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

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1. Abstract

Manuka honey is currently valued by consumers and produces based on its MGO content. The origin of MGO is currently thought to be due to DHA chemical conversion. However, the serendipitous discovery of polymeric glyoxal in Manuka pollen whilst performing MALDI TOF MS analysis on royal jelly protein colloidal nanoparticles isolated from Manuka honey provides an alternative explanation for the origin and formation of MGO and DHA via radical chemistry in Manuka honey induced by light and photo-fenton chemistry. This report outlines preliminary findings to support this hypothesis. Analysis of pollen and the changes that occur during Manuka honey maturation are observed utilizing MALDI TOF MS, fluorescence microscopy and fluorescence spectrophotometry. The putative origin of MGO is questioned and understanding this alternative origin provides further

evidence toward phenolic transformation into precursor molecules that are involved in tissue regeneration.

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 system (Figure 1), to generate D-lactate a neuron energy source (Silva et al., 2013). The anti-microbial role of MGO is questionable. In addition, MGO modification of proteins has been associated with diabetes and cardiovascular disease (Hannsen et al., 2017). MGO reaction with protein 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). MGO would appear to have both positive and negative effects. MGO content for the Manuka honey industry has, none the less, remained important and honey with higher MGO content fetches a greater 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 devalues 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. 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 with heat is used to promote MGO generation as it also results in increased concentrations of 5-HMF.

Manuka honey has gained 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). This unique combination facilitates Manuka honeys bioactivity. The pollen when exposed to UVA light produces fluorescence. This fluorescence increases during honey maturation as identified in this paper, as well as the number of 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 discoveries were made that indicated that a potential source of methylglyoxal may not be from glucose conversion into DHA but occurring 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.

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 and centrifuged for 1 minute at 13,200 rpm in a bench top centrifuge. Isolated pollen grains from yound 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. 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. The excitation at 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 increase during maturation of Manuka honey

The presence of pollen in honey has been used extensively in melissopalynology to identify the nectar source of the honey. Manuka (*Leptospermum scoparium*) and Kanuka (*Kunzea ericoides*) pollen grains look identical and prevent the determination of nectar origin. It was noted that the pollen from Manuka honey had some unique properties with respect to its fluorescence profile (Figure 2), which changed during the maturation of Manuka honey (Figure 3). A consistent increase in pollen fluorescence was observed in the aging process. The pollen went from having fluorescence around the edge to highly fluorescent central sphere that glowed in an unusual way.

MALDI TOF MS analysis of isolated pollen

Pollen was isolated from various honeys including immature and aged Manuka, clover and Kanuka. As phenolics are present in the pollen grains and these compounds absorb the MALDI TOF laser light it was decided analyse the pollen grains directly within the addition of matrix ions (Figure 4). This allowed full spectral analysis from 0 to 2000 Daltons. The fingerprint analysis could be performed without

complications associated with matrix ions. Maximal laser intensity was used to observe the spectra. Characteristic peaks were observed for Manuka honey derived pollen grains at 337.37 Da. Clover had a characteristic peak at 381.29 Da, and Kanuka appeared to have a unique peak at 345.12 Da. Interestingly, the shape of Manuka and Kanuka pollen is identical which has prevented pollen morphology being used to identify the nector source. Current regulatory methods use RT PCR methods for DNA present in Manuka pollen to identify the nectar origin. The isolation of the pollen and MALDI TOF MS provides an alternative approach to identify pollen origin and therefore potential nector origin. The changes in fluorescence profile of the pollen grains also provides another indicator for MGO maturation.

The aged Manuka honey pollen appeared to have a modified fingerprint profile with a raised background envelope. Further investigation revealed an interesting polymeric compound that had a repeating mass of 58.04 Da (Figure 5). The 58.04 Da polymeric series appeared to correspond to a polymeric glyoxal. It is suggested that the loss of 1 glyoxal unit (58.04 Da) occurred due to fragmentation of the polymer by methyl and hydroxyl radicals to generate MGO and DHA respectively. The estimated length of the polymer corresponded to 17 glyoxal units, with an average of 10-12 glyoxal units. Two different series of polymers were evident in the spectra. The difference in mass between these two polymers was 42 Da which may have resulted from the loss of formaldehyde (30.02 Da) from bound glyoxal polymer.

4.1 Radical chemistry role in phenolic formation and degradation

New Zealand's environment along with the high iron content, phenolics present in the honey suggest the formation of hydroxyl radicals, which were confirmed by analyzed using 3'-(p-aminophenyl) fluorescein (APF) as the (•)OH trap and superoxide by NBT analysis. The effect of UV light on radical generation and changes in composition of key compounds (MGO, DHA, HMF and the phenolics) in diluted Manuka honey was determined (data not shown). No changes occurred in MGO, DHA and HMF content, however, methyl syringate concentration increased. The generation of the hydroxyl radicals induced by UV light and the phenolic anti-oxidant activity resulted in methyl syringate formation. Iron binding to the pi electrons in the aromatic ring of benzoic acid would position the radicals in close proximity to allow OH and methyl radical reactions to form methyl syringate (Figure 6).

Radical chemistry can not only be utilized to create compounds but also deconstruct molecules back down into their environmentally benign precursors CO_2 and H_2O (Figure 6). In this process a number of interesting molecules are produced including glyoxal. The generation of the glyoxal polymer is postulated and the formation of methyl and hydroxyl radicals which have been detected in Manuka honey are implicated in cleaving such a polymer, leading to the formation of MGO and DHA.

5. Discussion

The origin of MGO in Manuka honey has been investigated over the years using a range of approaches including artificial systems which have proven difficult to demonstrate successful formation of MGO from DHA (Grainger et al., 2016). The Manuka honey industry has investigated a wide range of approaches to increase

MGO content 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.

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, 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 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

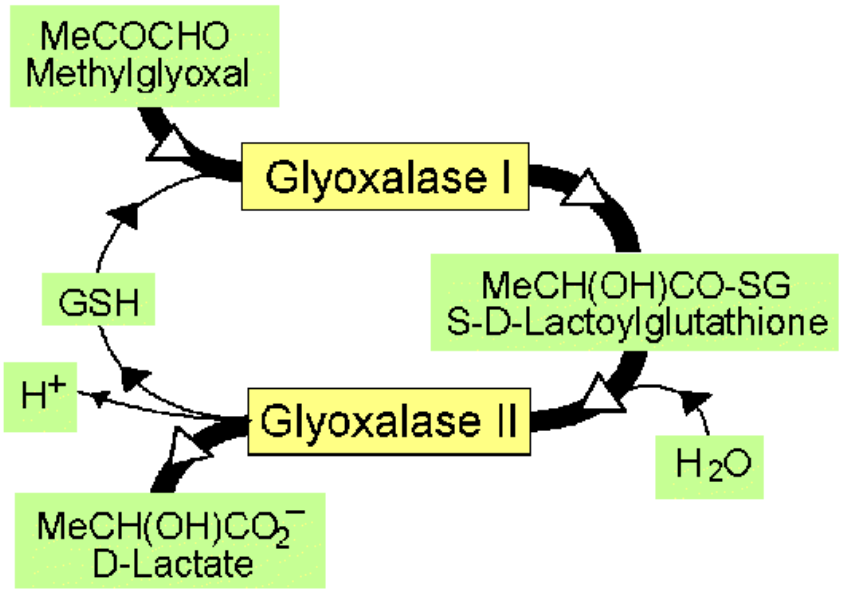


Figure 1: Glyoxalase pathway a proposed biological energy system rather than dicarbonyl stress detoxification system

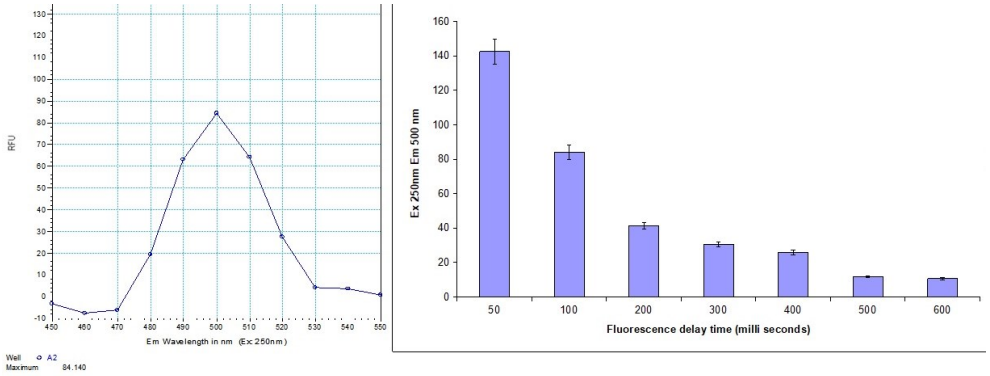


Figure 2: Fluorescence analysis of mature Manuka pollen and time resolved fluorescence

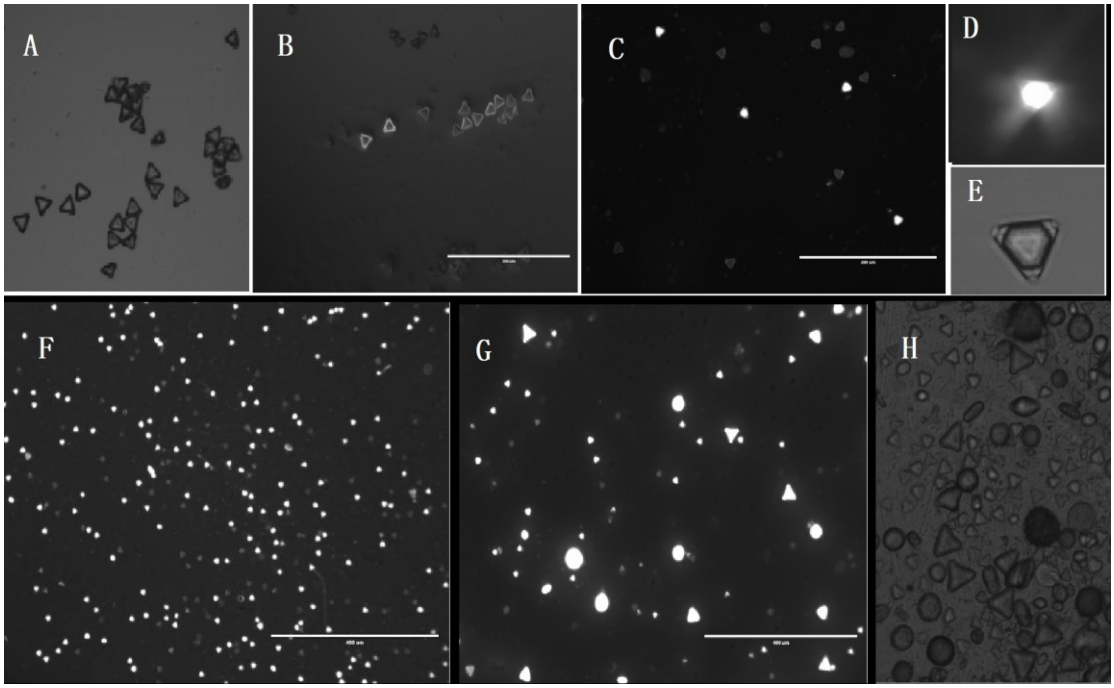


Figure 3: A) Manuka pollen isolated from the flower under bright field, B) Manuka pollen isolated from the flower under DAPI LED light source fluorescence analysis, C) A young Manuka honey pollen isolated and fluorescence analysis, D) Single pollen grain isolated from an aged Manuka honey fluorescence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

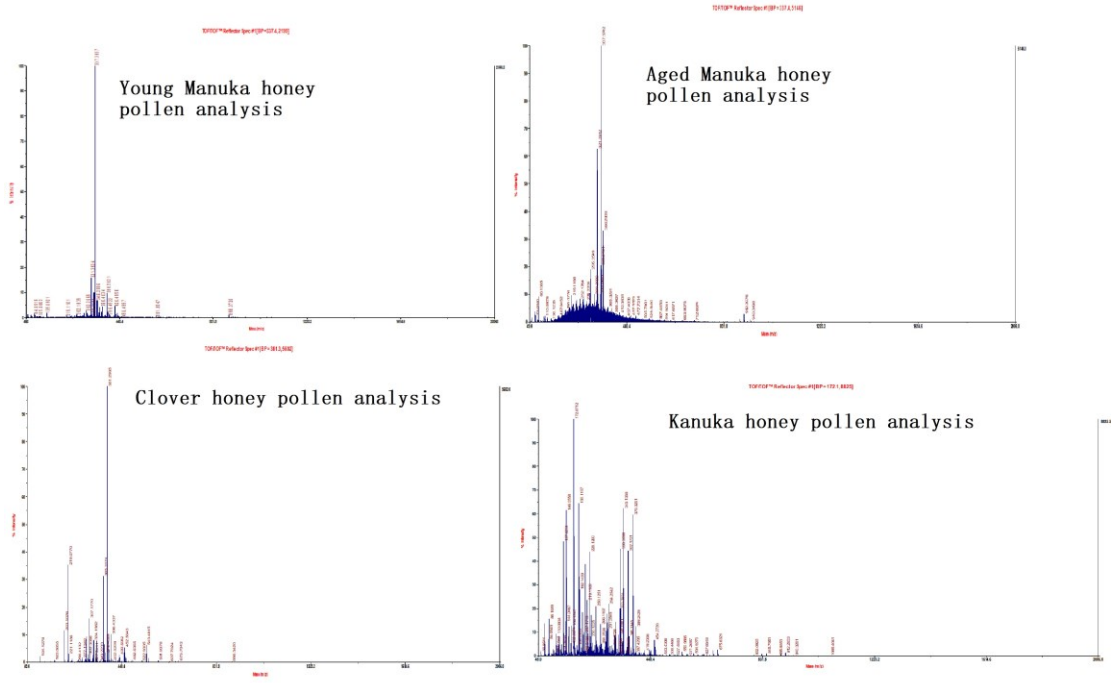


Figure 4: MALDI TOF MS analysis of pollen isolated from various honeys

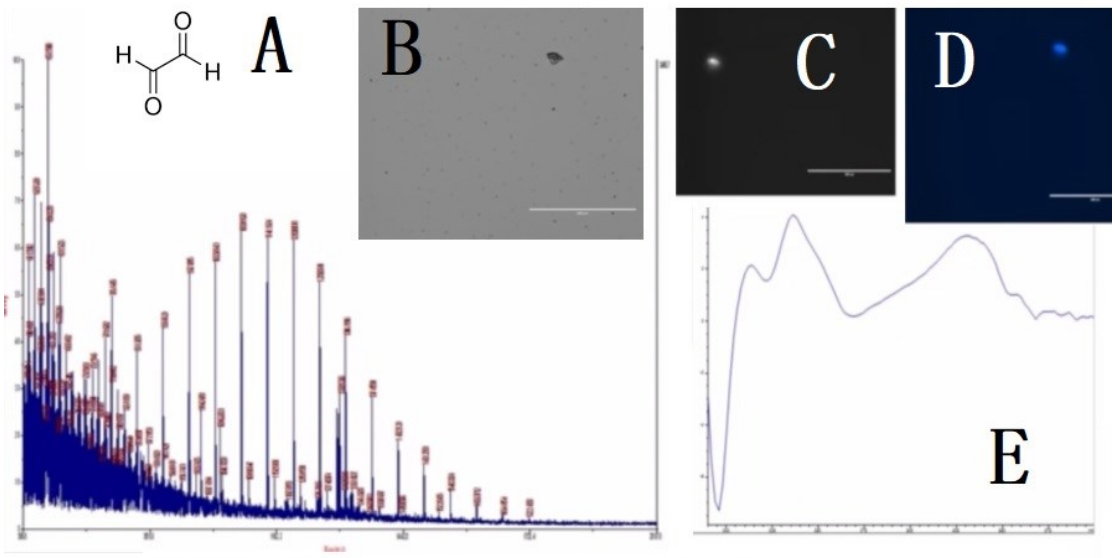
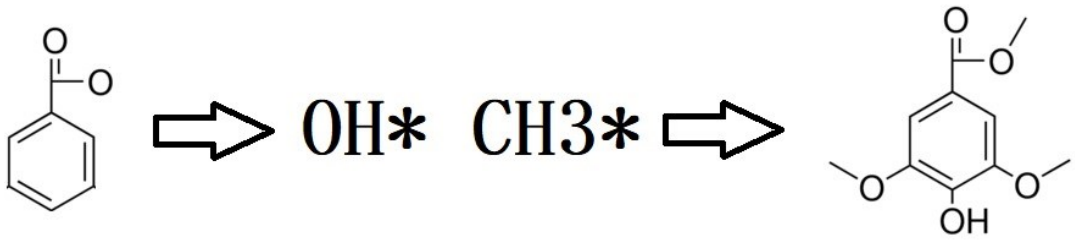


Figure 5: Polymeric glyoxal present in Manuka honey pollen. A) glyoxal structure and MALDI TOF MS polymer, B) bright field analysis of pollen grain on LED Evos FL microscope, C) Qdot longpass fluorescence analysis of pollen grain isolated from manuka honey on LED Evos FL microscope, D) DAPI filter set 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.



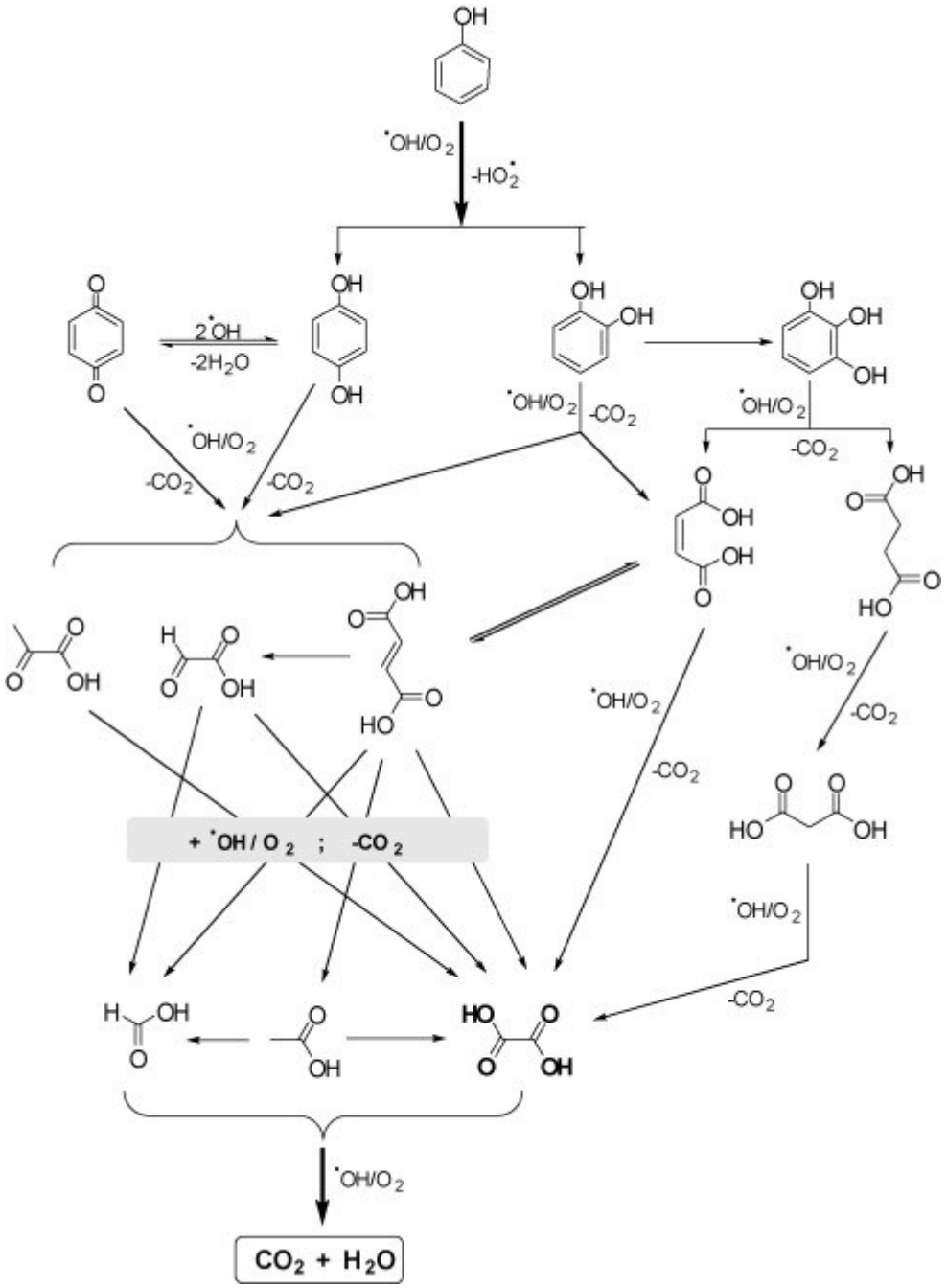


Figure 6: UV induced formation of methyl syringate from benzoic acid in Manuka honey pollen grains and radical chemistry deconstruction of phenolic aromatic ring structure into CO_2 and H_2O as well as glyoxal with the potential to form MGO and DHA.

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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

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