Manuka pollen as the potential source of methylglyoxal and dihydroxyacetone in Manuka honey

Dr Keryn Johnson

Quantum Technologies Limited

39a Bombay Street,

Ngaio

Wellington 6035

New Zealand

*corresponding author: Dr Keryn Johnson quantum.biologist1972@gmail.com

+64 22 199 8782

1. Abstract

Manuka honey is currently valued based on its MGO content. The origin of MGO is currently thought to be due to DHA chemical conversion into MGO over time in the honey as it matures. The low water content of honey means that DHA is present in a dimer and unable to react to form MGO. This puts into question the current thinking around MGO formation in Manuka honey. The serendipitous discovery of polymeric glyoxal in Manuka honey being present in pollen grains whilst performing MALDI TOF MS analysis on colloidal nanoparticles isolated from Manuka honey provides an alternative explanation as to the origin of MGO and DHA in Manuka honey and identifies novel methods to increase MGO content and therefore value of the honey. The mechanism responsible for the formation of MGO are also potentially

responsible for its non-peroxide based antibacterial activity. This report outlines preliminary findings to support this alternative origin of MGO and the antimicrobial mode of action. Analysis of pollen changes that occur during Manuka honey maturation were observed utilizing MALDI TOF MS, fluorescence microscopy and fluorescence spectrophotometry. These methods provide evidence of an alternative origin of this important molecule and Manuka honey special place as a premium product with beneficial healing properties in the health and wellness market.

2. Introduction

The Manuka honey industry is of considerable value to the New Zealand economy. Manuka honey differs from other honeys due to the presence of methylglyoxal (MGO), which has been shown to inhibit glucose oxidase effecting hydrogen peroxide generation (Majtan et al., 2014). The Fenton reaction with hydrogen peroxide generating hydroxyl radicals has been identified as the anti-microbial agent present in peroxide producing honeys (Brudzynski and Lannigan, 2012). The unique anti-microbial properties of Manuka honey has been partly attributed to MGO (Molan, 2008), however, MGO is broken down by the glyoxalase biochemical pathway (Figure 1), to generate D-lactate which neurons can use to produce energy (Silva et al., 2013). The anti-microbial properties of MGO is therefore questionable if it is unstable in our body due to the glyoxalase biochemical pathway. In addition, MGO modification of proteins has been associated with diabetes and cardiovascular disease (Hannsen et al., 2017). MGO reaction with proteins in honey and amino acids generates a range of radicals (Hyung-Soon et al., 1995; Galano et al., 2004;

Nakayama et al., 2007) that have anti-microbial properties (Johnston et al., 2018). Therefore, MGO appears to be a double edged sword having both positive and negative effects in the biology of health and well-being. MGO levels are important for the Manuka honey industry, and honey with higher MGO content fetches a higher economic return. The global industry has realized this fact which has led to some detrimental practices especially when the literature suggests that DHA is the source of MGO and DHA is a cheap commercially available chemical. Blending hydrogen peroxide positive honey with MGO containing Manuka honey generates lactate, reducing MGO content and results in the devaluation of the biological benefits of the honey. Adulteration of honey prevents the product from being exported and labeled as honey. Understanding the chemistry responsible for MGO formation and its many potential reactions within Manuka honey has significant economic potential. Therefore, the range of methods employed to enhance the MGO content is limited to physical approaches. Temperature and time are standard industry tools used to increase MGO content in the honey. Pressure has also been attempted as well as exposure to light, but both have fallen short as feasible approaches to accelerate MGO formation (Fauzi and Farid, 2014). Serious complications occur when heat is used to promote MGO generation as it also results in increased concentrations of 5-HMF, which has regulatory limits set for it to be exported to China.

Manuka honey has gained considerable international attention because of its health giving properties. The functional properties of Manuka honey are attributed to a range of ingredients including MGO. The content of dihydroxyacetone (DHA) and

MGO, minerals (iron) and phenolics provide an intriguing story regarding Manuka honeys bioactivity. The role of MGO in the formation of hydroxyl radicals is outlined by Galano et al., (2004), which occurs during the reaction with amino acids in proteins (lysine, arginine and cysteine) (Nakayama et al., 2007). The high molecular weight protein adducts that are resistant to dithiolthreitol reduction present in Manuka honey demonstrate MGO's covalent cross-linking reactivity (Stephen et al., 2017). This suggests that hydroxyl radicals produced by MGO protein cross-linking are responsible for the anti-microbial properties attributed to Manuka honey.

The government regulations around the certification of the Manuka honey combines genetic testing of Manuka pollen and LC MS MS analysis of four pollen phenolics (3-phenyllactatic acid, 2'-methoxyacetophenone, 2-methoxybenzoic acid and 4-hydroxyphenyllactic acid). The work of Adams et al., (2009) indicated that MGO originated from DHA and that DHA was present in the nector of the Manuka plant. The origin of MGO in biological systems has been attributed to glucose and glycolysis production of DHAP and its dephosphorylation. Grainger et al., (2017) also showed the effects of temperature and free amino acids on the conversion of glucose via dehydration generated 5-HMF but they were unable to show DHA conversion into MGO in their artificial honey system.

Manuka is a pioneering plant and grows in soil with high iron content. New Zealand skies are pollution free and the ozone layer is thinner than usual, which effects the electromagnetic spectrum present in New Zealand (Aoteroa), which contributes to our increased skin cancer rates. This unique combination of factors is suggested to

play a role in Manuka honeys bioactivity. The pollen present in Manuka honey when exposed to UVA light produces fluorescence. This fluorescence changes during the Manuka honey maturation process. The fluorescence intensity increases during honey maturation as identified in this paper, as well as an increase in the number of high intensity fluorescent pollen grains. Two novel fluorescent compounds have previously been detected in Manuka honey (Stephens et al., 2017) and have been used for authentication of nectar origin. Whilst working on the identification of Manuka honey anti-inflammatory compounds a number of additional discoveries were made that indicated that a potential source of methylglyoxal may not be from glucose conversion into DHA or from nectar but originating within the unique environment of a pollen grain within Manuka honey. Pollen has not previously been identified as a potential source of polymeric glyoxal, DHA and MGO and evidence supporting this hypothesis is outlined in the present study. The mechanism responsible for production of polymeric glyoxal, DHA and MGO appears to originate from radical chemistry induced by photo-fenton chemistry that generates hydroxyl radicals and is part of a light-based photo-reduction system present in Manuka honey and the potential anti-microbial agent in Manuka honey.

3. Materials and Methods

3.1 MALDI TOF MS analysis of pollen isolated from various honeys

The pollen grains were harvested rapidly by dissolving between 0.1 to 0.2 g of honey in 1 mL of nanopure water. The honey was rapidly dissolved by vortex then the sample was centrifuged and the pollen pellet collected and washed three times with distilled water. The pellet was finally suspended in 10 microL of water and 1 microL

of the sample was spotted on to the MALDI TOF MS plate and allowed to dry without adding matrix material. Maximal lazer intensity (7900), was used to obtain spectra from 0 to 2000 daltons using Applied Biosystems 5800 MALDI TOF instrument.

3.2 Fluorescence analysis of pollen from manuka flower and from various honey sources

Direct analysis of pollen isolated by dissolving honey in water followed by centrifugation for 1 minute at 13,200 rpm in a bench top centrifuge. Isolated pollen grains from young and mature Manuka, Kanuka and Clover honeys were analyzed by Epi-fluorescent microscopy using an Evos FL microscope (Invitrogen) using a range of magnifications from 2 to 60 times magnification. Manuka honey isolated pollen grains 10 times magnification QD long pass filter setting 70% light intensity and 60 milliseconds.

3.3 Spectraphotometric analysis of pollen grains isolated from an aged Manuka honey

Aged manuka pollen grains were isolated as outlined above an suspended in 100 microL of nanopure water (Mllipore). Fluorescence analysis was performed using a SpectaMax M3 plate reader (Molecular Devices). Excitation 250 nm and emission over 450 to 550 nm. Time resolved fluorescence was performed using a delay of 50 to 600 milliseconds and excitation of 250 nm and an emission of 500 nm.

4. Results

Pollen fluorescence increases during maturation of Manuka honey

145 The presence of pollen in honey has been used extensively in melissopalynology to
146 identify the nectar source of the honey. Manuka (*Leptospermum scoparium*) and
147 Kanuka (*Kunzea ericoides*) pollen grains look identical and prevent the determination
148 of nectar origin. It was noted, however, that the pollen from Manuka honey had
149 some unique properties with respect to its fluorescence profile (Figure 2), which
150 changed during the maturation of Manuka honey (Figure 3). A corresponding
151 increase in pollen fluorescence was observed during Manuka honey aging, indicating
152 that changes were taking place in the pollen grains during the aging process. The
153 pollen went from having fluorescence around the edge (bifringence) to becoming
154 highly fluorescent as a central sphere that glowed for a long time in an unusual way.
155
156 *MALDI TOF MS analysis of isolated pollen*
157 Pollen was isolated from various honeys including young and aged Manuka, clover
158 and Kanuka. As phenolics are present in the pollen grains and these compounds
159 absorb the MALDI TOF laser light it was decided analyse the pollen grains directly
160 without the addition of matrix ions (Figure 4). This allowed full spectral analysis from
161 0 to 2000 Daltons. The fingerprint analysis could be performed without
162 complications associated with matrix ions allowing low MW species to be observed
163 without having the complications of matrix ions. Maximal laser intensity was used to
164 observe the spectra in order to release the contents of the purified pollen grains.
165 Characteristic peaks were observed for Manuka honey derived pollen grains at
166 337.37 Da. Clover had a characteristic peak at 381.29 Da, and Kanuka appeared to
167 have a unique peak at 345.12 Da. Interestingly, the shape of Manuka and Kanuka
168 pollen is identical which has prevented pollen morphology being used to identify the

169 nector source. Direct MALDI TOF MS analysis is both rapid and potentially diagnostic
170 as a screening tool to identify pollen origin. The current regulatory methods use RT
171 PCR methods for DNA detection present in Manuka pollen to identify the nectar
172 origin of the honey as well as phenolic content determined by MS methods. The
173 changes in fluorescence profile of the pollen grains also provide valuable information
174 related to MGO maturation stage.
175
176 The aged Manuka honey pollen appeared to have a modified fingerprint profile with
177 a raised background envelope. Further investigation revealed an interesting
178 polymeric compound that had a repeating mass of 58.04 Da (Figure 5). The 58.04 Da
179 polymeric series appeared to correspond to a polymeric glyoxal. It is suggested that
180 the loss of 1 glyoxal unit (58.04 Da) occurred due to fragmentation of the polymer by
181 methyl and hydroxyl radicals to generate MGO and DHA respectively. The estimated
182 length of the polymer corresponded to 17 glyoxal units, with an average of 10-12
183 glyoxal units. Two different series of polymers were evident in the spectra. The
184 difference in mass between these two polymers was 42 Da which may have resulted
185 from the loss of oxygen (16 Da) from bound glyoxal polymer.
186
187 *4.1 Radical chemistry role in phenolic formation and degradation*
188 New Zealand's environment with its high UV levels along with the high iron content,
189 and phenolics present in the honey suggests the formation of hydroxyl radicals
190 based on photo-fenton chemistry, the presence of which was confirmed by analysis
191 using 3'-(p-aminophenyl) fluorescein (APF) as the (•)OH trap and superoxide by NBT
192 analysis. The effect of UV light on radical generation and changes in composition of

193 key compounds (MGO, DHA, HMF and the phenolics) in diluted Manuka honey was
194 determined (data not shown). No changes occurred in MGO, DHA and HMF content,
195 however, methyl syringate concentration increased. The generation of the hydroxyl
196 radicals induced by UV light and the phenolic anti-oxidant activity resulted in methyl
197 syringate formation. Iron binding to the pi electrons in the aromatic ring of benzoic
198 acid would position the radicals in close proximity to allow OH and methyl radical
199 reactions to form methyl syringate (Figure 6).
200
201 Radical chemistry can not only be utilized to create compounds but also deconstruct
202 molecules back down into their environmentally benign precursors CO_2 and H_2O
203 (Figure 6). In this process a number of interesting molecules are produced including
204 glyoxal. The generation of the glyoxal polymer is postulated and the formation of
205 methyl and hydroxyl radicals which have been detected in Manuka honey are
206 implicated in cleaving such a polymer, leading to the formation of MGO and DHA.
207 The short half-life of the hydroxyl radical makes it's detection extremely difficult but
208 this hypothesis seems possible considering the composition and functional activity as
209 antimicrobial.
210
211 **5. Discussion**
212 The origin of MGO in Manuka honey has been investigated using a range of
213 approaches including artificial systems which have proven difficult to demonstrate
214 successful formation of MGO from DHA (Grainger et al., 2016). The Manuka honey
215 industry has investigated a wide range of approaches to increase MGO content
216 without adulterating the honey, in attempt to maximize its value. The most

successful approach to date to increase MGO content is long term storage at specific temperatures. On shelf marketing claims for MGO content are carefully calculated as MGO concentration declines over time if sufficient DHA content is not present (Stephen et al., 2017). Models have been developed by testing laboratories to predict the maximal MGO content during prolonged storage, as well as expected time-frame to generate maximal MGO providing an indication of expected shelf-life. These models are based on MGO, DHA, 5-HMF testing and do not consider pollen content or pollen fluorescence as an alternative source for polymeric glyoxal and MGO.

The preliminary findings indicate an increase in pollen fluorescence and fluorescence lifetime as well as the physical location of the fluorescence from the edge to a central spherical shape within the pollen grain during the maturation process in Manuka honey, which was unusual and remarkable (Figure 3). The discovery of polymeric glyoxal in the pollen grain that had been damaged during the aging process within Manuka honey and its increased fluorescence correlating with the aging process suggests that MGO and DHA are most likely derived from a glyoxal polymer compound due to radical chemistry based decomposition that occurs over a prolonged period of time during storage. The pollen content of Manuka honey is therefore important in determining MGO content. The current testing methodologies do not account for the pollen containing polymeric glyoxal as a potential source of MGO. Future efforts by the industry to increase MGO content should focus on pollen and monitoring its fluorescence and methods that can release

pollen contents including the polymeric glyoxal into the honey in an effort to enhance the honeys MGO content.

It appears that Manuka honey is unique from the perspective of glucose oxidase inhibition (Majtan et al., 2014) preventing the formation of hydrogen peroxide which effects hydroxyl radical generation (Brudzynski and Lannigan, 2012). However, this appears to be compensated for by the higher phenolic and iron content in Manuka honey, which can produce hydroxyl radicals via a photo-Fenton mechanism. The reaction of MGO with proteins within the honey also generates various radicals (Hyung-Soon et al., 1995). It is suggested that radicals may generate polymeric glyoxal in pollen, methyl syringate and potentially DHA and MGO within Manuka honey. The encapsulated nature of the pollen grain means that glyoxal polymers are unable to be analyzed until the pollen either breaks down or germinates releasing it's contents.

6. Conclusions

It is postulated that MGO formation appears to be directly linked to radical chemistry, which produces polymeric glyoxal and further generation of the methyl radical and hydroxyl radicals maybe responsible for the production of MGO and DHA from this polymer. It is suggested that polymeric glyoxal content be evaluated from pollen present in Manuka honey utilizing either pollen fluorescence analysis or MALDI TOF MS as a screening tool to further investigate these preliminary findings.

Figures

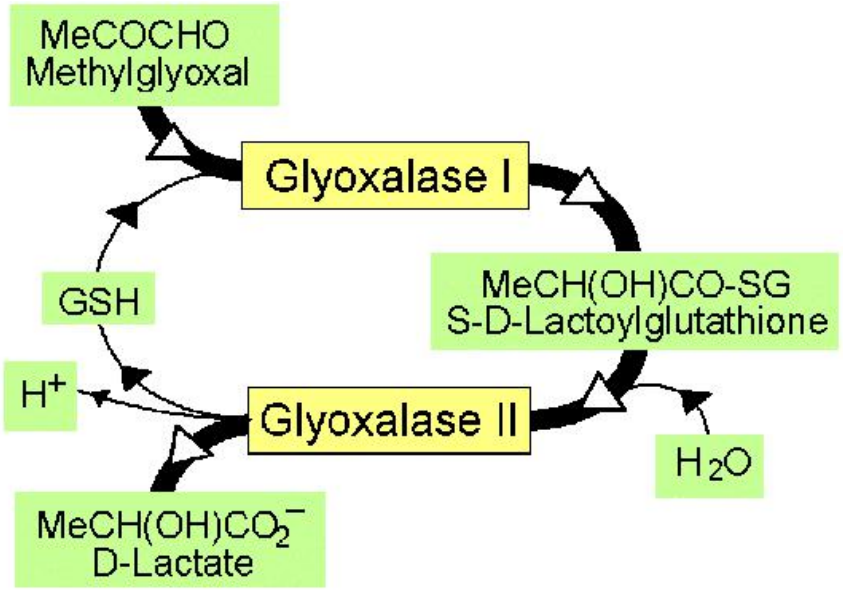


Figure 1: Glyoxalase pathway a proposed biological energy system rather than dicarbonyl stress detoxification system

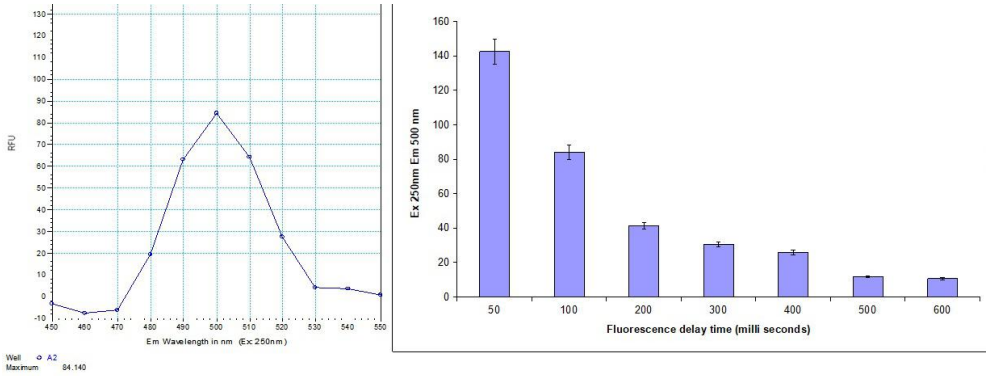


Figure 2: Fluorescence analysis of mature Manuka pollen and time resolved fluorescence

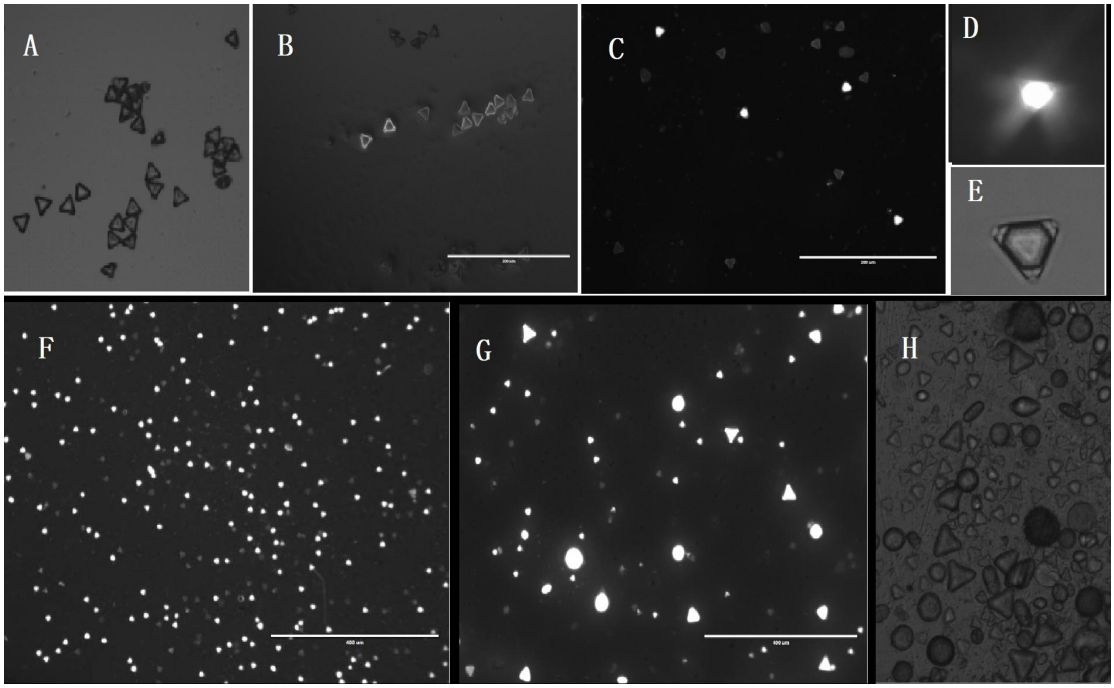


Figure 3: A) Manuka pollen isolated from the flower under bright field, B) Manuka pollen isolated from the flower under DAPI LED light source fluorescence analysis, C) A young Manuka honey pollen isolated and fluorescence analysis, D) Single pollen grain isolated from an aged Manuka honey fluorescence analysis, E) Single pollen grain isolated from an aged Manuka honey bright field analysis, F) Aged Manuka honey isolated pollen, G) Aged Manuka honey isolated pollen increased magnification fluorescence analysis and H) Aged Manuka honey isolated pollen increased magnification bright field analysis

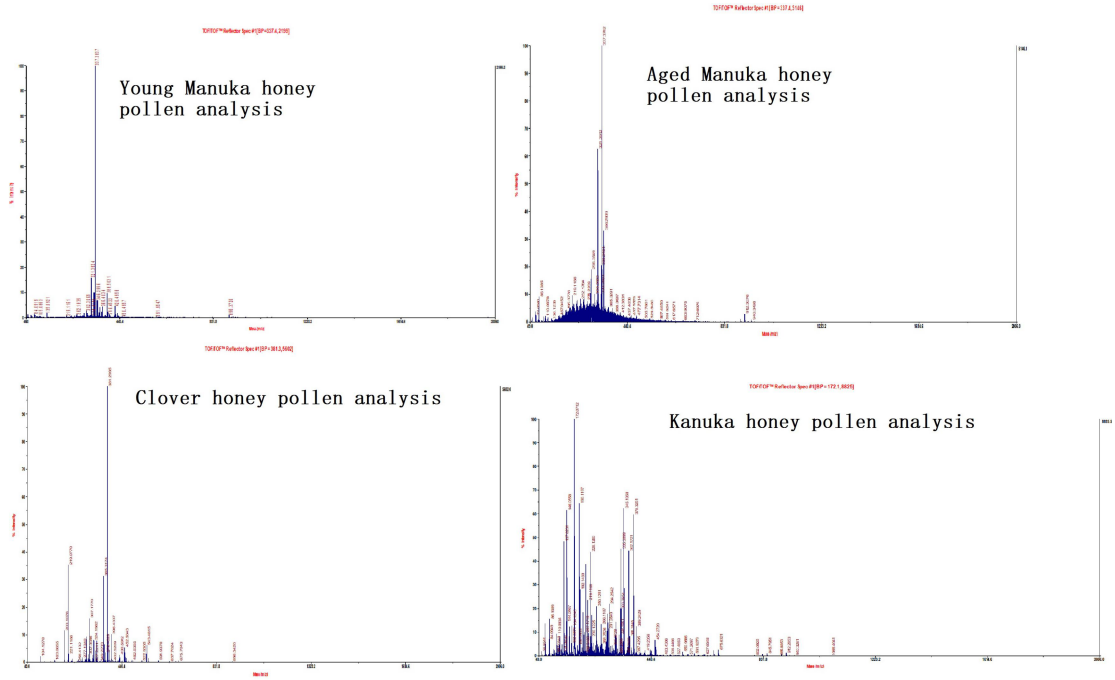


Figure 4: MALDI TOF MS analysis of pollen isolated from various honeys

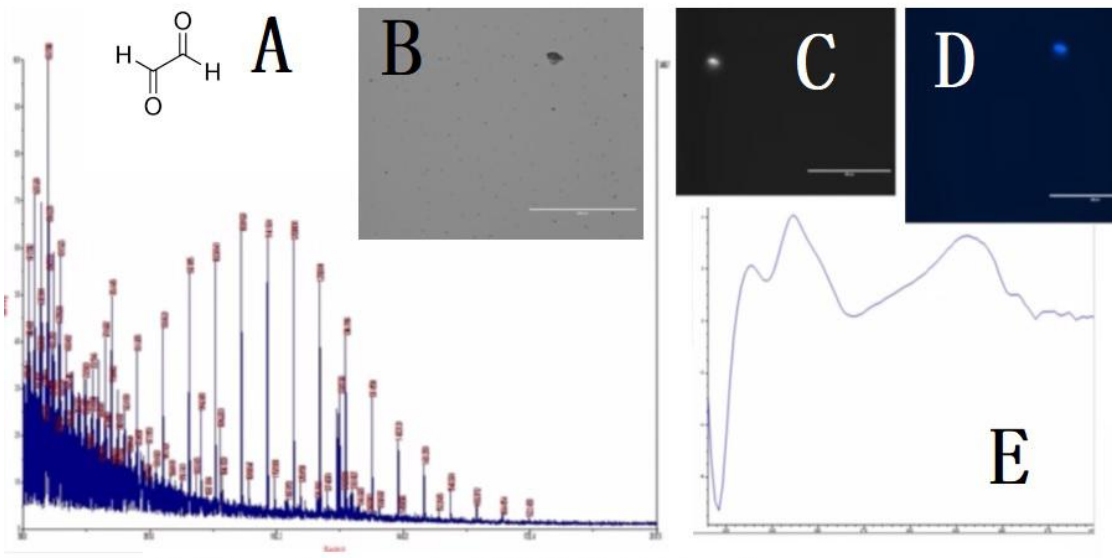
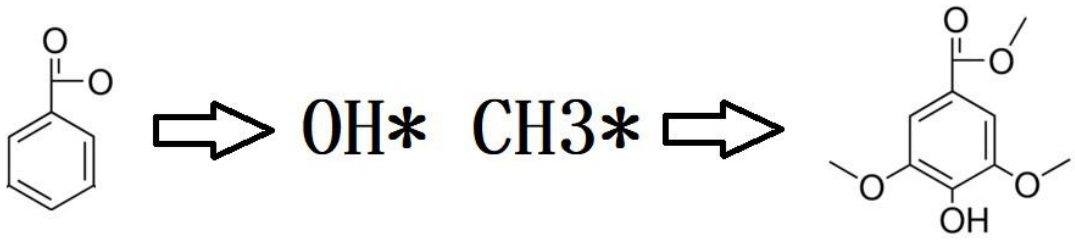


Figure 5: Polymeric glyoxal present in Manuka honey pollen. A) glyoxal structure and MALDI TOF MS polymer, B) bright field analysis of pollen grain on LED Evos FL microscope, C) Qdot longpass fluorescence analysis of pollen grain isolated from manuka honey on LED Evos FL microscope, D) DAPI filter set fluorescence analysis of pollen grain isolated from manuka honey on LED Evos FL microscope and E) spectral profile of fraction containing the pollen grain that gave the polymeric material, F) glyoxal polymer structure



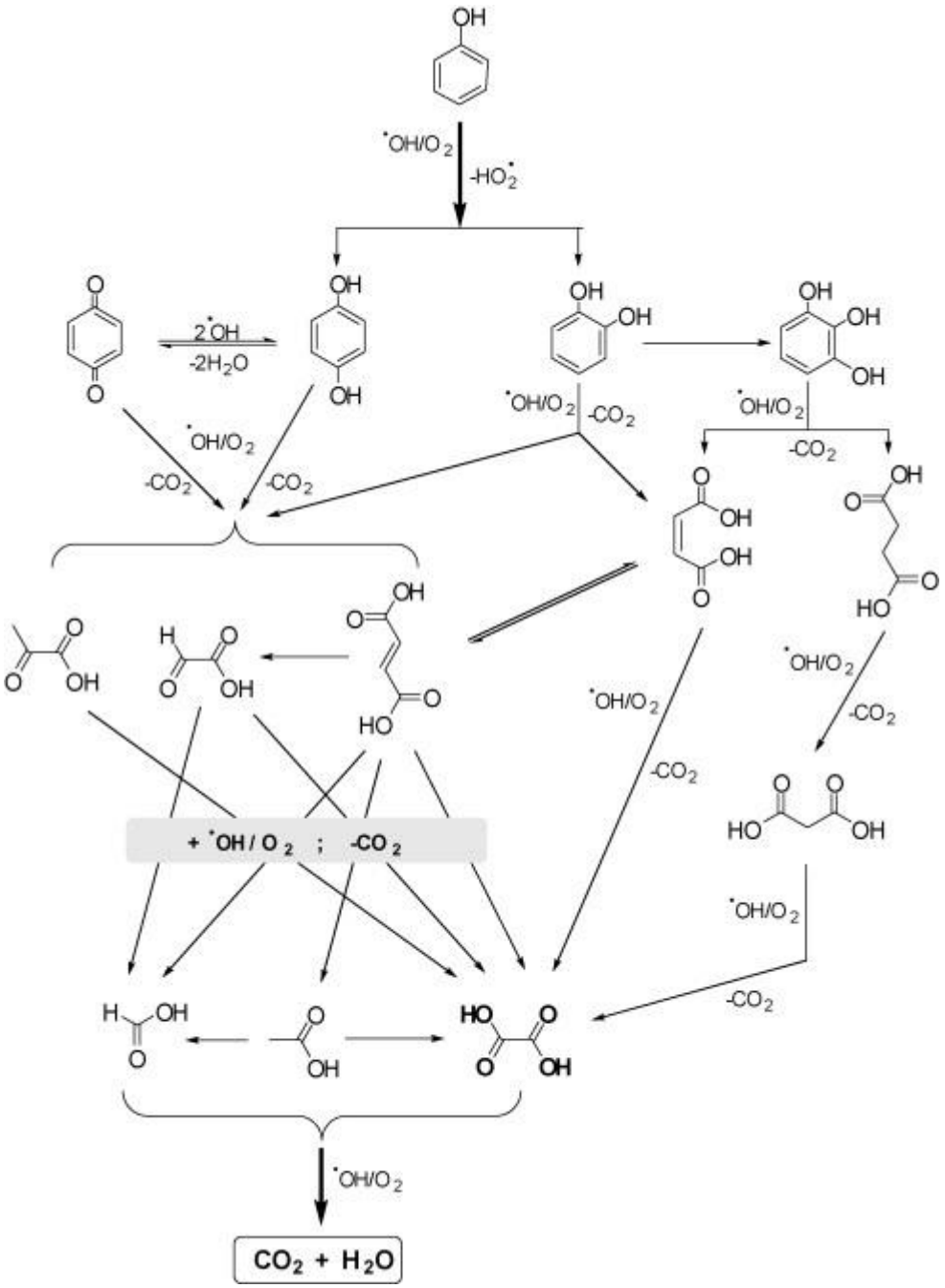


Figure 6: UV induced formation of methyl syringate from benzoic acid in Manuka honey pollen grains and radical chemistry deconstruction of phenolic aromatic ring structure into CO_2 and H_2O as well as glyoxal with the potential to form MGO and DHA.

References

Adams, C. J.; Manley-Harris, M.; Molan, P. C. The origin of methylglyoxal in New Zealand mānuka (Leptospermum scoparium) honey. Carbohydrate Research, 2009, 344, 1050-1053.

Brudzynski K, Lannigan R. (2012). Mechanism of Honey Bacteriostatic Action Against MRSA and VRE Involves Hydroxyl Radicals Generated from Honey's Hydrogen Peroxide. Front Microbiol. 2012 Feb 7;3:36.

Christopher J. Adams, Merilyn Manley-Harris, Peter C. Molan (2009). The origin of methylglyoxal in New Zealand manuka (Leptospermum scoparium) honey. Carbohydrate Research 344 1050–1053

Federoňko, M. Königstein, J. (1969) Coll. Czech. Chem. Commun. 34, 3881-3894.

Galano A, Alvarez-Ldaboy JR, Ruiz-Santoyo ME, Vivier-Bunge A. (2004). Mechanism and kinetics of the reaction of OH radicals with glyoxal and methylglyoxal: a quantum chemistry + CVT/SCT approach. Chemphyschem. Sep 20;5(9):1379-88.

Grainger, M.N.C.; Manley-Harris, M.; Lane, J.R.; Field, R.J. (2016) Kinetics of conversion of dihydroxyacetone to methylglyoxal in New Zealand mānuka honey: Part I - Honey systems. Food Chemistry, 202, 484-491.

Grainger, M.N.C.; Manley-Harris, M.; Lane, J.R.; Field, R.J. (2016) Kinetics of conversion of dihydroxyacetone to methylglyoxal in New Zealand mānuka honey: Part II - Model systems. Food Chemistry, 202. 492-499.

Grainger, M.N.C.; Manley-Harris, M.; Lane, J.R.; Field, R.J. (2016) Kinetics of conversion of dihydroxyacetone to methylglyoxal in New Zealand mānuka honey: Part III - a model to simulate the conversion. Food Chemistry, 202, 500-506.

Hanssen NMJ, Scheijen JLJM, Jorsal A, Parving HH, Tarnow L, Rossing P, Stehouwer CDA, Schalkwijk CG. (2017). Higher Plasma Methylglyoxal Levels Are Associated With Incident Cardiovascular Disease in Individuals With Type 1 Diabetes: A 12-Year Follow-up Study. Diabetes. Aug;66(8):2278-2283.

Hyung-Soon Yim, Sa-Ouk Kang, Yung-Chil Hah, P. Boon Chock, and Moon B. Yim. (1995).¶ Free Radicals Generated during the Glycation Reaction of Amino Acids by Methylglyoxal A MODEL STUDY OF PROTEIN-CROSS-LINKED FREE RADICALS. The Journal of Biological Chemistry. Vol 270 No. 47, issue 24 pages 28228-28233.

https://www.mpi.govt.nz/dmsdocument/17374-manuka-honey-science-definition-infographic

Matthew Johnston, Michael McBride, Divakar Dahiya, Richard Owusu-Apenten, and Poonam Singh Nigam (2018). Antibacterial activity of Manuka honey and its components: An overview. AIMS Microbiol. 2018; 4(4): 655–664.

Jonathan M. Stephens, Kerry M. Loomes, Terry J. Braggins, Jessie Bong, Bin Lin and Gordana Prijic (March 15th 2017). Fluorescence: A Novel Method for Determining Manuka Honey Floral Purity, Honey Analysis, Vagner de Alencar Arnaut de Toledo, IntechOpen, DOI: 10.5772/66313. Available from: https://www.intechopen.com/books/honey-analysis/fluorescence-a-novel-method-for-determining-manuka-honey-floral-purity

Majtan J, Bohova J, Prochazka E, Klaudiny J. (2014). Methylglyoxal may affect hydrogen peroxide accumulation in manuka honey through the inhibition of glucose oxidase. J Med Food. Feb;17(2):290-3.

Marta Sousa Silva, Ricardo A, Gomes, C. J. X. (2013). The glyoxalase pathway: the first hundred years….and beyond. Biochem J. 2013 Jul 1;453(1):1-15.

Molan P, (2008). An explanation of why the MGO level in manuka honey does not show the antibacterial activity. New Zealand Beekeeper May 11-13.

Nakayama M, Saito K, Sato E, Nakayama K, Terawaki H, Ito S, Kohno M. (2007). Radical generation by the non-enzymatic reaction of methylglyoxal and hydrogen peroxide. Redox Rep. 12(3):125-33.

Noor M Fauzi and Mohammed M Farid. (2014). High-pressure processing of Manuka honey: Brown pigment formation, improvement of antibacterial activity and hydroxymethylfurfural content. International Journal of Food Science & Technology 50(1)

Owens, A. (2016) The kinetics of the dissociation of the dihydroxyacetone dimer in aprotic media. MSc thesis, University of Waikato.

Thornalley, P.J. (1990). The glyoxalase system: new developments towards functional characterization of a metabolic pathway fundamental to biological life. Biochem J. 1990 Jul 1; 269(1): 1–11.

Cover letter

To the Editor,

The paper titled “Polymeric glyoxal discovered in Manuka pollen as the potential source of methylglyoxal and dihydroxyacetone in Manuka honey” provides the first evidence for the origin of DHA and MGO, which are key compounds in Manuka honey. A significant amount of research has been performed to develop models by testing laboratories in order to inform the companies how best to produce honey with high MGO content based on DHA concentration. The detection of polymeric glyoxal and a potential mechanism where MGO and DHA are produced from the polymer, which originate from pollen phenolics provides a paradigm shift in understanding the complexities of the MGO story. The highlights of the work include:

1) Discovery of polymeric glyoxal present in Manuka honey pollen.
2) Changes in pollen fluorescence during maturation correlation with MGO content.
3) MALDI TOF MS fingerprint analysis of pollen for determination of nectar origin.
4) An alternative mechanism for MGO and DHA generation in Manuka honey.
5) The role of radical chemistry in MGO generation.

Thank you for considering the inclusion of this publication in Food Chemistry. I feel that it will make a positive contribution to our current understanding of the complexities of food system as it introduces a conceptual shift into the understanding of food and its role in health and well-being in relation to the generation of high energy short lived radicals which appear to be involved in a biological recycling system.

Kind regards

Dr Keryn Johnson PhD MSc BSc

THE QUANTUM BIOLOGIST

Quantum Technologies Limited

https://quantum-technologies-ltd.myshopify.com