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Kinetics of conversion of dihydroxyacetone to methylglyoxal in New Zealand mānuka honey: Part I – Honey systems

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ABSTRACT

The kinetics of conversion of dihydroxyacetone (DHA) to methylglyoxal (MGO) were investigated in mānuka honeys and DHA-doped clover honeys stored between 4 and 37 °C. Both the disappearance of DHA and appearance of MGO were confirmed as overall, first order reactions, albeit probably composites of multiple reactions. Increasing the storage temperature accelerated the rate of DHA loss and the initial rate of formation of MGO, but better conversion efficiency was observed at lower temperature. At 37 °C, more MGO was lost at later times in mānuka honey compared to DHA-doped-clover honey. Thirty-seven New Zealand mānuka honeys and four clover honeys were analysed for various chemical and physical properties; comparison of rate constants and these parameters identified some positive correlations.

1. Introduction

Mānuka honey, from *Leptospermum scoparium* J.R. Forst. & G. Forst (Myrtaceae), has a unique, non-peroxide anti-bacterial activity (NPA) which predominantly arises from the presence of unusually large amounts of methylglyoxal (MGO) (Adams et al., 2008; Mavric, Wittmann, Barth, & Henle, 2008). Dihydroxyacetone (DHA), the precursor of MGO, derives from the nectar of mānuka flowers (Adams, Manley-Harris, & Molan, 2009) and converts to MGO as the honey matures. Nectar dihydroxyacetone varies from tree to tree and between seasons (Williams et al., 2014) and may therefore vary in immature honeys; MGO was not reported in mānuka nectar by Adams et al. (2009) or Williams et al. (2014) but trace amounts were reported by Stephens et al. (2010), this latter observation might possibly be an artefact of nectar storage. DHA converts to MGO as the honey matures; Adams et al. (2009) observed a decrease in DHA concentration and an increase in MGO concentration upon storage in both immature mānuka honey and in clover honey doped with DHA; similar behaviour in mānuka honey was observed by Atrott, Haberlau, and Henle (2012).

This conversion is a non-enzymatic dehydration (Adams et al., 2009; Fedoroňko & Königstein, 1969; Königstein, 1976) and is an irreversible first-order reaction for which the generally accepted mechanism involves an enediol intermediate (Fedoronko, Temkovic, Mihálov, & Tvaroska, 1980; Lookhart & Feather, 1978; Strain & Spoehr, 1930). The reaction, being chemical, is ongoing during storage and transport and thus there is interest in the behaviour of DHA and MGO in the honey matrix during all stages of production and marketing.

The honey matrix is a unique environment, and a number of reaction characteristics are affected by the viscous, dehydrating, acidic (~pH 4) matrix. The viscosity of honey may influence the speed at which molecules can move and is likely to be one cause of slower reaction rates in honey compared to reactions in aqueous solution. The low water activity (0.56–0.62 a_w) (Lin, Molan, & Cursons, 2010) in honey means that most water is likely to be bound to sugars and unable to react readily with other species. The pH of honey influences the state in which molecules are present, however, the concept of pH becomes problematic in such a strongly dehydrating environment; honey pH is measured in diluted solutions and may not reflect the actual availability of H^+ in the original matrix.

DHA exists as a dimer in the solid state but converts to a monomer in solution (Bell & Baughan, 1937; Davis, 1973; Yaylayan, Harty-Majors, & Ismail, 1999). This occurs rapidly in water (0.034 min^{-1}, 48.96 $days^{-1}$), but it is very slow in DMSO with only 50% converted after 64 h (Davis, 1973). It is likely that in the dehydrating honey environment, a large proportion of the DHA exists as the dimer and requires conversion to the monomer before it is reactive. This will affect the conversion rate which thus may not be directly comparable to the same reaction carried out in aqueous solution. It is likely that the conversion of the DHA dimer to monomer will be facilitated by the presence of proton donors and/or

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acceptors; various compounds besides water, which are naturally present in honey such as phenolic acids (Stephens et al., 2010) and amino acids (Hermosín, Chicón, & Dolores Cabezudo, 2003), may facilitate the conversion by acting in this capacity.

Phenolic acids and amino acids may also facilitate the removal of both MGO and DHA by Maillard-type side reactions either by acting in a catalytic capacity or by direct reaction (Martins, Jongen, & van Boekel, 2001). Inorganic ions have been shown to affect the course of low temperature conversion of biomass (Tchapda & Pisupati, 2014) and thus may also play a role in facilitating either MGO formation or side reactions.

This paper describes a study of the kinetics of conversion of DHA to MGO in honey matrices variously doped with DHA and attempts to relate this conversion to possible endogenous perturbants that might influence positively or negatively the rate and efficiency of DHA conversion to MGO.

2. Experimental

2.1. Honey samples

Thirty-seven mānuka honeys from various regions and years were kindly provided by Steens Honey Ltd (Te Puke, New Zealand), Gibbs Honeybees (Masterton, New Zealand) and New Zealand Honey Traders (Northland, New Zealand). The years of storage at ambient temperature ranged from freshly harvested through to eight years; on receipt, honeys were stored at −18 °C. Four clover honeys, were purchased from commercial outlets and therefore their origin and date of harvest is not known. There is no known benefit to storing clover honey, therefore it is likely that it was harvested in the same year as purchased. These samples were Happy Bee Clover Honey (Hamilton, New Zealand), Airborne Pure Natural New Zealand Clover Honey (Leeston, Canterbury, New Zealand), Katikati clover honey (Katikati, New Zealand) and Holland Clover Honey (Timaru, New Zealand). All honeys were analysed for DHA, MGO, 5-hydroxmethyl furfural (HMF), moisture, pH, acidity, trace elements and proline. In addition, eight mānuka honeys, which were harvested within the previous twelve months, plus the four clover honeys were also analysed for primary amino acids and phenolic compounds. The four clover honeys and four of the mānuka honeys harvested within the previous four months made up the matrices for the storage trials. Supplementary material 1 details all samples analysed and initial DHA concentrations used in the storage trials.

2.2. Chemicals and reagents

Methylglyoxal (MGO, 40% w/w), 1,3-dihydroxyacetone (DHA, 97%) and ninhydrin were obtained from Sigma–Aldrich. *O*-(2,3,4,5,6-Pentaflurobenzyl)hydroxylamine hydrochloride (PFBHA, 99+ %) was obtained from Alfa Aesar. Hydroxyacetone, (HA, $\geqslant$90%) was from Aldrich. Type I water (distilled and deionised) was obtained using a Barnstead E-pure system (18.0 MΩ cm). Acetonitrile was obtained from Honeywell, Burdick and Jackson or from Merck. Hydrochloric acid, propan-2-ol, and hydrogen peroxide were obtained from Ajax Fine Chem Pty Ltd. Nitric acid and formic acid (98–100%) were obtained from Merck. Sodium hydroxide pellets were obtained from Univar APS Fine Chem.

2.3. Moisture content

Moisture content of honey samples was determined using a Misco Palm Abbe PA203 digital refractometer. Samples were left at 20 °C for four hours and stirred before analysis. The samples were placed into the machine and left for one minute for the temperature to equilibrate. Readings were taken at 30 s intervals until three consecutive values were constant.

2.4. Determination of fructose, glucose and sucrose

Determination of fructose, glucose and sucrose was performed on an HPLC system fitted with a Waters 515 pump and 996 photodiode array (PDA) detector (240–400 nm), refractive index (RI) detector, column oven, Rheodyne 7725i injector fitted with a 20 μm loop and an Alltech Elite degassing system. Two columns were used in series for separation of compounds; these were Shodex KS-801 and Shodex KS-802 and were maintained at 50 °C. The system was controlled using Waters Empower™ 2 Chromatography software. The system was operated isocratically with 1 ml/min of water. Honey samples were thawed and thoroughly mixed before weighing into a vial (3 ± 0.2 g). Water (3.0 ± 0.2 g) was added to give a 50% solution (*w*/*w*). Vortexing and sonication were required to dissolve the honey. Samples were centrifuged at 4.4×10^3 rpm for 20 min. The supernatant was filtered through a 0.45 μm syringe filter before analysis. Samples were diluted 40-fold for sugar analysis to allow for separation of close eluting glucose and fructose peaks. Samples were analysed in triplicate.

2.5. Determination of pH and acidity of honey

The determination of pH and acidity in honey was based on the AOAC Official method 962.19 ("AOAC official method 962.19 Acidity (Free, Lactone, and total) of honey," 1995) and International Honey Commission (IHC) (Bogdanov, 2002) methods. Analyses were carried out in duplicate and the average is reported.

2.6. Trace element determination

Details are given in Supplementary material 2. Samples were analysed in triplicate and the average reported.

2.7. Primary amino acids determination

Primary amino acid analysis of honey samples was carried out by the commercial service of the Nutrition Laboratory at Massey University. The samples were analysed using AccQ Tag derivatisation, AOAC 994.12, using a Thermo Acclaim RSLC 120 C18 column (2.2 μm, 120A, 2.1 × 100 mm) for separation on a Dionex Ultimate 3000 HPLC equipped with Ultimate 3000 pump, autosampler, column compartment, and fluorescence detector. Sixteen free primary amino acids (aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, arginine and tryptophan) were analysed.

2.8. Proline (secondary amino acid) determination

The proline content of honey was analysed using the International Honey Commission Method (Bogdanov, 2009). Analyses were carried out in duplicate and the average is reported.

2.9. Determination of phenolic compounds

Leptosperin, methyl syringate, phenyllactic acid, 2-methoxybenzoic acid, 4-methoxybenzoic acid, syringic acid and luteolin were quantified by Ultra Performance Liquid Chromatography PhotoDiode Array Tandem Mass Spectrometry (UPLC-PDA-MS/MS). The samples were kindly analysed by Analytica Laboratories Ltd, Hamilton, NZ. Details are given in Supplementary material 2.

2.10. DHA, MGO and 5-hydroxymethyl-2-furaldehyde (HMF) determination

Determination of DHA, MGO and 5-hydroxymethyl-2-furaldehyde (HMF) was based on Windsor, Pappalardo, Brooks, Williams, and Manley-Harris (2012). Details are given in Supplementary material 2. Analyses were carried out in duplicate and the average is reported.

2.11. Determination of reaction order

The reaction order for the loss of DHA and gain of MGO was investigated in three mānuka and four clover honeys doped with either 2000 mg/kg or 10,000 mg/kg DHA and stored at 37 °C, as well as in a clover honey which was divided into six portions and doped with various levels of DHA (500, 1000, 2000, 4000 and 8000 mg/kg). All trials were carried out in duplicate.

2.12. Treatment of rate data for first-order formation of MGO

The rate treatment for first-order formation of MGO needs to reflect the probable loss of DHA to side reactions. The equation $\frac{d[\mathrm{MGO}]}{dt} = k'[\mathrm{DHA}]$ was used to describe the rate of MGO production in which k', the rate constant for the formation of MGO, is not equal to k, the rate constant for the disappearance of DHA. Substitution of $[\mathrm{DHA}] = [\mathrm{DHA}]_0 e^{-kt}$ and integration gave $k\frac{[\mathrm{MGO}]}{[\mathrm{DHA}]_0} = k'[1 - e^{-kt}]$. Thus a plot of $k\frac{[\mathrm{MGO}]}{[\mathrm{DHA}]_0}$ *versus* $\left[1 - e^{-kt}\right]$ gave slope k', the first-order rate constant for the appearance of MGO.

2.13. Storage trials of mānuka or clover honey matrices

Five fresh mānuka honeys with naturally occurring DHA (1539–3242 mg/kg), five fresh mānuka honey doped to achieve an initial DHA of 2000 mg/kg and three replicates of a clover honey doped with either 2000 mg/kg or 10,000 mg/kg DHA were divided into portions (two for each temperature) and stored at temperatures of 4, 20, 27, 32 and 37 °C in order to examine the effect of temperature on the conversion of DHA to MGO as well as the extent of possible competing reactions. Clover honey, in which DHA could not be detected, was used as a matrix not previously exposed to DHA so that potential perturbants were not previously depleted by reaction with DHA or MGO.

Mānuka or clover honeys (40.00 ± 0.02 g) had a DHA stock solution (1.00 ± 0.01 ml) added to them. The stock solution of DHA was made to have a final concentration of 2000 or 10,000 mg/kg when added to the honey. The pH of the DHA solution was adjusted to 3.8–4 using D-gluconic acid before being added to the honey. Honeys were incubated at 4, 20, 27 or 37 °C for various times extending up to two years. Duplicate sub-samples were periodically removed for analysis.

2.14. Statistical analyses

Statistical analyses were carried out in Microsoft© Excel 2007 and Minitab® 16 Statistical Software.

3. Results and discussion

3.1. Confirmation of reaction-order for conversion of DHA to MGO in honey matrices

The reaction orders for the loss of DHA and gain of MGO were investigated in three mānuka and four clover honeys doped with either 2000 mg/kg or 10,000 mg/kg DHA and stored at 37 °C; the higher value was chosen to replicate the work of Adams et al. (2009) for comparison and the lower value to approximate the normal values found in mānuka honey.

A further clover honey was divided into six portions and doped with various levels of DHA (500, 1000, 2000, 4000 and 8000 mg/kg) to encompass the range that might be expected in commercial mānuka honeys. All first-order plots for the disappearance of DHA showed linearity, and all second-order plots showed significant deviation from linearity (Fig. 1). Assignment of first-order loss of DHA concurs with studies carried out in acidic aqueous solutions (Fedoroňko & Königstein, 1969; Königstein, 1976). However, multiple reactions, for example Maillard-type reactions, in the complex honey matrix may be contributing to the loss of DHA and the overall order; the relative contribution of these individual reactions may vary with the matrix and the temperature and this will affect the value of the overall rate constant. Therefore loss of DHA in the honey matrix is not strictly comparable with an aqueous matrix containing clearly defined reactants.

DHA-doped clover honey obeyed first-order kinetics for both DHA loss and MGO formation for the duration of analysis, which was until the ratio of DHA:MGO reached 0.4:1.

In contrast, in the mānuka honeys overall first-order behaviour was only observed for the initial portion of the reaction. A simple plot of concentration of MGO *versus* time in the mānuka honeys yielded a plateau in MGO formation when the ratio was approximately 2DHA: 1MGO; Atrott et al. (2012) have previously reported a mean ratio of DHA:MGO of 2:1 in 18 commercial mānuka honeys at the point of sale. However, the plateau does not indicate an equilibrium or a steady-state but instead arises from the amount of MGO formed, being temporarily equal to the amount of MGO lost to side reactions at this stage of maturation. At 37 °C, a loss of MGO was observed after the plateau presumably due to reactions that consume MGO becoming more significant relative to the slowing rate of MGO formation from DHA; in some mānuka honeys the DHA: MGO ratio reached 0.6:1 by the end of data accumulation, accompanied by a net loss of MGO. These regions in the simple concentration *versus* time plot were reflected in the first-order plots for MGO appearance in which a transition from net gain to net loss of MGO is observed (Fig. 2); this transition corresponds to the plateau in the concentration *versus* time plot. Only the early linear portions of the plots were used to calculate the first-order rate constant of MGO appearance in mānuka honeys.

These features in the process were less obvious at lower temperatures; mānuka honeys stored at 32 °C showed only a slight deviation from linearity in first order plots after 250 days; this

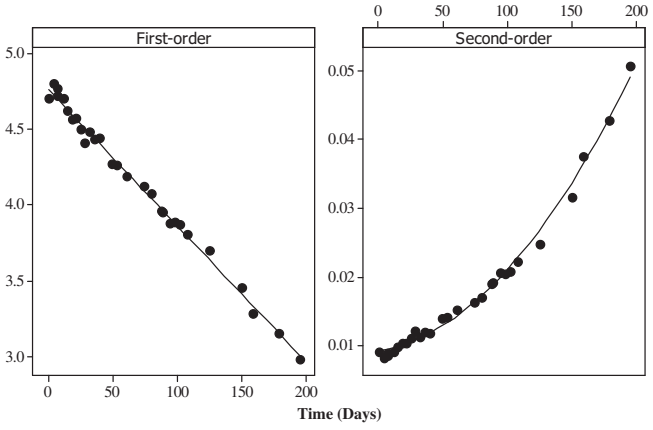

Fig. 1. Mānuka honey doped with DHA (10,000 mg/kg, 120 mmol/kg) and incubated at 37 °C.

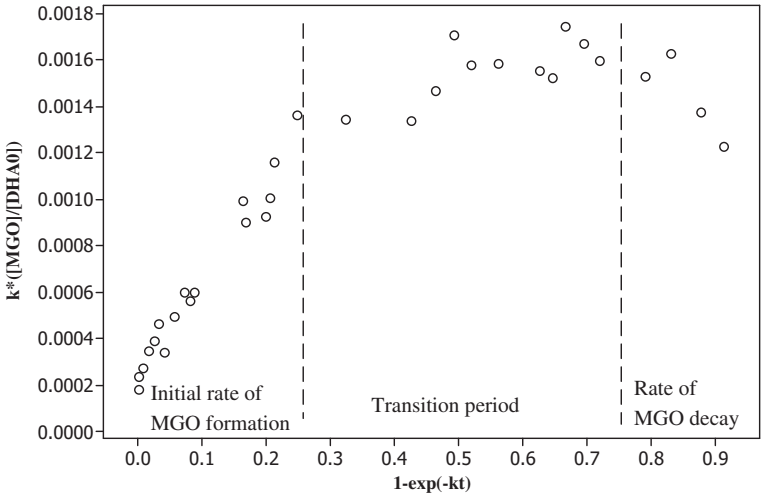

Fig. 2. First-order plot of MGO appearance in mānuka honey (2200 mg/kg/24 mmol/kg initial DHA) incubated at 37 °C. The quantity k is the first-order rate constant for disappearance of DHA.

difference is presumably attributable to lower rates of the reactions that compete with the conversion.

3.2. Influence of temperature on the loss of DHA and formation of MGO

Honey held at 4 °C showed no change in either DHA or MGO concentrations over one year, showing that at this temperature the conversion of DHA to MGO and any competing side reactions do not occur to any measureable extent.

Scatter was observed in the measured DHA concentration for doped samples stored at 20 and 27 °C which was more prominent in the early stages. This early scatter is probably due to a longer equilibration time of the system at lower temperatures because of the increased viscosity of the honey. There was no scatter in the MGO measurements at any time or temperature. Table 1 gives the mean first-order rate constants for disappearance of DHA and appearance of MGO; Supplementary material 3 contains rate constants for all samples analysed.

Higher temperatures appear to accelerate side reactions of MGO and DHA in mānuka honey more than the reaction of interest; thus, in mānuka honey, more MGO is gained overall at a lower temperature. In comparison, after 500 days, doped-clover honey stored at 37 °C had the highest MGO concentration of all honeys with 2000 mg/kg initial DHA because there was no loss of MGO at later times (Supplementary material 4). Thus clover honey is not a good matrix for modelling the fate of MGO at later times in the reaction since perturbants are obviously present in mānuka honey that are not found in clover honey.

As the temperature of storage increased, the first-order rate constant for DHA loss increased for both clover and mānuka honeys (Table 1). The measured rate constants for DHA loss were significantly larger for mānuka honeys compared to doped clover honeys at each temperature; this further reinforces the hypothesis that mānuka honey contains compounds which enhance the loss of DHA in various reactions and are not present in clover honey. The rate constant for MGO appearance also increased with increasing temperature; at 27 °C the average rate constant for MGO formation was larger in the clover honey but at 37° the rate constant for MGO formation was the same in both honeys because only the initial gain of MGO was considered in the mānuka honeys. It is important to remember that in mānuka honey, MGO loss through other pathways becomes dominant after an extended time period, which is not accounted for in the initial rate constant.

Background data from the Adams et al. (2009) paper was also analysed. The first-order rate constants for DHA loss in doped-clover honeys ($0.0041 \pm 0.0002\ day^{-1}$) and mānuka honeys (between 0.0057 and $0.0081\ day^{-1}$) stored at 37 °C were similar to that reported in this research. However, the first-order rate constant for MGO appearance reported by Adams et al. (2009) was smaller for both the doped-clover ($0.0017 \pm 0.0001\ day^{-1}$) and mānuka honeys ($0.0023\ day^{-1}$, $n = 3$), which is probably due to the compositions of the honeys varying due to the different regions and seasons in which they were collected.

In mānuka honey, large rate constants for DHA disappearance did not necessarily correspond to large rate constants for formation of MGO presumably due to a loss of DHA in side reactions. For example, one mānuka honey (2000 mg/kg initial DHA) had a large rate constant for DHA loss, but the rate constant for MGO appearance was one of the lowest recorded. The efficiency of the reaction (see below) in mānuka honey was not as good as in a clover matrix where the rate constants for DHA loss and MGO appearance were closer in value to each other.

Table 1
Average rate constants for DHA loss and MGO gain for mānuka and clover honey stored at different temperatures.

Temp (°C)	n	Rate constant for DHA disappearance (k, day^{-1})	Rate constant for MGO appearance (k', day^{-1})[a]	Stoichiometric factor ($x = k'/k$)	n	Rate constant for DHA disappearance (k, day^{-1})	Rate constant for MGO appearance (k', day^{-1})[a]	Stoichiometric factor ($x = k'/k$)
		Mānuka honey				Clover honey		
20		N/A			3	0.0009 ± 0.0001	0.0006 ± 0.0001	0.67
27	5	0.0023 ± 0.0005	0.0008 ± 0.0001	0.35	3	0.0014 ± 0.0003	0.0012 ± 0.0001	0.85
32	4	0.0042 ± 0.0005	0.0016 ± 0.0002	0.38		N/A	N/A	
37	10	0.0066 ± 0.0007	0.0037 ± 0.0010	0.56[b]	4	0.0048 ± 0.0003	0.0037 ± 0.0008	0.77

[a] Only the linear portion of the plot was used to calculate the first-order rate constant for MGO appearance.
[b] The stoichiometric factor for mānuka honey stored at 37 °C only takes into account the initial portion of the reaction.

The amount of DHA that converted to MGO was calculated as percent efficiency (overall MGO gain/DHA loss × 100) and also as a stoichiometric factor (rate constant for MGO gain/rate constant for DHA loss), with the expectation that the values should be similar. However, there are strengths and limitations to both calculations. The percentage efficiency for clover honey was constant, within experimental error, throughout the course of the reaction, but for mānuka honey it was intimately related to the stage of the reaction at which it was calculated. For example, at 63 days the average percent efficiency in four mānuka honeys stored at 37 °C was 38 ± 4% compared to 24 ± 7% at 241 days. In contrast, a clover honey stored in the same conditions had a similar efficiency throughout the time that it was monitored (56 ± 11%). The stoichiometric factor was restricted to reporting the efficiency of the initial portion of the reaction in mānuka honey, whereas for clover honey it encompassed the entire reaction. The average stoichiometric factor for the conversion of DHA to MGO was 0.5 ± 0.1 for mānuka honeys (n = 10) with 2000 mg/kg DHA at 37 °C in the first phase of the reaction (Supplementary material 3). This suggests that, even in the initial stage, only 50% of DHA is converted to MGO when incubated at 37 °C and supports the hypothesis that the first-order rate constant for DHA loss is actually a composite for multiple reactions.

The activation energy (E_a) and pre-exponential factor (A) for DHA loss and MGO gain in mānuka and doped-clover honeys were calculated (Table 2). These quantities take into consideration all reactions in which the two compounds are involved and not just the reaction of interest. Therefore rate parameters (e.g. E_a and A) were derived from the early stages of the reaction when side reactions of MGO should be minimal; this gives a better indication of the early chemistry occurring than when more complex rate parameters, derived at later times in the reaction, are used. Side reactions of MGO may have different activation energies from those of the initial conversion of DHA to MGO and thus the apparent E_a and A may change as the extent of the reaction increases and side reactions are more prominent. Arrhenius plots were linear for DHA and MGO rates. For doped-clover honeys, average activation energies and pre-exponential factors are the same within experimental error for DHA loss and MGO gain indicating that the reaction is likely to be a simple first order reaction of DHA converting to MGO. The average activation energy for DHA disappearance for mānuka honey is similar to that reported by Fedoronko et al. (1980) in aqueous solution and concurs within experimental error with the results for clover. The average activation energy for appearance of MGO was higher for mānuka honey compared to doped-clover honey (173 ± 17 compared to 78 ± 3 kJ mol^{-1}) and was significantly higher than that for DHA loss. This difference may indicate that even at early stages of reaction the rate constant for MGO appearance is composite and incorporates MGO or DHA loss to side reactions; for this reason caution should be exercised when interpreting this type of data.

3.3. Implications of the existence of DHA as a dimer in honey

The viscous, dehydrating, acidic matrix of honey is unique, and reactions and rates in this matrix may differ from those in aqueous solution. In the solid state, DHA exists as the dimer, but dissociates into its monomeric form upon dissolution (Davis, 1973). The slower conversion in a honey matrix may arise from the equilibrium of the DHA dimer and monomer lying further toward the dimer due to limited availability of a proton donor/acceptor. Fig. 3 postulates the steps between the dimer and monomer of DHA; the dissociation requires both acid and base catalysis, which in aqueous solution would be supplied by the water. The dissociation may be the rate limiting step in the conversion of DHA to MGO in honey, compared to a different rate limiting step for the corresponding reaction in aqueous solution. Dissociation of the dimer may be enhanced by general acid or base catalysis by other components (perturbants) in the matrix. For example, undissociated acetic acid has been reported to play a role in the conversion of DHA to MGO (Fedoroňko & Königstein, 1969).

3.4. Influence of perturbing compounds present in the honey matrix on the loss of DHA and formation of MGO

DHA, MGO, HMF, fructose, glucose, sucrose, pH, free acidity, lactone, total acidity, moisture content, proline, primary amino acids, trace elements, leptosperin, methyl syringate, phenyllactic acid, 2-methoxybenzoic acid, 4-methoxybenzoic acid, syringic acid and luteolin were quantified in mānuka honeys from various regions of New Zealand and in commercially purchased clover honeys. The honeys spanned ages from freshly harvested up to eight years of storage. The results for mānuka and clover honeys are summarised in Supplementary material 5.

The rate constants for disappearance of DHA and appearance of MGO for honeys stored at 37 °C were plotted against the values measured to identify those parameters that might influence the disappearance of DHA or appearance of MGO. Sample 78 was an outlier in almost all plots because the rate constant for DHA loss was higher than expected; a compound that was not examined in this research may be the cause of the large loss of DHA in this sample and this compound may not be present in large quantities in the other samples tested. Sample 78 was therefore excluded from the following discussion. Table 3 summarises the correlations between the rate constant for DHA disappearance (k) and either total acidity, phenolic acids or various amino acids singly or collectively.

Total acidity (17.09–37.34 meq/kg) had a positive correlation (R^2 = 87%) with the rate constant for DHA loss. This correlation may be due to the influence of proton donation on shifting the DHA dimer: monomer equilibrium; however, the correlations was not as strong as that for some individual phenolic acids. No

Table 2
Activation energies for disappearance of DHA and formation of MGO between 27 and 37 °C in mānuka honeys and 20 and 37 °C in clover honeys doped with DHA.

Sample	Honey matrix	Temp range (°C)	E_A for disappearance of DHA (kJ mol^{-1})	E_A for appearance of MGO (kJ mol^{-1})	A for disappearance of DHA (day^{-1})[a]	A for appearance of MGO (day^{-1})[a]
14	Mānuka	27–37	70	152	22	54
25	Mānuka	27–37	85	189	28	68
32	Mānuka	27–37	93	184	31	66
41	Mānuka	27–37	93	167	31	59
HB	Clover	20–37	81	81	26	21
AB	Clover	20–37	78	78	25	26
Hol	Clover	20–37	74	74	23	29
Average	Mānuka	27–37	85 ± 11	173 ± 17	28 ± 4	62 ± 6
Average	Clover	20–37	78 ± 3	78 ± 3	25 ± 2	25 ± 4

[a] A was calculated as the intercept of the plot.

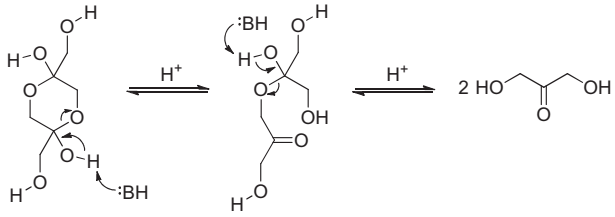

Fig. 3. Possible mechanism for the dissociation of DHA dimer to two monomeric DHA, requiring both acid and base catalysis.

correlation was observed between the first-order rate constant for MGO appearance and total acidity.

A positive linear correlation between the first-order loss of DHA and proline concentration (82%) was observed for the six mānuka honeys and three doped clover honeys analysed. Proline is a known catalyst with four modes of action – bifunctional acid/base catalysis, metal catalysis, iminium catalysis and enamine catalysis (List, 2002). The higher the concentration of proline, the faster DHA was consumed, but this did not equate to a higher rate constant for MGO formation. No trend was observed between the rate constant for MGO appearance and proline concentration.

There was a strong correlation between total primary amino acids and the rate constant for DHA loss when only the mānuka honey samples with 2000 mg/kg DHA were plotted (R^2 = 86%). Furthermore, total amino acids (including proline) also gave a correlation of 92%. When clover honeys were also plotted, a different trend was observed, most likely due to the natural differences in concentrations of amino acids; this may be influenced by an observed much higher phenylalanine concentration in the clover honeys compared to mānuka honey; this observation may also account for the tabulated differences in rate constants in different clover honeys. Correlations between k and individual amino acids were examined for the mānuka honey samples (Table 3). It is possible that the differential ability of amino acids to donate a proton might depend upon the R group. Serine, alanine, valine, phenylalanine and tyrosine had the highest correlations (R^2 > 90%). Alanine and valine had R^2 values of 96% compared to isoleucine and leucine which only had R^2 values of 78%, although all four amino acids have similar R groups. Methionine has a similar R group, but was the least abundant amino acid analysed, and no trend was observed. Tyrosine and phenylalanine have similar hydrophobic R groups and had similar positive correlations with the rate constant for DHA loss (k). Tryptophan also has a similar R group, but no correlation could be observed because there was only a small variation in concentration over the samples analysed. There was no correlation between k and aspartic acid or glutamic acid, which have very similar structures; their R groups contain COO^- groups, which may H bond with the amino group preventing the H from being labile. Bonsignore, Leoncini, Ricci, and Siri (1972) previously reported relationships between amino acid functional groups and the conversion of glyceraldehyde to MGO; –OH groups spatially proximate to the $-NH_3^+$ group were reported to slow down the reaction.

No apparent trend between the rate constant for formation of MGO (k') and the concentrations of individual primary amino acids or total amino acids was observed. Only tryptophan had a positive correlation with k' (R^2 = 72%). Aspartic acid and glutamic acid both have weak negative correlations with the appearance of MGO (k') for mānuka honeys (R^2 = 35 and 43% respectively). Adams et al. (2009) reported that lysine and serine enhanced the production of MGO; however, these compounds were doped at much higher concentrations than those which occur naturally in honey. These results suggest that the concentration of amino acids at time zero are not a good predictor, by themselves, of the rate of MGO appearance, and that there are one or more other compounds that enhance the conversion of DHA to MGO.

Phenolic compounds were plotted against k and correlations are summarised in Table 3. All acidic phenolic compounds measured can act as moderately strong acids (low pKa values), hence they are expected to act as proton donors. The rate constant for the disappearance of DHA, k, has the strongest correlations with phenyllactic acid (R^2 = 98%, pKa = 3.72) and 4-methoxyphenyllactic acid (R^2 = 89%, the pKa for 4-methoxyphenyllactic acid is unknown,

Table 3
Summary of R^2 (%) for correlation between perturbants and the first-order rate constant for DHA loss and MGO loss.

Compound	pKa	R^2 (%) for correlation between compound and first-order rate constant for DHA loss (k)[a]	R^2 (%) for correlation between compound and first-order rate constant for MGO loss[a]
Phenyllactic acid	3.72	98[a]	58
Serine	9.15	99	98
Alanine	9.87	96	92
Leucine	9.74	96	79
Phenylalanine	9.24	93	55
Total amino acids	N/A	92	74
Tyrosine	9.11	92	80
Sum of phenyllactic acid and 4-methyoxyphenyllactic acid	N/A	91	80
Sum of phenolic acids		91	71
4-Methyoxyphenyllactic acid	b	89	54
Total acidity	N/A	87	89
Total primary amino acids	N/A	86	56
Lysine	8.95	86	75
Syringic acid	4.34, COOH; 9.49, 4-OH	86	73
Glycine	9.78	85	77
Proline	10.6	82	94
Valine	9.72	78	88
Isoleucine	9.76	78	90
Histidine	9.18	73	58
Methyl syringate	8.7, 4-OH	70	99
Arginine	9.04	68	29
Threonine	9.1	60	55
2-Methoxybenzoic acid	4.09	66 (negative)	–

[a] R^2 values $\geqslant$70% are significant based upon a two-tailed p value of 0.05.

but it is expected to be close to that for phenyllactic acid). Furthermore, these two compounds are found in high abundance in mānuka honey (720 ± 156 mg/kg and 303 ± 282 mg/kg respectively). These compounds probably account for the larger rate constant for DHA loss in mānuka honey compared to clover honey. The sum of phenyllactic acid and 4-methoxyphenyllactic acid was plotted against the rate constant for the loss of DHA, k; this gave a very strong correlation (R^2 = 91%). Furthermore the sum of all phenolic acids also had a strong correlation with k (R^2 = 91%). Syringic acid also had a good correlation with k (R^2 = 86%), but is only found in low levels in mānuka honey. Methyl syringate does not have a COOH group and the pKa of the OH is high, however, it still had a good correlation with k (R^2 = 70%), but this is most likely due to its self-correlation with 4-methoxybenzoic acid (R^2 = 80%). 2-Methoxybenzoic acid had a negative correlation with k (R^2 = 66%); the reason for this is unknown. There was no correlation between k and leptosperin or luteolin; this is expected because neither compound has the ability to donate a proton. The compounds that have the largest effect on the loss of DHA, do not necessarily positively influence the formation of MGO, but instead side products may be formed. Furthermore, these compounds may also influence the loss of MGO to side products at later times which would not be apparent by examining k', the rate constant for the linear portion of the reaction.

Phenyllactic acid had the best correlation with the rate constant for appearance of MGO, k' (R^2 = 66%) and, because of its large concentration in the mānuka honey, probably has more of an effect than amino acids on the formation of MGO. The correlation of k' with all other compounds was 30% or less.

Five mānuka honeys were monitored past the time at which MGO began to decline. This portion of data fitted a first-order rate loss for MGO. Assignment of first-order is not as clearly delineated as for the early stage reactions because loss may be due to multiple reactions. Correlations between the rate constant for loss of MGO and various parameters were sought (Table 3). Honey 946 was an outlier and was removed from analysis. Serine, proline, alanine and isoleucine had a strong correlations with loss of MGO (90% or greater); however, amino acids are also present in clover honey in which loss of MGO was not observed. One or more compounds unique to mānuka honey must be correlated with the rate constant for loss of MGO since a loss was not observed in doped-clover honey. Total acidity was strongly correlated (89%) to loss of MGO and is higher in mānuka than clover honeys. Since many reactions of MGO are catalysed by acid this correlation seems logical. There were strong correlations between the loss of MGO and methyl syringate (R^2 = 99%), which, as mentioned previously is unlikely to be a strong proton donor, however there were no strong individual correlations with phenyllactic acid (R^2 = 58%) or 4-methoxyphenyllactic acid. It is possible that some as yet unidentified compound, which is present in mānuka but not in clover honey, is partly responsible for the disappearance of MGO; alternatively the reason may be that there are multiple reactions occurring with a variety of proton donors.

No apparent trends were seen between Ca, P or Al concentrations and k or k', but there was a positive trend between Mg concentration and k. Ions of alkaline and alkaline earth metals are well-known for their catalytic effect in low-temperature conversion of biomass, (Tchapda & Pisupati, 2014) and many of the reactions occurring there may be similar to those occurring here to consume DHA and MGO, for example Maillard reactions. Honeys doped with 2000 mg/kg DHA had very similar potassium levels so a trend of potassium concentration with the rate constant for DHA loss (k) and MGO appearance (k') could not be examined. Honeys doped with 10,000 mg/kg DHA had varying potassium concentrations (322–1097 mg/kg). A positive linear correlation between potassium and disappearance of DHA was seen.

4. Conclusion

Overall rate constants have been determined for loss of DHA and formation of MGO in mānuka and DHA-doped clover honeys. The conversion appears to follow first-order kinetics in DHA-doped clover honey but in mānuka honey the kinetics are complex and vary during the course of the reaction due to the influence of endogenous perturbants in side reactions of both DHA and MGO. More MGO was gained overall at lower temperaturesin mānuka honey; however, in clover honey the highest concentration of MGO was in the honey incubated at the highest temperature because there was no loss of MGO at later times. Activation energies and pre-exponential factors were similar for DHA loss and MGO gain in DHA-doped clover honey, but differed for mānuka honey. Concentrations of proline, total primary amino acids, phenyllactic acid and methoxyphenyllactic acid as well as total acidity had positive linear correlations with the first-order loss of DHA. Phenyllactic acid and tryptophan had moderate correlations with the first-order rate constant for MGO appearance.

The equilibrium of dimeric and monomeric DHA is suggested as a significant influence on the rate of DHA loss.

The use of model compounds as perturbants is discussed in Part II (Grainger, Manley-Harris, Lane, & Field, 2016a) and the simulation of the conversion of DHA to MGO is described in Part III (Grainger, Manley-Harris, Lane, & Field, 2016b).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.02.029.

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