Food Chemistry 119 (2010) 1000–1005

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Hydroxymethylfurfuraldehyde and amylase contents in Australian honey

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ARTICLE INFO

Article history:
Received 26 September 2008
Received in revised form 30 July 2009
Accepted 31 July 2009

Keywords:
Hydroxymethylfurfuraldehyde
Gothe unit = diastase number
Amylase activity
Processed honey
Fresh honey

ABSTRACT

The quality of Australian honey samples (processed and unprocessed) was assessed using HPLC techniques. 5-Hydroxymethylfurfuraldehyde (HMF) was used as the main quality indicator. Sampling included four commercially-processed honeys (Australian rainforest, Beechworth, Homebrand and Leabrook) and three unprocessed (Banksia, Grey box and Mallee). All honey samples, except Leabrook and Beechworth, showed an initial HMF content less than the Codex Alimentarius and International Honey Commission standard (40 mg/kg). HMF contents in Leabrook and Beechworth were 50.8 ± 1.34 and 74.9 ± 2.34 mg/kg, respectively. Heating unprocessed honey at 85 °C for 2 min caused significant ($p \leqslant 0.05$) increment in HMF contents. The amounts of HMF in Mallee samples increased from 34.0 ± 0.31 to 42.3 ± 0.37 mg/kg after 2 min at 85 °C. All honey samples showed amylase activity above the minimum limit (8 Gothes). The physiochemical properties of honey showed significant variations among samples. The results revealed also that heating was not the only factor influencing HMF formation in honey, but also honey composition, pH value and floral source can contribute to these variations. Consequently, the amount of HMF may be an insufficient sole indicator of honey quality.

1. Introduction

Food Standard Australia New Zealand (FSANZ, 2006) defines honey as "The natural sweet substance produced by honey bees from the nectar of blossoms or from secretion of living parts of plants". It must contain at least 60% reducing sugars and no more than 21% moisture. Honey composition is highly influenced by the types of flowers used by the bees as well as regional and climatic conditions (Mendes, Proenca, Ferreira, & Ferreira, 1998).

Australia is the world's fourth largest exporter of honey. The honey industry is worth at least $65 million per annum in Australia. New South Wales is the major producer of honey, accounting for 45% of the total production. Half of the honey produced is consumed domestically, while the remainder is exported (Honeybee Industry Council of Australia, 2004). Eucalyptus represents the main native flora (78%) for honey production in Australia (Gibbs & Muirhead, 1998). Eucalyptus constitutes about 95% of Australian vegetation, dominating the woodlands with species and varieties consisting of 550–600 more or less distinct forms, plus many hybrids (Kelly, 1983).

Fresh honey is usually heated in order to facilitate processing and to maintain good quality. However, excessive heat treatment leads to the formation of 5-hydroxymethylfurfuraldehyde (HMF) and reduced honey quality. HMF value is virtually absent or very low in fresh honey and is high in honey that has been heated, stored in non-adequate conditions, or adulterated with invert syrup (Nozal, Bernal, Toribio, Jimenez, & Martin, 2001). Chemical properties of honey such as pH, mineral content and total acidity also affect HMF content. The presence of organic acids and low water activity also favours the production of HMF (Kalabova, Borkovcova, Smutna, & Vecerek, 2003). The Codex Alimentarius (2001) and International Honey Commission. (2002) set the maximum concentration of HMF to 40 mg/kg for honey from non-tropical regions and 80 mg/kg for honey from tropical regions. Extremely high (>500 mg/kg) HMF values demonstrate adulteration with invert syrup (Coco, Valentini, Novelli, & Ceccon, 1996).

Codex Alimentarius (2001) proposed two quality indicators for honey, namely, 5-hydroxymethylfurfuraldehyde and amylase (diastase) activity to measure the freshness of honey. Many countries have set the national limit for HMF content in honey to 40 mg/kg.

Honey production in Victoria, Australia is largely reliant on access to native flora and various crops, such as apples, pears, cherries, berries, nashi, kiwi fruit and vegetables. Particular flora species and crops produce honey with specific characteristics, in terms of colour and flavour. Honey can be classified by the floral source of the nectar from which it was made, or by the name of the town producing a specific type of honey. Most of the Australian natural honey is produced using a particular flora as the main source of nectar. For example, Grey box honey is named after *Eucalyptus microcarpa* (Grey box), which is a medium-sized tree

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doi:10.1016/j.foodchem.2009.07.057

growing up to 25 m in height. Buds appear in early December for flowering at the beginning of March and last until late May, sometimes flowering into winter (Heathmont Honey., 2009). The reddish-tinged orange–yellow flowers of *Banksia spinulosa* are the exact colour of Banksia honey. *Banksia* is a genus of around 170 species in the plant family Proteaceae. Iconic Australian wild flowers and popular garden plants, they are easily recognised by their characteristic flower spikes and fruiting "cones" and can vary from prostrate woody shrubs to trees up to 30 m tall (Banksia Integrifolia., 2009). However, Mallee honey is named after a town of northwestern Victoria, Australia, where a narrow belt of irrigated land supports vineyards, citrus orchards, wheat fields, and dairy and sheep farming. Mallee is said to be derived from an aboriginal term denoting species of eucalyptus, hence we have Mallee honey, which depends mostly on the Eucalypt family as a source of bee nectar (Britannica Encyclopedia., 2009). Additionally, some commercial honey products are often made from a mix of flower sources and then get labelled as wildflower honey (Australian rainforest) or garden honey (Beechworth and Leabrook), or blended after extraction (Homebrand honey).

Despite many international scientific investigations into the physicochemical and enzymatic properties of honey, standard quality guidelines are still lacking on Australian honey. Additionally, adulteration of pure honey with synthetic honey has become much more prevalent in recent years and more difficult to identify, due to the availability of cheap fructose corn syrup. Consequently, this project was developed to thoroughly examine the physiochemical properties of Australian honey and to identify a standard for HMF content in comparison to the Codex Alimentarius standard.

2. Materials and methods

2.1. Experimental design

Fresh unprocessed honey samples were obtained from Department of Primary Industry (DPI) in northeastern Victoria (Mallee), and included Mallee, Banksia and Grey box honey. Each fresh unprocessed honey sample weighed 500 g, and was from season 2006. The commercially-processed Australian honey samples (500 g each) were purchased from a local supermarket in Melbourne in July 2006, and included Australian rainforest, Banksia, Leabrook and Beechworth. The samples were kept in the original containers and stored in a dark room at room temperature throughout the analysis that was completed in mid November 2006. Each honey sample was divided into three sub-samples, to represent three replicates. All analyses were performed at least three times.

2.2. Heat treatments of honey samples

Honey samples, 5 g in Falcon™ tubes (BD Biosciences, Franklin Lakes, NJ), were submerged in a water bath (Thermoline, Australia) at 65 °C, 77 °C and 85 °C for 2 min and cooled down in an ice water bath for 4 min. Heat-treated samples were subjected to HMF, and amylase activity analyses.

2.3. Materials

Analytical standard-grade HMF was obtained from Sigma–Aldrich (Steinheim, Germany). Sulfuric acid was of analytical-reagent grade and supplied by Univar (Sevenhills, NSW, Australia). Methanol of HPLC grade was purchased from Merck (Kilsyth, VIC, Australia). Water was purified by using a Milli-Q water system from Millipore (Bedford, MA). All other chemicals were purchased from the local chemical store on campus.

2.4. Determination of moisture, pH, free acidity, lactones and total acidity contents in honey

Moisture content was determined using a standard direct drying method. In this method, 2.5 g of each honey sample were placed in an aluminium dish, mixed with 2 g of analytical-grade acid-washed sand, and dried in the oven at 105 °C, following the method of the International Honey Commission (2002). The pH was measured using a solution containing 10 g honey in 75 ml carbon dioxide-free water in a 250 ml beaker (PHM 210 Standard pH meter, MasterLab, Radiometer, Copenhagen). The free acidity lactones and total acidity were determined by a titrimetric method (International Honey Commission, 2002), and the following equations:

$$\text{Free acidity} = (\text{ml } 0.05\ \text{NNaOHfrom burette} - \text{ml blank}) \times 50/\text{g sample}$$

$$\text{Lactone} = (10.00 - \text{ml } 0.05\ \text{NHCl from burette}) \times 50/\text{g sample}$$

$$\text{Total acidity} = \text{free acidity} + \text{lactone}$$

2.5. Measurement of colour

Colour was measured using a Minolta colour meter (Tokyo, Japan), with a specific presentation of honey samples to avoid variations. The honey sample (15 g) was poured into a small disposable petri dish (55 mm × 14 mm), covered with the lid and left at room temperature for 20 min before taking the colour measurement (Ajlouni, 2006). Readings were taken at three different points with the lid on and the average values were calculated.

2.6. Evaluation of amylase activity

Heat-treated honey (5 g) was dissolved in 15 ml Milli-Q water and 5 ml acetate buffer solution (pH 5.3), transferred to a 50-ml volumetric flask containing 3 ml sodium chloride solution and diluted to volume. Using a volumetric pipette, 10 ml of honey solution were transferred into a 50-ml flask and placed in a 40 °C water bath along with a second flask containing 10 ml of 1% starch solution. After 15 min, 5 ml starch solution were added to the honey solution, mixed and timed. At periodic intervals, for the first time after 5 min, 0.5 ml aliquots of the mixture were mixed with 5 ml diluted iodine solution and 22 ml Milli-Q water, vortexed and immediately measured at 660 nm against a water blank. A plot of absorbance against time was used to determine the time, t_x, at which the specified absorbance of 0.235 was reached. The diastase number was calculated following the method of the International Honey Commission (2002).

2.7. Determination of disaccharide, glucose and fructose contents

The sugar composition was determined by a HPLC (Shimadzu, Tokyo, Japan) fitted with a refractive index detector (HPLC-RI) at 30 °C. A honey sample (1 g) was dissolved in 19 ml Milli-Q water, filtered through a 0.22 μm nylon filter into an HPLC vial, capped and injected (20 μl) into the HPLC. The HPLC column was Supelcogel C-610H, 30 cm × 7.8 mm I.D. fitted with a guard column Supelguard C-610H 5 cm × 4.6 mm I.D. Col: H+11,855. The mobile phase was 0.005 M sulfuric acid at a flow rate of 0.75 ml/min. External calibration curves constructed from standard solutions were used to quantify the amount of sugars in the sample (International

Honey Commission, 2002). Results were expressed as gram sugar per 100 g of honey.

2.8. Quantitation of HMF

A honey sample (5 g) was dissolved in approximately 25 ml Milli-Q water and transferred quantitatively to a 50-ml volumetric flask. To clarify the honey samples and to stop HMF breakdown, 0.5 ml of a 15% (w/v) Carrez I (potassium hexacyanoferrate) solution and 0.5 ml of a 30% (w/v) Carrez II (zinc acetate dehydrate) solution were added and made up to 50 ml with Milli-Q water. The solution was filtered through Whatman filter paper, and the first 10 ml of the filtrate was discarded. The filtrate was passed through a 0.22 μm membrane filter before injection on the HPLC for HMF analysis (International Honey Commission, 2002). The HPLC consisted of a SCL-10A VP system controller, a LC-10AT VP liquid chromatograph, a FCV-10AL VP pump, a DGU-14A degasser and an auto-injector SIL 10AD VP from Shimadzu (Tokyo, Japan). The column used was a Supelcosil LC-18, reverse phase (Supelco, Bellefonte, PA) stainless steel column (25 cm × 4.6 mm i.d.; film thickness 5 μm), and operated at 30 °C along with a C18 guard column. The mobile phase was water:methanol (90:10, v/v), and the flow rate was 0.75 ml/min with an injection volume of 20 μl. Serial standard solutions of HMF (1–50 mg/l) were made in Milli-Q water, to generate a calibration curve at 285 nm.

2.9. Statistical analysis

Minitab 14 was used to perform statistical analyses of the data obtained. ANOVA (one-way analysis of variance) was performed to study the effect of heating at different temperatures on HMF, amylase activity and sugars. *F*-test ($\alpha = 0.05$) was used to examine for any significant differences among honey samples. The differences among the means were determined for significance at the 5% level using Tukey's test.

3. Results and discussions

3.1. pH of honeys

The pH of the honey samples varied from 4.02 to 4.69. These values were within the pH range of 3.81–6.32 reported by Chandler, Fenwick, Orlova, and Reynolds (1974) for Australian honey. Banksia honey was found to have the highest pH value of 4.69 (Table 1). However, that high pH value was still below the highest value reported for Australian honey of pH 6.32 (Chandler et al., 1974). pH was found to be statistically different for all samples ($p \leqslant 0.05$), and varied from 4.02 ± 0.01 to 4.69 ± 0.01 for Leabrook and Banksia, respectively. The pH values of honey are of great importance during extraction and storage, since acidity can influence the texture, stability, and shelf life of honey (Terrab, Diez, & Heredia, 2003).

3.2. Total acidity of honeys

Total acidity of the honey ranged from 33.5 ± 0.35 to 53.5 ± 0.18 milliequiv acid/kg in Homebrand and Banksia, respectively (Table 1). These values were larger than those reported by Mossel (2003) of 13.1–31.9 milliequiv acid/kg, and smaller than the 60 milliequiv acid/kg in *Eucalyptus lanceolatus* honey recorded by Bath and Singh (1999). The acidity of honey is due to the presence of organic acids, particularly gluconic acid, in equilibrium with their lactones or esters, and inorganic ions, such as phosphate and chloride. El-Sherbiny and Rizk (1979) reported that total acidity was higher in cotton honey than in clover honey. Furthermore, Fallico, Zappala, Arena, and Verzara (2004) found that eucalyptus honey had the highest concentration of free acids, lactones and total acidity than orange, chestnut and Sulla honeys. These data illustrate the significant influence of floral type on the total acidity of honey. The variation in acidity among different honey types may be attributed also to variation due to harvest season (Singh & Bath, 1996).

3.3. Free acidity of honey

Free acidity of all seven samples fell within the permitted range proposed by Codex Alimentarius (2001) of no more than 50 milliequiv acid/kg. The free acidity of honey samples in this study ranged from 10.25 ± 0.01 to 20.34 ± 0.18 milliequiv acid/kg in Grey box and Banksia, respectively (Table 1). High free acidity values may indicate the fermentation of honey sugar by yeasts. It is well known that during fermentation, glucose and fructose are converted into carbon dioxide and alcohol. Alcohol is further hydrolysed in the presence of oxygen and converted to acetic acid, which contributes to the level of free acidity in honey. The mean free acidity values in Leabrook, Beechworth and Mallee were not significantly different ($p > 0.05$).

3.4. Lactones contents

Lactone contents in all honey samples except Homebrand (18.5 milliequiv/kg) showed an average lactone content of >30 milliequiv/kg. These values are similar to those reported by Bath and Singh (1999) of 35 milliequiv acid/kg. A simple comparison between lactone and total acidity contents revealed that the honey sample with the lowest lactone content (Homebrand) had also the lowest total acidity (Table 1). These observations clearly support the view that lactones are among the main contributors to the total acidity in honey.

3.5. Moisture content

All the moisture values were under the allowed limit of 21% moisture content permitted by FSANZ (2006). The moisture content of the studied honey samples ranged from 10.6% to 17.8% (Table 1). As with acidity, the literature shows wide variation among the reported moisture contents in honey. Chandler et al.

Table 1
Physiochemical parameters of various processed and unprocessed honey samples. Results represent the average of three measurements ± SD.

Honey type	pH	Total acidity (milliequiv/kg)	Free acidity (milliequiv/kg)	Lactone (milliequiv/kg)	Moisture content (%)
Leabrook	4.02 ± 0.01	48.3 ± 0.28	17.6 ± 0.37	30.7 ± 0.27	12.6 ± 0.42
Beechworth	4.04 ± 0.01	49.2 ± 0.68	17.9 ± 0.63	31.3 ± 0.18	14.1 ± 0.43
Australian rainforest	4.07 ± 0.01	42.4 ± 0.59	10.3 ± 0.37	32.1 ± 0.28	14.9 ± 0.52
Homebrand	4.39 ± 0.00	33.5 ± 0.35	15.0 ± 0.00	18.5 ± 0.35	10.6 ± 0.30
Grey box	4.26 ± 0.01	41.8 ± 0.41	10.3 ± 0.01	31.5 ± 0.40	13.6 ± 0.29
Mallee	4.47 ± 0.01	50.6 ± 1.25	18.2 ± 0.75	32.4 ± 0.50	10.7 ± 0.69
Banksia	4.69 ± 0.01	53.5 ± 0.18	20.3 ± 0.18	33.2 ± 0.00	17.8 ± 0.37

(1974) reported the range of moisture contents in Australian honeys to be 13.6–17.4%. These values were smaller than 21.6–22.8% reported by Gidamis, Chove, Shayo, Nnko, and Bangu (2004) in Tanzanian honey, and the 22.0–23.1% reported by Joshi, Pechhacker, William, and Ohe (2000) in Philippine honey. These findings along with results from this study clearly show that moisture content in honey is influenced by botanical source, geographical and climatic conditions and the season. Fallico et al. (2004) reported that the principle Australian floral source is eucalypts which generally have low moisture contents. High moisture (>21%) honey indicates a premature extraction or extraction under high humidity conditions.

3.6. Analysis of colour

The values of L^*, a^* and b^* of honey samples are shown in Table 2. L^* value indicates degree of lightness, positive a^* indicates red, negative a^* indicates green component, positive b^* indicates yellow, and negative b^* indicates blue component. Leabrook had green and yellow components while Australian rainforest and Grey box had red and blue components only. Beechworth, Homebrand, Banksia and Mallee revealed some degree of redness and yellowness. These results are in agreement with those reported by Anupama, Bhat, and Sapna (2003) who indicated that 11 brands of Indian honeys had red and yellow colour components. Australian rainforest exhibited the largest degree of brightness as seen from the large L^* value (101.27 ± 0.34), while Grey box sample exhibited the least brightness of 80.81 ± 0.06. Generally eucalyptus honeys are darker than other honeys (Chandler et al., 1974).

3.7. Sugar content

All honey samples, except Banksia, had similar amounts of total sugars ranging from 82.4% in Grey box to 86.0% in Leabrook. Banksia contained the lowest level of total sugars of 68.1% (Table 3). Similar results were also recorded for fructose and glucose contents in Banksia. However, results of sugar analysis revealed no significant ($p > 0.05$) differences between processed and unprocessed honeys. Except for Banksia honey with a fructose content of 30.8%, results of fructose analyses are in agreement with those reported by Mossel (2003), who found the average fructose concentration of 15 types of Australian unifloral honeys to vary from 32.6 to 41.7 g/100 g. The calculated ratio of fructose:glucose ranged from 1.1 in Homebrand to 1.27 in Beechworth (Table 3). This general trend of higher fructose ratios in honey from different sources has been well documented. White (1980) reported an average fructose:glucose ratio of 1:2, which is similar to the average value (1.17) obtained in this study. Honey with high fructose:glucose ratio would remain liquid for longer periods because of the modification of the saturated level of glucose by the presence of the larger amount of fructose (White, Kushnir, & Subers, 1964). The actual proportion of fructose to glucose in any particular honey depends largely on the source of the nectar (Anklam, 1998). The fructose:glucose ratio may also have an impact on honey flavour since fructose is much sweeter than glucose.

Unlike to the amounts of monosaccharides recorded in various processed and unprocessed honey samples, the disaccharides contents were much smaller, and ranged from 10.1% in Mallee to 15.2% in Leabrook. These results are in agreement with those reported by Terrab et al. (2003) who indicated that fructose and glucose represented 92% of the total quantified sugars, in comparison to 73% of the disaccharides.

3.8. Analysis of amylase activity

The amylase activity is usually expressed as diastase number, symbol DN, and also known as Gothe units. A Gothe unit is defined as ml of 1% starch solution hydrolysed at 40 °C for one hour by the enzyme present in 1 g of honey (International Honey Commission, 2002). The initial amylase activity ranged from 9.43 ± 0.30 to 22.1 ± 1.09 in the tested commercial honeys and from 17.6 ± 0.35 to 25.4 ± 0.54 in unprocessed honeys (Table 4). These values were in agreement with those reported by Chandler et al. (1974) for Australian honeys (9–44 Gothe units).

Two of the commercial honey samples (Australian rainforest and Homebrand) showed relatively high initial DN, in comparison with the fresh samples, while Leabrook and Beechworth contained the lowest initial DN values (9.43 ± 0.3 and 10.6 ± 1.05, respectively). Such low DN values in some commercial honey samples may indicate severe heat treatments that caused a significant decline in amylase contents. The unprocessed Mallee sample had the highest initial DN (25.4 ± 0.54), followed by Banksia (18.4 ± 0.13), and Grey box (17.6 ± 0.35).

Heating honey samples in a water bath for 2 min at 65 °C, 77 °C and 85 °C revealed a positive correlation between temperature and level of amylase destruction. Heating at 85 °C caused the largest decline in amylase activities in all honey samples (Table 4). However, the initial DN values did not affect the level of amylase inactivation. Both the honey samples with the smallest initial DN

Table 2
L^*, a^* and b^* values of honey samples measured using a chroma meter. Results represent the average of three measurements ± SD.

Type of honey	L^*	a^*	b^*
Leabrook	98.3 ± 0.02	−0.20 ± 0.05	3.16 ± 0.08
Beechworth	96.1 ± 0.37	0.72 ± 0.05	5.92 ± 0.46
Australian rainforest	101 ± 0.34	1.83 ± 0.04	−10.3 ± 0.23
Homebrand	90.7 ± 0.21	3.21 ± 0.01	5.90 ± 0.20
Grey box	80.8 ± 0.06	3.68 ± 0.04	−9.79 ± 0.02
Mallee	89.9 ± 0.21	5.13 ± 0.04	9.11 ± 0.18
Banksia	90.3 ± 0.06	4.20 ± 0.00	6.22 ± 0.06

Table 3
The concentration of glucose, fructose and disaccharides in the honey samples (g/100 g) and fructose/glucose ratio.

Honey type	Sugar (g/100 g)				
	Glucose	Fructose	Disaccharides	Total sugar	Fructose/glucose
Leabrook	32.7	38.1	15.2	86.0	1.17
Beechworth	31.1	39.5	15.1	85.7	1.27
Australian rainforest	32.8	39.0	13.0	84.9	1.19
Homebrand	34.5	37.8	12.9	85.2	1.10
Grey box	31.9	37.4	13.3	82.5	1.17
Mallee	33.6	40.0	10.1	83.7	1.14
Banksia	26.5	30.8	10.7	68.1	1.16
Average	31.9	37.5	12.9	82.3	1.17

Table 4
Analysis of amylase activity, expressed as diastase number (Gothe unit), in various honey samples heated at different temperatures for 2 min. Results represent the average of four measurements ± SD.

Honey type	Gothe units			
	Initial	65 °C	77 °C	85 °C
Leabrook	9.43 ± 0.30	8.80 ± 0.29	8.77 ± 0.07	7.62 ± 0.03
Beechworth	10.6 ± 1.05	9.76 ± 0.12	8.88 ± 0.01	8.48 ± 0.07
Australian rainforest	22.1 ± 1.09	21.4 ± 1.04	20.7 ± 0.23	15.4 ± 0.09
Homebrand	19.9 ± 1.15	16.9 ± 0.22	15.7 ± 0.04	16.8 ± 1.18
Grey box	17.6 ± 0.35	15.4 ± 1.04	15.1 ± 0.06	11.3 ± 0.08
Mallee	25.4 ± 0.54	22.3 ± 1.16	21.8 ± 1.32	20.4 ± 0.37
Banksia	18.4 ± 0.13	17.3 ± 0.20	16.1 ± 0.41	13.1 ± 0.42

values (9.43, Leabrook), and the largest (25.4, Mallee) lost similar proportions (19–19.5%) of their amylase activities, when heated for 2 min at 85 °C. The same data showed also that all tested honey samples, except Leabrook, could be heated at 85 °C, and the remaining DN was still above the minimum limit (8 DN). Consequently, it has been recommended that other quality indicators, such as invertase activity, which is more heat-sensitive than amylase, should be used (Oddo, Piazza, & Pulcini 2006). A study by Tosi, Re, Lucero, and Bulacio (2004) showed that destroying all amylase activity required heating honey at 80 °C for 1.2 h, while it only took only 8.6 min for the inactivation of invertase present.

3.9. HMF contents in commercial honey samples

Australian rainforest and Homebrand honey revealed initial HMF contents of 2.22 and 17.7 mg/kg, respectively, which fell within the international limit of 40 mg/kg (Table 5). These two samples also had larger amylase activities than Leabrook and Beechworth samples (Table 4). Consequently, it was concluded that Australian rainforest and Homebrand honey samples were treated under appropriate temperature and storage conditions.

On the contrary, Beechworth and Leabrook contained excessively high initial HMF levels of 50.8 and 74.9 mg/kg, respectively, which were above the international limit. Furthermore, these two honeys also revealed low amylase activity (Table 4). Since HMF can be formed either by Maillard reaction (heating of reducing sugars in the presence of proteins), or by dehydration under acidic conditions, it can be concluded that the high HMF concentrations and low diastase number of both Leabrook and Beechworth samples could indicate improper heat treatment and storage conditions. Additionally, as a large majority of the national honey crops is harvested from eucalypts, and because the State of Victoria has been experiencing drought in the past few years, this might have affected the flowering ability of various eucalypts, thus, leading bee keepers to feed the bees with cheap fructose corn syrup. Honey bees can be fed various foodstuffs to supplement inadequate supplies of pollen or honey. Kerkvliet and Meijer (2000) reported that honey adulterated with 50% cheap fructose syrup contains HMF twice as high as pure honey. In early spring, before pollen and nectar are available, or at times of the year when these materials are in short supply, supplementary feeding may help the colony to survive or make it more populous and productive (Ozcan, Arslan, & Ceylan, 2006). Another factor that can affect the HMF content of honey is the tropical climate. Hot weather can increase the HMF level of honey in the bee hive. Consequently, Codex Alimentarius (2001) and International Honey Commission (2002) have increased the HMF limit in honey from tropical regions to 80 mg/kg.

3.10. HMF contents in fresh (unprocessed) honey

As expected, all fresh honey samples contained HMF within the recommended food authority limit (40 mg/kg). Grey box and Banksia samples contained very low amounts of HMF of 1.35 and 1.12 mg/kg, respectively (Table 5). These results were in agreement with those reported by Airborne Honey (2001), who indicated HMF contents were below 10 mg/kg in freshly extracted honey. On the contrary, the Mallee sample showed a high HMF value of 34 mg/kg, but still below the international standard limit (40 mg/kg). Considering the amylase activity in Mallee sample (25.4 DN), which was larger than those in Grey box and Banksia (Table 4) clearly illustrated that the high HMF value in Mallee was not heat-related, otherwise the amylase would be much lower. Consequently, high HMF level in the unprocessed Mallee honey may be due to different floral sources.

These results clearly illustrated that 50% (two out of four) of tested commercial honey samples that are readily available in the market did not meet the international standards for HMF content (40 mg/kg). Usually food produced locally or imported into Australia must comply with FSANZ's food standard codes. However, since the current FSANZ code does not have a specific identified guide for the HMF in honey, the honey industry has adopted the international standard of 40 mg/kg as a guideline (Capilano Honey, 2005).

Table 5
HMF (mg/kg) in commercial and fresh honeys heated (2 min) at 65 °C, 77 °C and 85 °C determined by HPLC method. Results represent the average of four measurements ± SD.

Honey type	Temperature			
	Initial	65 °C	77 °C	85 °C
Leabrook	50.8 ± 1.34	53.2 ± 1.37	51.3 ± 1.37	52.6 ± 1.78
Beechworth	74.9 ± 2.34	73.5 ± 1.46	74.9 ± 1.44	74.7 ± 1.10
Australian rainforest	2.22 ± 0.65	2.58 ± 0.44	3.39 ± 0.21	4.52 ± 0.37
Homebrand	17.7 ± 0.99	19.8 ± 0.10	19.9 ± 0.91	21.2 ± 0.85
Grey box	1.80 ± 0.42	2.03 ± 0.38	2.66 ± 0.60	4.09 ± 0.38
Mallee	34.0 ± 0.31	39.4 ± 0.58	41.8 ± 0.92	42.3 ± 0.37
Banksia	0.36 ± 0.07	1.74 ± 0.17	2.13 ± 0.37	3.56 ± 0.42

3.11. Effect of heating on HMF contents

Except for Leabrook ($p = 0.187$) and Beechworth ($p = 0.588$), heating honey samples for 2 min at 65 °C, 77 °C and 85 °C caused significant ($p < 0.05$) increment in HMF contents (Table 5). Both Beechworth and Leabrook samples had the largest initial level of HMF (50.8 and 74.9 mg/kg, respectively). Additionally, data from amylase analysis (Table 4) showed that these same honey samples had the lowest initial amylase activities. These findings may suggest that these honey samples had been heated to the extent that most amylase activities and the substrate for Maillard reaction had been exhausted. Another possible explanation for the insignificant impact of heating on HMF in Beechworth and Leabrook could be the floral sources (eucalyptus). Fallico et al. (2004) reported that heating eucalyptus honey at 70 °C did not yield detectable amounts of HMF in the first 24 h of heating.

It was interesting to notice that both Beechworth and Leabrook, which had the highest HMF contents, also had the lowest pH values (4.04 and 4.02, respectively) (Table 1). These findings were in agreement with those reported by Bath and Singh (1999), who demonstrated that honey with low pH usually has a high HMF content.

Considering the positive correlation between heat treatment and the increment in HMF contents in all fresh (unprocessed) honeys and two commercial samples (Australian rainforest and Homebrand), it can be suggested that HMF could be used as an indicator to judge honey quality.

4. Conclusion

Diastase number (amylase activity) varied between honey samples, had no uniform starting point, and was not very sensitive to applied heating (Table 4). Consequently, the suggested proposal by many researchers to use invertase instead of amylase as a quality indicator should be considered. Invertase has been reported to be more heat-sensitive than amylase. Bonvehi, Manzanares, and Vilar (2004) showed at least 80% reduction in invertase enzyme upon overheating ($\geqslant$77 °C for 20 s) in 21 honey samples.

HMF content in fresh honey should supposedly be very low, as seen in Grey box and Banksia samples. However, honey with HMF content up to 40 mg/kg can still be considered fresh (unprocessed), as in the case of Mallee sample, which exhibited 34 mg/kg initial HMF content. It is obvious that heating is not the only factor

influencing HMF formation in honey but also honey composition, pH value and floral source can contribute. Consequently, the statement that the amount of HMF is independent of honey type and composition reported by Fallico et al. (2004) could be misleading. This experiment found 40 mg/kg standard to be too strict on some honeys and too permissive for others. For example, this limit was found to be too strict on Mallee honey, which recorded 41.8 mg/kg after heating for 2 min at 77 °C.

This limit was also too permissive for Australian rainforest, Homebrand, Grey box and Banksia honeys. These honeys could be overheated ($\geqslant$77 °C for 2 min) without reaching the limit of 40 mg/kg. As a result, further research is recommended to examine the relationship between HMF formation and honey floral sources.

Acknowledgment

The authors wish to thank workers at the Horsham station, DPI, Victoria, for supplying the fresh honey samples.

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