RESEARCH

THE TRUE **RELATIONSHIP** OF **NPA AND MG LEVELS**

Peter Molan, formerly Professor in Biological Sciences and Director of the Honey Research Unit at the University of Waikato (now retired)

There has been up until now a major misunderstanding of the relationship between different levels of antibacterial activity in manuka honey. It has always been assumed that, for example, a honey with a non-peroxide antibacterial activity (NPA) of 20 is twice as potent as one with a rating of NPA 10, but that is in fact not correct.

The fallacy is due to it not being taken into account that the NPA rating starts at 8, not zero, a rating of 8 being the minimum level of activity that can be detected in the assay. This is the same situation as temperature measured on the Fahrenheit scale. A temperature of 100°F (38°C) is not twice as warm as 50°F (10°C), because the Fahrenheit scale starts at 32°F, not zero (see Figure 1), whereas a temperature of 100°C is exactly twice as warm as 50°C because the Centigrade scale starts at zero.

I got to thinking about this after being asked a simple question by someone new to the honey industry who wanted to calculate the blending of honey to obtain a desired NPA rating. I was asked whether it should be blended in proportion to the NPA ratings or in proportion to the methylglyoxal (MG) content. Intuitively I would have said to blend in proportion to the active ingredient (in this case methylglyoxal) as would be the case with any other product, but I knew that doing so would not give the desired result with manuka honey. That started me trying to work out why that was so.

Part of the issue is that the NPA rating is not a direct measure of antibacterial activity. The numbers show the concentration (as % in solution) of a standard reference antiseptic (phenol) that has the same level of antibacterial activity in the testing method as the sample of honey has. For the original research for which this testing method was developed, it was the best way of showing how honey compared for potency with other antibacterial substances. But just as the Fahrenheit scale starts at 32°F, not at zero, the antibacterial activity of phenol in the test method used starts at 8, not zero. (For all antibacterial substances there is a concentration below which they do not affect bacteria, known as the 'minimum inhibitory concentration' for each substance. Phenol at a concentration of 7% or less has no antibacterial activity at all in the testing method used.)

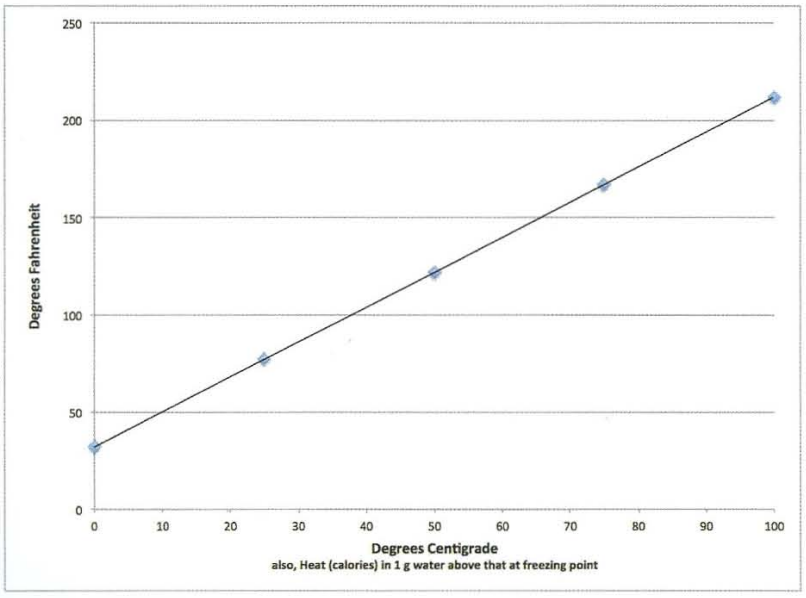

Figure 1: A graph showing the relationship between temperature measured on the Fahrenheit and Centigrade scales. (The Centigrade scale also shows the heat content.)

Thus whilst the NPA rating shows the antibacterial activity of honeys as being higher or lower than each other, it does not show their relative activity in direct proportion. A temperature of 100°F (38°C) is actually about four times as warm as 50°F (10°C), not twice as warm, because the starting temperature of 32°F on the Fahrenheit scale has to be subtracted first before looking at the numbers in proportion to each other (i.e., 100° - 32° = 68° is compared with 50° - 32° = 18°). A honey rated NPA 30 has a bit over three times as much antibacterial potency as one rated NPA 15, not twice as much. (i.e., 30 - 8 = 22 is compared with 15 - 8 = 7). This consideration brings it in line with the proportions of the active ingredient, methylglyoxal (i.e., 1,600 mg/kg compared with 500 mg/kg, a bit over three times as much). So the level of methylglyoxal present is actually a much better indicator than the NPA rating for consumers to see the relative potency of manuka honeys on sale.

Although the methylglyoxal rating scale does not start on zero either, we found that when we measured it in the laboratory, the minimum inhibitory concentration in the standard test method was low (about 50 mg/kg). Subtracting this relatively small number would have only a small effect on proportionality on a scale going up to numbers well over 1,000 mg/kg.

Another part of the issue is the belief by some that the graph of the correlation between NPA and the level of methylglyoxal is a curve and not a straight line. (See Figure 3 below for an example.) When I was first shown the graph produced by my colleagues in the Chemistry Department at the University of Waikato, I expressed the opinion that the data points sat as two

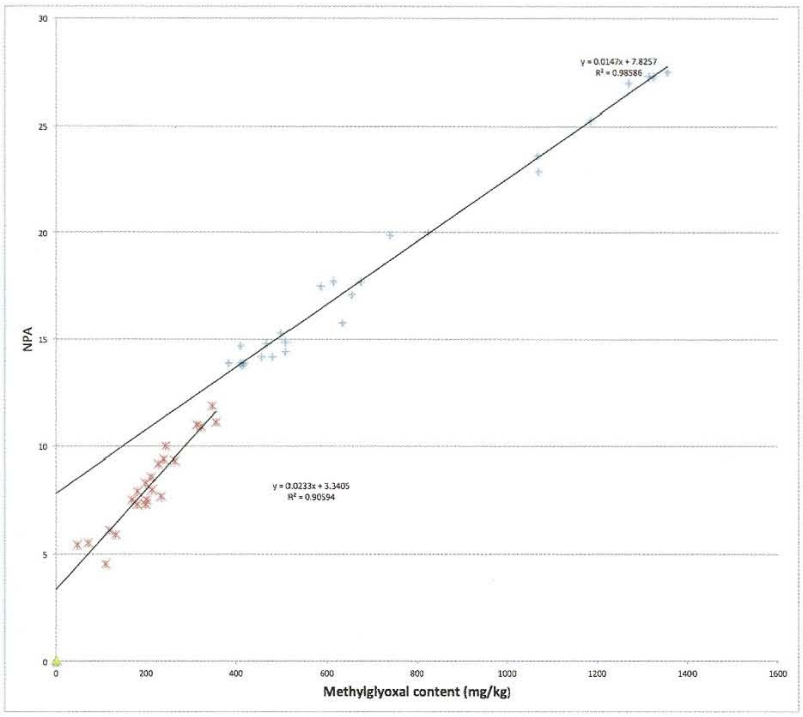

Figure 2: A graph showing the correlation between the level of NPA and methylglyoxal in various samples of manuka honey. (The data is from Adams, C. J., et al. (2008), Carbohydrate Research, 343(4): 651–659, corrected in accordance with the Erratum by Adams, C. J., et al. (2009), Carbohydrate Research, 344(18): 2609.)

separate straight lines and should not have a curve fitted to the total (see Figure 2). The realisation now that the NPA rating does not start at zero makes it very definite that the graph should be two separate straight lines.

The higher NPA values were obtained by assaying honey by the published method (Allen, Molan, & Reid, 1991), which has become the de facto standard method used internationally. In this method the honey is diluted to a 25% solution to get the optimum level of activity for accurate measurement. This method has a minimum level of detection of activity of 8% phenol, but it is very common for samples of honey with a rating of 11 or lower to give only partial inhibition of the bacterial growth and thus their activity rating cannot be measured. To get a measurement for these and for honeys with NPA below 8, it is necessary to test a more concentrated solution of honey to have a higher level of activity on the test plate. This is done with a 50% solution of honey, so the minimum level of detection then is 4% phenol. That means that the rating scale for honeys tested as a 50% solution starts at NPA 4, not NPA 8 as in the standard method. This can be clearly seen in Figure 2, where the fitted straight lines at the minimum detectable level of activity due to methylglyoxal (about 50 mg/kg) are at values of about 8% phenol for the higher set of data where NPA was measured with the standard 25% solution of honey, and 4% phenol for the lower set measured with 50% solutions of honey.

Another reason why the correlation graph should have straight lines fitted to the data points rather than a curve is that the relationship between both methylglyoxal and phenol and their strength of antibacterial activity is linear, so their relationship to each other must be linear. On the agar assay plates the area of the zone of inhibition of bacterial growth is a direct measurement of antibacterial potency (i.e., it is not relative to a reference standard; it is directly proportional to the strength of the antibacterial activity). This is a principle that applies to all antibacterial substances. Whereas with some antibacterial substances their potency may increase with concentration in an upward or downward curve, examination of the zone sizes in a random selection of the many assays we have conducted shows perfect straight lines for both phenol and methylglyoxal over a wide range of levels of activity.

The implication of this confirmation that the graph for correlation of methylglyoxal with NPA is not a curve is that the estimation of NPA from assays of methylglyoxal that is done commercially gives incorrect results. The NPA values being given are too low when below NPA 15. Above this level, the curve and the straight line start to be in about the same place on the graph. The discrepancy is most marked below NPA 8, where the curve is drawn through data that is not on the same scale of measurement (i.e., has been obtained by measurement with 50% solutions of honey).

Also, there is quite a large error involved in this region because when tested as double-strength honey solutions (i.e., 50% instead of the standard 25%) to detect the low level of activity, the results obtained for activity are not double, so an adjustment factor is used to get an approximately correct value. However, I have found by assaying a random collection of samples of manuka honey as both 25% and 50% solutions that the adjustment factor is quite different for each sample.

Another consideration is that whereas the standard method (testing as a 25% solution) is an internationally accepted published method, testing as a 50% solution is an ad hoc method with no established protocol. (The method was developed simply to give producers a guide as to whether a batch of honey was worth retaining for blending with honey of higher activity, or if it had potential to achieve an activity rating above 8 on storage. It was never intended to be used for rating honey for sale.)

The whole set of correlation values as are widely used appears to be incorrect anyway. Figure 3 shows the correlation curve used based on the corrected results of Adams et al. that are shown in Figure 2. Superimposed in Figure 3 are the mean results obtained by many testing laboratories in multiple countries who assayed the NPA and/or the methylglyoxal content of a standard set of honeys sent to them by Global Proficiency as an inter-laboratory comparison exercise. *[Editor's note: Global Proficiency's website states that it is "a specialist provider of proficiency testing, reference materials, and related services."]* It can be seen that the correlation curve gives values for NPA that are about 2 units (2% phenol equivalent) too high for the corresponding level of methylglyoxal. This is not surprising considering that in the past couple of years changes have been made

continued...

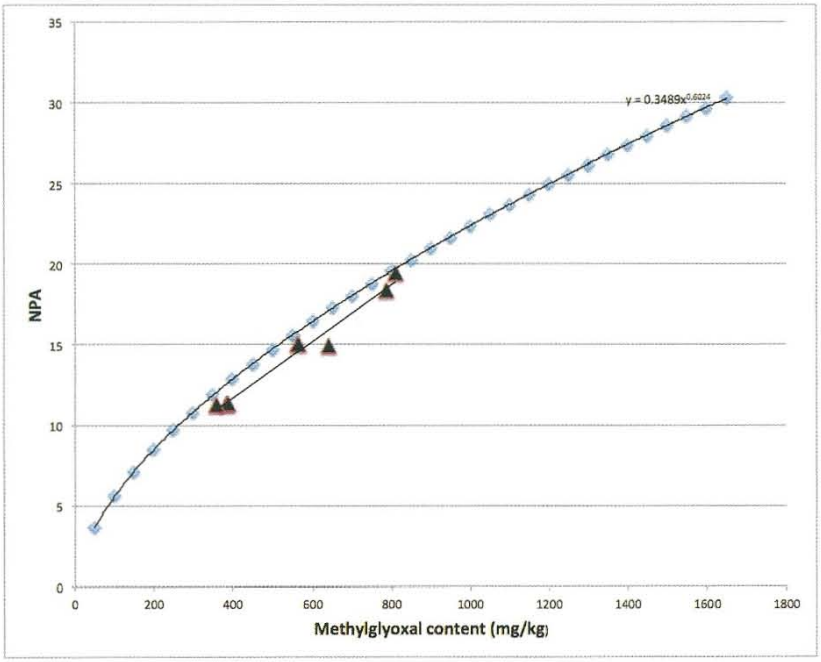

Figure 3: A graph showing as a curve the correlation between the levels of NPA and methylglyoxal in manuka honey. This graph has been plotted from the data generated from the calculator for converting between NPA and methylglyoxal content that was until recently on the UMFHA website. That calculator is based on a smooth curve fitted to the scattered data from the publication by Adams et al. (2008) after correction, as shown in Figure 2 above. Superimposed (with the triangular symbols) are the mean results obtained from the many laboratories that participated in the 2013 and 2014 rounds of the Inter-laboratory Comparison run by Global Proficiency.

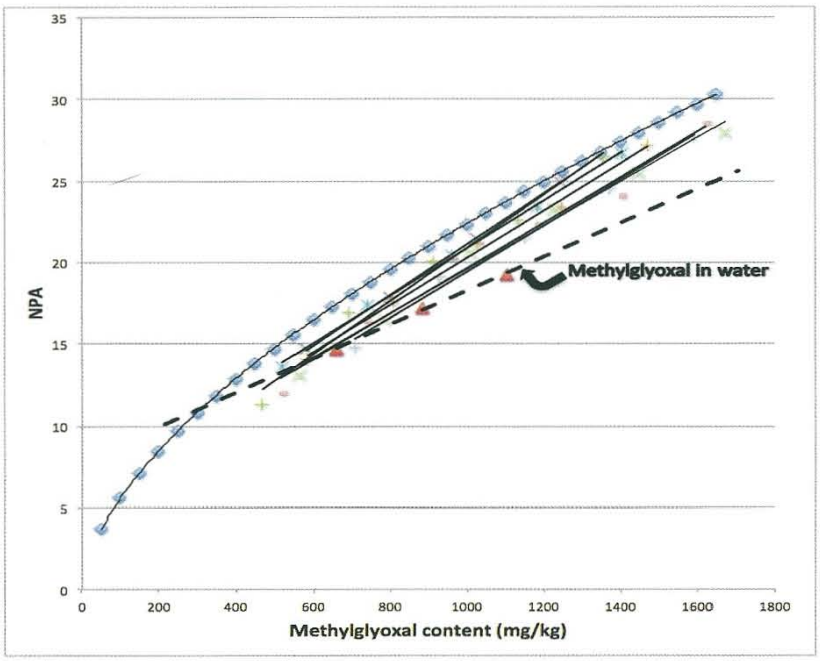

Figure 4: A graph showing the levels of methylglyoxal and NPA obtained when various amounts of methylglyoxal were added to a randomly selected variety of seven samples of manuka honey with different starting levels of methylglyoxal. The lines are superimposed on a copy of the correlation curve shown in Figure 3. Also shown (as a dashed line) are the results obtained from adding various levels of methylglyoxal to water rather than to honey.

in the assay of methylglyoxal to improve its reliability. It is my opinion that the industry should engage Global Proficiency or some other independent organisation such as the Bee Products Standards Council (BPSC) to obtain a new correlation graph with a range of honeys with NPA values from 10 upwards, using a laboratory assaying methylglyoxal with proven reliability.

In an article I wrote which was published in *The New Zealand BeeKeeper* (Molan, 2008), I hypothesised that the explanation for the values for NPA being on a straight line about 8 units (i.e., 8% phenol equivalent) higher than the activity of the corresponding level of methylglyoxal was that there was a synergist present in manuka honey, a component with no antibacterial activity itself but which boosted the antibacterial activity of methylglyoxal.

I was misled into this line of thinking by not realising at the time that methylglyoxal is an unstable substance. In a graph included in that article I showed that methylglyoxal dissolved in water (i.e., not in honey) displayed a much lower level of antibacterial activity than that in honey with the same level of methylglyoxal in it. I later found that the actual content of methylglyoxal in solution in the bottle of reagent we were using was much lower than was stated on the label. (It was failure to take into account this instability of methylglyoxal, giving decomposition of the content in the reference standard used, that was the cause of the failure to get consistent results reported for assays of methylglyoxal in the first couple of rounds of inter-laboratory comparison of methylglyoxal assays.)

I was further misled by the results of a subsequent experiment we conducted, where we added various amounts of methylglyoxal to honeys with different starting levels of activity. Not realising that the actual amounts of methylglyoxal added were a lot lower than believed, I had interpreted these results incorrectly. I had expected the added methylglyoxal to raise the NPA along the line of the graph as shown in Figure 2, but it did not. Instead, the NPA increased less steeply from whatever level to which methylglyoxal had been added. I hypothesised that this was because there were different amounts of synergist present in the honeys of different activity levels to which the methylglyoxal was being added.

I was incorrectly of the view that in honeys with a higher level of NPA, the amounts of synergist would be greater because the proportion of manuka nectar source in the honeys would be greater.

We repeated this experiment more recently using freshly purchased methylglyoxal, with the content of methylglyoxal in it verified by assay in the same analytical laboratory at the same time as the methylglyoxal content of the honey samples was assayed. This time we found that the level of NPA of the honey was increased as steeply as would be expected if accounted for by the antibacterial activity of its methylglyoxal content. These results are shown in Figure 4. Although the lines on the graph for each sample of honey do not coincide with the line of the correlation curve, they are close to the position of the line shown in Figure 3 that shows the values for correlation obtained from Global Proficiency's inter-laboratory comparison, which are more likely to show the correct values.

There is some synergism involved, as can be seen by looking at the line in Figure 4 that shows that lower levels of NPA result from adding various levels of methylglyoxal to water rather than to samples of honey. It has been reported by other researchers also that the antibacterial activity of methylglyoxal is greater when in honey than when on its own in water. We found the likely explanation of this when carrying out experiments to measure the minimum inhibitory concentration of methylglyoxal with bacteria in nutrient broth culture medium rather than on agar plates. We got very variable results depending on the time of exposure of the methylglyoxal to the broth.

Methylglyoxal is a reactive chemical that interacts with proteins and peptides to form addition compounds known as advanced glycation end-products (AGEs). In doing this with peptides in the bacterial culture medium, the level of methylglyoxal present will decrease. (In the agar diffusion assay there is much shorter exposure of methylglyoxal to the nutrient broth because fresh solution is diffusing out all the time from the wells cut in the agar plate.)

It is well known that antioxidants prevent the formation of AGEs from methylglyoxal. It is most likely that the antioxidants in honey are working in this way to preserve the methylglyoxal present. The variation between different samples of honey in their antioxidant content would account for the lines in Figure 4 obtained from adding methylglyoxal to a range of honeys not all being in exactly the same position, although the differences are not large.

We examined a large range of honeys of types other than manuka and found this synergism in all of them, as would be expected since all honeys contain antioxidants. The highest level of synergism was found to be 28% more than the lowest. The important point to keep in mind about this, though, is that whereas the synergism increases the NPA, the antibacterial activity is due entirely to methylglyoxal, which is the only significant antibacterial component present in manuka honey in the testing done with hydrogen peroxide removed.

Whilst the NPA rating shows the antibacterial activity of honeys as being higher or lower than each other it does not show their relative activity in direct proportion.

Conclusion

It is my opinion, formed from consideration of all the points made here, that it would be best by far for the rating of activity in manuka honey to be done by the whole industry as originally stated by the Ministry for Primary Industries (MPI) in their guidelines, which was that only the content of methylglyoxal be shown. This would then simply require education of consumers to have them realise that the antibacterial potency is directly proportional to the level of methylglyoxal.

Although in New Zealand and Australia there may be restrictions on marketers making reference to antibacterial activity, it could be done by non-commercial educators like myself. In other countries the Australia New Zealand Food Standards (ANZFS) Code does not apply, so there would be no restriction on such educating. Rating the content of methylglyoxal would overcome the problem of marketers using misleading rating numbers that are not actually for NPA. (The MPI guidelines will now allow numbers to mean anything the marketer defines them as meaning, which could be nothing to do with NPA.) It would also curb the freedom of marketers to mislead consumers by giving rating numbers that are actually higher than the true equivalent to NPA ratings. Additionally, it would allow consumers to see the actual value of honey on sale rated "MGO 80" when they see it up against manuka honey on sale with methylglyoxal ratings of 800 to 1,200. Furthermore, rating the methylglyoxal content of manuka honey will let consumers see that honey rated as NPA 5 (83 mg/kg methylglyoxal) has only one tenth of the activity of honey rated NPA 20 (830 mg/kg methylglyoxal).

Acknowledgements

I would like to thank Watson & Son for providing funding to allow the employment of Stacey Meyer as a research assistant to carry out some of the experiments mentioned in this article. I would also like to thank Brenda Tahi for asking the perceptive question about blending which started off my thinking about the relationship between NPA and methylglyoxal. The skilful work of Stacey Meyer and Kerry Allen in carrying out the experimental work is gratefully acknowledged.

References

Adams, C. J., Boult, C. H., Deadman, B. J., Farr, J. M., Grainger, M. N. C., Manley–Harris, M., et al. (2008). Isolation by HPLC and characterisation of the bioactive fraction of New Zealand manuka (*Leptospermum scoparium*) honey. *Carbohydrate Research, 343*(4), 651–659.

Adams, C. J., Boult, C. H., Deadman, B. J., Farr, J. M., Grainger, M. N. C., Manley–Harris, M., et al. (2009). Corrigendum to "Isolation by HPLC and characterization of the bioactive fraction of New Zealand manuka *(Leptospermum scoparium)* honey" [Carbohydr. Res. 343 (2008) 651]. *Carbohydrate Research, 344*(18), 2609.

Allen, K. L., Molan, P. C., & Reid, G. M. (1991). A survey of the antibacterial activity of some New Zealand honeys. *Journal of Pharmacy and Pharmacology, 43*, 817–822.

Molan, P. (2008, May). An explanation of why the MGO level in manuka honey does not show the antibacterial activity. *The New Zealand BeeKeeper, 16*(4), 11–13.