



Influence of proline and methylglyoxal on the formation of 5-hydroxymethylfurfural in manuka honey

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Abstract

Manuka honey, originating from the nectar of *Leptospermum scoparium*, is known for its antibacterial activity, which is due to the presence of methylglyoxal (MGO). As every honey, manuka honey has to fulfil legal requirements, e.g. must not be heat treated in a way that affects composition or quality changes and may not contain more than 40 mg/kg 5-hydroxymethylfurfural (HMF). To study the influences of MGO and proline as the most abundant amino acid in honey on the HMF formation, artificial honeys with up to 500 mg/kg MGO and up to 1000 mg/kg proline, as well as authentic honeys spiked with MGO, have been stored for 10 weeks at 37 °C and the formation of HMF and its precursor 3-deoxyglucosone (3-DG) have been analysed via HPLC-UV. For both MGO-spiked authentic honeys and artificial honeys, the content of HMF increased to significantly higher concentrations, depending of the added amount of MGO. Higher MGO levels induced an increased HMF formation due to higher 3-DG levels present in the honey. The addition of proline led to the contrary effect. Lower HMF levels were observed, although an addition of proline induced higher 3-DG levels, indicating a scavenging effect on the conversion of 3-DG to HMF. Based on these findings, it seems appropriate to review the current limit of 40 mg/kg for MGO-containing manuka honey.

Keywords Manuka honey · Methylglyoxal · Proline · 5-hydroxymethylfurfural (HMF) · Honey authenticity

Abbreviations

2-AP	2-acetylpyrroline
3-DG	3-deoxyglucosone
3,4-DGE	3,4-dideoxyglucosone-3-ene
DHA	Dihydroxyacetone
HMF	5-hydroxymethylfurfural
MGO	Methylglyoxal

Introduction

Manuka honey, derived from the nectar of *Leptospermum scoparium*, provides a significant non-peroxide antibacterial activity which is mainly due to the presence of

methylglyoxal (MGO) and other synergistically active compounds [1, 2]. It is noteworthy that the MGO contents in manuka honey, reaching levels up to 1541 mg/kg [3, 4], are about 100-fold higher than the MGO contents in non-manuka honeys, which may only contain up to 24 mg/kg [5]. The elevated MGO levels in manuka honey originate from dihydroxyacetone (DHA) in the nectar, which undergoes a non-enzymatic dehydration to form MGO during the process of maturation and storage [6].

In general, honeys are not allowed to be heated such that enzymes are partially or completely inactivated. Therefore, Codex Alimentarius and several national legal regulations contain requirements to prove that the honey was not heat-treated. The content of 5-hydroxymethylfurfural (HMF) must not exceed a threshold of 40 mg/kg, or 80 mg/kg for selected tropical honeys [6, 7].

As HMF is formed during caramelization and Maillard reaction, and due to the low water activity and high content of reducing sugars in honey, this food matrix provides excellent reaction conditions for HMF formation [9]. The precursor of HMF is 3-deoxyglucosone (3-DG), which is formed during caramelization from enolization and

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dehydration reactions from reducing sugars as glucose or fructose, or during Maillard reaction after the cleavage of Amadori and Heyns products, resulting from the reaction of glucose and fructose, respectively, with free amino acids [10]. Especially at elevated temperatures and under acidic conditions, 3-DG dehydrates to 3,4-dideoxyglucosone-3-ene (3,4-DGE) which then forms HMF after a cyclisation and subsequent dehydration (see Fig. 1) [11].

In contrast to the legal regulations, first studies and anecdotal evidence indicate that manuka honeys may contain HMF contents higher than 40 mg/kg without being heated [12–14]. However, specific influencing parameters are unclear until now, beside suggestions that the water content, the pH value and the Ca^{2+} , K^{+} and other metal ion content may influence the concentration of HMF in honey [15, 16].

Therefore, it is the aim of this study to elucidate a possible influence of MGO on the formation of HMF in order to evaluate whether the strict HMF limit of 40 mg/kg for manuka honey can continue to be maintained.

Materials and methods

Chemicals and reagents

Methanol (LC-MS grade) was purchased from VWR (Darmstadt, Germany). Methylglyoxal and ortho-phenylenediamine were obtained from Sigma Aldrich/Merck (Steinheim, Germany). 5-Hydroxymethylfurfural was purchased from Tokyo Chemicals Industries (Eschborn, Germany). Hydrochloric acid, ammonia, sodium hydroxide and AmberChrom 50WX8 resin were obtained from Fisher Scientific (Schwerte, Germany). Sodium dihydrogenphosphate and disodium hydrogenphosphate were purchased from Grüssing (Filsum, Germany). Fructose, glucose, sucrose, gluconic acid and proline were obtained from Carl Roth (Karlsruhe, Germany). All lithium citrate buffers for amino acid analyses were obtained from Sykam (Fürstfeldbruck, Germany). The standard of 3-deoxyglucosone was

synthesised in our laboratory according to [17]. Identity and purity were checked via HPLC-UV. For HPLC solvents double-distilled water (Bi 18E double distillation system, QCS, Maintal, Germany) was used.

Honey samples

For the storage experiment concerning influence of MGO on HMF formation commercially available robinia, linden, rapeseed and kanuka honey have been used. Furthermore, to exclude matrix effects from other compounds present in honey, artificial honey was prepared according to literature [18]. Therefore, for the preparation of 100 g artificial honey, 44 g of fructose, 37 g of glucose and 2 g of sucrose were dissolved in 17 g water. The pH value of the water was previously adjusted to 5 with gluconic acid. The suspension was stirred and heated to 45 °C until complete dissolving of the sugars. For the incubation experiments, the authentic honeys were spiked with MGO solution to a concentration of 500 mg/kg after checking their initial MGO contents with HPLC-UV (see 2.3). The artificial honeys were spiked with MGO in concentrations between 0 and 500 mg/kg, with proline in concentrations between 0 and 1000 mg/kg or with both. All honeys were stored for 10 weeks at 37 °C to simulate an accelerated storage and sampled subsequently. The samples have been stored until analysis at 7 °C.

Quantitation of 3-DG and MGO in honey samples

The quantitation of dicarbonyl compounds was performed according to literature with slight modifications [6]. Therefore, 500 µL of a 10% (w/v) honey solution in phosphate buffer, pH 6.5, were incubated with 150 µL phosphate buffer and 150 µL 1% (w/v) ortho-phenylenediamine solution in phosphate buffer over night in the dark at room temperature. After membrane filtration (0.45 µm, regenerated cellulose), measurements were performed using HPLC-UV. A system containing a pump with included online-degasser and mixing chamber P 6.1 L, an autosampler AS 6.1 L, a

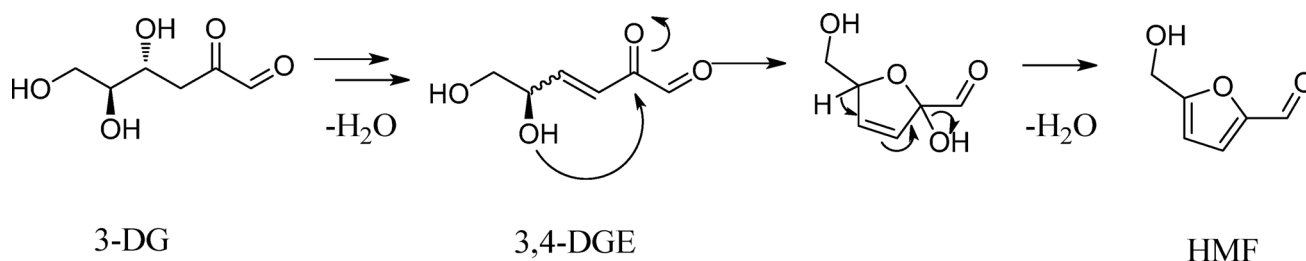


Fig. 1 Pathway of the formation mechanism of HMF

thermostat CT 2.1 and a diodearray detector DAD 2.1 L, all from Knauer (Berlin, Germany), was used for separation and detection. The separation was accomplished on a 250×4.6 mm Eurospher C-18 column with 5 µm particle size and integrated precolumn (Knauer, Berlin, Germany) as the stationary phase and a mobile phase containing 0.075% (v/v) acetic acid in water as solvent A and a mixture of 20% solvent A and 80% methanol as solvent B. A flow of 0.9 mL/min at 30 °C was used. After 1 min of the measurement, the composition of the mobile phase was changed from 40% B to 100% B within 20 min, changed back to the initial conditions in 4 min and was held there for further 5 min. 20 µL of the derivatized sample has been injected into the system. Quantification of the resulting quinoxalines was achieved at a wavelength of 312 nm with an external calibration with commercial MGO solution in concentrations between 0.6 and 30 µg/mL and solutions of 3-DG in concentrations between 0.81 and 16.2 µg/mL. All measurements have been performed in triplicates. The data for 3-DG were analysed using analysis of variance (ANOVA, Origin Pro 2021b) to determine different in the 3-DG content of the samples after incubation.

Quantitation of 5-hydroxymethylfurfural in honey samples

The quantitation of 5-hydroxymethylfurfural was performed according to literature with slight modifications [19]. Briefly, after membrane filtration (0.45 µm, regenerated cellulose) 20 µL of a 10% (w/v) honey solution in phosphate buffer, pH 6.5, were injected into a HPLC-UV system with a C-18 column (see 2.3). For the mobile phase an isocratic mixture containing 84% 50 mM phosphate buffer, pH 5.5, and 16% methanol. A flow rate of 0.8 mL/min was used. The signals were evaluated at 284 nm. Quantification was achieved using an external calibration of HMF in concentrations between 0.25 and 10 µg/mL. All measurements have been performed in triplicates. The data were analysed using analysis of variance (ANOVA, Origin Pro 2021b) to determine different in the HMF content of the samples after incubation.

Determination of free proline in honeys

For the quantification of free proline in honeys, cation exchange chromatography and amino acid analysis were performed according to literature with slight changes [20, 21]. In brief, 10 g AmberChrom 50WX8 resin was filled in a chromatography column with 1 cm diameter after activation

with 50 mL 2 M hydrochloric acid. Then, the resin was neutralized with ultrapure water and 10 mL of a 10% (w/v) sample solution in 0.1 M phosphate buffer, pH 2.1, was applied on the column. For removal of the sample matrix, the column was washed with 200 mL ultrapure water. The amino acids were slowly eluted with 150 mL 7 M ammonia solution and the resin was regenerated by washing with 50 mL 2 M sodium hydroxide solution, neutralizing with ultrapure water and washing with 50 mL 2 M hydrochloric acid.

After removing the solvent of the eluate containing the amino acids in the rotary evaporator, the residue was reconstituted in 10 mL ultrapure water, lyophilized and dissolved in 1 mL 0.12 M lithium citrate buffer, pH 2.2. The proline content was determined by injecting 90 µL of the solution into the amino acid analyser S4300 and analysing on a Cation Separation Column (LCA K07/Li 4.6×250 mm), all from Sykam (Fürstfeldbruck, Germany), according to the instructions of the manufacturer. The ninhydrin derivatives were detected at 440 nm. All measurements have been performed in triplicates.

Statistical treatment

To compare the means of datasets a one-way ANOVA with a Tukey posthoc test was used. All statistical analyses were performed using the software Origin 2021b.

Results and discussion

Formation of HMF in manuka honeys and MGO-containing model honeys

To evaluate the potential influence of MGO on the formation of HMF in authentic honey samples, robinia honey, linden honey, rapeseed honey and kanuka honey were spiked with MGO to a concentration of 500 mg/kg. The MGO content of the honeys before and after MGO addition was checked using HPLC-UV (see 2.3). The unspiked and spiked samples have been stored at 37 °C for 10 weeks to simulate a forced storage and to accelerate HMF formation. The storage temperature was based on the highest possible temperature to which honey is naturally exposed, in particular the temperature in the bee hives [22]. After the incubation, the contents of HMF have been compared between the samples without and with MGO addition (see Table 1).

Generally, it can be seen that the HMF content in all honey samples was below 40 mg/kg before the incubation and, therefore, fulfilled the legal threshold, though the HMF

Table 1 Contents of HMF in authentic honey samples without and with the addition of MGO to 500 mg/kg before and after incubation for 10 weeks at 37 °C, measurements were carried out in triplicates, measurement uncertainties show standard deviation

	HMF before incubation [mg/kg]	HMF after incubation, without added MGO [mg/kg]	HMF after incubation, with added MGO [mg/kg]	Increase of HMF formation by MGO [%]
Robinia honey	1.1±0.2	10.2±0.9	19.4±0.7	90
Linden honey	5.2±0.3	41.9±0.5	57.5±1.6	37
Rapeseed honey	1.9±0.1	6.1±0.4	13.8±3.2	126
Kanuka honey	16.0±0.2	35.5±1.8	55.8±4.4	57

content in Kanuka honey was slightly elevated. Additionally, it can be seen that the addition of MGO to the honey samples increased the HMF formation in all honeys during the storage. Although all honeys contained the same amount of added MGO at the start of the storage period, differences in the extent of HMF formation were observed. The increase in HMF after storage varied between 37 and 126% when compared to the starting values. Therefore, it can be concluded that other compounds beside MGO have to be taken into consideration when evaluating influences on the HMF formation. In the following experiments with model honeys, 3-deoxyglucosone as the precursor of HMF and proline as the most abundant free amino acid in honey additionally will be considered [23].

Influence of MGO on the HMF and 3-DG levels in artificial honey

Artificial honey was spiked with MGO to concentrations of 100, 250 and 500 mg/kg, respectively, and stored for up to 10 weeks at 37 °C. Levels of HMF and 3-DG were measured using HPLC and compared to a blank artificial honey which has been stored under the same conditions.

During the incubation, the HMF content in the blank artificial honey increased from 2 mg/kg to 46 mg/kg within 10 weeks. In artificial honey supplemented with MGO, the concentration of HMF formed depended directly on the amount of added MGO (see Fig. 2A). In model honeys supplemented with 100, 250 and 500 mg/kg MGO, the HMF concentrations increased to 58 mg/kg, 62 mg/kg or 74 mg/kg, respectively. This can be explained with the slightly elevated contents of 3-DG in the model honeys with higher MGO content, though the differences of the HMF contents only occur after 50 days of incubation, while the differences of the 3-DG contents were observable after 10 days of storage (see Fig. 2B). According to this, it can be concluded that the chosen temperature of 37 °C in this study is too low to directly convert 3-DG to HMF, however, it points to the fact

that MGO induces a slightly increased conversion of 3-DG which then is slowly converted to HMF.

This is in line with previous results published by Weigel et al. (2004) [19], who observed an elevated HMF formation in artificial honey during storage only after the temperature rose from 35 °C to 45 °C. Whereas 30 mg/kg HMF were formed within 70 days at 35 °C, at the higher temperature the same amount was formed within only 28 days. Furthermore, after 70 days at 45 °C 230 mg/kg were formed. The authors explained this phenomenon by assigning HMF as the thermodynamically controlled reaction product, whereas the formation of 3-DG is kinetically controlled.

In addition, the content of 3-DG remained constant after 50 days of incubation, though the HMF contents still increased. This indicates an equilibrium between the formation of 3-DG and its reaction to HMF. In particular, it can be hypothesized that the amounts of newly formed 3-DG is the same as the amount of 3-DG converting to HMF during that time. However, the mechanism how MGO influences the formation of 3-DG and HMF is unclear until now. Further experiments with isotopically-labelled MGO and thus a proposed formation of isotopically-labelled HMF could clarify possible reaction pathways and if the higher amount of formed HMF at high MGO levels is directly or indirectly caused by MGO or intermediates. Furthermore, incubations at a lower temperature, e.g. 20 °C, should be performed to show if the obtained results are transferable to honeys stored at ambient temperature.

Influence of proline on the HMF and 3-DG levels in artificial honey

The HMF precursor 3-DG is an intermediate formed in caramelization processes and in Maillard reaction by the cleavage of Amadori products. As the formation of Amadori products depends on the presence of free amino acids and of glucose, fructose or other reducing sugars, the influence of proline as the most abundant free amino acid in honeys, on the HMF formation was investigated [23]. Proline mainly

originates from bee secretions and its concentrations in floral honeys may vary from 207 to 970 mg/kg. Proline in manuka honey has been determined in concentrations up to 540 mg/kg [12, 23, 24]. Therefore, to simulate realistic proline levels in floral honeys, artificial honey was spiked with different amounts of proline between 0 and 1000 mg/kg and stored for 10 weeks at 37 °C.

In contrast to MGO, the addition of proline to artificial honey led to a decrease in HMF formation during storage. While the blank artificial honey contained 46 mg/kg HMF after the incubation of 10 weeks, in the artificial honey containing 100 mg/kg only 34 mg/kg HMF was formed. Higher proline additions were accompanied by a further decrease in the HMF content after the incubation up to an addition of proline of 500 mg/kg. A further increase in proline to 1000 mg/kg had no further effect. For 500 and 1000 mg/kg proline, a HMF content 20 mg/kg HMF resulted (see Fig. 2C). Although the HMF formation decreased at higher proline levels, more 3-DG was formed (see Fig. 2D). While the 3-DG content in the pure artificial honey increased by 91 mg/kg within the incubation, 201 mg/kg 3-DG was formed in the model honey with 1000 mg/kg proline. This should be mainly due to a promoted Maillard reaction, in particular an accelerated formation of Amadori and Heyns products, e.g. fructosylproline and glucosylproline, and an increased 3-DG release. It can be seen that the 3-DG contents continuously increased during the complete incubation period and that higher proline levels induce a higher increase of the 3-DG content. Therefore, it is hypothesized that proline not just induces 3-DG formation in honey, but also scavenges HMF, such that apparently less HMF was formed in the process despite high 3-DG levels. This is underlined by data in the literature where it was found that HMF and proline form Schiff base adducts and decarboxylated derivatives under pyrolysis conditions of 250 °C for 20 s [25]. This reaction should also be possible under milder conditions which are closer to honey storage conditions, however, further studies, e.g. incubation studies of HMF and proline, have to be performed to prove this.

Influence of MGO and proline on the HMF and 3-DG levels in artificial honey

As MGO and proline have opposing influences on HMF formation, it is open which effect has a dominating effect on HMF formation in honey. As can be seen from Fig. 2E and F, the artificial honeys with MGO contents of 100, 250 and 500 mg/kg no longer differed in HMF formation after the addition of 1000 mg/kg proline, but the HMF contents were

still increased compared to the blank sample without MGO. It can be concluded that at such high concentrations of proline, its inhibiting effect on HMF formation dominates over the HMF-increasing effect of MGO. A possible explanation of this phenomenon could be a reaction between proline and MGO, e.g. as discussed in [12] and [26] for the formation of acetylpyrroline. This is underlined by the fact that the contents of MGO in the honey decreased over the time in the presence of proline, e.g. from initially 500 mg/kg to 338 mg/kg after the incubation, while the MGO contents in the models without proline remained constant (see Figures S1 and S2). Additionally, proline may not just scavenge HMF, thus leading to lower HMF levels in the honey, but may also scavenge 3-DG, e.g. via formation of Schiff bases. These Schiff bases would block the further reaction to HMF. As Schiff bases might be unstable during sample cleanup, 3-DG still would be detectable. It is assumed that OPD as derivatisation agent displaces proline from the Schiff base as its amino groups have a higher affinity to (di-)carbonyl compounds than (secondary) amino acids due to a mesomeric effect during the reaction and due to the formation of quinoxalines with an additional aromatic ring.

However, when comparing the HMF formation in authentic honey samples with adjusted MGO (see Table 1), it becomes clear that other compounds beside MGO and proline, e.g. other amino acids, polyphenols or metal ions also have to be taken into account. Though the MGO content of every honey sample was adjusted to 500 mg/kg, and the proline contents were comparable, except robinia honey (linden honey: 206 mg/kg; rapeseed honey: 210 mg/kg, kanuka honey: 219 mg/kg, robinia honey: 126 mg/kg), the HMF content increased to a different extent in each honey, indicating that other compounds than MGO and proline influence HMF formation in honey as well. However, further studies have to be performed to elucidate additional substances which have an impact on the HMF formation in honey.

Conclusion

We were able to show that the HMF content in honey is, beside others, clearly dependent on the MGO content. An increased HMF content above the limit value of 40 mg/kg is therefore not necessarily due to heating of the honey. Since Manuka honeys are generally classified as low-enzyme honeys [27] and the determination of the diastase number is therefore not very meaningful, there is a risk that Manuka honeys with HMF contents above 40 mg/kg will be wrongly

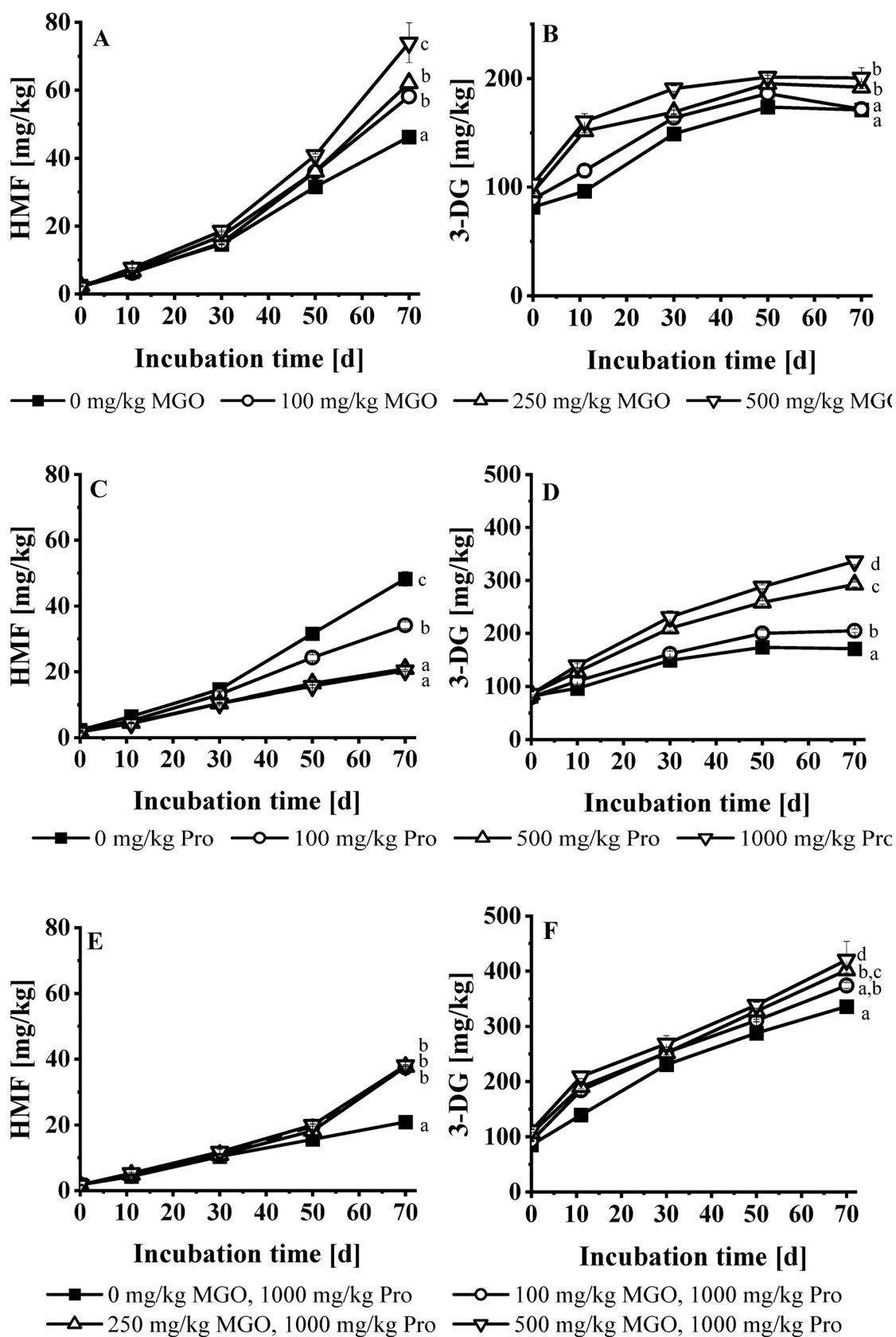


Fig. 2 HMF and 3-DG contents in artificial honeys spiked with 0, 100, 250 and 500 mg/kg MGO and 0, 100, 500 and 1000 mg/kg proline during an incubation at 37 °C for 10 weeks, measurements were carried out in triplicates, error bars show standard deviation, data that do not have a letter in common indicate significantly different levels of HMF and 3-DG, respectively, after 10 weeks of incubation (Tukey test at $p=0.05$)

classified as “heat treated” and, therefore, not marketable. We consider it necessary to conduct further systematic studies on the influence of MGO and other compounds on the formation of HMF in manuka honey with broader datasets and with lower temperatures and to examine whether a higher HMF content, e.g. of 80 mg/kg, comparable to tropical honeys, is acceptable for manuka honeys in the relevant regulations.

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Author contributions M.T., K.S., and T.H. planned the investigations. M.T. performed the analysis and laboratory work. M.T., K.S., and T.H. interpreted the results. M.T. wrote the first draft of the manuscript. T.H. and K.S. revised the manuscript. All authors reviewed the final version and approved it for publication.

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Data availability All data supporting the findings of this study are available within the paper. Original data (chromatograms, etc.) are available on request.

Declarations

Conflict of interest The authors M.T. and T.H. declare that they have received research support from Manuka Health Ltd., New Zealand. This financial support did not prevent the authors from publishing positive and negative results. Author K.S. receives a salary from Manuka Health Ltd. where he is Head of R&D.

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