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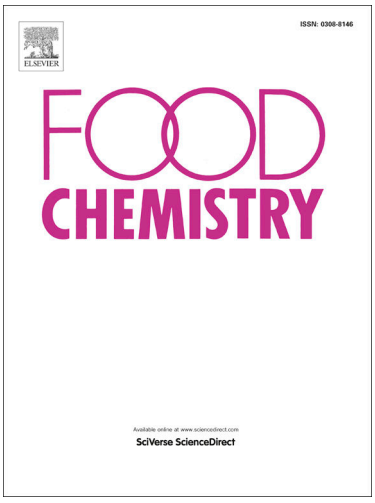

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Methylglyoxal binds to amines in honey matrix and 2'-methoxyacetophenone is released in gaseous form into the headspace on the heating of manuka honey

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Abstract

Reports on the thermal stability of manuka honey in terms of food processing have been few. This study investigated changes in nine characteristic chemicals of manuka honey during heating. Among these, methylglyoxal (MGO) and 2'-methoxyacetophenone (MAP) were significantly decreased by heating at 90 °C. To elucidate the mechanism for this decrease, artificial honey was prepared from sugars and water with MAP or MGO and then heated. The decrease of MGO was enhanced with L-proline, lysine, or arginine derivatives, accompanied by formation of 2-acetyl-1-pyrroline, MGO-derived lysine dimer, or argpyrimidine, respectively, suggesting that an amino–carbonyl reaction is one pathway for the loss of MGO. The decrease of MAP in the artificial honey depended on the volume of headspace in a vessel. MAP from heated manuka honey was also detected in the gas phase, indicating that MAP was vaporized. Heating could thus reduce the beneficial and/or signature molecules in honey.

Keywords

Manuka honey, heating process, methylglyoxal, amino–carbonyl reaction, 2'-methoxyacetophenone, vaporization

1. Introduction

Honey has been used as a food and in folk medicine since ancient times. Honey contains sugars, vitamins, hydrogen peroxide, amino acids, proteins, and phytochemicals. Today, honey is often eaten on toast, with tea, and used as a part of a sauce or dressing. Honey is also used as an additive for preparing secondary food products like candy. This means that honey-derived components occasionally receive heat treatment during food processing or in recipes that use honey as a seasoning.

Leptospermum honey (including manuka (*Leptospermum scoparium*) honey) comes from the nectar of *Leptospermum* flowers gathered by honeybees. Manuka honey is a premium product that has been used as a type of folk medicine; it has been used to prevent microbial infections because the honey has strong antibacterial activity derived from a characteristic component, methylglyoxal (MGO) (Atrott & Henle, 2009; Mavric, Wittmann, Barth, & Henle, 2008). Manuka honey often contains several hundred milligrams of antibacterial MGO per kilogram honey. MGO is not present in the nectar of *Leptospermum* flowers (Adams, Manley-Harris, & Molan, 2009), but nectar contains dihydroxyacetone (DHA), its precursor, in varying amounts (Noe, Manley-Harris, & Clearwater, 2019). This indicates that honeybees transport DHA to a beehive and then store it. Moderate heat (warm conditions) and the period of storage cause a chemical transformation of DHA to MGO in the beehive and also in storage drums after humans collect the honey.

Manuka honey is a premium product that is typically marketed at a high price; as such, it is frequently disguised and/or adulterated. Manuka honey contains other unique chemicals, including four chemicals, 3-phenyllactic acid (PLA), 4-hydroxyphenyllactic acid (4HPA), 2-methoxybenzoic acid (MBA), and 2'-methoxyacetophenone (MAP). The four chemicals are used as a set of markers, along with pollen-derived DNA, for the authenticity of manuka honey by the Ministry for Primary Industries (MPI), New Zealand (McDonald, Keeling, Brewer, & Hathaway, 2018; Ministry for Primary Industries, 2018). These four chemical markers were selected based on a comprehensive analysis of their specificity, amount detected, and thermal/storage stability (4 °C, 20 °C, and 35 °C for 68 days), among other properties; however, these characteristics, most notably the stability of MAP, have not been fully validated (Bong, Loomes, Lin, & Stephens, 2018). Of the four chemicals, MAP is specifically found in

the nectar from manuka, *Leptospermum scoparium*. Manuka honey also contains methyl syringate (Beitlich, Koelling-Speer, Oelschlaegel, & Speer, 2014; Kato, Fujinaka, Ishisaka, Nitta, Kitamoto, & Takimoto, 2014; Stephens et al., 2010), leptosperin (Daniels et al., 2016; Kato et al., 2014; Kato, Umeda, Maeda, Matsumoto, Kitamoto, & Kikuzaki, 2012; Lin, Loomes, Prijic, Schlothauer, & Stephens, 2017), pteridine (Beitlich, Lubken, Kaiser, Ispiryan, & Speer, 2016), and lepteridine (Beitlich et al., 2016). Of these, leptosperin has been used for the grading and certification of manuka honey but the thermal stability of leptosperin is not well validated (Bong et al., 2018; Kato et al., 2014; McDonald et al., 2018). As these unique chemicals could have certain beneficial functions for humans, it is important to maintain their levels during processing, shipment, storage, and cooking.

Honey may be pasteurized from 30 min to several hours to eliminate contaminating pathogens, extend shelf life, and increase its fluidity to facilitate pouring. Manuka honey is frequently stored in a drum for a few years without any temperature controls, in some cases to enhance the conversion of MGO to DHA. As noted above, honey is often used as a component in secondary products that are processed with thermal treatments. As such, the levels of leptosperin, a signature component of manuka honey, vary widely among these secondary products, including manuka honey candy, toothpaste, and drinks (Kato et al., 2014). These findings suggest that products labeled as "containing manuka honey" may contain only trace amounts of manuka honey and/or its critical components. These differences in content could be explained by differences in the absolute amount of the honey added to the product and/or by changes generated by processing, including those involving heat. Several recent studies have addressed the specific changes in the chemical profile of manuka honey that are observed in response to incubation at approximately 20–37 °C (Chernyshev & Braggins, 2020; Grainger, Manley-Harris, Lane, & Field, 2016a, 2016b, 2016c; Grainger, Owens, Manley-Harris, Lane, & Field, 2017; Rogers, Grainger, & Manley-Harris, 2014). These studies focused primarily on the specific chemical composition of manuka honey, including changes in relative concentrations of MGO, of its precursor molecule, DHA, and/or of hydroxymethylfurfural (HMF); however, only a few studies have focused on the stability of the unique components of this product, including leptosperin and the four aforementioned chemical markers (Kato et al., 2014; McDonald et al., 2018). Notably, honey-supplemented candy is frequently produced using thermal processing that reaches temperatures as high as 100°C. A preliminary study revealed that

manuka honey candies may contain a sizable range of MGO concentrations, from traces to >100 mg/kg candy (unpublished observation). However, only a few published studies address the issue of the thermal stability of honey-associated chemicals at temperatures that exceed 37 °C (Atrott, Haberlau, & Henle, 2012). Given these findings, we hypothesized that the heating of manuka honey might affect the composition of its chemical components, due to distinct differences in their thermal stabilities. This study aimed to investigate changes in the chemical profiles of manuka honey elicited by heat together with the elucidation of the underlying mechanisms. We specifically examined the impact of heat on MGO and MAP and explored mechanisms underlying their apparent decreased concentration. Altogether, our results emphasize the importance of temperature control not only during the primary processing of manuka honey but also during pasteurization and further applications.

2. Materials and Methods

2.1. Materials

Manuka honey and milk vetch (Chinese milk vetch, *Astragalus sinicus*) honey were obtained from local retailers in Japan. For this study, the manuka honey was classified into four grades, from the lowest (I) to the highest (IV), according to the certification of manuka honey displayed on the label. The grades I–IV correspond to the non-certified manuka honey, unique manuka factor (UMF5+; ≥MGO 83 mg/kg), UMF10+ (≥MGO 263 mg/kg), and UMF15+ (≥MGO 514 mg/kg), respectively. Note that the samples of honey were obtained before the implementation of definitions by the MPI (February 2018) (Ministry for Primary Industries, 2018). The manuka honey samples used in this study were imported from New Zealand, where they were stored in the stockroom of a retail store. On arrival at our facility, the honey samples were stored at 4 °C in our laboratory for a few years during experimentation. It is possible that concentration of chemicals (including MGO) in manuka honey have changed since the product was originally packed in New Zealand. Methyl syringate (methyl 3,5-dimethoxy-4-hydroxybenzoate) was obtained from Alfa Aesar (Heysham , UK). Leptosperin was prepared as described previously (Aitken, Johannes, Loomes, & Brimble, 2013). MGO, HMF, DHA, MAP, PLA, and MBA were purchased from

Sigma-Aldrich Japan (Tokyo). Anisole was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). *O*-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA), forchlorfenuron (1-(2-chloro-4-pyridyl)-3-phenylurea, FCF), and 4HPA were purchased from Tokyo Chemical Industry, Co., Ltd. (Tokyo, Japan). 2-Acetyl-1-pyrroline was obtained from Toronto Research Chemicals (North York, Canada). *N*-α-*tert*-Butoxycarbonyl-L-lysine (Boc-Lys) was purchased from Calbiochem-Novabiochem AG (Merck Millipore). Anti-MGO monoclonal antibody, which recognizes argpyrimidine residue, was purchased from the Japan Institute for the Control of Aging, Nikken SEIL Co., Ltd. *N*-α-Acetyl-L-arginine (*N*-Acetyl-Arg) was obtained from MP Biomedicals, Inc.

2.2. Processing of honey

Typically, each manuka honey sample (approximately 0.1 g), of grades III and IV, was placed in a 1.5-mL tube and capped. Grades I to IV honeys from the same brand were also used to compare the difference among the four grades of manuka honey. For a comparison of thermal dependency, the tubes were tightly capped and then incubated at 4, 36, 60, or 90 °C for 1 h. For the time course experiment, a tube containing honey (grade III) was heated at 90 °C for various periods of time. After these incubations, each sample was dissolved in water at 0.1 g/mL for MAP or at 0.05 g/mL for MGO and the components were then quantified as described in *Sections 2.4* and *2.5*.

2.3. Thermal treatment of chemicals in artificial honey

Artificial honey was prepared from water (36 g), D-fructose (93 g), D-glucose (68 g), and sucrose (3 g) without pH adjustment (Wahdan, 1998). MGO or MAP was then added to the artificial honey with or without another additive (i.e., L-proline, Boc-Lys, or *N*-acetyl-Arg) and mixed well using a mixer before the heating process. The thermal treatments were performed as described in *Section 2.2*.

To examine the effect of the headspace on vaporization of MAP or MGO by heating, 200 µL of artificial honey containing 1 mM MAP or 400 mg/kg MGO were added into various microtubes (0.2-mL, 0.6-mL, 1.5-mL, and 2.0-mL tubes) using a dispenser with a Visco Tip® (Eppendorf), and the tubes were capped, sealed, and then heated at 90 °C for 1 h. The volumes of headspace in the tubes (0.2, 0.6, 1.5, and 2.0-mL) with 200 µL of honey were approximately 95 µL, 720 µL, 1580 µL, and 1930 µL,

respectively. The volumes were separately estimated by measuring the weight before and after filling the space with water. A 0.2-mL tube filled with the artificial honey matrix containing MAP or MGO was also prepared (approximately 295 µL matrix in the tube) to exclude the headspace and heated. After heating, a sample (matrix) was taken and dissolved in water before the analyses as in *Sections 2.4* and *2.5*.

2.4. Measurement of methylglyoxal, dihydroxyacetone, and hydroxymethylfurfural

The treated honey was diluted to 0.05 g/mL with water. The solution was then vortexed and sonicated. After this, 100 µL of a sample or mixture of standards (DHA, MGO, and HMF) were mixed with 500 µL of derivatizing agent (2% PFBHA) and shaken (TAITEC Deep-Well Maximizer, M-BR-022UP) for 1 h at 50 °C at 600 rpm. To this sample, 25 µL of 0.27% anisole (dissolved in water/CH_3CN (25/75)) were added as an internal standard (Pappalardo, Pappalardo, & Brooks, 2016). The tube was centrifuged, and the supernatant was then transferred into a vial. Derivatized components in the sample (5 µL) were then quantified using ultra-high-performance liquid chromatography (UHPLC) with a photodiode-array detector (PDA; UltiMate 3000, Thermo Fisher Scientific). Separation was performed using a Hypersil Gold C18 column (2.1 × 100 mm, 1.9 µm, Thermo Fisher Scientific) at a flow rate of 0.7 mL/min with two solvents, 0.1% formic acid (liquid chromatography-mass spectrometry grade) in Milli-Q water (solvent **A**) and CH_3CN (HPLC grade) (solvent **B**), while monitoring at 215 nm (for DHA) or 262 nm (for MGO, HMF, and anisole). The column oven was set to 50 °C. The gradient program was as follows: initial 20% **B**, 0.6 min 20% **B**, 1.3 min 70% **B**, 3.0 min 100% **B**, 3.5 min 100% **B**, 4 min 20% **B**. Typical chromatograms of standards and manuka honey samples are shown in Supplemental Figure 1.

2.5. Measurement of leptosperin, methyl syringate, and 2'-methoxyacetophenone by UHPLC

Leptosperin, methyl syringate, and MAP in the sample (0.1 g/mL of water) were measured by UHPLC–PDA. A sample (2.5 µL) was injected into the UHPLC. Separation was performed using a Hypersil Gold C18 column at a flow rate of 0.4 mL/min with two solvents, 0.1% formic acid in water (solvent **A**) and CH_3CN (solvent **B**), with monitoring at 262 nm (for leptosperin), 275 nm (for methyl syringate), and 307 nm (for MAP). The column oven was set to 40 °C. The gradient program was as

follows: initial 10% **B**, 4 min 40% **B**, 5 min 10% **B**, 10 min 10% **B**. Amounts of components were calculated by comparison with standard curves.

2.6. Measurement of 3-phenyllactic acid, 4-hydroxyphenyllactic acid, 2-methoxybenzoic acid, and 2'-methoxyacetophenone by LC–MS/MS

The four marker chemicals were measured using liquid chromatography connected with a quadrupole tandem mass spectrometer (API3000, AB Sciex) (LC–MS/MS (QqQ)) according to a method provided by the MPI (McDonald et al., 2018; Ministry for Primary Industries, 2018) with some modifications. In brief, the incubated honey sample (0.1 g/mL) was further diluted 100 times in water and mixed with an equal volume of FCF (10 ng/mL in ethanol) as an internal standard. The sample was then analyzed by LC–MS/MS (QqQ). Separation was performed using HPLC (Agilent HP1100) with a Develosil C30-UG-5 column (2 × 150 mm, 5 µm; Nomura Chemical. Co.) at 0.2 mL/min using 0.1% formic acid in water (solvent **A**) and CH_3CN (solvent **B**). The gradient program was as follows: initial 5% **B**, 1 min 5% **B**, 3 min 15% **B**, 10 min 70% **B**, 12 min 98% **B**, 12.5 min 98% **B**, 13 min 5% **B**, 30 min 5% **B**. Five microliters of the sample were injected for positive and negative ionization separately. Multiple-reaction monitoring (MRM) transitions were used as follows: [Positive] MBA 153.0/77.0, 153.0/92.0 (to confirm), or 153.0/135.0 (to quantify); MAP 151.0/79.1 (to confirm), 151.0/105.1 (to quantify); FCF 248.0/93.1, 248.0/111.3, 248.0/155.1 (to confirm), or 248.0/129.1 (to quantify). [Negative] PLA 165.0/103.0 (to confirm) or 165.0/119.0 (to quantify); 4HPA 181.0/73.0, 181.0/119.1 (to confirm), or 181.0/135.05 (to quantify); FCF 246.0/91.0 (to confirm) or 246.0/127.0 (to quantify).

2.7. Measurement of 2'-methoxyacetophenone by headspace GC–MS

Gas chromatography–mass spectrometry (GC–MS) was performed using the Agilent 7890 GC and 5975 MSD with the 7697A headspace sampler (HSS). Samples (approximately 10 mg) containing MAP in a bar tolerance vial were heated in the HSS at indicated temperatures for 60 min, then injected (50 mL/min for 1 min) with a split ratio of 5:1 into the GC through the inlet, maintained at 250°C. GC separation was performed with a DB-WAX column (30 m × 0.25 mm, 0.25 µm), at an initial temperature of 35 °C for 1 min, which was increased by 5 °C/min to 120 °C, 26 °C/min to 250 °C, and then

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maintained at 250 °C for 10 min. Helium was provided as the carrier gas at 1.1 mL/min constant flow. The ion source (with electron ionization) and quadrupole were maintained at 230 °C and 150 °C, respectively. MAP was detected at 21.93 min. Levels of MAP were estimated by external standard methods.

2.8. Measurement of 2-acetyl-1-pyrroline as its quinoxaline derivative

Standard 2-acetyl-1-pyrroline quinoxaline (2APQ) was prepared according to a previously published method (Jost, Heymann, & Glomb, 2019) with some modification. Briefly, 2-acetyl-1-pyrroline (26 mg) was mixed with *o*-phenylenediamine (OPD) (36 mg) in 2 mL of 0.1 M phosphate buffer (pH 7.4) at 37 °C for 2 days. The reaction mixture was fractionated using a Develosil ODS-HG-5 column (8 × 250 mm, 5 µm; Nomura Chemical Co.) and eluted with 0.1% formic acid in water/methanol (7:3) at a flow rate of 2.0 mL/min with monitoring at 317 nm. The major peak (retention time 7 min) was repeatedly collected and then the collected portion was concentrated. Identification was performed using LC connected with a quadrupole time-of-flight high resolution mass spectrometer (LC–Q-TOF, SCIEX X500R) to measure the precise molecular weight (*m/z* 202.1341 $[M + H]^+$ (found), *m/z* 202.1344 $[M + H]^+$ calculated for $C_{12}H_{16}N_3$) and also its fragmentation pattern from 202.1 ($[M + H]^+$ 185.1073, 157.0762, 143.0604, 130.0402, 102.0339) (Jost et al., 2019). The fragmentation signals were mostly matched with the predicted numbers from the chemical structure of APQ using SCIEX OS software (version 1.6). The concentration of the synthetic 2APQ was calculated using 6700 $M^{-1}cm^{-1}$ as a molar extinction coefficient (Jost et al., 2019).

The standards and samples with the internal standard, FCF, were analyzed using the LC–Q-TOF as follows. Honey was dissolved in water at 0.05 g/mL and then mixed with an equal volume of 20 mM OPD in 0.1 M phosphate buffer (pH 7.4) and incubated at 37 °C for 2 days. The derivatized solution was diluted 5 times further and then mixed with an equal volume of the internal standard solution (10 ng/mL). The mixture was then separated using a Kinetex PS C18 column (2.1 × 50 mm, 2.6 µm; Phenomenex, Torrance, CA) for gradient elution using 0.1% formic acid in water (solvent **A**) and methanol (solvent **B**) at a flow rate of 0.4 mL/min. The gradient program was as follows: initial 0% **B**,

4.5 min 100% **B**, 5 min 0% **B**, 8 min 0% **B**. The eluate was introduced to the Q-TOF mass spectrometer. Positive MRM–high resolution (MRM^{HR}) combinations for APQ (202.1/185.1077 ± 0.01 (to quantify), 202.1/143.0607 (to confirm), 202.1/102.0341 (to confirm)) and FCF (248.0/129.0218 ± 0.01 (to quantify)) were selected (Ishisaka et al., 2017; Jost et al., 2019).

2.9. Measurement of L-proline by LC–MS/MS

Free L-proline in the honey matrix was measured using LC–MS/MS (QqQ) according to a previously published method (Nakamura, Kawai, Kitamoto, Osawa, & Kato, 2009) with some modification. Honey was diluted to 5 mg/mL in water and mixed with an equal volume of 5 μg/mL of internal standard (mixture of stable-isotopic amino acids, Spectra Gases Inc.), and was then applied to LC–MS/MS with a Develosil ODS-HG-3 column (2 × 50 mm, 3 μm; Nomura Chemical Co.) as gradient elution using 5 mM heptafluorobutyric acid (HFBA) in water (solvent **A**) and 5 mM HFBA in water/CH_3CN (9/1) (solvent **B**) at a flow rate of 0.2 mL/min. The gradient program was as follows: initial 0% **B**, 7 min 20% **B**, 10 min 20% **B**, 11 min 0% **B**, 15 min 0% **B**. For quantification of L-proline, the following combinations of positive MRM were selected: 116.0/70.0 (L-proline), 122.0/75.0 (stable-isotopic L-proline).

2.10. Measurement of methylglyoxal-derived lysine dimer (MOLD) as Boc derivative

Boc-Lys (10 mM) was incubated with the artificial honey matrix in the presence of MGO (400 mg/kg) at 90 °C for various periods of time. After the incubation, the sample (matrix) was diluted to 5 mg/mL with 0.1% formic acid/methanol (1:1) and then analyzed as follows: LC–MS/MS (QqQ) was performed with a Develosil ODS-HG-3 column (2 × 50 mm, 3 μm) and gradient elution using 0.1% formic acid in water (solvent **A**) and CH_3CN (solvent **B**) at a flow rate of 0.2 mL/min. The gradient program was as follows: initial 5% **B**, 3.9 min 95% **B**, 4 min 5% **B**, 12 min 5% **B**. For detection and calculation of peak areas of Boc-MOLD, the following combinations of positive MRM were selected: 541.3/341.5 (to quantify), 541.3/441.6 (to confirm), and 541.3/485.4 (to confirm).

2.11. Measurement of MGO-modified N-acetyl-arginine

N-Acetyl-arginine (10 mM) was incubated with the artificial honey matrix in the presence of MGO (400 mg/L) at 90 °C for various periods of time. After the incubation, samples (matrix) were diluted to 0.05 g/mL with 0.1% formic acid/methanol (1:1) and then analyzed by LC–Q-TOF as follows. Separation was performed using a Hypersil Gold column (2.1 × 100 mm, 1.9 μm) with gradient elution using 0.1% formic acid in water (solvent **A**) and CH_3CN (solvent **B**) at a flow rate of 0.4 mL/min. The gradient program was as follows: initial 0% **B**, 4 min 40% **B**, 5 min 0% **B**, 10 min 0% **B**. *N*-Acetyl-argpyrimidine ($[M+H]^+$ 297.1562) and *N*-acetyl-methylglyoxal hydroimidazolone (MG-H) ($[M+H]^+$ 271.1403) from *N*-acetyl-arginine were measured by scanning with the TOF-MS in positive mode. The identifications were performed by comparing the actual fragmentation patterns with the predicted patterns for the respective chemical structures using Sciex OS 1.6 software.

2.12. Reaction of MGO with a protein in artificial honey or with endogenous proteins in honey

Bovine serum albumin (BSA, 20 mg/kg) was mixed with MGO (400 mg/kg) in artificial honey and dispensed into several tubes. The tubes were then heated at 90 °C for 0–120 min. Manuka and milk vetch honeys were also dispensed into tubes and heated. Milk vetch is a popular plant in Japan, Korea, China, and East Asia; the plant is widely used as a green manure in rice fields (Sasakawa, 1987). After the heating, proteins in the manuka or milk vetch honey were dialyzed against water at 4 °C for 2 days and then freeze-dried. The protein was dissolved in 0.1 M phosphate buffer (pH 7.4) at a concentration of 0.5 mg/mL. For the control antigen, MGO-modified BSA (MGO-BSA) was prepared as follows: MGO (10 mM) and BSA (5 mg/ml) were mixed and incubated in 0.1 M phosphate buffer (pH 7.4) at 37 °C for 24 h. The modified protein was dialyzed against water at 4 °C for 2 days and then was lyophilized. The protein was dissolved in 0.1 M phosphate buffer (pH 7.4) and was used as the control antigen (MGO-BSA) for the following gel experiments.

2.13. Immunochemical detection of argpyrimidine residues in a protein

The honey protein samples were mixed with sodium dodecyl sulfate (SDS) loading buffer with 2-mercaptoethanol and boiled for 3 min. The proteins were applied to two gels for SDS polyacrylamide

gel electrophoresis. In one gel, the protein was stained with Flamingo™ fluorescent protein gel stain (Bio-Rad, Hercules, CA). The protein gel stain was captured by a Lumino Image Analyzer LAS-1000plus (FUJIFILM) and analyzed with Image Gauge software (ver. 3.4). The protein in the other gel was electrotransferred onto an Immobilon-P transfer membrane (Millipore). The membrane was then blocked with an aqueous solution of 4% Block Ace (KAC Co.) for 1 h at room temperature. After washing the membrane three times for 5, 10, and 10 min with Tris-buffered saline containing 0.05% Tween-20 (TTBS), the membrane was incubated with the anti-MGO monoclonal antibody (Nikken Seil Co., Ltd), which recognizes the argpyrimidine residue, at 0.04 μg/mL dilution in a signal enhancer, HIKARI-A (Nacalai Tesque), for 2 h at room temperature. After washing, the membrane was incubated with anti-mouse immunoglobulin horseradish peroxidase (HRP) conjugate (secondary antibody) (DAKO) at 1:5000 dilution in another signal enhancer, HIKARI-B, for 1 h at room temperature. After washing, the membrane was visualized using Chemilumi One Super (Nacalai Tesque). Immunostaining was detected by GeneGnome (Syngene) with GeneSys software (ver. 1.6.9).

For the artificial honey, after incubation at 90 °C, each sample was diluted at 0.1 g/mL and then mixed with SDS loading buffer and boiled as already described. Proteins were stained with SYPRO Orange (Invitrogen) according to the manufacturer's recommendations. For blot analysis, the anti-MGO antibody and the secondary antibody were respectively dissolved in TTBS at the concentrations already described.

2.14. Enzyme-linked immunosorbent assay (ELISA) for argpyrimidine residues

Proteins were coated onto the wells of a microtiter plate (Nunc, Maxisorp®) at a concentration of 0.01 mg/mL in phosphate-buffered saline (PBS) at 4 °C for overnight. After blocking with 1% Block Ace in water at 37 °C for 1 h, the anti-MGO antibody at 0.04 μg/mL in PBS containing 0.05% Tween-20 (TPBS) was added and incubated for 2 h at 37 °C. The wells were then incubated with a solution of the secondary antibody (1/5000) in TPBS for 1 h at 37 °C. Color development was achieved using the 3,3',5,5'-tetramethylbenzidine reagent (Nacalai Tesque POD Substrate TMB kit) and was terminated by the addition of 1 M phosphoric acid; color was then measured at 450 nm.

2.15. Statistics

All data presented are representative of two or more independent experiments. All experiments were performed using $n = 3$ samples unless otherwise indicated, and results are expressed as mean ± standard deviation. The significance of differences was assessed by Student's *t*-test using Excel or Kaleida Graph (version 4.5.2). The minimum level of statistical significance was set at $p < 0.05$.

3. Results

3.1. Temperature dependency of compositional changes for chemicals of manuka honey

In this experiment, honey was classified into four grades, from the lowest (I) to the highest (IV) grade, according to the certification of manuka honey displayed on the label. The grade I product was labeled as manuka honey but the label included no certification. Grades II to IV corresponded to UMF5+ (≥83 mg/kg MGO), UMF10+ (≥263 mg/kg MGO), and UMF15+ (≥514 mg/kg MGO), respectively. The changes in the key chemicals, MGO, DHA, HMF, leptosperin, and methyl syringate, along with the four signature chemicals (McDonald et al., 2018) (Fig. 1A), were measured in response to different thermal treatments.

At first, manuka honey (grade III), as an intact medium, was placed in a sealed microtube and treated with heat at various temperatures, 4, 36, 60, and 90 °C, for 1 h and then analyzed. Results showed that leptosperin was stable for 1 h during all thermal treatments examined, and methyl syringate was slightly decreased at 90 °C (Supplemental Fig. 2). Among the four chemical markers chosen for the manuka honey definition, both MBA and 4HPA were stable under the treatments but PLA tended to increase compared to the 4°C control sample. On the other hand, MAP decreased with increasing temperature; approximately 16% (60 °C) and 53% (90 °C) of MAP were lost in 1 h compared to the 4 °C sample. The antibacterial key compound MGO was significantly decreased 17% under 90 °C treatment. Conversely, HMF, which is a known indicator of heating or longer storage of honey, increased by 140% in 1 h at 90 °C. The content of DHA, which is the known precursor of MGO (Adams et al., 2009), was slightly decreased at 90 °C albeit with a statistically significant difference ($p < 0.05$). While DHA is a

precursor of MGO, and the loss of DHA may imply the generation of MGO, this does not account for the MGO loss incurred by heating. However, we cannot rule out the possibility that the conversion of DHA to MGO proceeded more slowly than the loss of MGO observed at 90 °C.

3.2. Time dependency of compositional changes for chemicals of manuka honey

The temperature was fixed at 90 °C and time-dependent changes in chemical profile in a grade III honey were examined (Fig. 1B). MGO decreased in a time-dependent manner; after 2 h of incubation at 90 °C, more than 55% of MGO was lost. On the other hand, MAP also decreased approximately 63%, from approximately 5 to 2 mg/kg, in 2 h. Methyl syringate and 4HPA were slightly decreased. HMF was gradually increased across 2 h. Leptosperin, PLA, and MBA were stable over time.

3.3. Comparison of thermal stability of chemicals among different grades

The changes in chemical compositions of the four different grades of commercial manuka honey (the same brand) were compared between incubations at 4 °C and 90 °C. As shown in Supplemental Fig. 3, MGO and MAP decreased and HMF increased for almost all the grades on heating. It is noteworthy that even if it is marketed as a single brand, the honey used to generate the final product might be from different parts of New Zealand and have completely different compositions. This factor could have a distinct impact on the pattern of changes observed.

3.4. Molecular mechanism for loss of methylglyoxal by thermal treatment

The mechanisms for the decreases of MGO under heating were investigated by model experiments. First, MGO at 400 mg/kg (corresponding to 5.5 mmol/kg, equivalent to UMF 12+) was dissolved in an artificial honey matrix consisting of water/D-fructose/D-glucose/sucrose. The solutions (200 μL) were dispensed into tubes of different sizes, which were sealed and then heated at 90 °C for 1 h. At this point, MGO was not decreased in the artificial honey matrix (Supplemental Fig. 4), suggesting that MGO was not volatilized under the heating conditions. It is often mentioned that under warmer conditions, an aldehyde reacts with an amino moiety, which is known as an amino–carbonyl reaction, that is, the Maillard reaction. Honey contains various free amino acids; among these, L-proline is found in

abundance (Bernal, Nozal, Toribio, Diego, & Ruiz, 2005). It has been reported that the reaction between MGO and L-proline generates 2-acetyl-1-pyrroline (Fig. 2A) (Ruckriemen, Schwarzenbolz, Adam, & Henle, 2015). When L-proline at 5 mmol/kg (575 mg/kg) (Bernal et al., 2005) was added to the artificial honey matrix solution with MGO, the amount of MGO in the presence of L-proline was significantly decreased (50%) by heating at 90 °C for 120 min, compared to artificial honey with added MGO alone (Fig. 2B). Fig. 2C shows that 2-acetyl-1-pyrroline, quantified as its quinoxaline derivative (Fig. 2A) (Jost et al., 2019), was formed in a time-dependent manner when heating the artificial honey matrix in the presence of both MGO and L-proline. Accordingly, the L-proline level in artificial honey was gradually decreased to 70% (Fig. 2D). MGO in manuka honey (of grades III and IV) was also decreased by heating at 90 °C for 2 h (Fig. 2E), and 2-acetyl-1-pyrroline was generated in the honey matrix time-dependently (Fig. 2F). During the heating process, L-proline in manuka honey was gradually decreased to approximately 65% (grade III) and 38% (grade IV) by 2 h (Fig. 2G). These patterns observed in response to heating the natural manuka honey were similar to those observed in artificial honey prepared with MGO and L-proline. It was noteworthy that even before heating, 2-acetyl-1-pyrroline was found in both manuka honeys, and a similar result has already been reported in another study (Ruckriemen et al., 2015). These results might be explained by the longer period of storage experienced by the manuka honey used in this experiment. Nonetheless, this chemical may ultimately be used as an indicator of product deterioration.

Honey also contains some proteins derived from bees and/or plants. In honey, these proteins may be modified by reactive chemicals including MGO *via* the amino–carbonyl reaction (Adams et al., 2009), followed by the formation of well-known MGO–lysine adducts such as MOLD (Supplemental Fig. 5A) (Frye, Degenhardt, Thorpe, & Baynes, 1998). In this study, to investigate the contribution of the amino–carbonyl reaction under the heating conditions, first, Boc-Lys (10 mM) was used as a model of lysine residue in a protein and the effect of Boc-Lys on the MGO decrease was examined. As shown in Supplemental Fig. 5B, there was a significant loss of 95% of MGO with the addition of Boc-Lys. Indeed, along with this decrease, in the artificial honey supplemented with both MGO and Boc-Lys, Boc-MOLD was also formed (Supplemental Fig. 5C). In addition, a considerable amount of Boc-MOLD was observed at the starting time, probably because the process of mixing of artificial honey with MGO and

Boc-Lys in the matrix caused adduct formation. It has been reported that the MOLD is formed by the reaction of MGO with a protein within a few hours (Nagaraj, Shipanova, & Faust, 1996).

MGO also reacts with the arginine residue, accompanied by the formation of adducts, including argpyrimidine, MG-H1, and others (Supplemental Fig. 6A) (Sousa Silva, Gomes, Ferreira, Ponces Freire, & Cordeiro, 2013). As a model of arginine residue, *N*-acetyl-Arg was mixed with MGO in artificial honey and then heated. With longer heating time, the levels of both *N*-acetyl-Arg and MGO gradually decreased (Supplemental Fig. 6B). Formation of *N*-acetyl-argpyrimidine (*m/z* 297.1562, theoretical 297.1563 from $C_{13}H_{21}N_4O_4$ as a protonated form) and MG-H isomers (*m/z* 271.1403, theoretical 271.1406 from $C_{11}H_{19}N_4O_4$ as a protonated form) was observed along with fragmentation patterns that matched with predictions from the chemical structures (data not shown). Because standards for the two products were not available, the generation was evaluated as percent of peak area compared to the 240-min sample (Supplemental Fig. 6C, D). Furthermore, this study could not distinguish the isomers of MG-H1–3 (*N* δ-(5-hydro-5-methyl-4-imidazolon-2-yl) ornithine [MG-H1], 2-amino-5-(2-amino-5-hydro-5-methyl-4-imidazolon-1-yl)pentanoic acid [MG-H2], and 2-amino-5-(2-amino-4-hydro-4-methyl-5-imidazolon-1-yl)pentanoic acid [MG-H3]) because their molecular weights are the same . The increase of MG-H was proportional to the incubation time. MG-H was presumably generated by the direct reaction of one MGO molecule with one arginine with dehydration (Klopfer, Spanneberg, & Glomb, 2011). In contrast, that of argpyrimidine was exponential, possibly because dihydroxy-imidazolidine as an intermediate was first generated by adduct formation with one MGO molecule added to arginine and then further conjugated with an additional MGO to generate argpyrimidine as previously proposed (Bhattacherjee, Dhara, & Chakraborti, 2017). The results indicated that under thermal treatment, argpyrimidine and the MG-H moiety were generated by the reaction between arginine and MGO.

When albumin in artificial honey was incubated with MGO, the protein molecules were observed to aggregate in a time-dependent manner (Fig. 3A). Accordingly, an immunoreactive stain for argpyrimidine residue was observed after 30 min of heating (Fig. 3B). The formation of immunoreactive materials, argpyrimidine residues, was also semiquantitatively measured by ELISA and a result similar to that of the blot analysis was observed (Fig. 3C). Heated manuka honey also generated immunoreactive

bands, suggesting that the honey proteins were targeted by endogenous MGO (Fig. 3D, E). By contrast, heating of milk vetch honey did not generate immunoreactive bands, in parallel with the lack of MGO in milk vetch honey. Minimally immunoreactive protein bands were also detected in unheated manuka honey (Fig. 3E); this suggests that these proteins were partly modified by MGO and other reactive carbonyls compounds, including DHA (Chernyshev et al., 2020) prior to the start of the experiment.

3.5. Molecular mechanism of MAP loss by thermal treatment

MAP was dissolved in the artificial honey matrix at 1 mM (150 mg/kg) and dispensed into various sizes of tubes as described in the Materials and Methods section. The tubes containing the matrix were then heated at 90 °C for 1 h. Contrary to effects on MGO, the decrease of MAP is dependent on the headspace volume of the container for the extent of reaction (Fig. 4A); it was difficult to eliminate air bubbles from a filled 0.2-mL tube, and the MAP in the sample was also decreased. No generated products in the heated artificial honey matrix were detected by UHPLC–PDA and LC–Q-TOF (data not shown). These results suggested that the MAP loss was mostly because of the vaporization under the thermal condition of 90 °C. Interestingly, even at 37 °C for 2 days, a decrease in MAP in artificial honey was also observed (Supplemental Fig. 7). The addition of amines (Boc-Lys or L-proline) into the matrix containing MAP had a negligible effect on the loss of MAP under heat treatment (data not shown). These results suggest that vaporization of MAP by heating is the major reason for the loss observed in heated manuka honey (Fig. 1).

To confirm the vaporization of MAP from artificial honey and manuka honey matrix by heating, the headspace gas from the honey was analyzed by GC–MS. The signal for MAP in the gas phase from the artificial honey supplemented with MAP was significantly increased with increasing temperature (Fig. 4B). Using the artificial honey supplemented with known concentrations of MAP, a standard curve for MAP at 90 °C in the gas phase was prepared (Fig. 4C). When samples of manuka honey were used (n = 5), the grade III and grade IV honeys generated MAP in the gas phase at 90 °C at concentrations of 21.9 ± 15.3 and 51.5 ± 10.0 μM, corresponding to 3.2 ± 2.3 and 7.7 ± 1.5 mg/kg honey, respectively.

4. Discussion

Manuka honey is often incorporated into secondary products, such as manuka honey candy; this is perceived as increasing the marketability of this product based on putative health benefits. Chemicals that are characteristic of manuka honey are often subjected to heating while undergoing incorporation into secondary products. We examined the changes in the chemical content of manuka honey in response to heat. As shown in Figure 1, PLA, leptosperin, and MBA remained unchanged when manuka honey was heated to 90 °C for 2 h. Small reductions in DHA and MSYR content were detected (10–20%). In contrast, we observed significant decreases in the concentration of both MGO and MAP in response to heating to 90 °C. Similar observations of MGO loss were reported previously (Adams et al., 2009; Grainger et al., 2016a, 2016c); specifically, Adams et al. (2009) reported marked decreases in MGO at 8 h (68–77% remaining) and 24 h (36–43% remaining) at 70 °C. That report also mentioned that the original level of MGO in manuka honey decreased approximately 10% with storage at even 37 °C for 12 weeks, and the decrease was enhanced by preheating at 70 °C for 10 min. To understand the mechanism underlying MGO loss, an artificial honey matrix was used in this experiment. The study found the formation of an MGO adduct with lysine, MOLD, in the artificial honey matrix containing MGO and Boc-Lys (Supplemental Fig. 5C) and also found an increase of *N*-acetyl-argpyrimidine/MG-H moieties from *N*-acetyl-Arg (Supplemental Fig. 6C, D). This study also confirmed that argpyrimidine residues in a protein were generated by heating manuka honey (Fig. 3E). An earlier study reported the presence of an MG-H1 moiety in protein-bound Maillard reaction products in manuka honey (Hellwig, Ruckriemen, Sandner, & Henle, 2017). As demonstrated in Fig. 2F, 2-acetyl-1-pyrroline was formed in the heated manuka honey, possibly through the reaction of MGO with L-proline (Ruckriemen et al., 2015), which is predominant in honey (Bernal et al., 2005). Yields of 2-acetyl-1-pyrroline of <1% were detected in association with the loss of L-proline from both manuka and artificial honey; these results suggested that other products might be generated along with the vaporization of 2-acetyl-1-pyrroline. Overall, our findings indicate that MGO is unstable at 90 °C in the presence of amino moieties. Likewise, flavonoids in honey could react covalently with MGO as previously reported (Shao, Bai, He, Ho, Yang, & Sang, 2008), and MGO could be converted into dimeric/oligo/polymeric products or undergo complete breakdown in response to heat. The mechanisms and pathways underlying the loss of MGO are probably varied. We conducted this study using artificial honey that was prepared without pH

adjustment. Because pH is a key factor facilitating a covalent amino–carbonyl reaction, the results obtained should be validated in future studies.

This study analyzed the thermal change in the amounts of four chemicals that are used as markers for the official definition of manuka honey (McDonald et al., 2018). Among these, the level of MAP tended to decrease at 60 °C and significantly decreased by more than half at 90 °C compared to 4 °C, when held at that temperature for 1 h (Supplemental Fig. 2), without detection of any products formed. This experiment suggested that MAP in manuka honey was vaporized into the headspace when heated. The phenomenon was also observed by heating the artificial honey with MAP. Headspace sampling with GC–MS confirmed the vaporization of MAP from heated manuka honey at 60 ºC and 90 °C across 1 h. Some beekeepers may heat their honey for extended periods of time in an effort to enhance generation of MGO; they may even pasteurize their honey products at high temperature (i.e., 70–80 °C) for 20–60 min or longer. The possibility of vaporizing MAP under these conditions should be noted, especially given the fact that MAP is one of the most important markers for the definition of manuka honey currently recognized by the government of New Zealand. It is noteworthy that the boiling point of MAP (272°C, 760 mm Hg) is far higher than 90 °C (ChemSpider, 2020). MAP vaporization may be due to the fact that it is relatively immiscible with water, certainly compared to water-soluble MGO or other phytochemicals, including leptosperin. In addition, our data also showed that MAP was lost from artificial honey even at 37 °C for 48 h (Supplemental Fig. 7). A similar observation in terms of MAP loss was reported for 37 °C incubation across more than 400 days (Bong et al., 2018), but the mechanism was not provided in the report. MAP was also found as one of the compounds in common in the headspace of three honey varieties, manuka, honeydew, and clover honey (El-Sayed, Unelius, & Suckling, 2018). This study is believed to provide the first report that MAP in manuka honey is possibly lost by vaporization under thermal treatment. Manuka honey is often kept in drums for a few years to maximize the MGO level. Because MAP is the most critical marker for "genuine" *Leptospermum scoparium* manuka honey (McDonald et al., 2018), beekeepers and those in the honey industry handling manuka honey should carefully select the storage conditions, in particular, the temperature and volume of headspace, to prevent vaporization of MAP. Because the constancy of MAP has been reported in one publication (McDonald et al., 2018), the stability of MAP needs further validation in the future.

Along with its use as a chemical marker for certified manuka honey, leptosperin could play an important role in the possible functionality of manuka honey through its *in vivo* metabolism *via* bioconversion to methyl syringate and syringic acid; this could be partly mediated by the microbiome in the gut (Kato et al., 2019). In this study, the levels of leptosperin in all four grades of manuka honey (the lowest to the highest grade) did not change under thermal treatment at 90 °C (Supplemental Fig. 3). Leptosperin is relatively stable during 1 month of storage at 37 ºC and 50 °C (Kato et al., 2014), and the level of leptosperin did not change significantly at 37 °C for more than 400 days (Bong et al., 2018). Thus, from a thermal (heating) point of view, leptosperin could be a suitable marker for certification of manuka honey supplemented in processed foods. By contrast, another study has reported the instability of leptosperin during 68-day incubation at 4, 20, or 35 °C (McDonald et al., 2018). This might mean that the stability of the chemical varies on its concentration and on the presence of other chemicals or enzymes. Therefore, the stability of leptosperin may require further validation in further studies.

5. Conclusion

We conducted this study in order to identify changes in the chemical profile of manuka honey that take place in response to heat. Specific loss of both MGO and MAP was observed when the honey samples were heated to 90 °C for 1–2 h. Decreases in MGO content may be attributed at least in part to amino–carbonyl reactions as characteristic conjugates of MGO with L-proline and other proteins were detected. In contrast, MAP was detected in the gaseous phase; its loss was most likely explained by vaporization. These findings contribute to our understanding of events that take place during processing of honey into secondary products but also with respect to the appropriate handling of the raw honey itself, given the desire to maintain the unique and beneficial chemicals underivatized and intact. The findings presented are not a full picture of the chemical changes associated with manuka honey in response to heating; a stoichiometric study that included kinetics would provide important additional understanding of these critical points.

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Conflict of interest:

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Fig. legends

Fig. 1

Changes in the profile of unique chemicals found in manuka honey. (A) Chemical structures of the unique chemicals examined. (B) Time course experiment for heat stability of chemicals in manuka honey (grade III) at 90 °C; the results are presented in two separate graphs based on the concentrations. (Left) The results of six chemicals showed relatively rich presence in the original honey (>20 mg/kg honey, each). (Right) The results of the other three chemicals showed less than 20 mg/kg for each in the honey. Significance of differences: * $p < 0.05$ vs. control (0 min); ** $p < 0.01$ vs. control (0 min).

Fig. 2

Effect of L-proline on thermal changes in artificial or manuka honey. MGO was added to artificial honey in the presence or absence of L-proline and heated at 90 °C. The matrices of two grades of manuka honey (III and IV) were also heated. Data are expressed as mg/kg honey. (A) Possible scheme for generation of 2-acetyl-1-pyrroline and successive derivatization to the corresponding quinoxaline APQ by *o*-phenylenediamine. (B) The residual amounts of MGO in artificial honey when heated. (C) Formation of 2-acetyl-1-pyrroline from artificial honey when heated. (D) The loss of L-proline in artificial honey when heated. (E) The residual amounts of MGO in two grades of manuka honey when heated. (F) Formation of 2-acetyl-1-pyrroline from the two grades of manuka honey. (G) The loss of L-proline in the two grades of manuka honey. Significance of differences: # $p < 0.05$ vs. control (0 min); ## $p < 0.01$ vs. control (0 min) for artificial honey or grade III honey; * $p < 0.05$ vs. control (0 min); ** $p < 0.01$ vs. control (0 min) for artificial honey plus L-proline or grade IV honey.

Fig. 3

Detection of argpyrimidine in protein by heating with MGO. (A) Protein staining for bovine serum albumin (BSA) heated with MGO in artificial honey. (B) Immunostaining of argpyrimidine residues in the BSA heated with MGO in artificial honey. (C) ELISA for argpyrimidine in BSA heated with MGO in artificial honey. Significance of differences: ** $p < 0.01$ vs. control (0 min). (D) Protein stain for honey proteins from manuka and milk vetch honey. (E) Immunostaining of argpyrimidine residues in

the honey proteins. Abbreviations: MGO-BSA, methylglyoxal-modified BSA (positive control); Marker, XP Magic marker.

Fig. 4

Thermally induced loss of 2'-methoxyacetophenone (MAP) from honey matrix. (A) Artificial honey matrix (200 μL) containing 1 mM MAP was placed in various tubes (0.2-mL, 0.6-mL, 1.5-mL, and 2.0-mL tubes) and then heated at 90 °C for 1 h. To exclude headspace in a tube, a tube filled with the matrix containing MAP was also prepared using a 0.2-mL tube and heated. The results are expressed as percent of the control, unheated sample. Significance of differences: ** $p < 0.01$ vs. control. (B) Chromatograms of MAP generated in the vapor phase from artificial honey supplemented with 1 mM MAP. (B, Inset) Comparison of MAP in vapor phase under three thermal conditions. Significance of differences: * $p < 0.05$ vs. 36 °C sample; ** $p < 0.01$ vs. 36 °C sample. (C) Correlation between the signal of vapor MAP and the dose of MAP supplemented in artificial honey (4.12–111.1 μM) when heated at 90 °C. The successive standard curve of signal vs. MAP concentrations was used for determination of vapor MAP from heated manuka honey.

References

Adams, C. J., Manley-Harris, M., & Molan, P. C. (2009). The origin of methylglyoxal in New Zealand manuka (*Leptospermum scoparium*) honey. *Carbohydrate Research, 344*(8), 1050-1053. https://doi.org/10.1016/j.carres.2009.03.020.

Aitken, H. M. R., Johannes, M., Loomes, K. M., & Brimble, M. A. (2013). Synthesis of leptosin, a glycoside isolated from mānuka honey. *Tetrahedron Letters, 54*(50), 6916-6919. https://doi.org/dx.doi.org/10.1016/j.tetlet.2013.10.042.

Atrott, J., Haberlau, S., & Henle, T. (2012). Studies on the formation of methylglyoxal from dihydroxyacetone in Manuka (*Leptospermum scoparium*) honey. *Carbohydr Res, 361*, 7-11. https://doi.org/10.1016/j.carres.2012.07.025.

Atrott, J., & Henle, T. (2009). Methylglyoxal in manuka honey - Correlation with antibacterial properties. *Czech Academy of Agricultural Sciences, 27*, S163-S165.

Beitlich, N., Koelling-Speer, I., Oelschlaegel, S., & Speer, K. (2014). Differentiation of manuka honey from kanuka honey and from jelly bush honey using HS-SPME-GC/MS and UHPLC-PDA-MS/MS. *Journal of Agricultural and Food Chemistry, 62*(27), 6435-6444. https://doi.org/10.1021/jf501818f.

Beitlich, N., Lubken, T., Kaiser, M., Ispiryan, L., & Speer, K. (2016). Fluorescent pteridine derivatives as new markers for the characterization of genuine monofloral New Zealand manuka (*Leptospermum scoparium*) honey. *Journal of Agricultural and Food Chemistry, 64*(46), 8886-8891. https://doi.org/10.1021/acs.jafc.6b03984.

Bernal, J. L., Nozal, M. J., Toribio, L., Diego, J. C., & Ruiz, A. (2005). A comparative study of several HPLC methods for determining free amino acid profiles in honey. *J Sep Sci, 28*(9-10), 1039-1047. https://www.ncbi.nlm.nih.gov/pubmed/16013831.

Bhattacherjee, A., Dhara, K., & Chakraborti, A. S. (2017). Bimolecular interaction of argpyrimidine (a Maillard reaction product) in in vitro non-enzymatic protein glycation model and its potential role as an antiglycating agent. *Int J Biol Macromol, 102*, 1274-1285. https://doi.org/10.1016/j.ijbiomac.2017.04.108.

Bong, J., Loomes, K. M., Lin, B., & Stephens, J. M. (2018). New approach: Chemical and fluorescence profiling of NZ honeys. *Food Chemistry, 267*, 355-367. https://doi.org/10.1016/j.foodchem.2017.07.065.

ChemSpider. (2020). ChemSpider ID13835161. Retrieved from: http://www.chemspider.com/Chemical-Structure.13835161.html Accessed March 11 2020.

Chernyshev, A., & Braggins, T. (2020). Investigation of temporal apparent C4 sugar change in Manuka honey. *Journal of Agricultural and Food Chemistry, 68*(14), 4261-4267. https://doi.org/10.1021/acs.jafc.9b06965.

Daniels, B. J., Prijic, G., Meidinger, S., Loomes, K. M., Stephens, J. M., Schlothauer, R. C., Furkert, D. P., & Brimble, M. A. (2016). Isolation, structural elucidation, and synthesis of lepteridine from manuka (*Leptospermum scoparium*) honey. *Journal of Agricultural and Food Chemistry, 64*(24), 5079-5084. https://doi.org/10.1021/acs.jafc.6b01596.

El-Sayed, A. M., Unelius, C. R., & Suckling, D. M. (2018). Honey norisoprenoids attract bumble bee, Bombus terrestris, in New Zealand mountain beech forests. *Journal of Agricultural and Food Chemistry, 66*(50), 13065-13072. https://doi.org/10.1021/acs.jafc.8b04175.

Frye, E. B., Degenhardt, T. P., Thorpe, S. R., & Baynes, J. W. (1998). Role of the Maillard reaction in aging of tissue proteins. Advanced glycation end product-dependent increase in imidazolium cross-links in human lens proteins. *Journal of Biological Chemistry, 273*(30), 18714-18719. https://doi.org/10.1074/jbc.273.30.18714.

Grainger, M. N., Manley-Harris, M., Lane, J. R., & Field, R. J. (2016a). Kinetics of

conversion of dihydroxyacetone to methylglyoxal in New Zealand manuka honey: Part I--Honey systems. *Food Chemistry, 202*, 484-491. https://doi.org/10.1016/j.foodchem.2016.02.029.

Grainger, M. N., Manley-Harris, M., Lane, J. R., & Field, R. J. (2016b). Kinetics of conversion of dihydroxyacetone to methylglyoxal in New Zealand manuka honey: Part III--A model to simulate the conversion. *Food Chemistry, 202*, 500-506. https://doi.org/10.1016/j.foodchem.2016.02.032.

Grainger, M. N., Manley-Harris, M., Lane, J. R., & Field, R. J. (2016c). Kinetics of the conversion of dihydroxyacetone to methylglyoxal in New Zealand manuka honey: Part II--Model systems. *Food Chemistry, 202*, 492-499. https://doi.org/10.1016/j.foodchem.2016.02.030.

Grainger, M. N. C., Owens, A., Manley-Harris, M., Lane, J. R., & Field, R. J. (2017). Kinetics of conversion of dihydroxyacetone to methylglyoxal in New Zealand mānuka honey: Part IV - Formation of HMF. *Food Chemistry, 232*, 648-655. https://doi.org/https://doi.org/10.1016/j.foodchem.2017.04.066.

Hellwig, M., Ruckriemen, J., Sandner, D., & Henle, T. (2017). Unique pattern of protein-bound Maillard reaction products in manuka (*Leptospermum scoparium*) honey. *Journal of Agricultural and Food Chemistry, 65*(17), 3532-3540. https://doi.org/10.1021/acs.jafc.7b00797.

Ishisaka, A., Ikushiro, S., Takeuchi, M., Araki, Y., Juri, M., Yoshiki, Y., Kawai, Y., Niwa, T., Kitamoto, N., Sakaki, T., Ishikawa, H., & Kato, Y. (2017). In vivo absorption and metabolism of leptosperin and methyl syringate, abundantly present in manuka honey. *Molecular Nutrition & Food Research, 61*(9). https://doi.org/10.1002/mnfr.201700122.

Jost, T., Heymann, T., & Glomb, M. A. (2019). Efficient analysis of 2-acetyl-1-pyrroline in foods using a novel derivatization strategy and LC-MS/MS. *Journal of Agricultural and Food Chemistry, 67*(10), 3046-3054. https://doi.org/10.1021/acs.jafc.9b00220.

Kato, Y., Fujinaka, R., Ishisaka, A., Nitta, Y., Kitamoto, N., & Takimoto, Y. (2014). Plausible authentication of manuka honey and related products by measuring leptosperin with methyl syringate. *Journal of Agricultural and Food Chemistry, 62*(27), 6400-6407. https://doi.org/10.1021/jf501475h.

Kato, Y., Kawai, M., Kawai, S., Okano, Y., Rokkaku, N., Ishisaka, A., Murota, K., Nakamura, T., Nakamura, Y., & Ikushiro, S. (2019). Dynamics of the cellular metabolism of leptosperin found in manuka honey. *Journal of Agricultural and Food Chemistry*. https://doi.org/10.1021/acs.jafc.9b03894.

Kato, Y., Umeda, N., Maeda, A., Matsumoto, D., Kitamoto, N., & Kikuzaki, H. (2012). Identification of a novel glycoside, leptosin, as a chemical marker of manuka honey. *Journal of Agricultural and Food Chemistry, 60*(13), 3418-3423. https://doi.org/10.1021/jf300068w.

Klopfer, A., Spanneberg, R., & Glomb, M. A. (2011). Formation of arginine modifications in a model system of Nalpha-tert-butoxycarbonyl (Boc)-arginine with methylglyoxal. *Journal of Agricultural and Food Chemistry, 59*(1), 394-401. https://doi.org/10.1021/jf103116c.

Lin, B., Loomes, K. M., Prijic, G., Schlothauer, R., & Stephens, J. M. (2017). Lepteridine as a unique fluorescent marker for the authentication of manuka honey. *Food Chemistry, 225*, 175-180. https://doi.org/10.1016/j.foodchem.2016.12.099.

Mavric, E., Wittmann, S., Barth, G., & Henle, T. (2008). Identification and quantification of methylglyoxal as the dominant antibacterial constituent of Manuka (*Leptospermum scoparium*) honeys from New Zealand. *Molecular Nutrition & Food Research, 52*(4), 483-489. https://doi.org/10.1002/mnfr.200700282.

McDonald, C. M., Keeling, S. E., Brewer, M. J., & Hathaway, S. C. (2018). Using chemical and DNA marker analysis to authenticate a high-value food, manuka honey. *npj*

Science of Food, 2(1), 9. https://doi.org/10.1038/s41538-018-0016-6.

Ministry for Primary Industries, N. Z. (2018). Mānuka honey. Retrieved from: URL (http://www.mpi.govt.nz/growing-and-harvesting/honey-and-bees/manuka-honey/) Accessed 01 June, 2019 2019.

Nagaraj, R. H., Shipanova, I. N., & Faust, F. M. (1996). Protein cross-linking by the Maillard reaction. Isolation, characterization, and in vivo detection of a lysine-lysine cross-link derived from methylglyoxal. *Journal of Biological Chemistry, 271*(32), 19338-19345. https://doi.org/10.1074/jbc.271.32.19338.

Nakamura, T., Kawai, Y., Kitamoto, N., Osawa, T., & Kato, Y. (2009). Covalent modification of lysine residues by allyl isothiocyanate in physiological conditions: plausible transformation of isothiocyanate from thiol to amine. *Chem Res Toxicol, 22*(3), 536-542. https://doi.org/10.1021/tx8003906.

Noe, S., Manley-Harris, M., & Clearwater, M. J. (2019). Floral nectar of wild mānuka (*Leptospermum scoparium*) varies more among plants than among sites. *New Zealand Journal of Crop and Horticultural Science, 47*(4), 282-296. https://doi.org/10.1080/01140671.2019.1670681.

Pappalardo, M., Pappalardo, L., & Brooks, P. (2016). Rapid and reliable HPLC method for the simultaneous determination of dihydroxyacetone, methylglyoxal and 5-hydroxymethylfurfural in Leptospermum honeys. *PLoS One, 11*(11), e0167006. https://doi.org/10.1371/journal.pone.0167006.

Rogers, K., Grainger, M., & Manley-Harris, M. (2014). The unique manuka effect: Why New Zealand manuka honey fails the AOAC 998.12 C-4 sugar method - Part 2. *Journal of Agricultural and Food Chemistry*. https://doi.org/10.1021/jf404767b.

Ruckriemen, J., Schwarzenbolz, U., Adam, S., & Henle, T. (2015). Identification and quantitation of 2-acetyl-1-pyrroline in manuka honey (*Leptospermum scoparium*). *Journal of Agricultural and Food Chemistry, 63*(38), 8488-8492. https://doi.org/10.1021/acs.jafc.5b03042.

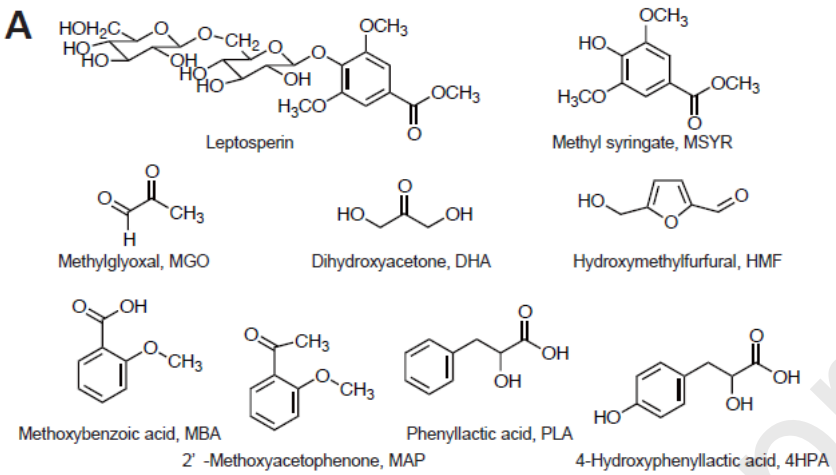
Sasakawa, H. (1987). Influence of environmental factors on nitrogen fixing activity in field grown Chinese milk vetch (*Astragalus sinicus* L.). *Japanese journal of crop science, 56*(4), 577-581. https://doi.org/10.1626/jcs.56.577.

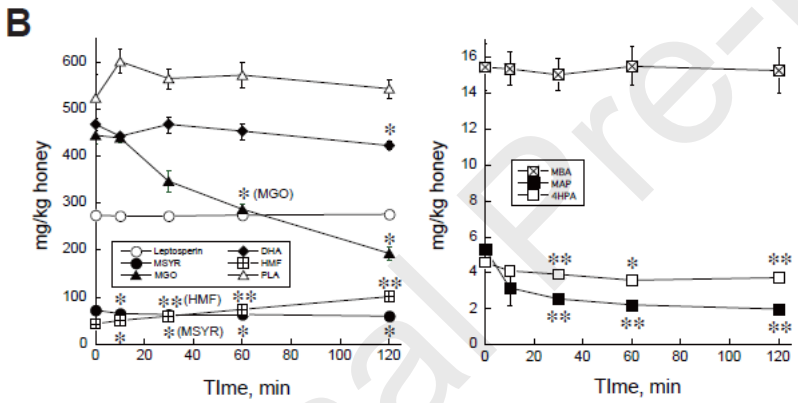
Shao, X., Bai, N., He, K., Ho, C. T., Yang, C. S., & Sang, S. (2008). Apple polyphenols, phloretin and phloridzin: new trapping agents of reactive dicarbonyl species. *Chem Res Toxicol, 21*(10), 2042-2050. https://doi.org/10.1021/tx800227v.

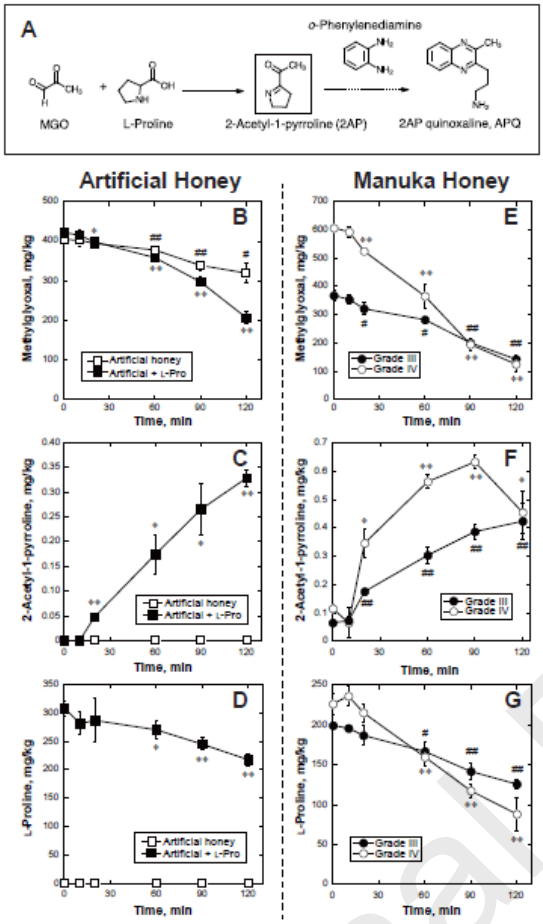
Sousa Silva, M., Gomes, R. A., Ferreira, A. E., Ponces Freire, A., & Cordeiro, C. (2013). The glyoxalase pathway: the first hundred years... and beyond. *Biochemical Journal, 453*(1), 1-15. https://doi.org/10.1042/BJ20121743.

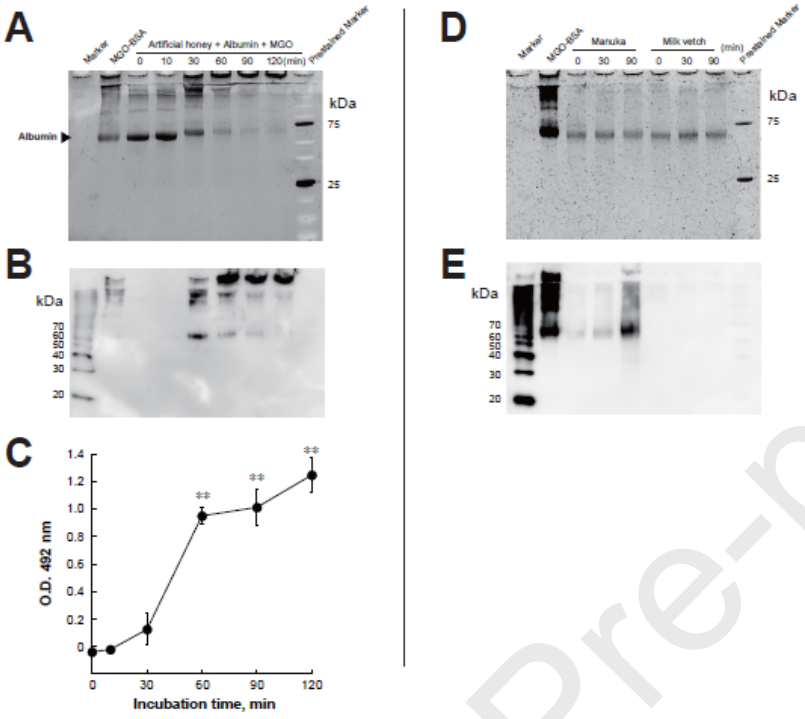
Stephens, J. M., Schlothauer, R. C., Morris, B. D., Yang, D., Fearnley, L., Greenwood, D. R., & Loomes, K. M. (2010). Phenolic compounds and methylglyoxal in some New Zealand manuka and kanuka honeys. *Food Chemistry, 120*(1), 78-86. https://doi.org/http://dx.doi.org/10.1016/j.foodchem.2009.09.074.

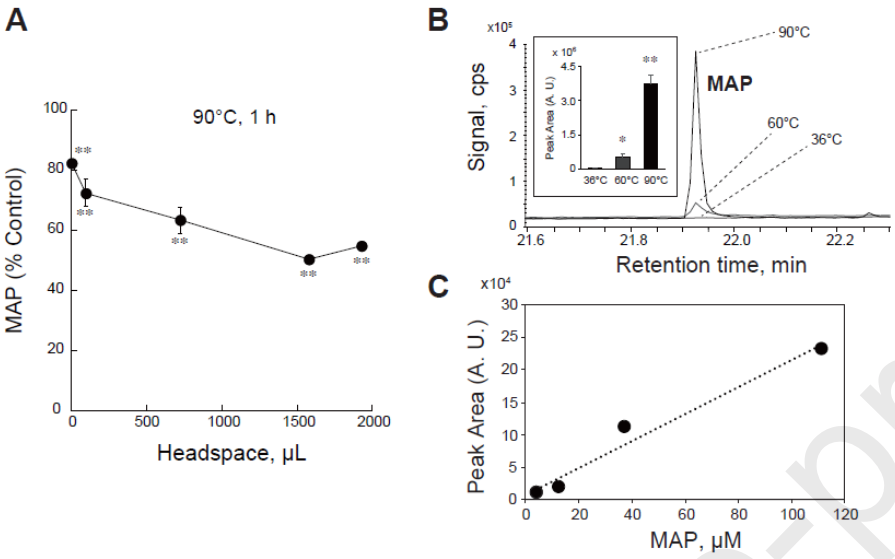
Wahdan, H. A. (1998). Causes of the antimicrobial activity of honey. *Infection, 26*(1), 26-31. http://www.ncbi.nlm.nih.gov/pubmed/9505176.











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Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- Changes in nine chemicals in manuka honey by thermal treatments were analyzed.
- Methylglyoxal (MGO) and 2'-methoxyacetophenone (MAP) were lost by high heating.
- MGO was lost by generation of adducts with L-proline, lysine, and arginine residues.
- MAP was released in the gaseous form into the headspace on heating.