Technical Note

3-in-1 Honey Test

Introduction

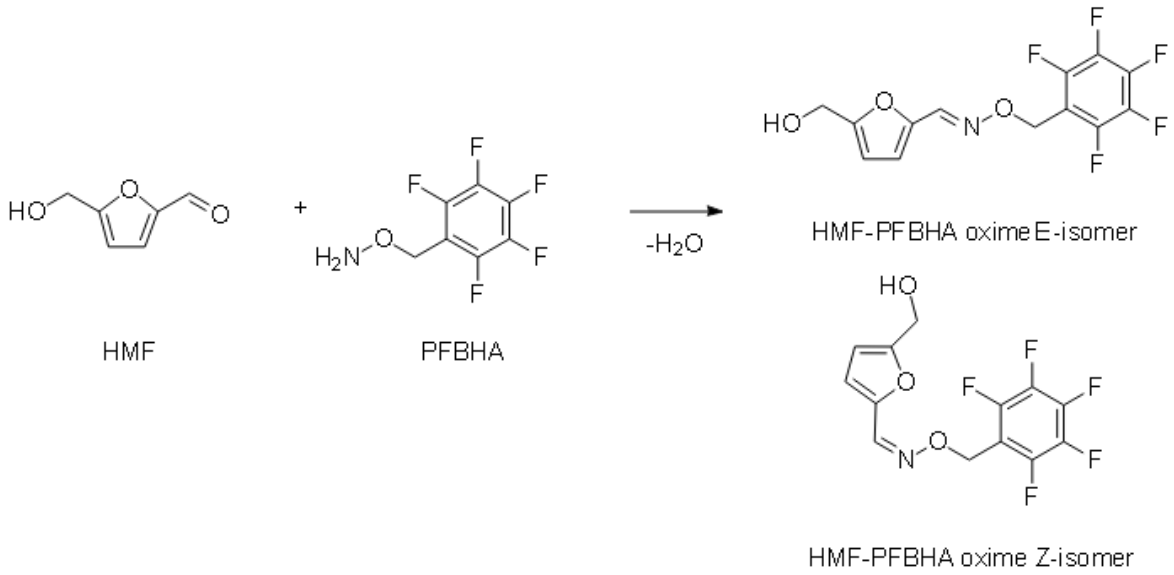
In December 2019, Hill Labs introduced a new version of the 3-in-1 honey method, measuring levels of methylglyoxal (MGO), hydroxymethylfurfural (HMF) and dihydroxyacetone (DHA) in Manuka honey. This was the result of a multi-year in-house R&D project, aimed at gaining more understanding of the derivatization reaction used, improving the accuracy, and reducing the uncertainty of measurement (UoM) of results reported to our honey customers.

Derivatisation is necessary in the 3-in-1 method for the measurement of MGO and DHA, as they are small polar (highly water soluble) molecules (e.g. for DHA in Scheme 1), not easily quantified in honey in their native form (see sidebar for further explanation of their derivatization).

HMF can be measured without derivatization, by high performance liquid chromatography (HPLC-UV), or spectrophotometry, or as part of the 3-in-1 method in derivatised form. Underivatised HMF methods are commonly used by laboratories worldwide, for HMF in high-sugar food products where MGO and DHA are not of interest (e.g. non-Manuka honeys). Examples of these “gold standard” reference methods are those published by the International Honey Commission (reference Error: Reference source not found) and in the AOAC journal (reference Error: Reference source not found).

An aim of developing the new 3-in-1 method was aligning HMF results with those of reference methods for underivatised HMF, and achieving this has given lower HMF results compared with the old 3-in-1 method used at Hills, meaning a relative drop in HMF values reported to customers.

Scheme 1: Derivatisation reaction of HMF with PFBHA



Improving the accuracy and UoM of the 3-in-1 method has been achieved by improvements to:

- Calibration standard accuracy.
- Robotic pipetting protocols.
- Derivatization reaction conditions.
- HPLC chromatography and UV wavelength choices.

For more information, freephone 0508 HILL LAB 44 555 22 mail@hill-labs.co.nz hill-labs.co.nz

Calibration standards are needed for a method of this type, as references to measure the concentrations of MGO, HMF and DHA against. High purity certified standards of HMF and DHA are available from ISO 17025/17034 accredited chemical suppliers, however MGO is only available as a “40 % weight/weight” solution in water. In a collaborative study with Analytica Laboratories (Now known as ALS), Scion Research and with support from Dr. Peter Brooks (University of the Sunshine Coast, Australia), using quantitative nuclear magnetic resonance spectroscopy (qNMR), we have been able to set a more accurate value for this MGO solution, improving the accuracy of MGO results.

UoM for the 3-in-1 method results in part from variability in volumetric transfers in the lab (e.g. when pipetting a solution of honey dissolved in water). The reliability and completion of the derivatization reaction also contributes to UoM, as most reactions are rapid in the initial stages and slow down when nearing completion (Figure 1), so it is easy to lose the last few percent of analyte, giving inaccurate results (e.g. if HMF was taken at 30 minutes in Figure 1).

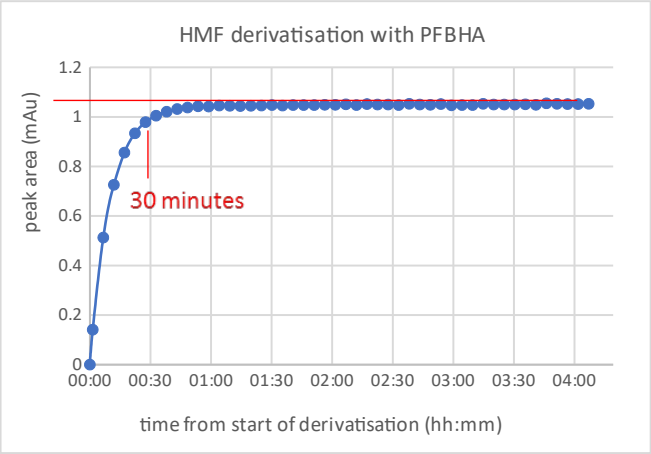


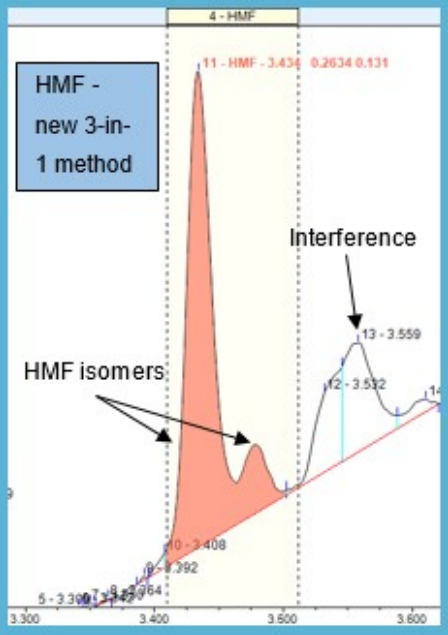
Figure 1: Reaction of HMF with PFBHA at 20 °C in acetonitrile/water. The red arrow indicates 30 minutes. Approximate completion of derivatisation is 1 hour 20 minutes.

Differing reactions conditions for the new 3-in-1 method, that are precisely timed on a computerized robotic pipetting station, and greater precision of pipetted volumes has resulted in lower UoMs for MGO, HMF and DHA.

HPLC chromatography also contributes to the accuracy of results. By optimizing the HPLC-UV analysis, the new 3-in-1 method gives cleaner peaks, allowing more accurate integration (see sidebar).

CHROMATOGRAPHY

Chromatography allows separation of compounds in a complex mixture so they can be quantified (measured) accurately. HPLC used for the 3-in-1 method separates compounds based on their solubility in water/ acetonitrile mixtures versus their retention on a solid packing in a column (C-18 derivatised silica particles). Derivatised MGO, HMF and DHA are retained more than compounds (interferences) that are retained to a similar degree. The improved separation of our new 3-in-1 method gives more accurate quantification. Below is an example for a potential HMF interference in a honey sample, which is now well separated from the HMF peaks (both integrated for HMF quantitation).



Results and Discussion

New 3-in-1 methods vs. old

Extensive comparison of the results from the new 3-in-1 method compared with the old (Table 1, for 80 monofloral, multifloral and non-Manuka honey samples), has shown that the MGO and DHA values have remained similar (both < 1% different). However our new derivatization conditions mean that HMF results are significantly lower (around - 15 % near the Codex MRL of 40 mg/kg, and approximately - 35 % at lower levels (less than 10 mg/kg). These changes are graphically displayed for our long-term In-house QC honey shown in Figure 2, which has mid-range MGO, DHA and HMF levels for a Manuka honey. This honey shows a greater than average drop in DHA, due to improved chromatography enabling the separation of a minor interference, included in the DHA result for the old method.

Table 1: Average change in results moving from Hill Labs old 3-in-1 method to the new method (80 honey samples tested).

	Average change in results (new 3-in-1 method – old method)
MGO (98 – 1280 mg/kg)	-0.4 %
DHA (430 – 3100 mg/kg)	-0.4 %
HMF (31 – 41 mg/kg)	-15.2 %
HMF (4.1 – 10 mg/kg)	-37.2 %

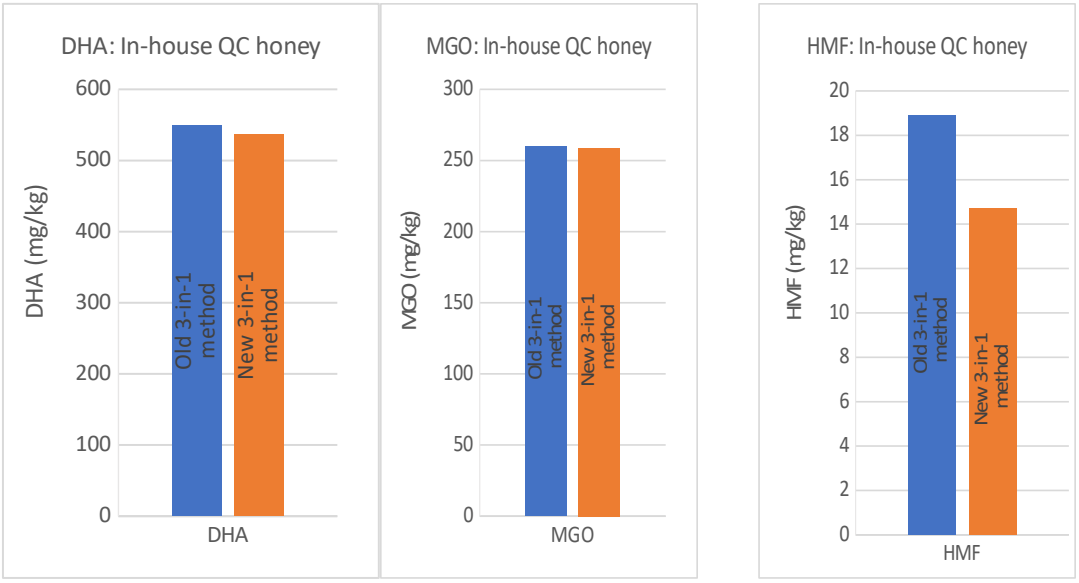


Figure 2: Change in results for Hill Labs In-house QC honey between the old 3-in-1 method and the new method. (Average of 30 results each).

HMF comparison with reference value

The drop in HMF results means they are now well aligned with reference methods for underivatised HMF (references 2 and 3); these, along with spectrophotometry, are the main methods used for HMF by international laboratories. This alignment is shown in Figure 3 by comparing HMF results with a method based on reference 2, where the new 3-in-1 method is on average 3.6 % higher than the reference method and the old method is 24.4 % higher. Table 2 shows a comparison with results from FAPAS ILCPs (inter-laboratory comparison programs). FAPAS is based in the UK, and typically sends out honey samples to around 50 laboratories worldwide for its ILCPs, most of which use underivatised methods for HMF. Results from the new 3-in-1 method showed excellent agreement with ILCP averages.

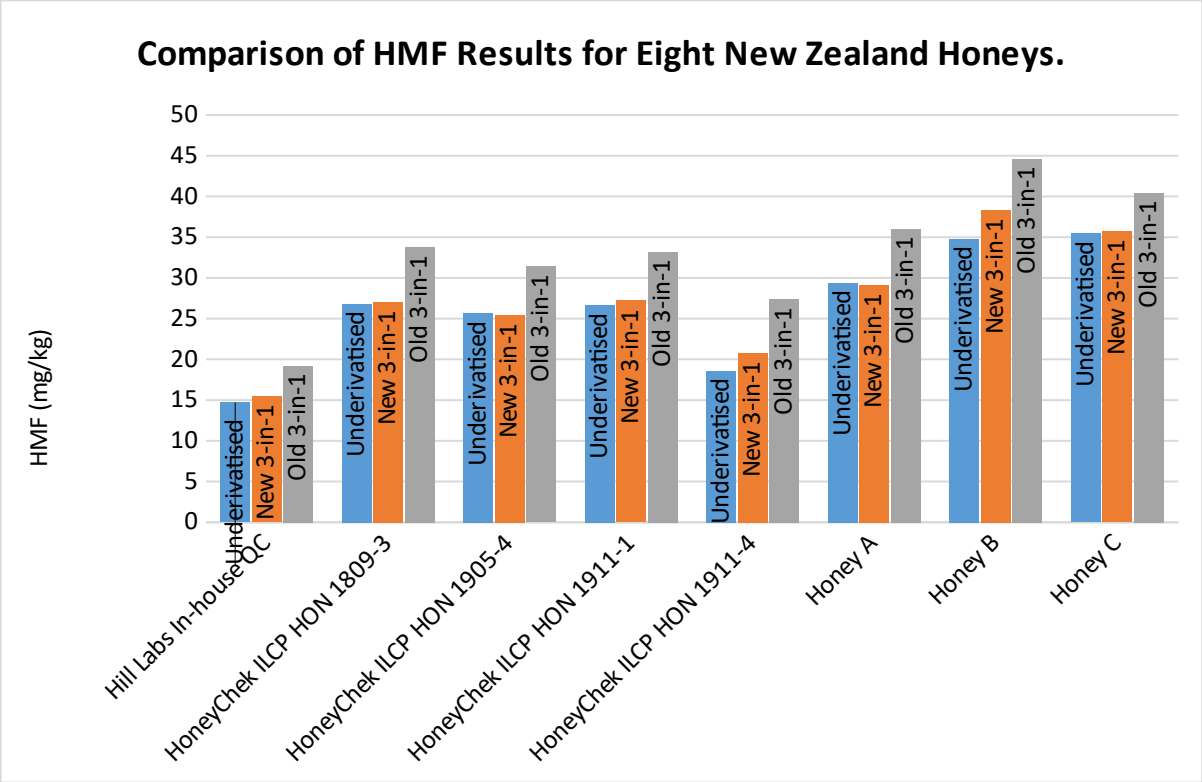


Figure 3: Comparison of HMF results for 8 honeys, using a method for underivatised HMF (based on reference 2), and Hill Lab's new and old 3-in-1 methods. HoneyChek is a New Zealand based ILCP provider. All except ILCP HON 1911-4 (non-Manuka) were classified as monofloral Manuka honeys by the MPI Manuka Honey Classification criteria.

Table 2: HMF results for FAPAS ILCP samples, comparing the new and old 3-in-1 methods with ILCP averages

A Z-score > 2 is regarded as unacceptable. *N* is the number of labs participating.

Sample	ILCP averages HMF (mg/kg)	HMF - New 3-in-1 method		
		Result (mg/kg)	% diff. from ILCP mean	Z-score
FAPAS ILCP 2843	8.46 (*N* = 43)	8.98	5.96%	0.53
FAPAS CRM T2830[a]	40.86 (*N* = 51)	41.36	1.22%	0.199
FAPAS CRM T2829[a]	48.0 (*N* = 48)	46.65	-2.85%	-0.454

[a] purchased in 2015, stored frozen since without being used (these are the test honeys used in FAPAS ILCP rounds 2829 and 2830).

Uncertainty of Measurement (UoM)

The UoM has been reduced for all 3 compounds reported for the 3-in-1 method. This can be seen in the graph below for MGO in our in-house QC honey, which shows the deviation from the average value, for the last 35 runs using the old 3-in-1 method (in orange), before changing to the new 3-in-1 method (in blue).

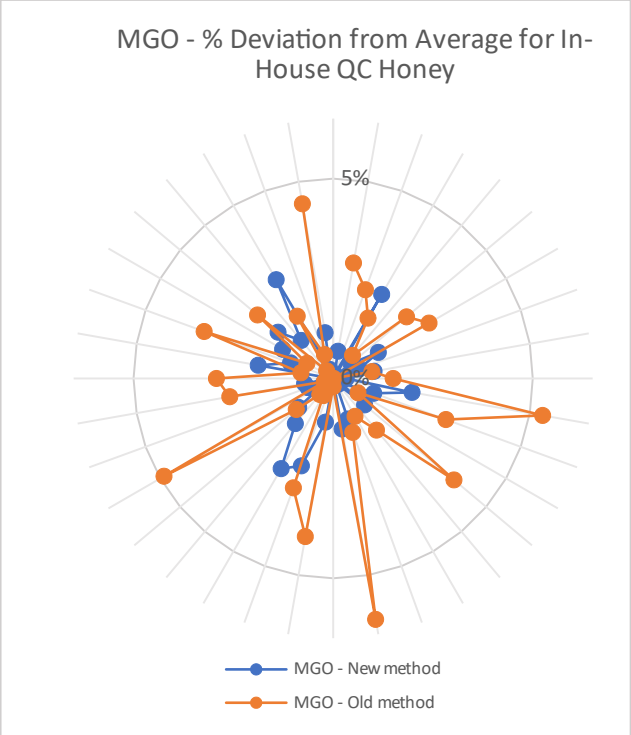
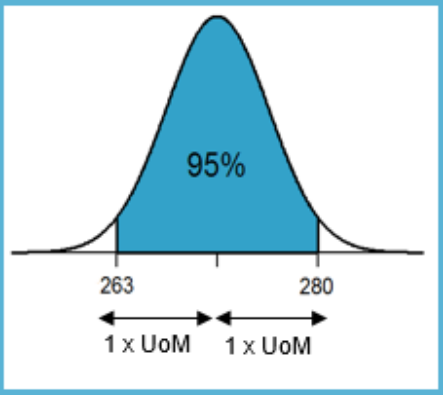


Figure 3: Deviation from the average value for MGO in Hill Labs in-house QC honey, run with each batch of customer samples. Orange points were run using the old 3-in-1 method, blue points using the new method. N = 35

Our reported UoM for MGO at 300 mg/kg (just above NPA 10) is now ± 3.2 % (it was 5.1 %). For HMF at 40 mg/kg the UoM is now 9.2 %, whereas it was previously 11.1 %, and for DHA at 500 mg/kg the UoM is now lowered to 4.2 % from 8.1 %. These lower UoMs should help honey producers meet overseas market requirements, and aid blending.

UOM

Uncertainty of measurement (UoM) for 3-in-1 is based on repeated analysis of a honey, finding its average result, then calculating the 95 % confidence interval (CI). The 95 % CI is the range of results within the Normal (or Gaussian) distribution for which there is 95 % probability of a new result falling. Therefore 19 out of 20 analyses should fall within a 2 × the UoM range (e.g. between MGO = 263 mg/kg (> NPA 10) and MGO = 280 mg/kg if the UoM is 3.2 %).



Summary

A new 3-in-1 method for measuring MGO, HMF and DHA in honey was introduced by Hill Labs in December 2019. This was the result of studying several critical aspects of the method and making significant changes to improve the accuracy and reproducibility of results sent out to customers.

The key improvements are:

- A significant change in HMF results, with the lower results now in line with “gold standard” reference methods for HMF.
- Improved UoM for all three analytes (i.e. greater method precision), allowing customers to more readily maximize the value of their Manuka honey and meet overseas market requirements.
- Increased robustness and reliability of the method means customers can have a high level of confidence in results.

For more information, freephone 0508 HILL LAB 44 555 22 mail@hill-labs.co.nz hill-labs.co.nz

References

1. Windsor, S., Pappalardo, M., Brooks, P., Williams, S. and Manley-Harris, M.
A convenient new analysis of dihydroxyacetone and methylglyoxal applied to Australian Leptospermum honeys.
J. Pharmacognosy Phytother. 4 (2012), 6-11.

2. Harmonised methods of the International Honey Commission (2009), 26-27.
http://www.ihc-platform.net/ihcmethods2009.pdf.

3. Driffield, M., Chan, D., Macarthur, R., Macdonald, S. and Brereton, P.
Single Laboratory Validation of a Method for the Determination of Hydroxymethylfurfural in Honey by Using Solid-Phase Extraction Clean-up and Liquid Chromatography. J. AOAC Int., 88 (2005), 121-127.

For more information, freephone 0508 HILL LAB 44 555 22 mail@hill-labs.co.nz hill-labs.co.nz