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Studies on the formation of methylglyoxal from dihydroxyacetone in Manuka (*Leptospermum scoparium*) honey

Julia Atrott, Steffi Haberlau, Thomas Henle *

Institute of Food Chemistry, Technische Universität Dresden, D-01062 Dresden, Germany

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ABSTRACT

Dihydroxyacetone (DHA) and methylglyoxal (MGO) are unique carbohydrate metabolites of manuka honey. A method for the reliable quantification of DHA in honey samples was established, based on derivatization with *o*-phenylenediamine (OPD) and subsequent RP-HPLC with UV detection. The previously unknown reaction product of DHA and OPD was identified as 2-hydroxymethylquinoxaline by spectroscopic means. DHA was exclusively determined in 6 fresh manuka honeys originating directly from the beehive as well as 18 commercial manuka honey samples, ranging from 600 to 2700 mg/kg and 130 to 1600 mg/kg, respectively. The corresponding MGO contents varied from 50 to 250 mg/kg in fresh and 70 to 700 mg/kg in commercial manuka honey samples. A good linear correlation between DHA and MGO values in commercial manuka honeys was observed, resulting in a mean ratio of DHA to MGO of 2:1. In contrast to this, the DHA-to-MGO relation was much higher in fresh manuka honeys but approximated to a ratio of 2:1 while honey ripening. Heating experiments revealed that MGO formation based on thermal treatment as a consequence, for example, of caramelization in honey does not occur. DHA and MGO can serve as suitable unique quality parameter for manuka honey.

1. Introduction

The presence of 1,2-dicarbonyl compounds such as 3-deoxyglucosone (3-DG), glyoxal (GO), and methylglyoxal (MGO) in honey was first described in 2004 by Weigel et al.[1] as a consequence of sugar degradation or caramelization. Over a wide range of different honey types, 3-DG, which is the precursor for 5-hydroxymethylfurfural (HMF), could be determined in high amounts, varying between 80 and 1270 mg/kg and correlating with heating or storage conditions.[1–3] Contents of GO and MGO generally were very low (up to 5 mg/kg). However, surprisingly high amounts of MGO (up to 760 mg/kg) were found exclusively in manuka honey.[2] This honey is derived from the flowers of the manuka tree (*Leptospermum scoparium*) in New Zealand. It was clearly demonstrated that the pronounced antibacterial activity of New Zealand manuka honey directly originates from MGO.[2] The amount of MGO correlates with the antibacterial activity in manuka honey,[4,5] and, therefore, can be used as a tool for labelling the bioactivity of manuka honey.

Manuka honey was shown to inhibit a wide range of microorganisms, including multiresistant strains.[6–8] Furthermore, wound dressings containing manuka honey seem to be useful supports in clinical applications for wound healing.[9,10]

The origin and in particular the high concentration of MGO in manuka honey cannot be explained by sugar degradation in the course of caramelization. In a recent report, Adams et al.[11] identified dihydroxyacetone (DHA) as direct precursor for MGO formation in manuka honeys. In this study, the authors had determined DHA in nectar and fresh honey derived from manuka plants and proved that DHA is non-enzymatically transferred to MGO during honey storage. However, the origin of DHA stills remains enigmatic.

DHA is a well-known degradation product of carbohydrates and was detected in caramelized mixtures next to other compounds as a result of the Maillard reaction.[12] DHA was also found in naturally aged wine samples.[13] Moreover, its presence was described in relation to fermentation processes of selected microorganisms.[14] Furthermore, DHA is discussed as food additive, for example to enhance browning in baked foods,[15] or for food preservation.[16] In certain cosmetic products, namely self-tanning creams, DHA is the functional ingredient.[17]

Knowledge about the amount of DHA and MGO in honey is of prime importance for manufacturers as well as for labelling purpose. In the present study, a new method for the reliable quantification of DHA via RP-HPLC using pre-column derivatization with *o*-phenylenediamine (OPD) is reported. The previously unknown derivative resulting from DHA and OPD is identified by spectroscopic means. DHA and MGO was measured for a number of commercially available and freshly produced manuka honeys in order to obtain information about the ratio between DHA and MGO and the transformation of DHA to MGO during storage. Furthermore, the influence of a thermal treatment on MGO content in

* Corresponding author. Fax: +49 351 463 34138.
E-mail address: Thomas.Henle@chemie.tu-dresden.de (T. Henle).

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manuka honey was studied in order to clarify whether the bioactive compound can be produced artificially in the final product.

2. Results and discussion

2.1. RP-HPLC analysis of DHA and characterization of DHA quinoxaline

The analysis of 1,2-dicarbonyl compounds such as 3-desoxyglucosone (3-DG) or methylglyoxal (MGO) in honey is generally achieved by RP-HPLC of the corresponding quinoxalines resulting from derivatization with *o*-phenylenediamine (OPD). Under conditions applied for optimal derivatization of these compounds (phosphate buffer pH 6.5, incubation at room temperature), dihydroxyacetone (DHA) also formed an OPD-derivative eluting in the chromatogram at about 13 min (Fig. 1A). This reaction product was isolated using semi preparative RP-HPLC. The molar mass of the isolate was determined with 160 g/mol by LC-ESI-TOF-MS. The UV maximum at 319 nm was observed, varying only slightly when compared to the absorption maximum of 312 nm generally reported for quinoxalines. Based on the NMR data, the structure was identified as 2-hydroxymethylquinoxaline (2-quinoxalinemethanol, compound VII in Fig. 2).

2-Hydroxymethylquinoxaline has already been described as reaction product formed during incubation of sugars like glucose or ribose, respectively, with OPD.[18,19] The identified quinoxalines derived from this reaction mixture were explained by the buffer-induced degradation of hexoses to 1,2-dicarbonyl fragments.[19] However, the authors did not propose DHA being involved in quinoxaline formation. Up to now, it was generally accepted that DHA does not react with OPD to a stable derivative, and, therefore, cannot be quantified by means of RP-HPLC following derivatization with OPD.[20]

A possible reaction mechanism for the formation of 2-hydroxymethylquinoxaline from DHA and OPD is shown in Figure 2. First, a nucleophilic addition of one amino group of OPD (I) to the carbonyl carbon of DHA (II) occurs. The iminol (III) is formed by dehydration and is able to convert via the enaminol (IV) into the ketoamine (V) according to the conversion of Amadori products. A second dehydration results in formation of an intermediate (VI), which is converted into the quinoxaline (VII), putatively by oxidation. Compared to dicarbonyl compounds like MGO or 3-DG, complete derivatization of DHA with OPD takes more time for development of the quinoxaline. Therefore, it can be supposed that the formation of the keto group in (V) via enaminol (IV) is the rate determining step of the reaction. The oxidation of intermediate (VI) to the quinoxaline cannot be proven yet, but it may due to the oxidative properties of the reaction mixture, for example the influence of oxygen.

Kinetic studies with varying incubation time, temperature, pH value, and molar ratio of DHA to OPD were performed in order to optimize derivatization of DHA to compound (VII) (data not shown). Finally, it could be shown that best results are obtained after incubation for 16 h at 37 °C using an acetate buffer with a pH value of 4.0 for sample preparation. Due to these differences in methodical conditions, reliable simultaneous quantification of DHA together with MGO was not possible. At pH value of 4.0, acid-induced formation of the MGO–quinoxaline was observed in carbohydrate solutions, and hence, MGO values would be overestimated. Otherwise, reaction of DHA and OPD is too slow at neutral pH, and therefore, DHA does not react completely with OPD under these conditions. Consequently, to reach optimal quinoxaline formation and best sensitivity for both analytes, two independent sample preparations and two chromatographic runs per sample are necessary.

Performing DHA determination under these conditions, it became clear that notable amounts of DHA are also formed from the honey matrix itself, namely from glucose and fructose breakdown, during derivatization (Fig. 1B and C). This reproducible basis level of DHA has to be taken into account by using an artificial honey matrix as blank value.[21] Calibration of (VII) using this sugar matrix as 10% solution in acetate buffer showed a good linearity (regression equation $y = 0.15x + 5.66$, $R^2 = 0.9944$) in the range from 0.18 to 2.20 mmol/L DHA in sample solution, equivalent to DHA concentrations between 165 and 1980 mg/kg in honey. Quantification of DHA in manuka honey (Fig. 1D) was finally performed

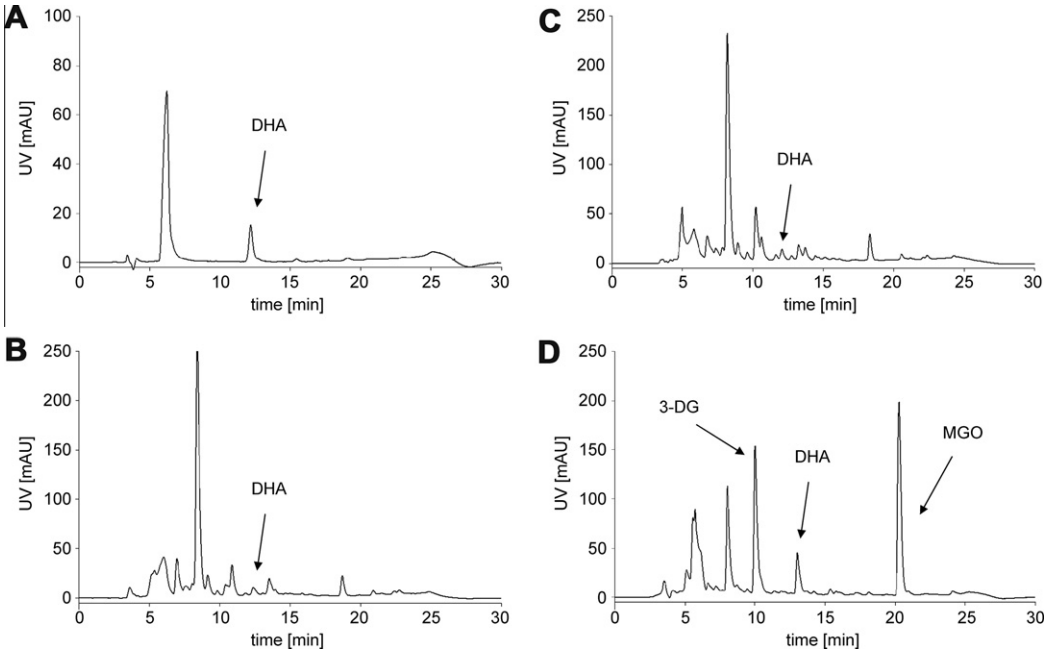

Figure 1. RP-HPLC after derivatization with OPD of (A) DHA standard solution in water, (B) solution of fructose and glucose simulating honey matrix, (C) rape honey, (D) manuka honey.