Identification and Quantitation of 2-Acetyl-1-pyrroline in Manuka Honey (Leptospermum scoparium)

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ABSTRACT: Manuka honey from New Zealand is known for its exceptional antibacterial activity, which is due to high amounts of the 1,2-dicarbonyl compound methylglyoxal (MGO). MGO in manuka honey is formed via non-enzymatic dehydration from dihydroxyacetone (DHA) during honey maturation. MGO and DHA are highly reactive substances, leading to a variety of unique chemical reactions. During Strecker reaction between proline and MGO, 2-acetyl-1-pyrroline (2-AP), an important aroma compound, is formed. Using liquid–liquid extraction and gas chromatography–mass spectrometry analysis, 2-AP was identified unambiguously in manuka honey for the first time. Quantitation was carried out via external matrix calibration, using a synthetic 2-AP standard and artificial honey. The 2-AP concentration in 11 commercial samples of manuka honey ranged from 0.08 to 0.45 mg/kg. For manuka honey samples containing MGO in concentrations above 250 mg/kg, significantly higher amounts of 2-AP were found when compared to non-manuka honeys. When high amounts of MGO were artificially added to non-manuka multifloral honey, an increase of the 2-AP concentration from 0.07 to 0.40 mg/kg after 12 weeks of storage at 37 °C was observed, concomitant with a significant increase in the concentration of 5-hydroxymethylfurfural (HMF). No increase of 2-AP was found during storage at ambient temperature. 2-AP together with MGO can be a suitable parameter for the quality control of manuka honey.

KEYWORDS: manuka honey, 2-acetyl-1-pyrroline, methylglyoxal, aroma, Maillard reaction, Strecker degradation, 5-hydroxymethylfurfural

INTRODUCTION

Manuka honey from New Zealand, originating from the floral source Leptospermum scoparium, is well-known for its unique antibacterial properties.1 The 1,2-dicarbonyl compound methylglyoxal (MGO) was found to be responsible for the antibacterial activity of manuka honey.2,3 MGO is formed non-enzymatically from dihydroxyacetone (DHA), a compound present in the nectar of the manuka plant, during storage of the nectar and ripening of the honey. Manuka honeys may contain MGO and DHA in concentrations up to 800 and 1600 mg/kg, respectively. In non-manuka honeys, only negligible amounts ranging from 5 to 10 mg/kg of these compounds are found.4 Reactive carbonyl species, such as MGO, play an important role in flavor chemistry. During Strecker reactions of amino acids and MGO, various aroma compounds are generated.5 Intermolecular reactions of MGO can lead to the formation of furaneol via the Cannizzaro reaction.5 The volatile compound 2-acetyl-1-pyrroline (2-AP) originates from the reaction of MGO with the amino acids ornithine or proline, respectively.6 2-AP is the main aroma compound in aromatic rice varieties and bread crust.7–9 Its smell is described as “popcorn-like” or “roasted”, with an odor threshold of 0.1 μg/kg.10 Besides MGO, honey contains other 1,2-dicarbonyl compounds, such as 3-desoxyglucosone (3-DG) or glucosone, resulting from sugar degradation or caramelization.1,11,12 1,2-Dicarbonyl species quickly interact with amino groups of proteins or amino acids and form Maillard reaction products. This process is affected by water activity, temperature, and pH value.13 Here, we followed the hypothesis that the presence of MGO can lead to the formation of characteristic reaction products in manuka honey, in particular, during storage, depending upon the concentration of the dicarbonyl compound. Proline is the most abundant amino acid in honey, accounting for 260–890 mg/kg, which represents 70% of the total free amino acids.14 The aim of this study, therefore, was to investigate the possible occurrence of 2-AP, a direct reaction product of proline and MGO, in manuka honey and its dependency upon the MGO content. Moreover, kinetic studies of 2-AP formation in non-manuka multifloral honey with artificially added MGO were conducted. The identification and quantitation of 2-AP in honey contributes to a better understanding of the unique chemistry of manuka honey and offers possibilities for verifying the quality of manuka honey.

MATERIALS AND METHODS

Honey Samples. All samples were commercially available and stored at 4 ºC until analysis. In total, 11 manuka honeys with varying MGO contents and 8 non-manuka honeys were investigated. The group of non-manuka honeys consisted of 2 multifloral honeys and 6 monofloral honeys, namely, buckwheat, lime, cornflower, Scottish heather, rape, and thyme honeys.

Chemicals. Dichloromethane and methanol [both high-performance liquid chromatography (HPLC) grade] were obtained from VWR (Darmstadt, Germany). The commercial standard of 2-AP [10% (w/w) in dichloromethane] was from Toronto Research Chemicals (TRC, Toronto, Ontario, Canada). Sodium acetate, sodium dihydrogen
phosphate, disodium hydrogen phosphate, and anhydrous sodium sulfate were purchased from Grünig (Filsium, Germany). MGO (40%, w/w), DHA, ortho-phenylendiamine (oPD), and 2,4,6-trimethylpyridine (TPD) were obtained from Sigma-Aldrich (Steinheim, Germany). 5-Hydroxymethylfurfural (HMF) was provided by Alfa Aesar (Karlsruhe, Germany). δ−Fructose, δ+−glucose, δ−−saccharose, and acetic acid were purchased from Carl Roth (Karlsruhe, Germany). Sodium hydroxide pellets were purchased from Fisher Scientific (Schwerte, Germany). For all experiments, ultrapure water was used, prepared by an ELGA LabWater PURELAB Plus water system (Celle, Germany).

Reversed-Phase High-Performance Liquid Chromatography with Ultraviolet Detection (RP-HPLC–UV) Analysis of MGO and DHA after Derivatization to Quinoxalines. The determination of MGO and DHA was performed according to the literature,1 with some modifications. Briefly, for quantitation of MGO, 1 mL aliquots of 10% (w/v) honey solutions in 0.5 M phosphate buffer (pH 6.5) were mixed with 300 μL of phosphate buffer (pH 6.5) and 300 μL of oPD solution (1% in phosphate buffer at pH 6.5). Samples were incubated in the dark at room temperature overnight and membrane-filtered (0.45 μm). For quantitation of DHA, 1 mL aliquots of 10% honey solutions in 0.5 M acetic buffer (pH 4.0) were mixed with 300 μL of acetic buffer (pH 4.0) and 300 μL of oPD solution (1% in acetic buffer at pH 4.0), followed by incubation for 16 h at 37 °C and membrane filtration (0.45 μm).

HPLC analyses were performed using an Äkta basic system from Amersham Pharmacia Biotech (Uppsala, Sweden) with a pump P-900 and an online degasser K-5004 (Knauer, Berlin, Germany) as well as an UV detector UV-900 and an auto sampler A-900. Peak evaluation was managed using the software UNICORN 4.11. The separation of quinoxalines was realized on a stainless-steel column filled with Eurospher 100 RP18 material (250 × 4.6 mm, 5 μm particle size, with integrated pre-column, Knauer, Berlin, Germany). The mobile phase was 0.075% acetic acid in water (solvent A) and a mixture of 80% acetic acid and 20% fructose (solvent B). The elution was carried with 0.05 M phosphate buffer (pH 4.0) was set to 40 °C and held for 2 min, then raised to 225 °C/min to 235 °C, and held for 5 min. The mass spectrometer was working in EI mode at 70 eV in the scan (mass range of m/z 40.0–300.0) and selected ion monitoring (SIM) mode. For quantitation, the SIM mode was used with the monitoring of the following ions: m/z 111 and 83 (2-AP) and m/z 121 and 79 (TMP). The ions underlined were used for quantitation. The retention time of the analytes was 15.0 min for 2-AP and 17.9 min for TMP, respectively. Data acquisition and evaluation were performed with MSD ChemStation software (Agilent, Germany).

Model Studies on 2-AP Formation in Honey. Commercial non-manuka multifloral honey from Germany was mixed with MGO alone (100 mg/kg, labeled as “low”), or 500 mg/kg, labeled as “high”), respectively or MGO and DHA in a 1:2 ratio (100 and 200 mg/kg, labeled as “low”, or 500 and 1000 mg/kg, labeled as “high”, respectively). The samples were stirred vigorously and stored in the dark at room temperature and 37 °C. The storage temperature of 37 °C was chosen to simulate accelerated ripening and to study chemical reactions that occur during longer storage. Samples were taken after 0, 4, and 12 weeks of storage. A control (multifloral honey without added MGO) was stored under the same conditions, and samples were taken after 0, 4, and 12 weeks of storage. The samples were analyzed according to the extraction procedure mentioned above.

Statistics. Results are expressed as mean values of two separate measurements, except otherwise indicated. Hypothesis testing was performed via a two sample t test with a significance level of 0.01. Intraday variation of 2-AP analysis was investigated using five replicate injections of a 2-AP standard solution. Intraday precision was determined using four calibration standards, which were analyzed during 8 months.

RESULTS AND DISCUSSION

The aim of this study was to investigate whether 2-AP, a specific flavor compound formed from MGO and proline, is present in manuka honeys from New Zealand. Identification and quantitation of 2-AP in manuka honey was achieved using GC–MS based on a method recently described in the literature for 2-AP analysis.18 For sample preparation, a crucial step for reliable quantitation of 2-AP, solid-phase microextraction (SPME), or solvent extraction methods are reported.7,18
Because 2-AP formation in manuka honey is probably enhanced by a high temperature, an extraction procedure using dichloromethane without heating was chosen. Furthermore, concentration steps like drying with nitrogen were avoided to prevent a loss of 2-AP during sample preparation. TMP was used as an internal standard to check the recovery during the extraction procedure. Comparing peak areas for 2-AP standard samples extracted from aqueous solutions or from honey matrix calibration resulted in an estimated 60% recovery of 2-AP. Therefore, quantitation was carried out via external matrix calibration, using a synthetic 2-AP standard and artificial honey. The actual 2-AP concentration of the commercial standard was checked with RP-HPLC and chemiluminescent nitrogen detection (CLND) to be 83.3% of the specification given by the supplier. A regression coefficient $R^2$ of 0.998 was calculated for the linear calibration range from 0.1 to 2.0 mg/kg. The limit of detection (LOD) was 0.04 mg/kg, and the limit of quantitation (LOQ) was 0.09 mg/kg. LOD and LOQ were calculated on the basis of the signal-to-noise-ratio. Inter- and intraday precisions were 3.1 and 6.1%, respectively. 2-AP was unequivocally identified in commercial samples of manuka honey by comparing retention time and corresponding mass spectra of honey samples and standard solutions (panels A and C of Figure 1). The comparison of the fragmentation pattern and the characteristic ratio of the quantifier ion to the qualifier ion ($m/z$ 83 and 111, respectively) of the 2-AP standard and the 2-AP peak in honey yields identical relative intensity ratios. The hypothesis that 2-AP in manuka honey results from the reaction of MGO and proline was confirmed by significantly smaller signals of 2-AP in non-manuka honeys without MGO (Figure 1B) and the detection of 2-AP in an artificial honey containing MGO and proline after 20 weeks of incubation (Figure 1D).

For 11 commercially available manuka honeys, amounts of 2-AP ranging from 0.08 to 0.45 mg/kg were measured (Figure 2). In contrast to this, the majority of non-manuka honeys analyzed in this study contained 2-AP only in very small amounts between 0.08 and 0.11 mg/kg. However, some aromatic non-manuka honeys, namely, buckwheat, lime, and thyme honey, contained 2-AP in concentrations ranging between 0.20 and 0.31 mg/kg. According to the literature, 2-AP has been found in different sorts of food as an aroma active compound. The concentrations vary between 78 μg/kg for white-bread crust and 0.8 mg/kg for cooked rice. Concerning honey, 2-AP has already been detected in lime honey from the linden tree. The authors pointed out that 2-AP is an important odorant of linden honey but did not report on quantitative amounts of 2-AP. Interestingly, no 2-AP was detected in the blossoms of the linden tree, what supports the hypothesis that 2-AP originates from reactions in the honey matrix. The 2-AP concentration of manuka honey with a MGO concentration below 250 mg/kg is comparable to the non-manuka honeys. However, the concentration of 2-AP in manuka honeys with a MGO concentration above 250 mg/kg was significantly increased in comparison to the non-manuka group (Figure 2). The average amount of 2-AP in manuka honeys with MGO contents above 250 mg/kg was 0.36 mg/kg, and the average amount of 2-AP in non-manuka honeys was 0.16 mg/kg. For this samples, the 2-AP content of the analyzed manuka honeys increased with increasing MGO concentrations up to a MGO value of approximately 500 mg/kg honey (see Figure S1). For concentrations of 600−1000 mg of MGO/kg of honey, no further increase of 2-AP was found. Proline is the most abundant amino acid in honey, accounting for 260−890 mg/kg, which represents 70% of the total free amino acids. The analysis of the proline concentration of the studied honeys showed no significant difference between the non-manuka and manuka honeys with varying MGO contents. The proline concentration ranged between 200 and 540 mg/kg and did not correlate with the MGO content (for data on MGO and
In fact, MGO appears to be the crucial factor for 2-AP formation. The observation that MGO contents between 600 and 1000 mg/kg do not lead to a further increase of 2-AP in manuka honeys could be explained by competitive reactions. MGO can react with other honey substances or with another MGO molecule to form adducts or aroma-active compounds, such as furaneol, respectively. To assess whether storage of non-manuka honey containing artificially added MGO or DHA results in the formation of 2-AP, a commercial multifloral honey was spiked with MGO or a 1:2 mixture of MGO and DHA, respectively, and stored at room temperature and 37 °C for 12 weeks. Figure 3A shows the 2-AP concentration depending upon the incubation time. When high amounts of MGO (500 mg/kg) were added to the honey, 2-AP significantly increased after 4 weeks of storage at 37 °C. After 12 weeks, the 2-AP concentration reached the amount that was quantitated in the commercial manuka honey samples with high MGO contents, followed by no further increase. No increase of 2-AP was found during storage of non-manuka honey with artificially added MGO at ambient temperature and non-manuka honey without MGO (ambient temperature and 37 °C; data not shown). There was also no increase in 2-AP when low MGO concentrations (100 mg/kg) were added to non-manuka honey and stored at an elevated temperature for 12 weeks (see Figure 3). This is in accordance with the 2-AP amounts of commercial manuka honeys containing MGO below 250 mg/kg. The average 2-AP content of these samples was 0.14 mg/kg. The non-manuka honey with artificially added MGO (low MGO) had approximately the same amount of 2-AP, which did not increase after storage. This indicates that a “threshold level” of MGO of approximately 250 mg/kg is necessary to initiate a reaction of proline and MGO and significantly increase the 2-AP content in manuka honey. Competitive reactions as a possible reason for the disproportionate conversion of MGO and proline to 2-AP were discussed above. The 2-AP concentration in non-manuka honey was not influenced by the addition of DHA and subsequent storage (Figure 3B). For the spiked honey samples, which were stored for 8 weeks or longer at 37 °C, a significant increase in the concentration of HMF in addition to the formation of 2-AP was observed. HMF is used as a quality parameter of honey and should not exceed 40 mg/kg. All commercial manuka honeys in this study were below this threshold level. This result points to the fact that, following “artificial” spiking of a non-manuka honey with MGO, 2-AP concentrations do only increase during storage at elevated temperatures, which also leads to increasing amounts of HMF. 2-AP, therefore, might be an interesting indicator to control the storage conditions and the quality of manuka honey. High amounts of 2-AP, as a result of high MGO levels, together with an increased concentration of HMF may point to a fraudulent addition of MGO and increased temperatures for “artificially” producing high-price manuka honey products. A manuka honey with a high MGO content together with a high 2-AP concentration and a low HMF level has likely been stored at low temperatures over a longer time period. This “natural” ripening process cannot be simulated by MGO addition followed by a high temperature storage. According to the literature, the storage of manuka honey initially leads to a MGO increase as a result of continuous formation from DHA, followed by a MGO decrease, probably as a result of reactions of MGO with other honey components. Further studies are needed to clarify the reaction pathways of MGO and its role in the unique chemistry of manuka honey. In addition to MGO and DHA, 2-AP as a direct reaction product of MGO and proline could be a suitable parameter to characterize the quality of manuka honey.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b03042.

MGO, HMF, proline, and 2-AP contents of non-manuka and manuka honeys (Table S1) and 2-AP concentration of commercial manuka honey samples (n = 11) with varying MGO contents (Figure S1) (PDF)
2-AP, 2-acetyl-1-pyrroline; MGO, methylglyoxal; DHA, dihydroxyacetone; GC–MS, gas chromatography–mass spectrometry; LOD, limit of detection; LOQ, limit of quantitation; HMF, 5-hydroxymethylfurfural; oPD, ortho-phenylenediamine; HPLC, high-performance liquid chromatography; TMP, 2,4,6-trimethylpyridine

REFERENCES