx.doi.org/10.1016/j.foodcont.2004.03.006

Funding: None

Received: Nov. 10, 2022; **Accepted:** Apr. 10, 2023

Section Editor: Marta Hiromi Taniwaki.