Hydroxymethylfurfural content of old honey samples – Does the sticky treat really last forever?

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ABSTRACT

Honey is thought to be a food source with indefinite shelf life - this statement is questioned in present work by analysing the 5-hydroxymethylfurfural (HMF) content of a unique series of old honeys from 1959 to 2020. Two special series are included where honey was produced and kept yearly by the same beekeeper for 30 years. Application development was carried out for White method by scaling down the volumes for the analysis. The HMF content of acacia honeys vary in a wide range (9–1320 mg/kg honey), but tendentially increase with age. However, rape and sunflower honeys show a remarkably high offset compared to the expected trendline and there is no observable pattern in their HMF level plotted against the year of collection. Even the youngest rape and sunflower honeys exceeded the threshold limit of 40 mg/kg and only acacia samples collected after 2015 remained below the accepted health value.

1. Introduction

The organic compound 5-hydroxymethylfurfural (HMF) is a cyclic aldehyde that can be formed in different foodstuffs from reducing sugars under acidic condition via the Maillard reaction, as well as a result of caramelisation (Adu et al., 2019; Capuano & Fogliano, 2011; Mehrotra et al., 2022). The natural processes responsible for its presence occur in most sugar-containing food products like breakfast cereals and beverages, and also in all types of honeys (Ball, 2007; Bharate & Bharate, 2014; Lee et al., 2019; Ortu & Caboni, 2018). Its concentration, however, is elevated upon heat processing – which is the most commonly applied treatment in food industry - and unsuitable storage circumstances thus the presence and level of HMF is an important indicator of these foodstuffs (Bodor et al., 2022; Lee et al., 2019). The concentration of this furanic compound is one of the parameters required to be determined routinely from apiarian products to assure their quality. Many adverse health effects are connected to HMF such as cytotoxicity, mutagenicity, carcinogenicity or chromosomal aberrations proved both

towards humans and animals (Alizadeh et al., 2017; Capuano & Fogliano, 2011; Choudhary et al., 2021; Kitts et al., 2012; Monien et al., 2012; Shapla et al., 2018; Zhao et al., 2013). HMF is also harmful for the health of the bees - contact may occur via high-fructose corn or invert sugar syrup given by the beekeeper as winter feed. Elevated concentration of the compound can cause dysentery and intestinal ulceration, as well as can result in the death of the individuals (Shapla et al., 2018).

Due to the negative impacts of ingesting HMF, international standards specify the maximum amount honeys can contain for safe consuming. According to the Codex Alimentarius Commission, limit level of HMF in honey is 40 mg/kg except originating from the tropical region for which 80 mg/kg is set. (Codex Alimentarius, 2019) Interestingly, not only adverse effects are caused by HMF and its derivatives when absorbed by the gastrointestinal tract - antioxidative, -allergic and –inflammatory impacts are also reported, among others (Zhao et al., 2013).Thus, remarkable amount of studies consider the evaluation of the processes in which HMF is formed, its degradation to different compounds and their health related effects (Martins et al., 2022) all

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requiring multifaceted research approach.

Since honey is a concentrated carbohydrate solution containing a mix of simple and complex sugars among water, it is very much affected by HMF formation. The non-enzymatic browning reaction resulting in elevated HMF level can occur during food-processing such as heating or long/inappropriate storage, yet other circumstances can contribute to its concentration (Fallico, Zappalà, Arena, & Verzera et al., 2004; Portillo Perez et al., 2019). The presence of certain acids, minerals, the botanical origin and even the material of the storage container can reasonably affect the extent of HMF formation in honey products (Gidamis et al., 2004; Gökmen & Morales, 2014; Khalil, Sulaiman & Gan et al., 2010). Its level is very low in fresh honeys and is reported to elevate upon aging. High HMF content in honeys may also be a sign of adulteration where its elevated concentration originates from the added invert syrup thus can indicate the artificial feeding of honeybees along with diastase activity and sucrose (Khalil et al., 2010).

The analysis of HMF from different foodstuff is of high interest since more than 200 papers discussed it in the last 20 years in higher impact factor journals based on the Web of Science database (Martins et al., 2022). While in most cases, chromatographic techniques were applied for the quantitative determination, the use of spectrophotometry has notably increased in the last few years due to its practical aspects: easy to use technique, cheaper instrumentation and lower measurement costs. Even the International Honey Commission recommends two spectrophotometric methods for HMF analysis along with the HPLC: determination after White and after Winkler (Zappalà, Fallico, Arena & Verzera et al., 2005). The Winkler method is reported to have the lowest precision of the three and avoidable due to the use of *p-toluidine* which is a carcinogenic substance (Martysiak-Żurowska, 2009). Repeatability and reproducibility of the White method as well as the HPLC technique are also equally better (Bogdanov, 1999).

In the present study, unique series of old honey samples were used for HMF determination that was collected between 1959 and 2020. The elemental concentration of the same samples was determined by microwave plasma atomic emission spectrometry (MP-AES) and their age was verified by radiocarbon dating (accelerated mass spectrometry – AMS) in our earlier studies (Sajtos et al., 2022; Varga et al., 2020). The aim was to prove that old honeys can be used as time capsules to reveal environmental- and apicultural-related composition changes. While the MP-AES results show certain correlation with the ages for specific elements in both rape, sunflower, and acacia honeys, the AMS dating agrees with the expected values only for the acacia samples. HMF concentration is an important quality indicator and no literature data is available regarding such an old series of honeys originating from different species. Moreover, the observed difference in the radiocarbon results without a reasonable explanation raises questions whether the HMF level correlates with the age in all sample types as can be expected. Spectrophotometric method, which is also recommended by the Hungarian Regulation (Codex Alimentarius Hungaricus, 2009), was chosen out of the several methods available to quantitatively measure HMF in honey samples (Martins et al., 2022).

Since only a limited amount of the unique samples was available, method development was necessary to minimize the required honey amount for the analysis by optimizing the volume and concentration of the applied reagents.

The novelty of the study is that it innovatively conducts a longitudinal analysis of HMF in unique, old honey samples from 1959 to 2020, two series of them collected yearly by the same beekeeper, ensuring a consistent source and eliminating location-based variances. The species-specific examination of acacia, rape, and sunflower honeys provides valuable insights into the temporal evolution of HMF thus into the aging process of different honey varieties. Unprecedentedly, this study is the first to measure HMF in honey samples spanning several decades, particularly those provided yearly from the same source. The quantification of HMF increase in honey over a long period of time, from a unique series of old samples with the same source, provides valuable

insights in terms of shelf life determination, quality control, consumer education, market differentiation, regulatory compliance, and further research opportunities. The spectrophotometry method optimized to handle low sample amount and wide range of HMF concentrations can be easily adapted by routine laboratories.

2. Materials and methods

2.1. Honey products

In present study, the HMF content of 82 honeys (acacia, sunflower, rape honeys) was determined. Unique, old samples were received from Hungarian beekeepers and honey museums dated from 1959 to 2020. The botanical and geographical origin along with the year of production are listed in Table 1.

Since nearly the same very special sample series were used previously for elemental analysis and AMS dating purposes (Sajtos et al., 2022; Varga et al., 2020), similar grouping of the samples was applied in present study as in the previous ones.

- *Acacia series:* acacia honeys containing the unique series of samples collected and stored yearly from 1994 to 2020 by the same beekeeper, originating from the same species and area. AMS measurements confirmed their date of origin (Varga et al., 2020).
- *Field crop series:* sunflower and rape honeys from 1996 to 2020 collected and stored yearly by the same beekeeper who provided the *acacia series*. AMS measurements cannot be performed successfully, random offsets from the expected C-14 values were observed (Sajtos et al., 2022).
- *Acacia samples:* old acacia honeys with different geographical origin and collected by different beekeepers (2002–2015).
- *No data samples:* old honeys with known age but unknown botanical and/or geographical origin. They were provided mostly by honey museums of Gemenc and Gödöllő and they are dated between 1959 and 1987.

Samples were kept properly by the professional beekeepers and in the honey museums as defined by the Codex Alimentarius Hungaricus (2009). The same circumstances were used in the laboratory where samples were stored in centrifuge tubes prior to analysis.

2.2. Reagents and instrumentation

All reagents were of analytical grade. For the Carrez I solution, zinc acetate dihydrate and 96% acetic acid were purchased from Scharlab (Debrecen, Hungary). The potassium hexacyanoferrate (II) for the Carrez II solution was from Avantor (Radnor, PA, USA). Sodium bisulfate (Honeywell-Fluka, Charlotte, NC, USA) was freshly made for the determination. Solid HMF standard was used from Sigma Aldrich (Budapest, Hungary). The solutions were prepared with ion-exchanged and ultrafiltered water using Synergy Millipore MilliQ water purification system (Darmstadt, Germany). For the spectrophotometric measurements, Agilent Technologies Cary 60 type photometer was applied (Santa Clara, USA).

2.3. Method optimization

A modified spectrophotometric method of White was applied for the quantitative determination of the HMF concentration in old honey samples (White, 1979). Since a unique sample collection with limited amount of honey was used for this research, the amount of honey recommended by the original method of White had to be reduced. For the optimization, a commercially available honey product (acacia honey) was applied. The original method by White uses 5 g of honey sample which we aimed to reduce and optimize the needed reagents accordingly. Instead of the 50.00 mL of stock solution instructed in White's

Table 1
Year of collection, as well as the botanical and geographical origin of the studied old honey samples.

No.	Year of collection	Geographical origin	Botanical origin
1	1987	Nyírlugos	Acacia
2	1993	Gór	Acacia
3	1994	Gór	Acacia
4	1995	Gór	Acacia
5	1996	Gór	Acacia
6	1996	Gór	Acacia
7	1996	Gór	Acacia
8	1997	Gór	Acacia
9	1998	Gór	Acacia
10	1999	Gór	Acacia
11	1999	Gór	Acacia
12	2000	Gór	Acacia
13	2001	Gór	Acacia
14	2001	Gór	Acacia
15	2001	Mesterháza	Acacia
16	2002	Gór	Acacia
17	2002	Dunavecse	Acacia
18	2002	Dombóvár	Acacia
19	2003	Gór	Acacia
20	2004	Gór	Acacia
21	2004	Zalaegerszeg	Acacia
22	2005	Gór	Acacia
23	2006	Gór	Acacia
24	2006	Gór	Acacia
25	2007	Gór	Acacia
26	2007	Gór	Acacia
27	2007	Tét	Acacia
28	2008	Gór	Acacia
29	2009	Gór	Acacia
30	2009	Gór	Acacia
31	2010	Gór	Acacia
32	2011	Gór	Acacia
33	2012	Gór	Acacia
34	2013	Gór	Acacia
35	2014	Gór	Acacia
36	2015	Gór	Acacia
37	2015	Gór	Acacia
38	2015	Szakcs	Acacia
39	2016	Gór	Acacia
40	2017	Gór	Acacia
41	2017	Gór	Acacia
42	2018	Gór	Acacia
43	2018	Gór	Acacia
44	2019	Gór	Acacia
45	2020	Gór	Acacia
46	1958	Baja	no data
47	1962	Baja	no data
48	1974	Gödöllő	no data
49	1986	Nyírlugos	Rape
50	1997	Mesterháza	Rape
51	2000	Mesterháza	Rape
52	2000	Mesterháza	Rape
53	2001	Gór	Rape
54	2002	Mesterháza	Rape
55	2003	Mesterháza	Rape
56	2004	Mesterháza	Rape
57	2004	Mesterháza	Rape
58	2005	Mesterháza	Rape
59	2007	Mesterháza	Rape
60	2008	Mesterháza	Rape
61	2010	Mesterháza	Rape
62	2014	Mesterháza	Rape
63	2015	Mesterháza	Rape
64	2016	Mesterháza	Rape
65	2017	Mesterháza	Rape
66	2017	Mesterháza	Rape
67	2018	Mesterháza	Rape
68	2018	Mesterháza	Rape
69	2019	Mesterháza	Rape
70	2020	Mesterháza	Rape
71	1985	Nyírlugos	Sunflower
72	1996	Mesterháza	Sunflower
73	1996	Mesterháza	Sunflower

Table 1 (*continued*)

No.	Year of collection	Geographical origin	Botanical origin
74	1998	Mesterháza	Sunflower
75	1999	Mesterháza	Sunflower
76	2006	Mesterháza	Sunflower
77	2008	Mesterháza	Sunflower
78	2009	Mesterháza	Sunflower
79	2011	Mesterháza	Sunflower
80	2013	Mesterháza	Sunflower
81	2012	Mesterháza	Sunflower-honeydew
82	2012	Mesterháza	Sunflower-honeydew

description, smaller volumes (5.00 or 10.00 mL) were used to avoid the dilution of the samples. In addition, after filtration, the spectrophotometric measurement of the sample was not carried out in a 1:1 volumetric ratio, instead, a more concentrated sulfite reagent was added. Therefore, a smaller dilution of the analyte was achieved.

Each time, at least two parallel measurements were performed and the result was accepted if the standard deviation of the two analysis data fell within 5%. For method validation, HMF solution of known concentration was prepared from solid chemical and dilution series was used to determine its molar absorptivity. The effect of different sample amounts (5.0 g, 2.5 g and 0.5 g) after reagent optimization on the quantitative determination was tested by one-way analysis of variances (ANOVA) performed in IBM SpSS software package. Levene's test was used for confirming the homogeneity of variances within groups and Tukey Multiple Comparisons test was applied to evaluate differences. Significant difference was declared when the p value was <0.05.

2.4. HMF determination by spectrophotometric method

Honey samples were thoroughly homogenized without heating by a plastic stirring rod prior to the sample preparation. Analysis was carried out in a completely randomized design and since the age of the studied honey samples exceeded 4 decades, the HMF content significantly differed in the younger and older ones requiring the dynamic change of the measurement conditions (e.g. volume and concentration of stock solutions, ratios of the volumes of the reagents), especially the added sulfite concentration within the analysis.

According to the original method, a filtered honey solution without bisulfite is used as the reference and the same honey solution treated with 0.1% of sodium bisulfite served used as the sample. The difference in the spectra is due to addition reaction between bisulfite and the HMF present in the honey solution.

For the quantitative HMF determination, the absorbance data measured at 284 nm and 336 nm were used and the final results were calculated based on the following expressions:

$$\Delta A_{284} = A_{284,\text{ref}} - A_{284,\text{sample}} \qquad \text{(eq. 1)}$$

$$\Delta A_{336} = A_{336,\text{ref}} - A_{336,\text{sample}} \qquad \text{(eq. 2)}$$

$$A_{\text{corr}} = \Delta A_{284} - \Delta A_{336} \qquad \text{(eq. 3)}$$

$$c_{\text{cuvette}} = \frac{A_{corr}}{\varepsilon \times d} \qquad \text{(eq. 4)}$$

where $\varepsilon = 16830 \frac{1}{Mcm}$ and $d = 1.000$ cm.

$$c_{\text{stock}} = \frac{c_{\text{cuvette}} \times V_{\text{cuvette}}}{V_{stock}} \qquad \text{(eq. 5)}$$

where V_{stock} is the volume of the stock solution pipetted to the cuvette.

$$m_{\text{HMF}} = n_{\text{HMF}} \times M_{\text{HMF}} = c_{\text{stock}} \times V_{\text{tot}} \times M_{\text{HMF}} \qquad \text{(eq. 6)}$$

where V_{tot} is the total volume of the stock solution prepared and $M_{\text{HMF}} = 126.11$ g/mol.

$$c_{HMF} = \frac{m_{HMF} \; (mg)}{m_{honey} \; (kg)} \qquad (eq. \; 7)$$

Pearson correlation was used for evaluating the effect of honey age on the HMF concentration performed in PAST (Windows) statistical software package.

3. Results and discussion

3.1. Method development to downscale the required sample volume for the spectrophotometric analysis

From commercially available solid HMF, a dilution series was prepared and the absorbance values of the solutions were determined at 284 nm. The absorbance-concentration data pairs are plotted in Fig. 1A. From the slope of the straight line crossing the origin, the molar absorbance of the HMF was determined ($\mathcal{E} = (163 \pm 3) \times 10^2 \; dm^3/(mol \times cm)$) and a good agreement with the literature value was found ($16830 \; dm^3/(mol \times cm)$) (White, 1979).

White mentioned in this article that the precise quantitative determination is aggravated by the fact that bisulfite absorbs in the 284 nm region too. The obvious, yet rarely emphasized conclusion can be drawn that the bisulfite concentration in the cuvette may affect the quantitative results. If chosen to be lower than optimal, the 284 nm band's decrease will not be complete, thus the smaller change in the absorbance will result in the under-estimation of the HMF concentration. On the other, too high bisulfite concentration in the mixture will also cause an apparently lower calculated HMF level due to the own absorbance of the HSO_3^- at 284 nm (i.e. again the decrease in the absorbance is smaller

than expected). This issue is not further discussed in White's paper and rarely appears in later publications working with his method since the 0.1% bisulfite concentration chosen in the original description "provides an acceptable compromise between effectiveness and interference". Only a few authors attempted to solve the problem (Bogdanov, 1999) and more examples can be found regarding the opposite - that is to leave the issue out of consideration (Hoseney, 1984; Pasias et al., 2017). When the question of the measurement is to determine if the HMF is within the health limit but the exact value is not necessarily the purpose of the analysis, optimizing of the bisulfite concentration from sample to sample might not be reasonable. In such case, higher measurement error can be accepted (10–20%) except for those samples close to the threshold. However, when the goal is to recognise exact trends in the concentration, precise analysis cannot be avoided.

The effect of the bisulfite concentration observed in present study is shown in Fig. 1B. In this experiment, the same stock of honey sample was used, but after the filtration, mixtures with different amounts of bisulfite were analysed by spectrophotometry. The points show the apparent HMF content of the honey calculated by using eqs (1)–(7). Dots are connected with a third degree polynomial equation. Such fitting has no theoretical basis, yet leads the eye in estimating the optimal HSO_3^- concentration. As an example, the average of the three apparent HMF concentration values highlighted in the blue circle is 211 ± 1 mg/kg in the honey for which the optimal $c \; (HSO_3^-)$ is between 12 and 30 mmol/L. If the accepted deviation is ~5% then at this given HMF content, the bisulfite concentration of 5–40 mmol/L will not result in notable interference and measurement error. This seems to be a relatively wide range but in a series of experiment where the age of the honey samples embraces 4 decades, 2-3 orders of magnitude differences can occur in the
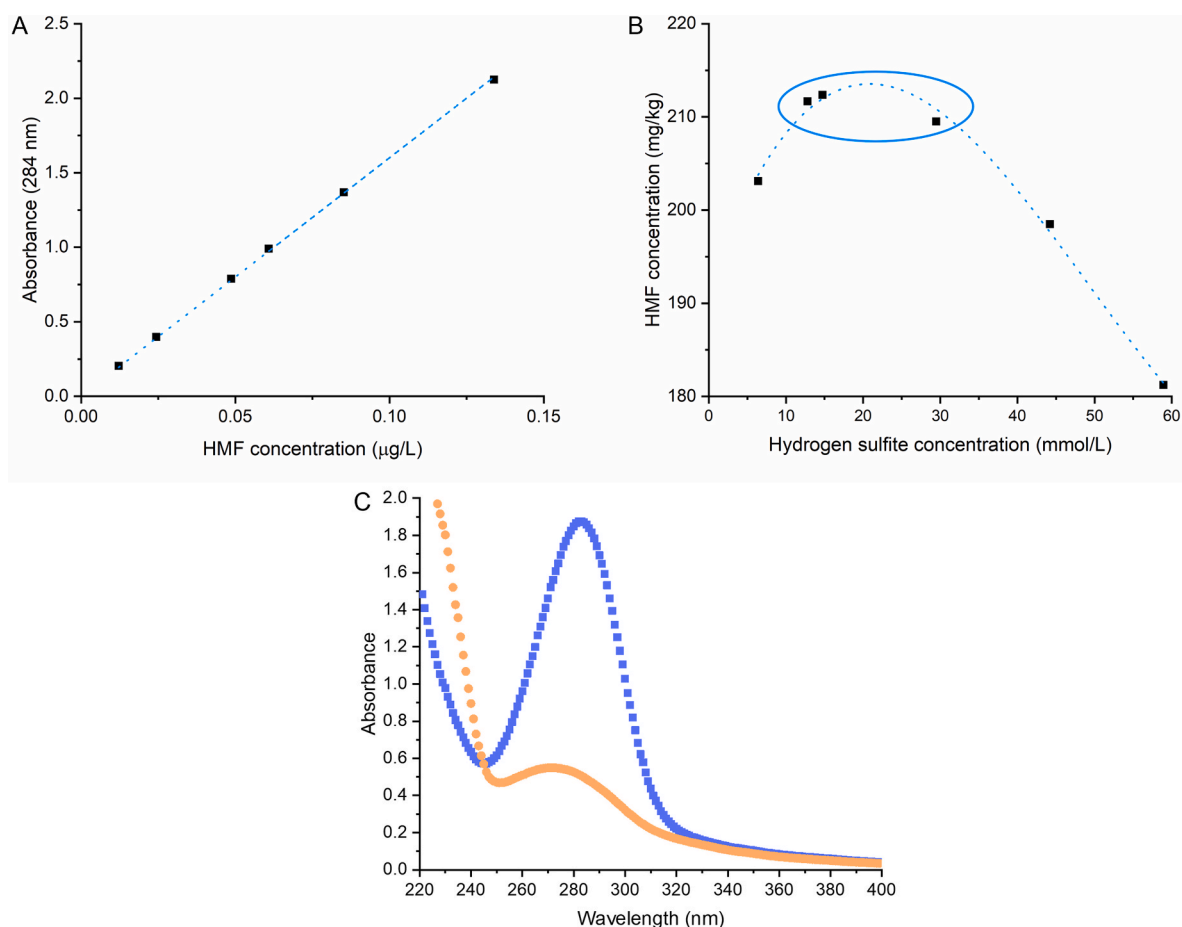

Fig. 1. HMF calibration at 284 nm (**A**) and the effect of the bisulfite concentration on the HMF determination (**B–C**). Legends (**C**): HMF (blue dots); HMF + Bisulfite (orange dots). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

expected HMF levels. In such case, a more prudent work is required since age dependent trends are to be explored. Thus, in the present work, the spectrophotometric determination of the HMF content was carried out by using at least 3 different bisulfite concentration in the mixture in order to gain a more reliable result. If the apparent concentration did not follow the maximum curve as indicated in Fig. 1B, additional mixtures were measured. The HMF stock solution was also used as standard solution during the measurements and its concentration was determined <3% RSD.

While in routine measurements, usually enough sample is available for the determination, in the present work, the considered special old honeys are precious enough to decrease the sample amount as much as possible.

HMF results of the same commercially available honey from three different sample amounts and optimized sulfite concentration is indicated in Fig. 2. No statistical difference based on ANOVA ($p < 0.05$) occurred between the HMF results using the original 5.0 g, and the lowered 2.5 g and 0.5 g of honey sample when the used reagents were optimized accordingly. Since the down-scaling gave identical results, one tenth of White's amount (0.5 g of honey) was used for further analysis along with the proportionally smaller volumes of the Carrez I and Carrez II solutions. The optimal bisulfite concentration was sought for every sample i.e. at least three different mixtures were used to find the maximum of the c (HMF)-c (HSO_3^-) curve (Fig. 1B).

In a typical analysis, 0.1 mL Carrez I. and 0.1 mL Carrez II. reagents were added to 0.5 g of honey sample, and the volume was completed up to 10 mL in a volumetric flask. This mixture was filtered by filter paper and the filtrate was either treated directly with bisulfite or deionized water was also added for dilution (if the measured absorbance was too large). The reaction with bisulfite occurred in the cuvette and the optimal sulfite concentration was sought by adding the reagent in a stepwise manner (Fig. 3). The volume of the bisulfite was increased until the total volume of the cuvette enabled us to do so. Thus, the effect of typically 5 different bisulfite concentrations on the same stock solution was studied without the need of preparing a new sample. In this way a fast, simple, cheap and reasonably precise method was developed for the routine analysis of honeys with highly altering HMF levels.
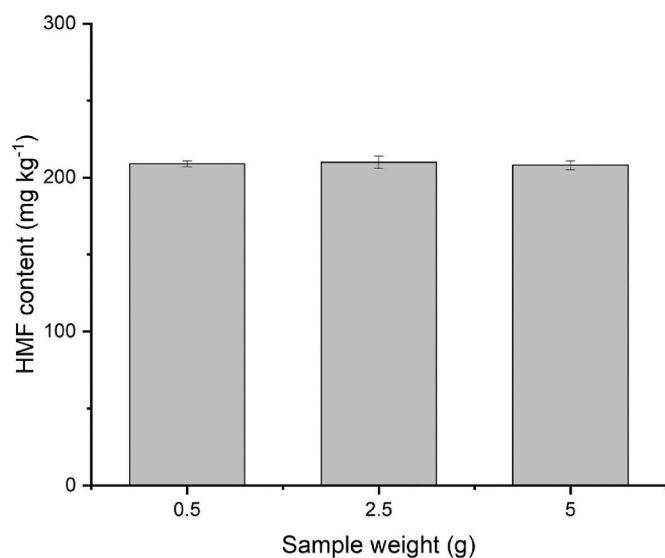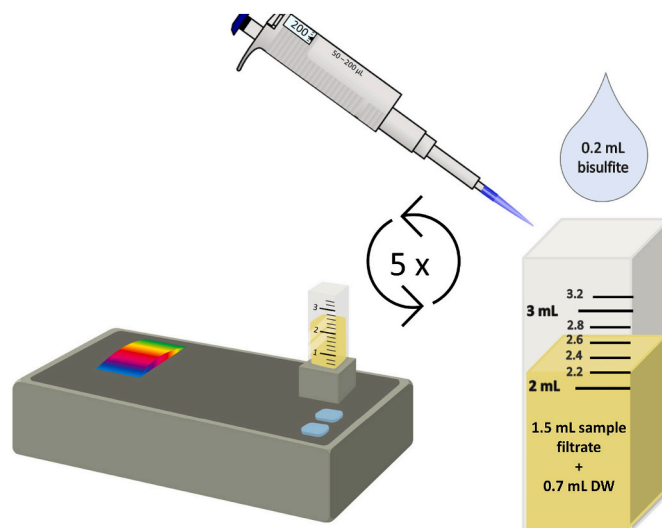

Fig. 3. *One-cuvette* method for the HMF determination of honey samples by using increasing bisulfite concentrations (sample filtrate: to 0.5 g of honey sample 0.1 ml Carrez I. and 0.1 ml Carrez II. solutions were added and completed up to 10 ml then filtered; DW: deionized and ultrafiltered water using Synergy Millipore MilliQ purification system).

3.2. HMF concentration of the unique old sample of honeys

The HMF content of the honey samples from the last 30 years have been quantified with the method optimized for the unique feature of present study: low sample amount and wide range of HMF concentrations.

For the acacia honeys, two sample types were studied: the *acacia series* with the same botanical and geographical origin, collected, processed and stored yearly by the same beekeeper and *acacia samples* with the same botanical but different geographical origin received from different beekeepers. As indicated in Fig. 4A and also expected based on literature data, the HMF content of the *acacia series* shows a decreasing trend with the year of collection (Pearson correlation r = −0.95972). The older honeys clearly have HMF levels that range from 431 to 9.31 mg/kg (samples from 1999 to 2020, respectively.) This observation confirms that aging is a very important factor in HMF formation. In this unique series of honeys, the effects of botanical and geographical aspects as well as the production and storage conditions can be excluded. This is the first study to show this finding on such a long time-scale. Also it is interesting to see that only acacia honeys collected after 2015 meet the health requirements for HMF and are within the threshold value of 40 mg/kg (Codex Alimentarius, 2019) Thus, acacia honeys older than 5 years might not be safe to consume considering the level of the studied substance.

A few honeys (indicated as *acacia samples* in Fig. 4C) were received from other nectar producing parts of Hungary collected by different apiarian specialists (2002–2015). The same trend was observed in their HMF concentration that it significantly decreased with their age (197–67.0 mg/kg, 2002–2015, Pearson correlation coefficient r = −0.98018). Thus, for the acacia honeys, the HMF concentration pattern depending on the year of collection was neither significantly influenced by the geography of their origin nor the beekeeper responsible for the handling and storage. In case of the *acacia samples,* the honey originated from 2015 contained HMF in a level (67.0 mg/kg) higher than the officially set health value. (Codex Alimentarius, 2019) The HMF results of these samples also confirm our previous statement that after 5–6 years of proper storage the acacia honeys will reach the consumable limit for HMF concentration.

Similarly to acacia honeys, the *field crop series* of sunflower and rape samples from 1996 to 2020 were yearly collected and stored by the same

Fig. 2. The effect of different sample amounts on the quantitative determination of HMF from honey products with optimized reagent volumes. White et al. recommended 5 g of honey, (White, 1979) in the present work, the conditions of down-scaling were optimized.
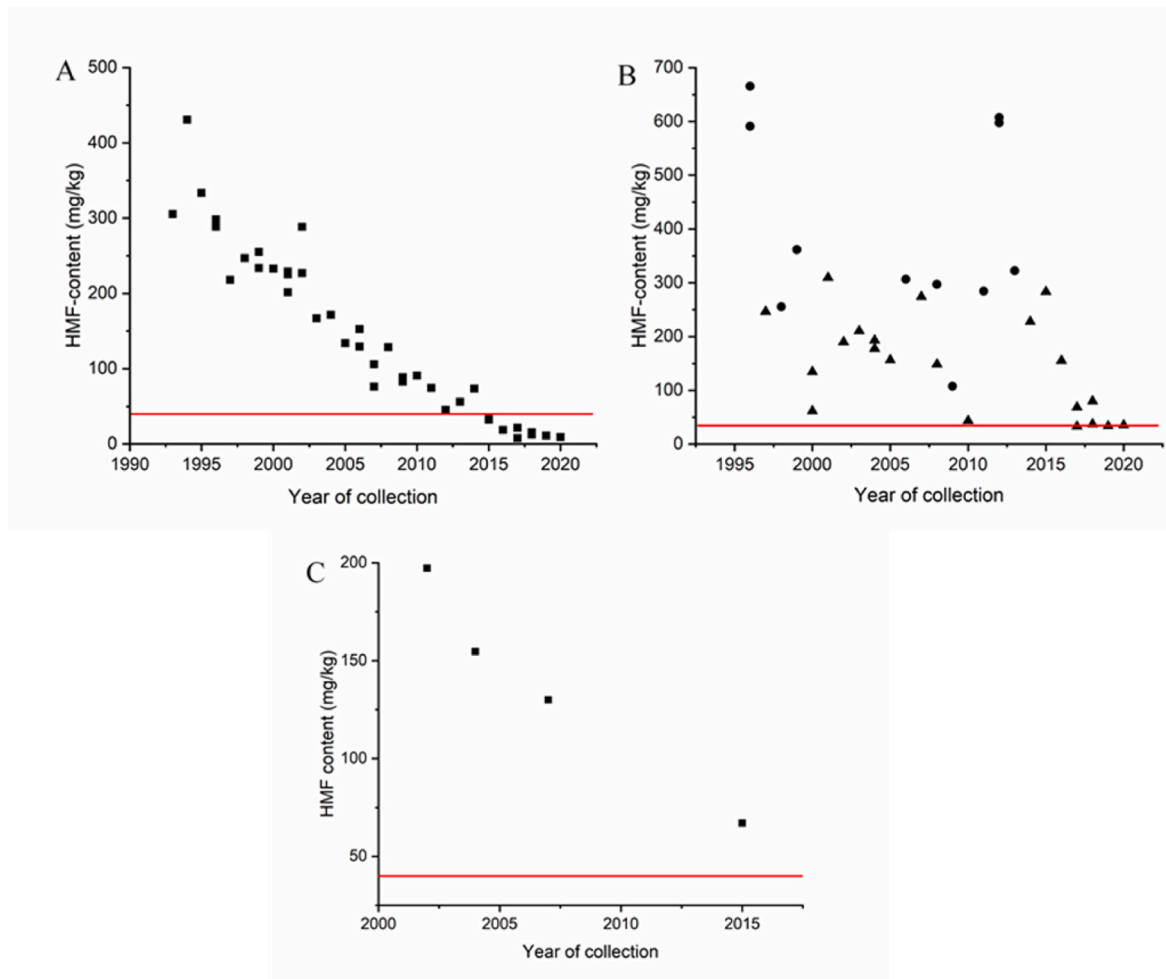
Fig. 4. HMF concentration of the *acacia series* (**A**) (acacia honeys containing the unique series of honeys collected and stored yearly from 1994 to 2020 by the same beekeeper, from the same species and area), HMF concentration of the *field crop series* (**B**) (rape and sunflower honeys collected and stored yearly from 1995 to 2020 by the same beekeeper from the same area) and HMF level of *acacia samples* (**C**) (old acacia honeys with different geographical origin and collected by different beekeepers). Red line indicates the health risk limit of 40 mg/kg (*Codex Alimentarius*, 2019). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

beekeeper (who provided the *acacia series*) thus their geographical origin, type of handling and storage were similar. Surprisingly, no tendency in the HMF level plotted against the age can be observed – the dots are spread without a pattern in Fig. 4B. (Pearson correlation coefficient r = −0.56632) Even the most recent samples produced after 2015 have HMF content close to the health limit (rape honey from 2020: 35.5 mg/kg) and some samples after 2010 have HMF values excessively higher (rape honey from 2014: 284 mg/kg). None of the samples had HMF level well below the threshold value of 40 mg kg^{-1} making their frequent consumption a health concern. This result is in accord with previous findings suggesting to consume honey within six months to one year of production regardless of honey type (Khalil et al., 2010).

The unexpected difference in the observed trends regarding the correlation of HMF level with the age between the *acacia* and *field crop series* of honey samples requires further analysis to interpret since literature data suggest that storage time is the most important factor affecting HMF level in honey products (Khalil et al., 2010). It was reported that the HMF content is strongly related to conditions such as the temperature: its level in fresh honey is very small but can elevate rapidly if the temperature is above 20 °C. However, the two studied series of honeys in the present work did not differ in storage conditions. Honey samples of 4 years old contained an average 52% higher HMF than the fresh ones in the study of Kesic et al. Other factors can also highly influence the HMF level in honeys such as the botanical origin. In the same

paper, acacia was reported to have the highest HMF concentration among the studied honeys while chestnut samples had a very low level. In our work, the HMF content of sunflower honeys are tendentially higher than acacia honeys of the similar age both from the same origin.

It was published by Fallico et al. that chestnut honey, for example, did not produce a measurable amount of HMF upon a 7 days of continuous heat treatment at 50 °C, while orange honey under the same circumstances exceeded the health limit after 4 days. The authors explained the observation by pH difference: at lower temperatures (<50 °C), the initial acidity of the honey products strongly affected the HMF formation while at higher temperatures (>100 °C), no species-related differences can be observed and only the time of treatment defined the HMF concentration. Chestnut honey, with the highest pH of the studied samples did not produce HMF after a prolonged period of moderate heating and this phenomenon was confirmed later in the literature (Chis & Purcarea, 2011). Species related difference can also cause the remarkable tendency difference in the HMF pattern of acacia and field crop samples found in our study. Not only the acidity but the glucose/fructose (G/F) ratio is also a factor to affect HMF formation which is strongly related to the botanical origin of the nectar producing plant. Since HMF is synthetized by the dehydration of fructose, a negative correlation is found with the HMF concentration and the G/F ratio in honeys by Kesic et al. HMF concentration is strongly related to the presence and level of the precursor molecules taking part in its

formation (Anese & Suman, 2013). Some of these are present naturally and are dependent on the floral type and geographical source such as the pH, total acidity and mineral content or meteorological aspects as humidity (Anam & Dart, 1995; Spano et al., 2006). The difference in HMF content between the *acacia* and *field crop series* may be derived from the different nectar producing origin but that does not explain why no exponential trend with age is observed in the latter group of honeys or the outlier values in the younger samples of the *field crop series*.

Another important parameter that may have an effect on the HMF content of the studied sample series is the tendency for crystallisation and the related handling. While crystallisation is a natural process in apiarian products, its rate depends mostly on the chemical composition of honeys. The G/F ratio is among the most important features: the greater the amount of the less water soluble glucose, the faster crystals form. (Assil, Sterling, & Sprons, 1991) Thus, high G/F and high glucose to water ratio honeys crystallise rapidly. Rape and sunflower samples belong to this group and they may grow crystals as early as 1–2 months (Amariei, Norocel, & Scripcă et al., 2020). In contrast, honeys naturally containing higher level of the more water soluble fructose (such as acacia honeys) tend to crystallise much slower. If processed and stored properly, crystallisation might be avoided completely (Conforti et al., 2007). The observable changes due to the crystals formed in apiarian products, such as colour and texture, makes crystallised honeys less favourable (Alias et al., 2018). Not only the market potential is lower of those products being crystallised but their processing is more difficult due to their higher viscosity. In order to make transferring and bottling easier, these honeys are often heat-treated to liquify them prior to further handling (Pasias et al., 2022). If the *field crop series* studied were randomly heated during the years to make sampling easier that could result in the observed scattering in **4B**. It should be noted that in a recent project of our group, the elemental composition along with the C-14 concentration of old honeys was investigated (Varga et al., 2020). The AMS data of the two series of samples showed distinctive patterns similar to the findings of the present work. In the *acacia series*, the AMS measurements confirmed the age of the honey products, as the results correlated with the atmospheric radiocarbon bomb-peak. Therefore, it is reasonable to conclude that acacia honeys are suitable materials for radiocarbon dating (Varga et al., 2020). However, the rape and sunflower samples originating from the same beekeeper proved to be less reliable materials for radiocarbon dating compared to acacia honeys as random offsets were observed in the specific radiocarbon activity (Sajtos et al., 2022). The difference in the AMS results of the acacia honeys and the rape/sunflower samples is proposed to be species related suggesting that non-photosynthetic substances present in the nectar of annual crops can shift C-14 values. The observations found in the two studies (i.e. radiocarbon dating and HMF) show similar patterns: acacia honeys gave results in accordance with the expectations, while rape and sunflower samples showed unexpected C-14 values and HMF levels. Heat treatment of honeys to prevent crystallisation should not interfere with AMS dating. Therefore, the answer could lie in the botanical origin. Species-related difference in the precursor molecules present in crops might differ annually, consequently, it can be responsible for the different rate of HMF formation over the years. However, further research is required to confirm or discard this assumption.

Sunflower honeydews from 2012 showed significantly higher concentrations of HMF (608 mg/kg and 598 mg/kg) than sunflower honeys collected in the previous and in the following year (285 mg/kg and 323 mg/kg, respectively). Fresh honeydew honeys are usually reported to have lower than 10 mg/kg HMF concentration (Seraglio et al., 2019) yet Kurtagic et al. found honeydew samples to have relatively high HMF content originating from fresh collection in Bosnia and Herzegovina.

Some very old honeys were received by our laboratory from different nectar producing plants and areas, each collected by different beekeepers. Since such apiarian products are very rare, their HMF content is also included in the present work – however, limited information is known about them. As seen is Table 2, the HMF content of these old honeys are high, but not in correlation with their age. The oldest acacia samples have lower HMF content (696 mg/kg from 1959 to 884 mg/kg from 1962) while relatively younger honeys of sunflower and rape have significantly higher HMF values (1272 mg/kg from 1985 to 1286 mg/kg from 1986, respectively).

Table 2
The HMF concentration of very old honey samples from 1959 to 1987 collected by different beekeepers at different nectar producing sites and from different species (SD indicated in parentheses).

Year of collection	Botanical origin	Geographical origin	HMF-content (mg/kg)
1959	Acacia	Baja	696 (10.6)
1962	No data	Baja	884 (33.9)
1963	No data	Baja	931 (8.5)
1974	No data	Gödöllő	1213 (7.1)
1985	Sunflower	Nyírlugos	1272 (51.6)
1986	Rape	Nyírlugos	1286 (13.4)
1987	Acacia	Nyírlugos	739 (24)

4. Conclusion

Honey is often described to have eternal shelf life and stay edible after long years of proper storage - such as the "liquid gold" found in Egyptian tombs after 3000 years that are claimed to be perfectly consumable. Present study is the first to consider the HMF content of old honey series from the last decades collected yearly by the same beekeeper. The HMF concentration of acacia honeys significantly increases with age - results were excellently approximated by a quadratic equation. In contrast, the HMF content of rape and sunflower honeys does not correlate with the year of collection showing remarkably high concentration even in the more recent samples. To decide if the difference in the HMF pattern is caused by heat treatment over the years in order to re-dissolve crystals for easier handling or species-related alterations can be assumed further investigations are required. Nevertheless, none of the old honeys studied can be considered edible based solely on the HMF level except for acacia honeys collected after 2015. The optimized method of White presented in our study provides a simple and cheap solution for the quantitative determination of HMF in a wide concentration range even if only a limited amount sample is available.

CRediT authorship contribution statement

Zsófi Sajtos: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Ágota Zsófia Ragyák:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Fruzsina Hódi:** Software, Formal analysis. **Viktória Szigeti:** Software, Formal analysis. **Gábor Bellér:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Edina Baranyai:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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