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Full Length Research Paper

The quantitation of hydroxymethylfurfural in Australian *Leptospermum* honeys

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This research extends the rapid high-performance liquid chromatographic (HPLC) analysis of *O*-(2, 3, 4, 5, 6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA) derivatives of methylglyoxal and dihydroxyacetone in Australian *Leptospermum* (L.) honeys to quantify hydroxymethylfurfural (HMF) content. Results showed that among the studied honeys stored at 4°C, all six *L. polygalifolium* and one *L. liversidgei* honeys exceeded the HMF upper limit (40 mg/kg) of the International Honey Commission (IHC), while four *L. liversidgei*, one *L. semibaccatum* and one *L. laevigatum* honeys satisfied this IHC regulation. It was found that all of the 13 heat treated (37°C for 60 days) *Leptospermum* honeys exceeded the IHC limit.

Key words: Hydroxymethylfurfural, *Leptospermum*, honey.

INTRODUCTION

Honey is prone to sugar crystallization. This rate of crystallization increases with increasing supersaturation ratio of glucose and viscosity. The former of these factors dominate at temperatures below 15°C, whilst the latter factor dominates at temperatures above 15°C (Venir et al., 2010). Commercial honey processing methods such as heating (significantly above 15°C), decrease viscosity and thus successfully decrease the rate of crystallization of honey (Turhan et al., 2008), and are also responsible for the production of undesirable compounds that reduce honey quality (Ajlouni and Sujirapinyokul, 2010). Hydroxymethylfurfural (HMF), a cyclic aldehyde, is one of such unfavourable compounds, which is virtually absent in fresh and untreated foods (Teixido et al., 2011). Although HMF is found in a variety of processed foods; honey is the only food for which there exists a recommendation on the allowable content of HMF (Arribas-Lorenzo and Morales, 2010). HMF is high in honeys that have been heat treated, stored in non-adequate conditions or adulterated with invert syrup (Ajlouni and Sujirapinyokul, 2010), thus HMF is a recognised parameter related to the quality of honey (Spano et al., 2006). The International Honey Commission (IHC) has stated that after processing and/or blending, HMF levels shall not exceed 40 mg/kg, unless the honey originates from regions with tropical ambient temperatures, in which case levels shall not exceed 80 mg/kg (Ajlouni and Sujirapinyokul, 2010).

The main concern surrounding HMF intake by humans is that, in both *in-vivo* and *in-vitro* experiments (Capuano and Fogliano, 2011), sulfotransferases (SULTs) metabolise HMF to its mutagenic derivative sulfomethylfurfural (SMF) by sulfonation of the allylic hydroxyl functional group (Arribas-Lorenzo and Morales, 2010; Husoy et al., 2008; Teixido et al., 2011). Differing views persist about the risk HMF and its metabolite SMF pose to human health (Spano et al., 2009). The cytotoxicity of HMF to humans has been demonstrated by reduced granulocyte metabolism (Nassberger, 1990).

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Epidemiological studies identified HMF in caramelised sugar as a possible dietary factor associated with the risk of colorectal cancer (Bruce et al., 1993). Single subcutaneous injections showed both HMF and SMF to be weak intestinal carcinogens in multiple intestinal neoplasia mice (Svendsen et al., 2009). There is the concern that humans may be more sensitive to HMF than rats and mice, because humans express SULTs in their extrahepatic tissue more extensively compared to rats and mice (Capuano and Fogliano, 2011; Husoy et al., 2008). In contrast, genotoxic and mutagenic effects were not observed in *in vitro* experiments of mammalian cells, except at high (≥ 1500 mg/kg) HMF concentrations (Janzowski et al., 2000).

Since consumer protection and quality control gained importance, the presence of potentially toxic compounds in foods has been attracting more attention (Spano et al., 2009). This attention has been directed to the determination of HMF in several food products (including honey), because HMF occurrence is an indication of quality deterioration (Schultheiss et al., 2000). The IHC has recommended three methods for determination of HMF (Zappala et al., 2005). Method 1 involved the measurement of ultraviolet (UV) absorbance of clarified aqueous honey solutions with and without bisulphite (White, 1979). Method 2 involved the measurement of UV absorbance of honey solutions with added barbituric acid and *p*-toluidine (Winkler, 1955). Method 3 involved the dissolution of honey in water and after filtration, HMF determination on a reversed phase-high performance liquid chromatography (RP-HPLC) column by isocratic elution with water and methanol mobile phases (Jeuring and Kuppers, 1980). Methods 1 and 3 usually gave similar values for HMF, but Method 2 consistently gave HMF values higher than the other methods (Zappala et al., 2005). Sulphuric acid was added to the water/methanol mobile phase of Method 3 in a new gradient method to completely resolve the peaks of HMF and homogentisic acid (the marker of strawberry tree origin of honey) (Spano et al., 2006). This adapted method has since been applied to concurrently determine HMF, 2-furfural, 3-furfural, 2-furoic acid and 3-furoic acid in strawberry tree, thistle cardoon and *Eucalyptus* honeys (Spano et al., 2009). No previous methods have concurrently determined potentially toxic and potentially beneficial compounds in honey.

This study extends previous PFBHA derivatisation and RP-HPLC determination of the beneficial antibacterial compound MGO and its precursor DHA in Australian *Leptospermum* (L.) honeys (Windsor et al., 2012) to the determination of the potentially toxic HMF in Australian *Leptospermum* honeys (a genus that has not previously been studied in relation to HMF concentrations). This study investigates the concentrations of HMF in Australian *Leptospermum* honeys and the effect of heating on these concentrations. This technique provides a sensitive and selective means for the simultaneous determination of potentially toxic HMF and also potentially beneficial bioactive compounds present in honey.

MATERIALS AND METHODS

The honey derivatisation and HPLC conditions were adapted from Windsor et al. (2012), a brief summary follows. Honeys were obtained from Tyagarah Apiaries, Tyagarah, New South Wales, Australia, 2841. Unprocessed honey samples were stored at 4°C until HMF analysis via HPLC, while a duplicate of each sample was heated at 37°C for 60 days prior to HMF analysis via HPLC. HPLC MilliQ grade water was used in all analyses. HPLC Chromasolv grade acetonitrile (ACN) was obtained from Merck, Kilsyth, Victoria, Australia, 3137. PFBHA (99%) and hydroxyacetone (HA) (90%) were purchased from Sigma-Aldrich, Castle Hill, New South Wales 1765, Australia.

Analyses were performed on a Perkin Elmer Series 200 Pump and Autosampler with a Flexar photo diode array detector (λ = 263 nm). HPLC separations were performed on a Synergi Fusion column (75 × 4.6 mm, 4 μm particle size). Mobile phase A was water:ACN (70/30, v/v) and mobile phase B was 100% ACN. The following 23 min gradient elution was employed: A:B = 90:10 (isocratic 2.5 min), graded to 50:50 (8.0 min), graded to 0:100 (1.0 min), 0:100 (isocratic 7.0 min), graded to 90:10 (1.0 min), 90:10 (isocratic 4.0 min), and detection at 263 nm.

The PFBHA derivatising reagent was 19.8 mg/ml in citrate buffer (0.1 M) adjusted to pH 4 with NaOH (4 M). The HA internal standard solution was prepared by dissolving HA (152.5 mg) in 50.0 ml of water. Honey samples (0.1 to 0.15 g) were weighed into 16 × 75 mm test tubes. HA standard solution (250 µl) and PFBHA derivatising solution (1500 µl) were added to each of these test tubes. Each of the test tubes was thoroughly mixed and was allowed to stand for 1 h for complete derivatisation. ACN (6 ml) was added to each test tube and mixed until all crystals dissolved. Water (2 ml) was added to each test tube and was mixed. A 1.5 ml aliquot of each sample was placed in an HPLC vial for analysis. Calibration was against a series of HMF standards with the same addition of HA internal standard.

RESULTS AND DISCUSSION

An HPLC method has previously been used to simultaneously separate a standard mixture of potentially toxic compounds (HMF, 2-furfural, 3-furfural, 2-furoic acid and 3-furoic acid) and successfully been applied to real samples of strawberry tree, thistle cardoon and *Eucalyptus* honeys (Spano et al., 2009). This work's HPLC method concurrently determines the potentially toxic HMF, and also the beneficial antibacterial compound MGO and its precursor DHA in Australian *Leptospermum* honeys. The PFBHA derivative of HMF has both a cis and trans isomer around its C=N double bond. These two isomeric forms of this potentially toxic compound were observed as two distinct peaks in the HPLC trace eluting at 10.87 and 11.25 min (Figure 1). The PFBHA derivatives of the beneficial antibacterial compound MGO (eluting at 16.4 min) and its precursor DHA (eluting at 4.7 min) also elute as clear peaks (Figure 1). Therefore, this technique could be used as a quantitation method for all of these three compounds. The HPLC trace also showed derivatised sugars (eluting at 1 to 2 min), internal standard, hydroxyacetone (eluting

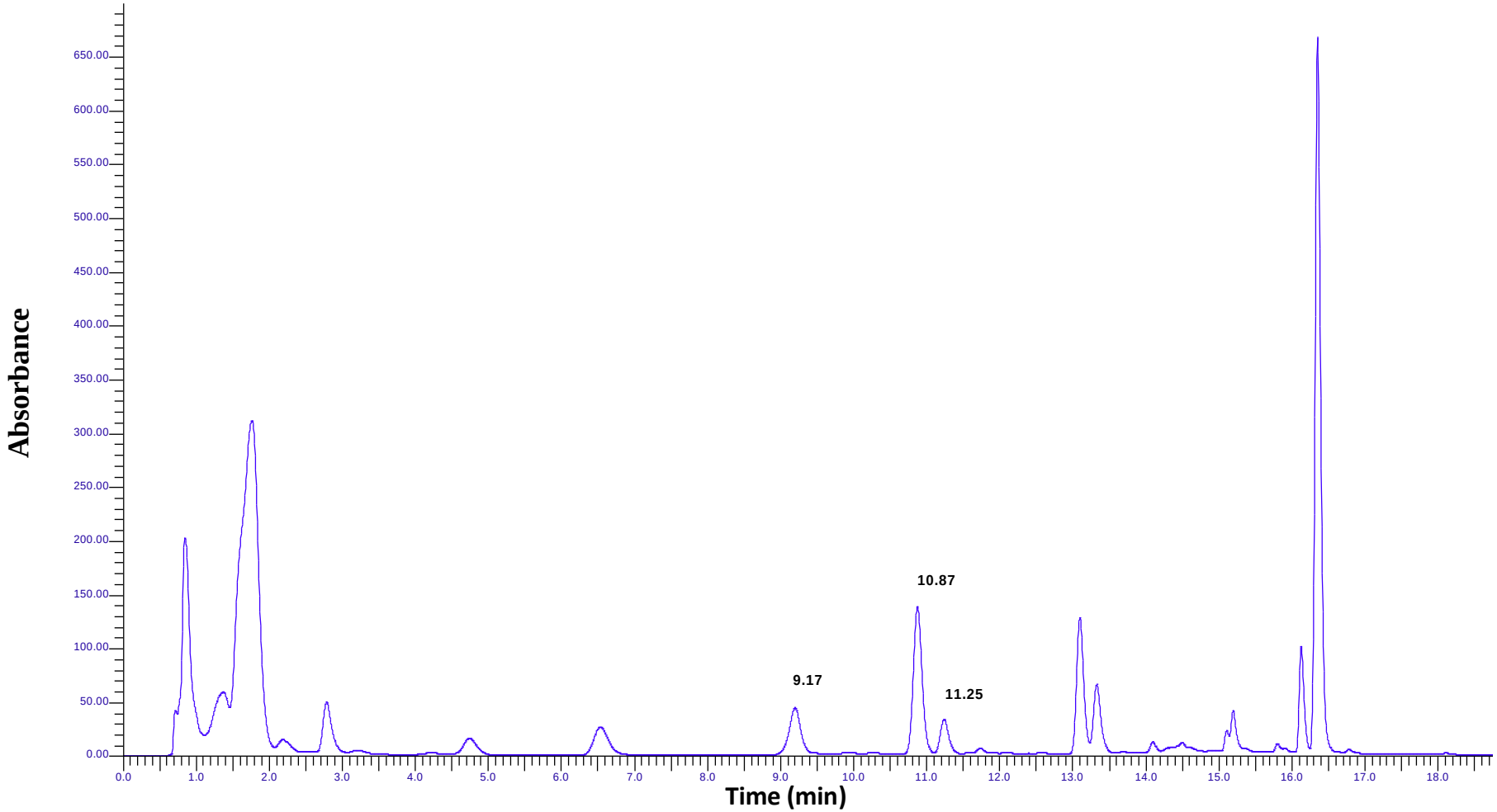

Figure 1. HPLC trace of heat treated sample number 1.

at 9.17 min) and the PFBHA derivatising agent at 6.5 min PFBHA peak shows that the derivatisation reaction of HMF and the other carbonyl compounds present in the honey went to completion. Unprocessed honeys stored at 4°C until HMF analysis via HPLC have HMF content largely determined by the botanical origin of the honey (Spano et al., 2009; Turhan et al., 2008). Eleven out of twelve strawberry tree honeys had HMF to honey concentrations greater than the IHC recommended limit of 40 mg/kg (Spano et al., 2009); whilst honeydew, thistle, *Eucalyptus*, cistus and chestnut honeys had HMF to honey concentrations less than the IHC recommended limit of 40 mg/kg (Spano et al., 2009; Turhan et al., 2008). This work's analysis of unprocessed Australian *Leptospermum* honeys stored at 4°C until HMF analysis via HPLC showed a genus average and

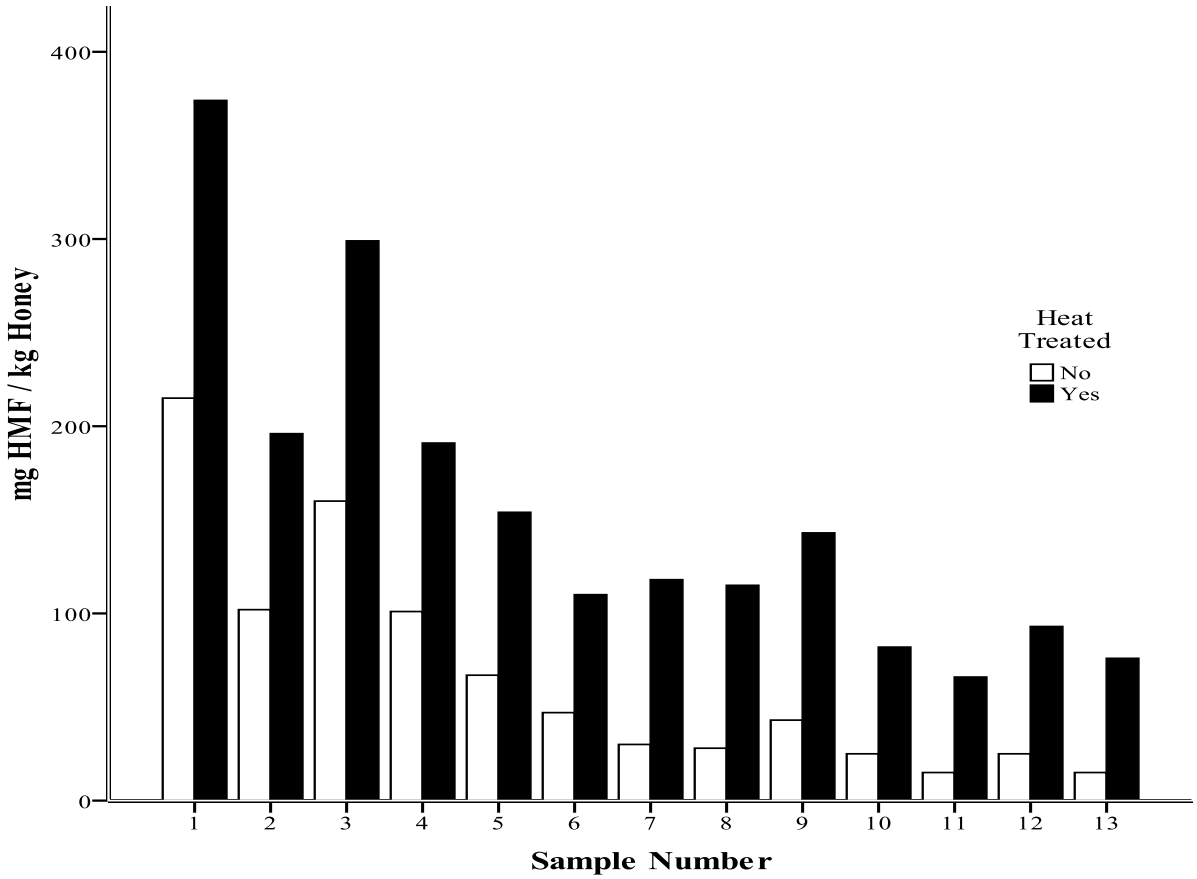

Figure 2. HMF content in untreated and heat treated honey samples.

standard deviation of HMF to honey concentrations of 67 ± 62 mg/kg, but also revealed a species specific determination of HMF content. All *Leptospermum polygalifolium* honey samples (sample numbers 1 to 6) and one (sample number 9) out of five *Leptospermum liversidgei* honey samples (sample numbers 7 to 11) had HMF to honey concentrations greater than the IHC recommended limit of 40 mg/kg; whilst *Leptospermum semibaccatum* and *Leptospermum laevigatum* honey samples (sample numbers 12 and 13, respectively) had HMF to honey concentrations less than the IHC recommended limit of 40 mg/kg (Figure 2).

The honeydew honey stored at 4°C until initial HPLC analysis mentioned earlier (Turhan et al., 2008), was then heated at 75°C for 90 min, but its HMF to honey concentration was still less than the IHC recommended limit of 40mg/kg (Turhan et al., 2008). Unprocessed Australian honeys from *Banksia* and *Eucalyptus* botanical origin stored at room temperature until initial HPLC analysis, then heated at 65°C for 2 min, had HMF to honey concentrations less than the IHC recommended limit of 40 mg/kg prior and post heat treatment (Ajlouni and Sujirapinyokul, 2010). Twenty samples of commercially available honeys in Spain stored at room temperature until initial HPLC analysis, then heated at 35°C for 29 days, had HMF to honey concentrations less than the IHC recommended limit of 40 mg/kg prior to heat treatment, but some samples showed levels greater than this limit towards the end of the heating period (Escriche et al., 2008). It is believed that the temperatures honey would be exposed to during collection and storage, range between 35 and 45°C (Escriche et al., 2008). Therefore, the temperature at which samples were heated in this study (37°C for 60 days) are realistic conditions for temperatures honey may be exposed to. Australian *Leptospermum* honeys subjected to this heat treatment showed an average and standard deviation of HMF to honey concentrations of 155 ± 91 mg/kg. A paired samples t-test revealed a statistically significant increase of HMF to honey concentrations of 88 ± 32 mg/kg (paired t = 10.071; one-tail P < 0.001; df = 12) post heat treatment. Figure 2 shows all heat treated Australian *Leptospermum* honeys had HMF to honey concentrations greater than the IHC recommended limit of 40 mg/kg.

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