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The Validity of Protein in Australian Honey as an Internal Standard for C4 Sugar Adulteration

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Notes

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ABSTRACT

Recent media reports claimed that a large proportion of honey sold in Australian supermarkets was adulterated, although no specific details of the nature of the adulteration were made public. To investigate the basis of these claims, the current study was conducted with samples of Australian honey obtained from supermarkets or direct from beekeepers/small scale producers. Without evidence to the contrary, it was assumed that the beekeeper honey would be unadulterated. Analysis of these samples using the official AOAC method (998.12) found that a proportion of both supermarket and beekeeper honeys had apparent C4 sugar contents greater than 7% and this proportion was similar to the failure rates reported by popular media. The AOAC test measures the difference in carbon isotopic composition between the honey (mostly sugar) and protein precipitated from the honey and relies on the assumption that nectar (the source of sugar) comes from the same plant species as pollen (the source of protein). Initial results found that most Australian honey failed the AOAC test because the protein was depleted in ^{13}C with respect to the sugar although the sugar was in the normal range ($\delta^{13}C$ more negative than –24‰). A series of experiments investigated the nature of the precipitate obtained from Australian honey and found the weight of precipitate was greater than the amount of protein measured in the honey and the precipitate contained non-protein material. Based on these findings a modification to the AOAC method is proposed whereby samples, after addition of sodium tungstate and acid, are left unheated for 48 hours for a floc to form. This procedure produced a precipitate that was mostly comprised of protein. It is also recommended that the acceptance criteria for Australian honey is increased from 1‰ to 2‰ difference between the carbon isotopic composition of the honey and the precipitate. Using the modified method and acceptance criteria the failure rate for Australian honey was no more than 8%, compared to 37% using the official method.

Key words: Australian honey, mānuka, AOAC 998.12, adulteration, carbon isotope, C4 sugar

INTRODUCTION

Honey is a viscous, golden liquid created by social insects from the nectar of blossoms, from secretions of living plants or from excretions of plant sucking insects. Bees, bumblebees and certain wasps collect these substances and combine them with substances from their own bodies to form honey which is then stored in a honeycomb to ripen and mature. As a sweet and naturally nutritious product *Apis mellifera* honey has found favour with human consumers. The colour, flavour and aroma of honey will vary depending on the source of the nectar, climatic conditions, and the methods of production handling and storage. These differences in "quality" lead to a wide range of market prices. [1] For example, there is a high consumer demand for mono-floral honey (derived predominantly from a single plant species) due to the flavour and aroma, and also to particular pharmacological attributes which increases the commercial value of these premium products. [2] Because of this, characterization of the floral and geographical origin of honey has become important due to the high costs of production and the potential for fraudulent substitution to meet consumer demand. [3] In this manuscript, however, the authors focus exclusively on tests to detect the adulteration of honey with C4 plant derived sugars.

Many foods have the potential to be deliberately adulterated and those that are expensive are most at risk. Over many years the practice of adulteration of honey with low-cost sugars has unfortunately become commonplace in numerous countries [4] particularly because of the availability of cheap high fructose corn syrup. [5] Although the adulteration of honey may not be injurious to health, problems of honey fraud negatively impact market growth by damaging consumer confidence. [4, 5-8]

Honey consists mainly of sugars, and this is reflected in the compositional requirements e.g. the Australia and New Zealand Food Standards Code requires only that honey contains no less than 60% reducing sugars and no more than 21% moisture. [9] The relative amounts of individual sugars will vary according to the nectar source but typically comprises fructose (30 to 44%), glucose (22 to 40%) and sucrose (0.25 to 7.7%). [10] It is, however, difficult to define the composition of honey as it is produced under wide fluctuations in weather and harvesting conditions. In a revised Codex standard for honey [11] the sugar composition, for example, fructose, glucose and sucrose content, can be used for honey authenticity [12], however, the chromatographic tests and others analytical procedures used are not sensitive enough to detect very low concentration of adulterating sugars. Indirect "adulteration" of honey can also occur as a result of feeding honeybees with commercial sugars to prevent starvation of the hive in

times of stored honey shortage or to artificially stimulate breeding and foraging. Such indirect "adulteration" can be extremely difficult to distinguish from direct adulteration. [13]

Isotope Ratio Mass Spectrometry (IRMS) presents one method used to detect adulteration. The carbon isotopic ratio of plant tissue ($^{13}C/^{12}C$) reflects the photosynthetic pathway of plants with C3 plants (the majority of flowering plants) being more depleted in ^{13}C than C4 plants (e.g. sugarcane and corn/maize). Typical $^{13}C/^{12}C$ ratios for C3 plants are –22 to –28‰ and for C4 plants –9 to –15‰. Variation in the isotopic composition of similar plants can occur due to differing availability of light, water and nutrients. [13] Because honey is ultimately derived from flowering plants, its carbon isotopic composition should be similar to the range for C3 plants, typically –25‰. [14] When C4 sugar is added to pure honey, the $^{13}C/^{12}C$ ratio will be altered and, in 1978, the first official method to detect the adulteration of honey with C4 sugars by stable carbon IRMS was established in the USA as AOAC 978.17. [15] This method was based on measurements of the stable carbon isotopic composition of whole honey and the preamble to the procedure implies that honeys with $\delta^{13}C$ compositions more negative than –23.5‰ are pure (later revised to –24‰).

As noted above, the composition of honey (including the isotopic composition) can vary as a function of many factors, including climate, geography and floral source. It has been reported that certain citrus honeys and honey derived from crassulacean acid metabolism (CAM) plants had $\delta^{13}C$ values significantly less negative than others, up to –12‰. [16] As a response to the need for a more robust test the official method was changed by adding an internal standard procedure. [17] The modified method considers the difference between the stable carbon isotopic composition of whole honey (mostly sugar) versus protein precipitated from the same honey (by heating with sodium tungstate and sulphuric acid), defined as

$$C4_{SUGARS}(\%) = \frac{\delta^{13}C_{PROTEIN-} \; \delta^{13}C_{HONEY}}{\delta^{13}C_{PROTEIN-}\,(-9.7)} \times 100$$

The method relies on the assumption that nectar and pollen (the presumed source of the protein in honey) from the same plant will have very similar stable carbon isotopic compositions and that the isotopic composition of typical C4 plant sugar is –9.7‰. According to AOAC 991.41 (later revised as 998.12) the value for the $\delta^{13}C$ value of the honey and its protein ($\Delta^{13}C_{HONEY\text{-}PROTEIN}$) should differ by no more than 1‰ with differences greater than 1‰ being indicative of greater than 7% adulteration with C4 sugar. [17]

Some questions arise as to whether honey protein is an appropriate "internal standard" for the authentication of the origin of the sugar in honey. According to some authors, the protein present in honey generally arises directly from pollen, or from an enzymatic reaction between plant pollen and bee saliva. [18-20] Other authors claim that proteins from pollens are broken down by the bee's digestive enzymes and re-assembled into the specific proteins and enzymes needed for apian body functions. [21-22] It has also been noted that the protein content of mixed flora honey would vary as a function of many ecological factors that influenced the flowering of the different plant species, [23] and that nectar and pollen are not necessarily derived from the same plants. More recently, authors have proposed to compare the carbon isotopic composition of individual sugars (disaccharides, trisaccharides, glucose and fructose) present in honey [12] by means of liquid chromatography-isotope ratio mass spectrometry (δ^{13}C LC/IRMS) as an alternative to protein. [24]

It has been reported that many, unadulterated New Zealand mānuka honeys fail the AOAC 998.12 (formally 991.41) test with apparent C4 sugar contents greater than 13%. Suspicions of adulteration arose despite bulk New Zealand mānuka honeys having $\delta^{13}C_{HONEY}$ values in the normal range (i.e. –27 to –24‰) and created a question mark of authenticity and impacted on market reputation and export revenue. [25] It was suggested that some New Zealand mānuka honeys contain insoluble components that lower the ^{13}C content of the extracted protein with the argument that these honeys fail the AOAC test, not because the sugar is ^{13}C enriched, due to adulteration, but because the precipitate has a naturally lower ^{13}C content. [26] New Zealand researchers, therefore, proposed an additional step to remove pollen and other materials using centrifugation and/or filtration before flocculation to ensure that a true comparison was made between the whole honey and its protein. [25] Even after filtration and when it could be proved that the colony had not been fed on cane sugar, some mānuka honey still had high apparent C4 sugar content (up to 15%). Researchers, therefore, proposed that some other mechanism contributed to more negative $\delta^{13}C_{PROTEIN}$ values and generated false-positive AOAC 998.12 results. [27]

Further research appeared to demonstrate that the apparent C4 sugar content of mānuka honey increased over time and with increased storage temperature. [28] These authors proposed a "unique mānuka effect" by which the higher levels of dihydroxyacetone (DHA) and/or methylglyoxal (MGO), found in bioactive mānuka honey, react with protein to cause isotopic fractionation. It was proposed that such a reaction would cause $\delta^{13}C_{PROTEIN}$ to become more negative by consuming acidic (^{13}C enriched) amino acids, and thereby artificially enhances the

apparent C4 sugar value during storage for longer periods at ambient temperature. The authors called for an exception to be considered for all New Zealand mānuka honey that has demonstrated DHA and/or MGO levels with the potential to produce MGO greater than 250 ppm; allowable apparent C4 sugar content should be at least 13% in mānuka honey ($\Delta^{13}C_{HONEY\text{-}PROTEIN} < 2‰$) Recent research [29] presented proof that neither DHA or MGO are responsible for the change in the apparent C4 sugar content of New Zealand mānuka honey and these authors speculated that an unknown substance binds to the protein and causes negative $\delta^{13}C$ changes over time.

In recent years, Australian honey has made infamous news headlines with claims such as “Almost 20 per cent of Australian honey samples found to not be pure” and "Fake honey scandal widens". [30-31] These headlines were based on the results of a Nuclear Magnetic Resonance test that, apparently, showed 12 out of 28 blended and imported honey samples being sold in Australian supermarkets were not 100% pure honey. The tests were instigated by a law firm, acting on behalf of an Australian horticulturalist, that had sent the samples to a German laboratory, specialising in the authentication of honey. [32]

What was less reported was that the same 28 samples were also tested using the official AOAC test for C4 sugar and all passed. [32]

Shortly afterwards newspapers, again, seized on a report of research at Macquarie University [33] that found 21 of 95 commercial Australian honey samples failed the AOAC apparent C4 sugar content criteria and were of “questionable authenticity” based on carbon isotopic and trace element analysis.

In this manuscript we consider why a significant proportion of Australian honey appears to exceed the 1‰ $\Delta^{13}C_{HONEY\text{-}PROTEIN}$ threshold set in the AOAC 998.12 test and, therefore, deemed to be adulterated with C4 sugar. We also propose a modification to the test to account for the unique floral and climatic inputs that characterise Australian honey. Our simple modification provides a protein precipitate which has less contamination, and thus a more accurate $\Delta^{13}C_{HONEY\text{-}PROTEIN}$ value.

MATERIALS AND METHODS

Samples. In total, 102 honey samples were purchased from shops, markets and online retailers in different parts of Australia between Oct 2016 and Oct 2018. Of these, 52 samples were from small-scale producers, sourced from markets or direct from beekeepers and were classified as “boutique”. Without evidence to the contrary it was assumed that these samples would be

representative of unadulterated, Australian honey. A further 31 samples were purchased from supermarkets and had been packaged by large commercial companies. Of these, one claimed to be mono-floral (ironbark) and the rest were assumed to be blended. The remaining 19 samples were mānuka honeys samples purchased in Oct 2018 from shops, markets and online retailers. Sixteen of the mānuka honeys were labelled as Australian and three as New Zealand. Samples were analysed between Feb 2019 and Jun 2020.

Protein Precipitation. Protein was precipitated from honey samples using AOAC Official Method 998.12 [34] and with a number of modifications summarised in Table 1.

Table 1. **Summary of the methods used to precipitate protein from honey sample. (RT = room temperature).**

method	H_2O (mL)	filtration	[H_2SO_4] (mol/L)	precipitation time
I (AOAC)	4	none	0.335	until floc [a]
II	4	none	1.34	1 h @ 80 °C
III	15	0.45 µm	1.34	1 h @ 80 °C
I*	4	none	0.335	48 h @ RT
II*	4	none	1.34	48 h @ RT
III*	15	0.45 µm	1.34	48 h @ RT

[a] Samples were heated at 80 °C and additional acid was added until a floc appeared.

All the methods followed a broadly similar protocol. To begin, 10 to 12 g of honey were weighed into 50 mL centrifuge tubes and high purity water (MilliQ 18 MΩ) was added to make a uniform solution (Table 1 column 2). For methods III and III* additional water (as shown in Table 1) was added to reduce viscosity, before filtration through a 1.2 µm glass fibre membrane filter followed by a 0.45 µm cellulose filter (Phenomenex, Lane Cover, NSW Australia). To each honey solution, 2 mL of 10% sodium tungstate (Sigma, Castle Hill, NSW, Australia) solution was added together with 2 mL of sulphuric acid of either 0.335 M or 1.34 M concentration (Table 1). Samples were then left under various conditions (Table 1, column 5) to allow floc/precipitate to form. After this time, the floc was centrifuged at 3000 rpm

(relative centrifugal force 3120 × g) for 5 min and the supernatant discarded. The precipitate was washed with 40 mL of high purity water and, after vortex mixing, this was centrifuged at 3000 rpm for 5 min and the supernatant discarded. This procedure was repeated a total of five times. The final supernatant was checked to ensure it was free of sugar using an Atago handheld refractometer (John Morris Scientific, Chatswood, NSW, Australia). A small amount of water was then added to the precipitate that was freeze dried overnight.

Protein Concentration Measurements. Protein concentrations in honey were measured using the Bradford assay, a colorimetric method based on a colour change from red to blue as the dye Coomassie Brilliant Blue G-250 binds to protein. [35] Protein standards were prepared over a concentration range from zero to 100 mg/kg from α-lactoalbumin (>85%) (Sigma) dissolved in 0.15 mM aqueous sodium chloride. In summary, 2.0 g of each honey were dissolved in 50 mL of high purity water. Aliquots (50 μL) of standard and sample solutions were transferred to a 96 well plate and 200 μL of Bradford reagent (Sigma) added to each well. The plate was incubated, with mixing, at room temperature for 5 minutes and the absorbance recorded at 595 nm using a Sunrise absorbance microplate reader (Tecan, Port Melbourne, Vic, Australia). Data were acquired and processed using Magellan 7.2 software.

Infrared Spectroscopy. Infrared spectra were recorded using a Spectrum 100 FT-IR spectrophotometer with a universal attenuated total reflectance (ATR) sampling accessory (Perkin Elmer, Melbourne, Australia). Spectra were recorded (4 scans, 4000 to 650 cm^{-1}) at approximately 40% transmission. Data were acquired using Spectrum 6.2.0 software with no ATR correction applied. Spectra was exported via ASCII format into The Unscrambler 10.5 software (Camo, Norway) for chemometric analysis. Principal component analysis (PCA) was carried out using cross validation (leave-one-out). Before PCA analysis, the spectra were pre-processed using Savistky-Golay second derivative (2nd polynomial order and 10 data points smoothing). PCA enables the detection of cluster formation or specific trends in the samples analysed as a result of the information provided by the analytical method used (precipitation methods, Table 1). This analysis has been achieved by describing relationships between samples (scores) and compounds (loadings) allowing the best treatment to be defined.

Stable Isotope Ratio Measurements. Stable carbon isotope ratio measurements were made using a Delta V^{PLUS} isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany) coupled to a ConFlo IV interface for sample dilution and working gas introduction. Approximately 1 mg aliquots of honey or protein were crimped into tin capsules (4 × 3.2 mm,

IVA Analysentechnik, Meerbusch, Germany). Samples were analysed in triplicate using a Flash 2000 elemental analyser (Thermo Scientific) in flash combustion mode at 950 °C, with a helium flow of 100 mL/min, followed by a post reactor gas chromatography column at 95 °C. The reactor was packed with chromium (II) oxide, electrolytic copper and silvered cobaltous (II / III) oxide (IVA). A water trap (magnesium perchlorate) was placed between the reactor and the GC column. Measurements were normalised to the VPDB scale by three point calibration using IAEA-CH-7 (International Atomic Energy Agency, Vienna, Austria) (–32.15‰) and two glucose solutions (+1.25 and –46.10‰) calibrated against the international reference materials NBS 19 and LSVEC. [36] Data were acquired and processed using ISODAT 3.0 software.

Samples of glucose with well-characterised isotopic composition (–24.75 ± 0.10‰) were analysed at the beginning of each acquisition sequence and after every five samples, for quality control purposes. These data are presented in the Supporting Information.

RESULTS AND DISCUSSION

Samples. In total 102 different samples of honey samples were collected from beekeepers, shops and online retailers in Australia including 19 samples labelled as mānuka honey. All samples were stored at room temperature prior to analysis.

AOAC method 998.12 (Method I). Figure 1a summarises the results for all 102 honey samples tested following the official AOAC 998.12 method for apparent C4 plant sugars in honey (Method I).

A total of six boutique honeys fell outside the basic AOAC requirement that $\delta^{13}C_{HONEY}$ should be more negative than –24‰, one sample having a $\delta^{13}C_{HONEY}$ value of –18.1‰ (#133). According to AOAC 998.12, $\delta^{13}C_{HONEY}$ values more positive than –24‰ can be considered evidence of either direct or indirect adulteration with C4 sugar. [34] Since many regions of Australia are currently undergoing a prolonged period of drought, Government agencies have recommended white cane sugar as a safe, cost effective product to feed honeybees. [37] It is, therefore, likely that some of these six samples present the result of indirect adulteration as a result of C4 sugar fed to bees.

Even excluding these ("failed") samples the boutique honeys had much wider ranges of both $\delta^{13}C_{HONEY}$ (–26.79 to –24.12‰) and $\delta^{13}C_{PROTEIN}$ (–28.59 to –24.85‰) compared to the

supermarket honeys ($\delta^{13}C_{HONEY}$ –26.09 to –24.25‰ and $\delta^{13}C_{PROTEIN}$ –27.80 to –24.80‰). This finding was consistent with boutique honey being produced at a single location with potentially limited floral sources. In contrast, supermarket honeys may be blended from honeys from multiple locations with potentially diverse floral sources (and more variability between samples from different locations. Commercial beekeeping in Australia is mainly nomadic and follows the seasonal flowering of diverse flora and, therefore, supermarket honey will very likely represent a time average of different sources of nectar and pollen. [38] The ranges of $\delta^{13}C_{HONEY}$ for the mānuka honeys were similar to the supermarket honeys (–26.83 to –24.99‰) but the $\delta^{13}C_{PROTEIN}$ values (–29.50 to –25.64‰) were more depleted than either supermarket or boutique honeys.

The critical parameter for the AOAC test i.e. a difference in the carbon isotopic composition of honey and protein ($\Delta^{13}C_{HONEY-PROTEIN}$) varied up to +2.76‰ for boutique honeys, +2.13‰ for supermarket honeys and +4.11‰ for mānuka honeys. Based on this parameter, the failure rates ($\Delta^{13}C_{HONEY-PROTEIN} > 1$‰) were 29% and 29% for boutique and supermarket honey respectively when tested by Method I. These percentages were comparable to the frequencies of suspected adulteration reported in the popular press and scientific literature. [32-33] The failure rate for mānuka honey was a significant 79% but was still less than the 97% failure rate previously reported for New Zealand mānuka honey. [25]

To investigate the cause of these high failure rates, 8 boutique honeys were selected for subsequent experiments using modified versions of the AOAC 998.12 method. These samples included one honey that passed the AOAC test (#163) ($\Delta^{13}C_{HONEY-PROTEIN} < 1.0$ ‰), two honeys that were classed as marginal failures (#621, #711) ($\Delta^{13}C_{HONEY-PROTEIN} < 1.5$ ‰), four honeys that failed by a significant margin (#11, #161, #264, #736) ($\Delta^{13}C_{HONEY-PROTEIN} > 1.5$ ‰) and one sample that failed the basic criteria (#133) with $\delta^{13}C_{HONEY}$ more positive than –24 ‰.

Results for these samples, analysed by the unmodified AOAC method (Method I), are shown in Fig 2a. For these samples, Method I $\Delta^{13}C_{HONEY-PROTEIN}$ ranged from –0.54 to +5.77‰ with an average value of +1.46‰.

Table 2 shows the concentrations of protein determined for the eight honeys together with the weights of precipitate obtained. The protein concentrations for the Australian honeys were consistent with those reported for Taiwanese and Thai honeys using the same methodology. [35] The precipitate weights, shown in Table 2, were highly variable (by all

methods) but show that the precipitates from Method I contained between approximately 3 to 13 times more material mass than would be expected if the precipitate was comprised only of protein. It must be noted that these values were derived from a single experiment and this calculation does not account for the inclusion of tungsten with the protein, which might make a significant contribution to the weight.

Acid concentration (Method II). A significant variable in the original AOAC method is the requirement to add an unspecified amount of 0.335 M sulphuric acid until "visible floc forms". For two of the boutique honeys in this study it was necessary to add a total of 40 mL of 0.335 M acid before a visible floc formed i.e. 20 times the initial amount. To investigate the effect of the volume of acid added, Method II used a fixed amount (2 mL) of a higher concentration acid (1.34 M). Experience of Method I suggested that this amount of acid would be sufficient to produce a floc from the majority of the honey samples. Results for the eight test samples, analysed by Method II, are shown in Fig 2b. Somewhat surprisingly, using this method all eight test samples failed with $\Delta^{13}C_{HONEY-PROTEIN}$ ranging from +3.40 to +4.75 ‰ (average +4.05 ‰). From Table 2 it is apparent that Method II (with more concentrated acid) produced approximately two to four times as much precipitate as Method I.

Table 2. The protein concentration of honey samples as determined by the Bradford reagent test compared to the weight of precipitate obtained by Method I, II and III.

sample	protein content (g/100g)		precipitate weight (g/100g)		
	unfiltered	filtered	Method I	Method II	Method III
#11	0.089	0.036	0.55	1.10	0.83
#133	0.043	0.018	0.41	1.59	0.14
#161	0.037	0.017	0.29	1.05	0.45
#163	0.100	0.058	0.31	1.11	0.50
#264	0.078	0.041	0.49	1.80	0.54
#621	0.091	0.064	0.66	1.48	1.27
#736	0.058	0.056	0.77	1.28	0.29
#771	0.061	0.031	0.29	1.18	0.10

Filtration (Method III). The official method 998.12 already includes an optional filtration step, if significant amounts of solid matter are present, but only through 100 to 150 mesh (100

to 150 μm). As noted above, a significant number of authentic New Zealand mānuka honeys have been reported to fail the standard AOAC method and one explanation put forward was that pollen and/or other insoluble components were extracted along with the flocculated protein. [25] To test this hypothesis the eight Australian test honeys were filtered through 1.2 μm glass fibre filter followed by a 0.45 μm cellulose filter as proposed by Cottee. [39] The filtered honey solutions were then precipitated using Method II.

Results for the eight test samples, analysed by the modified AOAC method (III), are shown in Fig 2c. This method produced a much wider range of $\delta^{13}C_{PROTEIN}$ (–32.62 to –25.24‰) than either Method I or II, and all but one sample failed the AOAC criteria with $\Delta^{13}C_{HONEY-PROTEIN}$ ranging from +0.04 to +7.08 ‰. This finding was in contrast to earlier research that reported a slight isotopic "enrichment" (ca 0.5‰) as a result of filtering European honey. [39] This difference in isotopic behaviour when filtering may point to differences in the protein content of European and Australian honey.

Table 2 shows that the weight of precipitate produced by Method III was of the same order as that produced by Method I, however, Table 2 also shows the effect of filtering on the protein concentration of the diluted honey samples as determined with the Bradford reagent. Filtration removed between approximately 30 and 60% of the protein. One exception was sample #736 for which filtration appeared to remove little protein. This sample was described as "grey ironbark" (*Eucalyptus paniculate*) and may have contained protein specific to that species.

Given that Method III produced a comparable weight of precipitate to Method I from only half the available protein this precipitate was also assumed to contain a high proportion of non-protein material.

To understand the nature of the precipitates from Methods I, II and III these precipitate samples were analysed by FTIR. Spectra from the precipitates contained absorbances, in the mid infrared region, associated with amide groups (1650 and 1540 cm^{-1}) (Fig 3a). [40] Comparing the intensity of these absorbances for precipitates obtained by Methods I, II and III, it was found that the relative protein content of the precipitate decreased in the order Method I > Method II > Method III.

The precipitates from both Methods II and III showed strong absorbance around 1710 cm^{-1} corresponding to ester groups that was not apparent in the precipitate from Method I. This may suggest that the non-protein component of the precipitates was, at least in part, due to

beeswax (reported to contain approximately 71% esters) [41] or sporopollenin from pollen exine (a complex polymeric mixture of fatty acids and phenolic compounds). [42]

The results from these initial experiments with Methods I, II and III can be summarised as (i) the weight of precipitate was greater than the measured amount of protein (although the precipitate may include a contribution from the inclusion of tungsten), (ii) increasing the amount of acid increased the proportion of non-protein materials in the precipitate and (iii) filtering the honey typically removed a significant amount of protein.

Heating time. As noted above, the unmodified AOAC method requires an unspecified amount of acid to be added until flocculation occurs and, because of this, the heating time for flocculation will also vary. Methods I, II and III typically required the samples to be heated for three hours before a floc formed. To test the effect of varying heating time, the eight test samples were also treated by Methods I and III using a one-hour heating time. Results for these experiments are presented in Table 3 together with results from the original approximate 3 h heating time. For Method I, the reduced heating time had a dramatic effect, the failure rate increasing from 3 of 8 samples to 7 of 8 samples. In contrast, for Method III the reduced heating time had little effect.

The $\Delta^{13}C_{HONEY-PROTEIN}$ value for sample #163, described as "Bloodwood" honey (*Corymbia terminalis*) did not change significantly with heating time for either method. This was surprising as the precipitates from this sample otherwise behaved in a similar manner to other honeys (shown in Table 3), and the precipitate did not exhibit any unusual features in the IR spectrum.

Table 3. $\Delta^{13}C_{HONEY\text{-}PROTEIN}$ obtained by Methods I and III with varied heating time (1h or 3h, as indicated). Values underlined exceed the AOAC recommendation for 7% apparent C4 sugar content.

sample	Method I		Method III	
	1 h	3 h	1 h	3 h
#11	1.53	0.65	3.08	1.75
#133	6.36	5.78	7.08	5.73
#161	3.45	1.00	6.70	6.91
#163	0.19	-0.54	0.04	-0.33
#264	1.53	0.82	3.33	1.66
#621	1.13	0.85	2.82	3.36
#736	1.60	0.66	6.84	7.15
#771	1.05	2.48	6.52	6.51

Figure 3b shows 2nd derivative mid infrared spectra of precipitates produced by Methods III with 1 h and 3 h heating times. For both methods, increased heating time resulted in an increase in the amount of protein observed in the amide I (1656 cm^{-1}) and amide II (1512 cm^{-1}) absorbances. The increase in protein was, however, much more apparent for Method III and the 1 h precipitate also showed absorbances corresponding to ester groups. The spectra obtained for precipitates using Method I with 1 h and 3 h heating were generally similar.

Without heating (Methods I*, II* and III*). From the experiments above, it was apparent that longer heating time resulted in lower failure rates ($\Delta^{13}C_{HONEY–PROTEIN} < +1‰$) but more extreme conditions (e.g. more acid) resulted in more non-protein material in the precipitate. As a compromise, Methods I*, II* and III* used the same protocols as Methods I, II and III with fixed amounts of acid but, instead of heating the samples at 80 ºC, the samples were allowed to stand at room temperature for at least 48 h. For these methods, 20 g of honeys were prepared with twice the volume of reagents in order to obtain sufficient precipitate for FTIR and IRMS analysis.

The results for the eight test samples analysed by Methods I*, II* and III* are summarised in Fig 2d to f, although only 7 results were obtained for Methods II* and III* as insufficient precipitate was obtained from sample #736 (“grey ironbark”). It may be noteworthy that this was the only sample tested that did not show a significant loss of protein when filtered.

From Fig 2 it was apparent that sample #133 failed by all the methods, modified or unmodified, which was taken as further proof that the sample contained a significant amount of C4 sugar. In contrast to the heated methods (Fig.2a, Fig.2b, Fig.2c), all variants of the

unheated methods (Fig.2d, Fig.2e, Fig.2f) had a smaller range of $\delta^{13}C_{HONEY-PROTEIN}$ values that fell closer to a 1:1 relationship. The addition of higher concentration acid (Method II*) resulted in a small negative offset in the $\delta^{13}C_{PROTEIN}$ values but significantly less than when the samples were heated with extra acid (Method II). Filtration also had a much less pronounced effect when the samples were unheated although the range of $\delta^{13}C_{PROTEIN}$ values was still wider than the unfiltered equivalents.

Methods I* (Fig 2d) and II* (Fig 2e) produced precipitates with similar $\delta^{13}C_{PROTEIN}$ values (–26.98 to –24.43‰ and –27.12 to –24.52‰ respectively) whereas the values obtained by Method III* (Fig 2f) were slightly more negative (–27.50 to –25.07‰). Again, this was in contrast to a small positive "enrichment" reported for filtered European honey. [40] Methods I* and II* also produced similar $\Delta^{13}C_{HONEY-PROTEIN}$ values (–0.31 to +6.27 and +0.51 to +6.36‰ respectively) whereas Method III* gave a slightly wider range (–0.71 to +8.11‰).

All three methods (I*, II*, III*) yielded amounts of precipitate that were too small to weigh accurately. The total amount of precipitate was typically only sufficient to obtain an FTIR spectrum and triplicate $\delta^{13}C$ measurements. This would suggest that approximately 20 to 25 mg of precipitate was obtained from 20 g of honey, equivalent to *ca*. 0.1%. This concentration would be similar to the protein concentration measured using the Bradford reagent (Table 2), and previously reported for other honey, and may imply that these precipitates were comprised mainly of protein.

Figure 3c shows the 2nd derivative IR spectra of precipitates obtained by Methods I and I*. Based on the intensity of the amide I and II absorbance, Method I* precipitate contained more protein than Method I precipitate, and neither sample contained the ester absorbance observed in other precipitates. Both precipitates also exhibited an absorbance at approximately 1740 cm^{-1} (*), that was distinct from the ester absorbance observed for some other precipitates and may be due to protein-containing compounds such as lipo-proteins or components of pollen exine.

Based on these experiments it was concluded that Method I* produced precipitates comprised primarily of protein and/or protein containing compounds and yielded more accurate $\Delta^{13}C_{HONEY-PROTEIN}$ values than the other methods.

Modified AOAC method for Australian honey. In summary, Method I* used a 20 g sample of honey with 4 mL of 0.335 M sulphuric acid and 4 mL of 10% sodium tungstate solution. Resultant solutions were kept at room temperature for at least 48 h to allow a floc to form.

Figure 1b shows the results for all 102 of the original samples (boutique, supermarket and mānuka) re-analysed using Method I*. Although visually, the data appear more constrained than the unmodified AOAC method (Method I) the range of $\Delta^{13}C_{HONEY\text{-}PROTEIN}$ values were similar; boutique up to +2.16‰, supermarket up to +2.59‰ and mānuka up to +2.57‰.

The failure rate for apparent C4 sugar content, based on $\Delta^{13}C_{HONEY-PROTEIN} < +1‰$, was 49, 35 and 42% for boutique, supermarket and mānuka honey respectively compared to the unmodified method (Method I) which gave failure rates of 48, 39 and 79%. If, however, the acceptance criteria was extended to $\Delta^{13}C_{HONEY-PROTEIN} < +2‰$, as proposed for New Zealand mānuka honeys, [27] the failure rate according to Method I* was 8, 6 and 5% for boutique, supermarket and mānuka honey respectively compared to the unmodified AOAC method (Method I) which gave failure rates of 13, 3 and 37% (at $\Delta^{13}C_{HONEY-PROTEIN} < +2‰$). Based on the work presented, the authors recommend that the authentication of Australian honey is undertaken using a modified procedure (Method I*) with the acceptance criteria of $\Delta^{13}C_{HONEY-PROTEIN} < +2‰$.

The protein in honey can be viewed as being derived from three sources; [39] pollen from the same plant as the nectar, pollen from adjacent flora, proteins from the secretions of harvesting bees. Although research has found that honey protein is consistent with bee protein (albumins, globulins, etc.) [43] these are ultimately derived from the proteins present in consumed pollen, broken down and reformed inside the bee. Australia's dominant flora, the eucalypts, are pollinated by birds, possums and fruit bats as well as insects and to attract larger animals, these native plant species often produces large quantities of nectar and pollen. Although European honeybees (*Apis mellifera*) undoubtedly enjoy a plentiful supply of nectar there are questions about the nutritional value of the pollen from these species. Government agencies have reported that "pollen from some Australian native flora is of no value to the imported bees" and "bees often do best when there are some European plants, usually weeds, in the vicinity of flowering eucalypts". [44] This would suggest that the sugar and protein in Australian honey are derived from different floral sources and might not automatically have similar isotopic composition. Although the presence of pollen is often used to characterise the flora origins of honey, it has been reported that bees collecting nectar from mānuka flowers do not actively collect pollen but can have pollen brushed onto their bodies that finds its way into honey. [45]

405 Although the European honeybee is not native to North America many of the crops that
406 depend on western honeybees for pollination have also been imported since colonial times. [46]
407 This evolutionary relation between honeybees and flora can certainly explain why the AOAC
408 998.12 method is applicable to both European and North American honey but not to honey
409 from Australia, New Zealand, China and other counties where honey is predominantly derived
410 from indigenous flora.

411 This research has demonstrated that even when high purity protein can be isolated from
412 Australian honey there remains a question over the application of AOAC 998.12 for the
413 detection of added C4 sugar. Australian honey is often marketed on the basis of character
414 imparted by unique flora such as Leatherwood, Ironbark, Blue Gum, etc. and, unfortunately, it
415 may be the nature for these plants or the European honeybee's disinterest in their pollen that
416 makes the unmodified AOAC 998.12 method unsuitable for Australian honey. The authors
417 propose a minor modification to method AOAC 998.12, by eliminating heating and extending
418 the flocculation time with acceptance criteria of $\delta^{13}C_{HONEY} < -24‰$ and $\Delta^{13}C_{HONEY-PROTEIN} <$
419 +2‰. This protocol has been shown to be generally applicable to the detection of C4 sugar in
420 Australiana and New Zealand honey including mānuka honey.

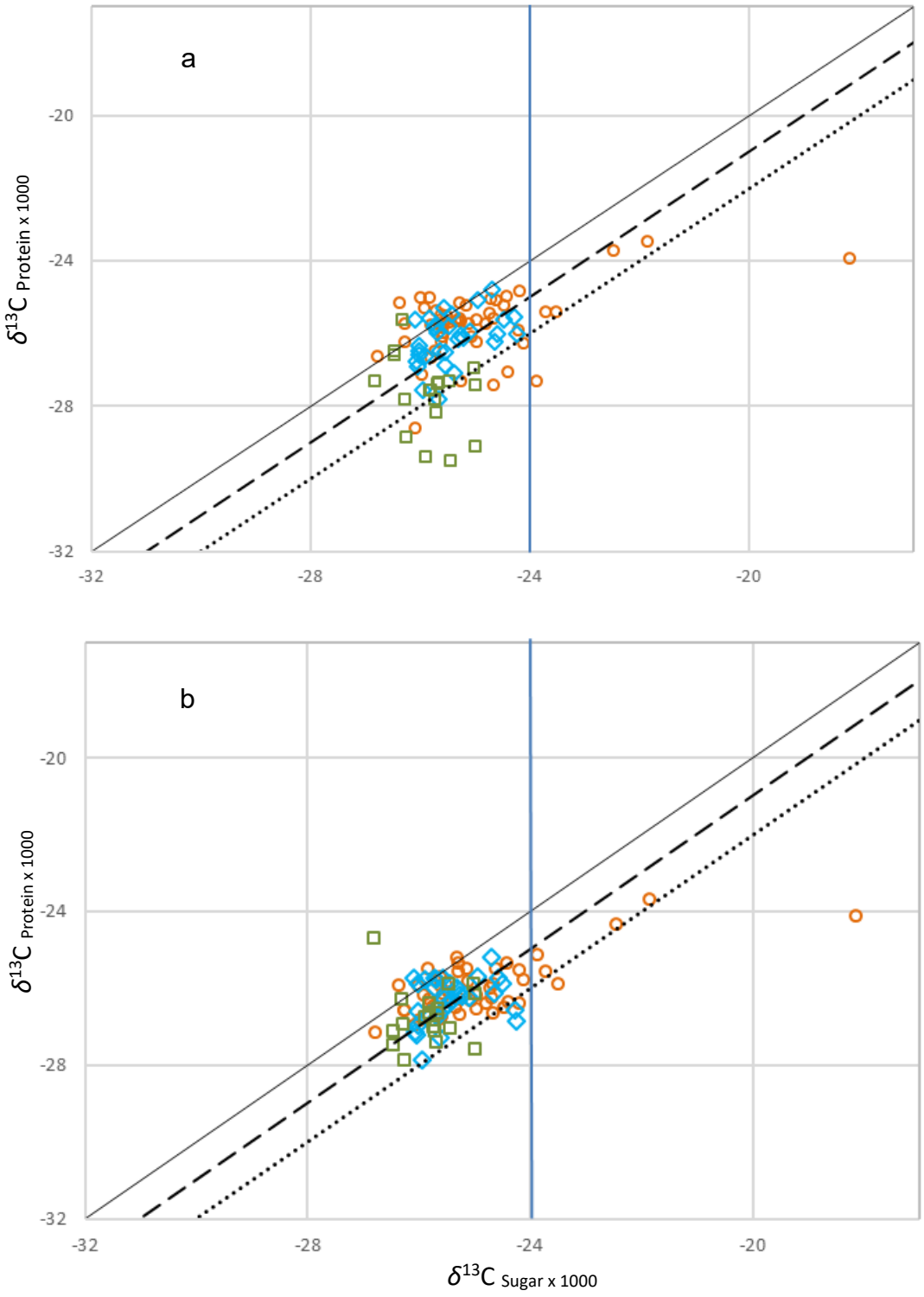

Figure 1. $\delta^{13}C_{PROTEIN}$ versus $\delta^{13}C_{HONEY}$ results for (a, upper) Method I and (b, lower) Method I*. Boutique honey (circle, red), supermarket honey (diamond, blue), mānuka honey (square, green). Solid line shows 1:1 relationship, dashed line shows $\delta^{13}C_{SUGAR-PROTEIN}$ = +1 ‰, dotted line shows $\delta^{13}C_{HONEY-PROTEIN}$ = +2 ‰. $\delta^{13}C_{HONEY}$ values more negative than -24 ‰ (vertical, blue line) are considered normal.[38]

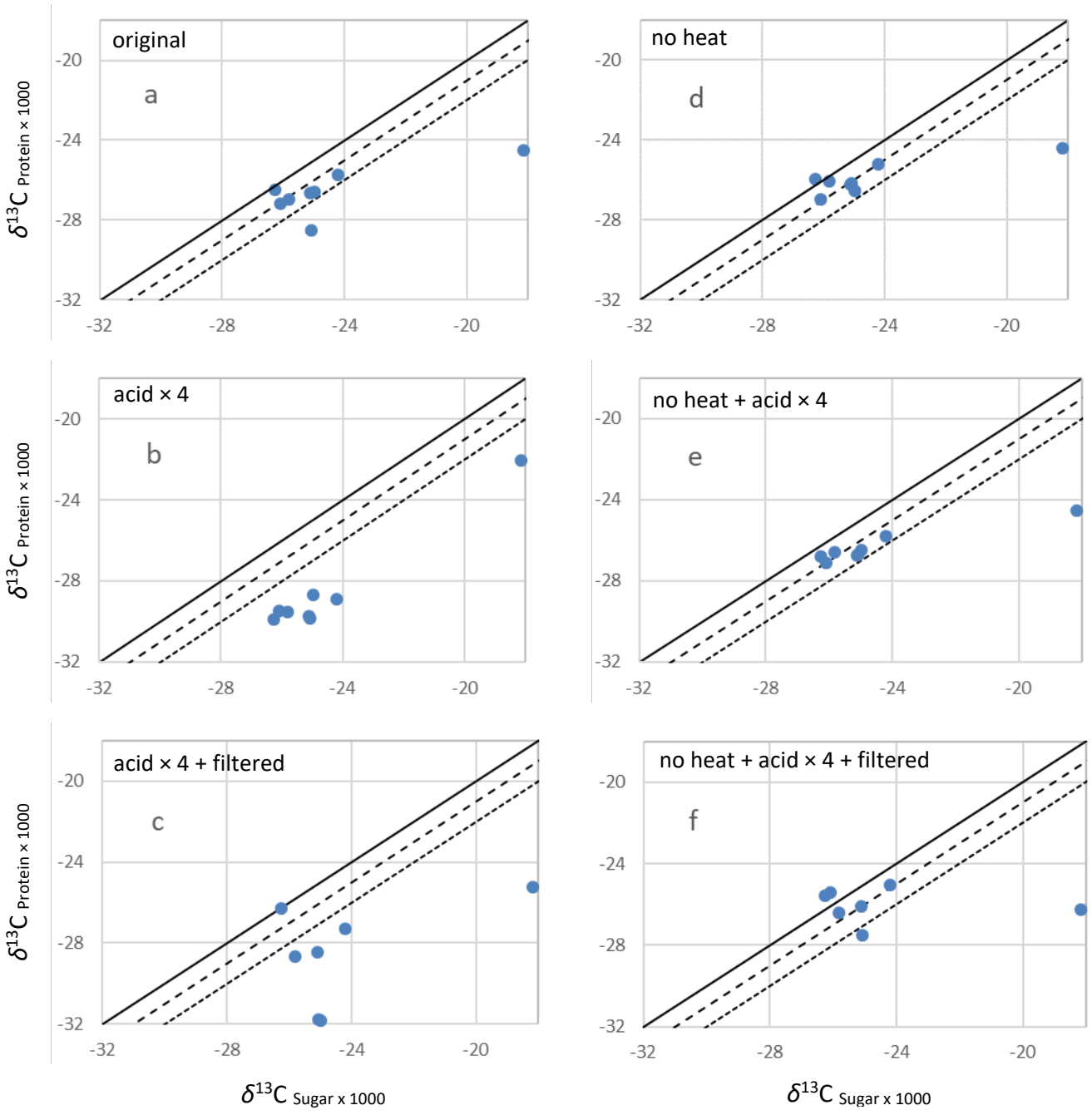

Figure 2. $\delta^{13}C_{PROTEIN}$ versus $\delta^{13}C_{SUGAR}$ results for 8 boutique honey samples tested by (a, upper left) AOAC 998.12, (b, middle left) with an increased amount of acid, (c, lower left) filtration step before precipitation with an increased amount of acid, (d, upper right) 48 h bench top precipitation, (e, middle right) increased amount of acid and 48 h bench top precipitation, (f, lower right) filtration step with an increased amount of acid and 48 h bench top precipitation. Solid line shows 1:1 relationship, dashed line shows $\delta^{13}C_{HONEY-PROTEIN}$ = +1‰, dotted line shows $\delta^{13}C_{HONEY-PROTEIN}$ = +2‰.

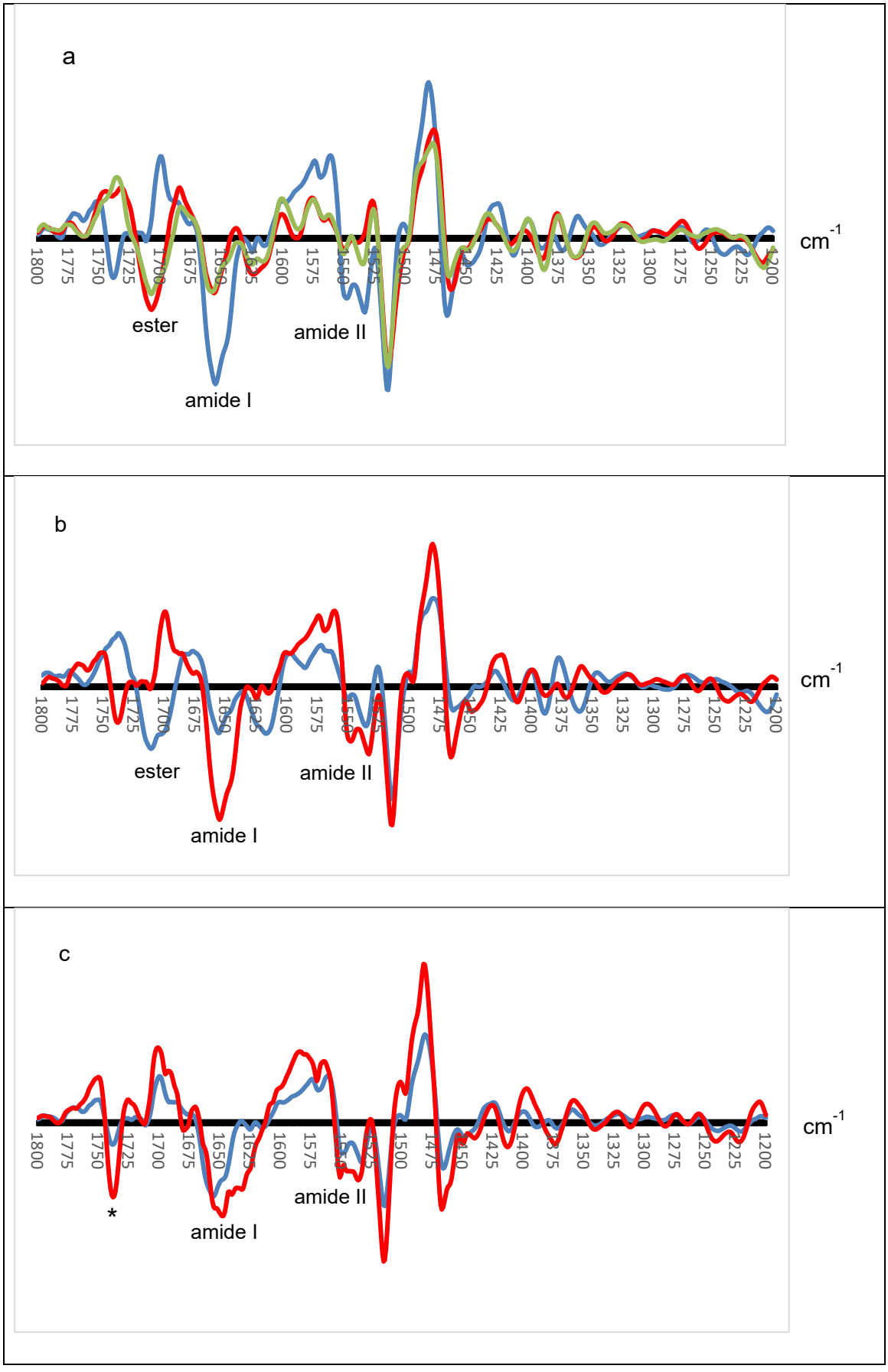
a
ester
amide I
amide II
cm-1
b
ester
amide I
amide II
cm-1
c
*
amide I
amide II
cm-1

Figure 3. Second derivative mid-infrared spectra of precipitates produced by (a, upper) Methods I (blue), II (red) and III (green). Method I (blue), (b, middle) Methods III with 1 h (blue) and 3 h (red) heating times and (c, lower) Method I (blue) and I* (red).

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