Improvements to the measurement of the antibacterial activity of honey

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The measurement of various characteristics of honey, such as antibacterial activity, antioxidant activity or the component methylglyoxal, have become an important part of the commercialisation, marketing, and sale of honeys.

Generally, whilst all honeys have antibacterial activity, honeys with higher antibacterial activity attract a higher sale price. With the discovery of methylglyoxal as an active antibacterial component of manuka honeys, pricing of these honeys is now based on MGO content rather than antibacterial activity measurements, with honeys with higher MGO content attracting a higher sale price. However, not all honeys contain MGO, so many other honeys still rely on antibacterial activity measurements to guide sale price.

In Australia and New Zealand, the Total Activity test has typically been used for determining the antibacterial activity of honey. The test was developed in New Zealand in 1991 (Allen et al., 1991) and is performed by a number of laboratories both locally and overseas. The assay involves adding a standardised number of bacteria (*Staphylococcus aureus*) to warm, molten agar, then pouring the agar into a large, square bioassay dish and allowing it to set. Wells are then cut into the agar and solutions of honey or phenol standards are added to wells. The assay plate is incubated and the next day, zones of inhibition are measured (Figure 1).

Figure 1. An example of zones of growth inhibition resulting from solutions of 25% honey placed into wells cut into agar seeded with Staphylococcus aureus.

A standard curve is plotted from the zones sizes obtained for the phenol standards and the Total Activity (TA) or Phenol Equivalence (PE) is calculated from the zones obtained for honey solutions. Non-peroxide activity (NPA) is also determined this way, but with the enzyme catalase added to denature any hydrogen peroxide activity.

There are a number of issues with this TA test, some of which relate to the test format itself, and some to how it is performed by different laboratories. These problems were even recognised by the developers of the methodology, with Peter Molan writing articles on how the methodology should be improved (Molan 2008a; 2008b).

The first major issue is that the test cannot detect antibacterial activity in all honeys, especially those with low or moderate activity. For example, in our laboratory at the University of Western Australia, we tested over 500 different honey samples and found that activity was undetectable using the Total Activity method for 29% of all honeys tested.

The second major issue requiring consideration is that the antibacterial measurement may not be representative of the activity of the honey, given that not all of the antibacterial components present in honey will diffuse through agar to an equal extent. The method may be well-suited to manuka honeys, where zone size has been shown to correlate closely with MGO content (Cokcetin et al., 2016), but any similar relationship between zone and antimicrobial components has not been established for non-manuka honeys.

For laboratory-based issues, the methodology followed by each laboratory is largely in-house methodology adapted from the original paper published by Allen and collaborators in 1991. As such, the method itself is not accredited and there are no publicly available quality control parameters that would allow for standardisation of methodology and results. This may be one of the reasons that beekeepers have reported receiving varying results for this test from different testing laboratories.

Lastly, as TA is becoming a major marketing tool for antibacterial activity (and honey quality) and relates to price, the scale of values obtained using the test is important. There is no consensus on how results for honeys with very high antibacterial activity should be reported, as the upper limit of the test has not been validated. Some laboratories only report values up to a phenol equivalence of 35, as this corresponds to the point where the standard curve generated for phenol is no longer linear (due to the limited solubility of phenol in water), whereas other laboratories extrapolate activity beyond the linear section of the phenol standard curve and report values above 35.

To address these methodological shortcomings, a new assay for measuring honey activity was developed in 2020 (Green et al). This method uses a broth (liquid) assay instead of agar and uses four different test bacteria instead of one. Bacterial growth is measured using optical density, which is both easy to measure and a non-subjective endpoint. The output of the assay is an Antibacterial Activity Value or AAV. An advantage of this test is that it is capable of measuring antibacterial activity in all honeys regardless of their activity level. The lowest activity honeys typically have AAVs of around 150, which is antibacterial activity due to the osmotic activity of the sugar content of honey, and the highest values that have been measured so far are up around 670 (Figure 2). It is recommended that AAVs are rounded to the nearest 50 as minor differences AAVS (for example 580 versus 590) do not represent truly significant differences in antibacterial activity.

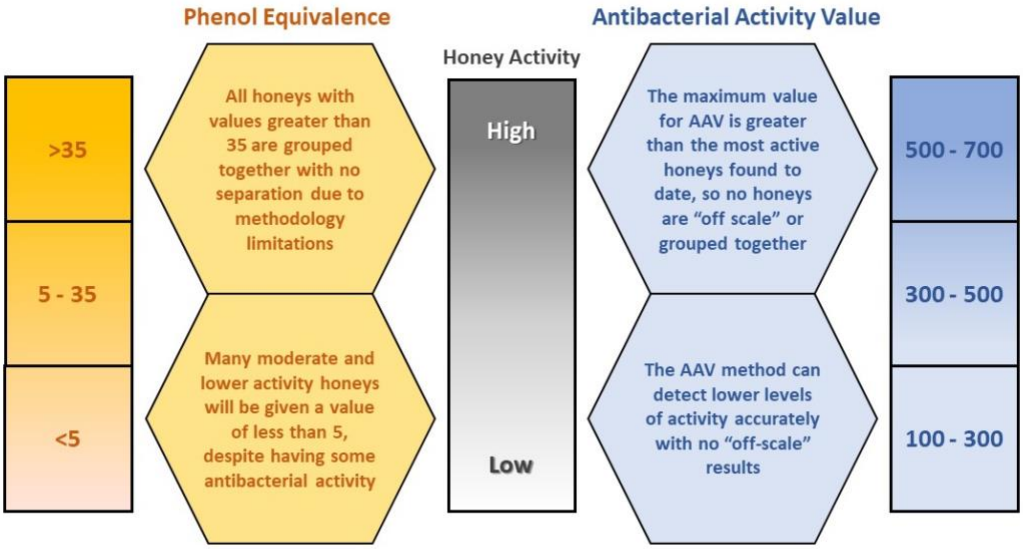

Figure 2. Comparison of the features of the phenol equivalence assay to the Antibacterial Activity Value methodology.

Verification of the new assay across different laboratories is an important step in method implementation. In 2021, an initial ring test was conducted where two honey samples were sent to several different commercial or research laboratories across Australia to each conduct the new test on the honeys. Each laboratory used the standard AAV methodology as published by Green et al (2020) and results are shown in Table 1.

Table 1: Results of the initial ring test for honeys

	Lab A (UWA)	Lab B	Lab C	Lab D
Jarrah honey	600	600	550	500
Artificial honey	200	150	250	50

Results of this initial ring test show that each laboratory found slightly different results for the two honeys. This is likely due to natural bacterial growth variation and not due to differences in methodology or subjective decisions. Looking at the raw optical density data, it was apparent that the bacterial organisms in some of the laboratories did not grow to the same optical density as others, which contributed to the differences in results. Addressing this issue, and then repeating the tests to generate more data would provide the necessary information to further standardise testing across different laboratories. This process occurs for the CODEX honey tests but has not been established the antibacterial test as yet.

The provision of quality control ranges for the expected optical densities obtainable for bacterial growth in the absence of honey (i.e. the positive growth control) would ensure that all laboratories are working within the same parameters, which will help to prevent inter-laboratory differences, such as those seen in the phenol equivalence assay.

The results of the ring test provide important insights and a level of assurance that the AAV methodology has good inter-laboratory reproducibility. This has provided areas for optimisation and further improvements will move towards a standard method for honey antibacterial testing.

An important step in transitioning to the new test methodology is to show how AAVs produced by the new test correspond to TA or PE values, so that one scale can be "translated" to the other scale. Bearing in mind that the two methods have major, fundamental differences in the ways that they measure activity, we would not expect results for the two methods to correlate perfectly. As part of research projects funded by the CRC for Honey Bee Products and AgriFutures Australia, we have tested over 500 honeys for both TA and AAV, and have also used a third method (the broth microdilution assay) to determine minimum inhibitory concentrations (MICs). MICs are determined using a broth assay similar to the AAV methodology, but the MIC endpoint is the lowest concentration of honey that completely inhibits bacterial growth, whereas the AAV assay measures degrees of bacterial growth inhibition via optical density. Since MICs measure the minimum inhibitory amount, the lower the value the higher the activity, whereas for both TA and AAVs the higher the value the higher the activity. By graphing scatter plots of AAV against TA, and AAV against mean MIC, we can see how well the two measurements correlate (Figure 3).

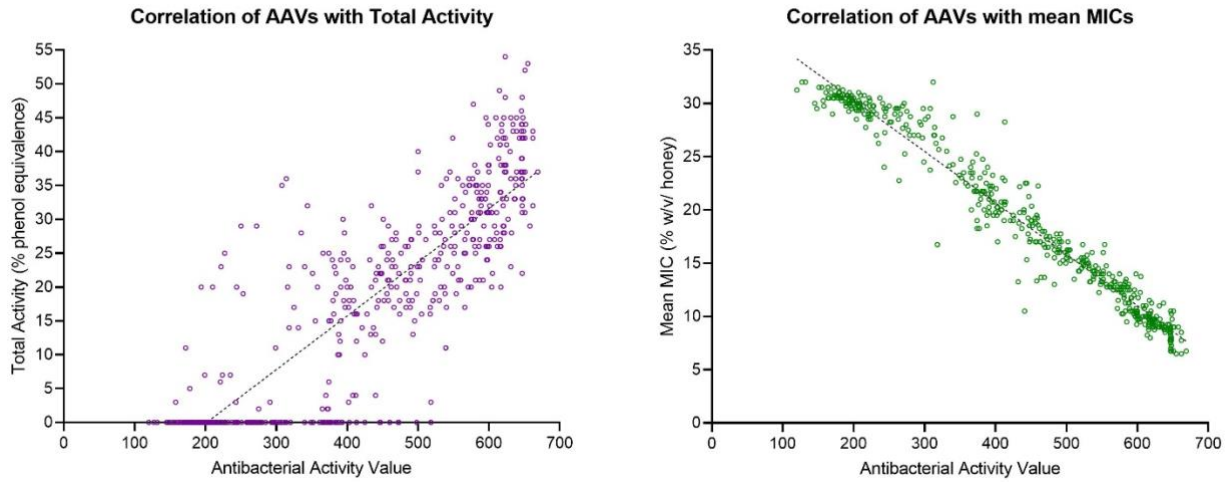

Figure 3. Correlation of AAVS with TA or mean MICs. Each data point represents a single honey, and there are 505 honeys shown in each scatter plot. The dashed line shows the linear trendline.

TA and AAVs show a moderate correlation, indicating that results are broadly (although not always exactly) in agreement. From this scatter plot, we were able to devise a scale showing how AAV results correspond to TA results (Figure 4).

TA	AAV
40+	610+
35+	570+
30+	540+
20+	460+
10+	370+
5+	260+
-	150+

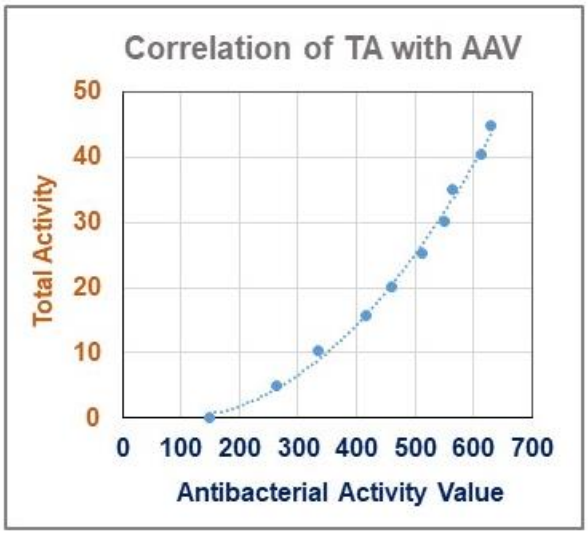

Figure 4. Correlation of TA values with AAVs

The development of AAV methodology represents a significant step forward in accurately measuring the antibacterial activity of honeys. A scale has been produced that correlates the existing TA scale with AAVs, meaning that results can be compared across the two scales. The next steps required

With enough industry support and demand, the AAV methodology could be adopted by laboratories as an improved method for measuring the antibacterial activity of honeys. Whilst the new methodology is an “in-house” laboratory method, as opposed to a standard reference method that is approved by a standards organisation, this should not be an impediment to its adoption. Similarly, the TA methodology in also an in-house, non-accredited method, but this has not prevented its use in commercial testing laboratories both in Australia and overseas. Replacement of TA methodology with AAV testing would be a significant improvement in antibacterial activity testing, as activity in both low and high activity honeys can be accurately measured.

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