Why does New Zealand Mānuka Honey Fail the AOAC C4 Sugar Method

Dr. Terry J. Braggins* & Dr. Anatoly Chernyshev
Analytica Laboratories Ltd, Ruakura Research Centre, Hamilton, New Zealand
Dr. Karyne Rogers
National Isotope Centre, GNS Science, New Zealand

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Elevated Foreign Sugar Levels

- Direct adulteration of honey with cane sugar (sucrose) [C4]
  - and other sugar syrups [C3]

- Feeding hives with sugar
  - Adulteration (sucrose → fructose + glucose)
  - Winter feeding
    - Balancing thrift with nectar flow

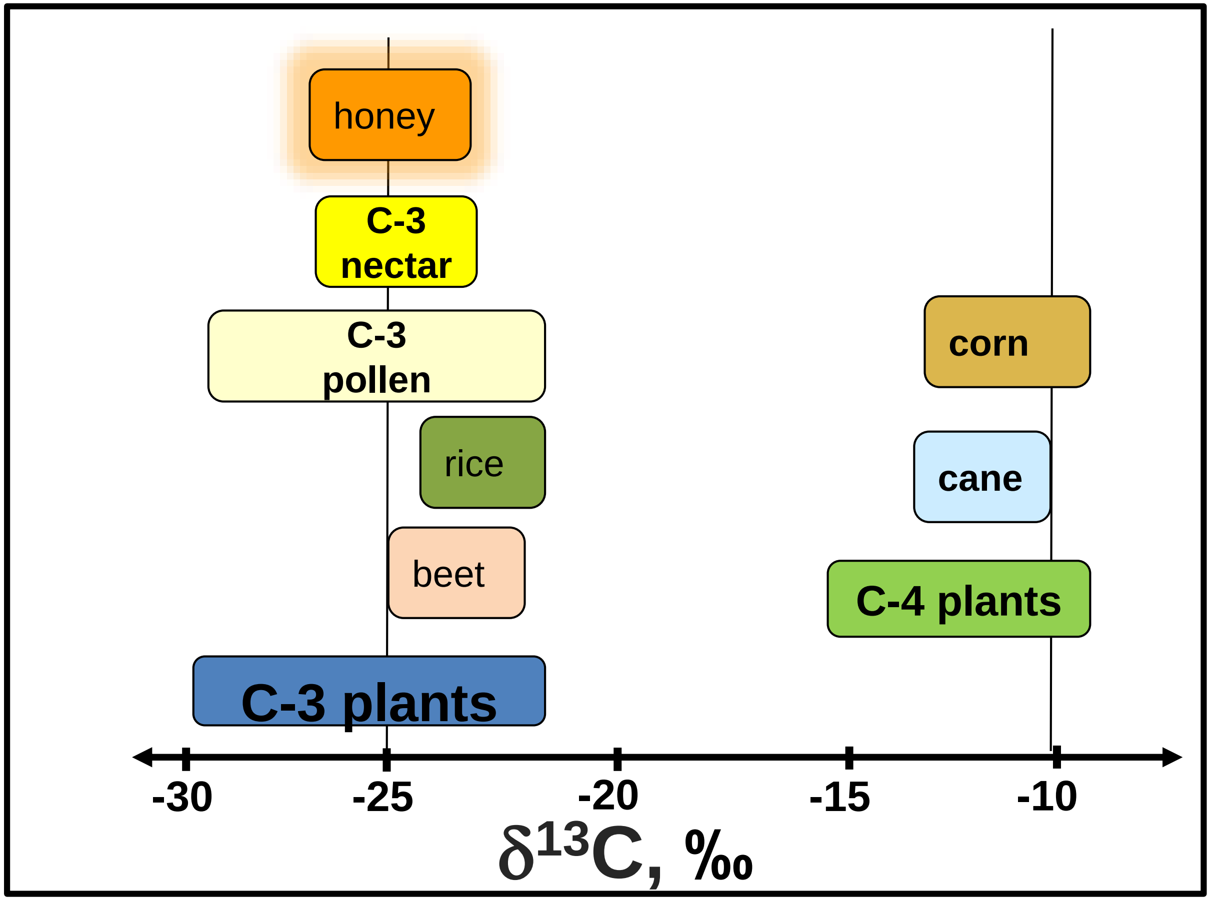
Methods of Analysis

- Sugar profiles (HPLC)
- C4 sugar analysis
  - Isotope Ratio Mass Spectroscopy (IRMS) and HPLC-IRMS;
  - Cavity Ringdown Spectrometry (CRDS)

  - C4 & C3 sugars
    - HPLC & GC Mass Spectroscopy
      - Oligosaccharides and trace components
      - HPLC-IRMS
  - Nuclear Magnetic Resonance (NMR)

AOAC – C4 Sugar Method

- Utilises the ratio of ^{13}C :^{12}C isotopes (in delta units, $\delta^{13}C$)



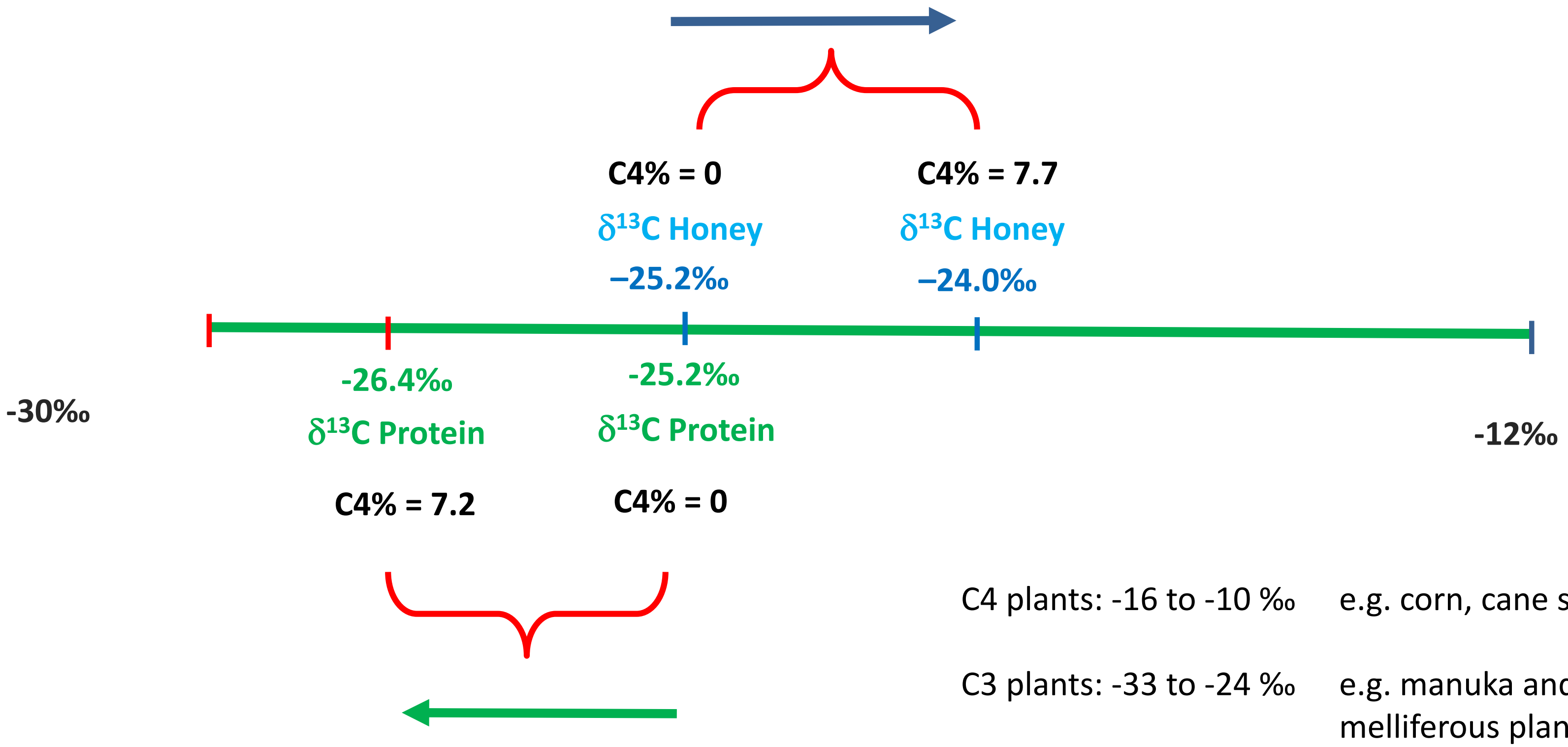
AOAC – C4 Sugar Method

- $$\% \text{ C4 sugar} = \frac{\delta^{13}C_{protein} - \delta^{13}C_{honey}}{\delta^{13}C_{protein} - (-9.7)} * 100\%$$
- Acceptance criteria ≤ 7.0% (6.4 to 7.6, UoM = 0.6)

AOAC 998.12 :
Pure honey (free of corn or cane sugars) with an exception of a few unusual varieties, yields a value of C-4 sugars of ≤7%. Some unusual varieties may slightly exceed the range, but will have $\delta^{13}C$ for honey which are in the normal range (more negative than –24.0‰).

Ways that a honey will return a C4 > 7%

[1] $\delta^{13}C_{honey}$ shifts positively
(when C4 sugar is added)

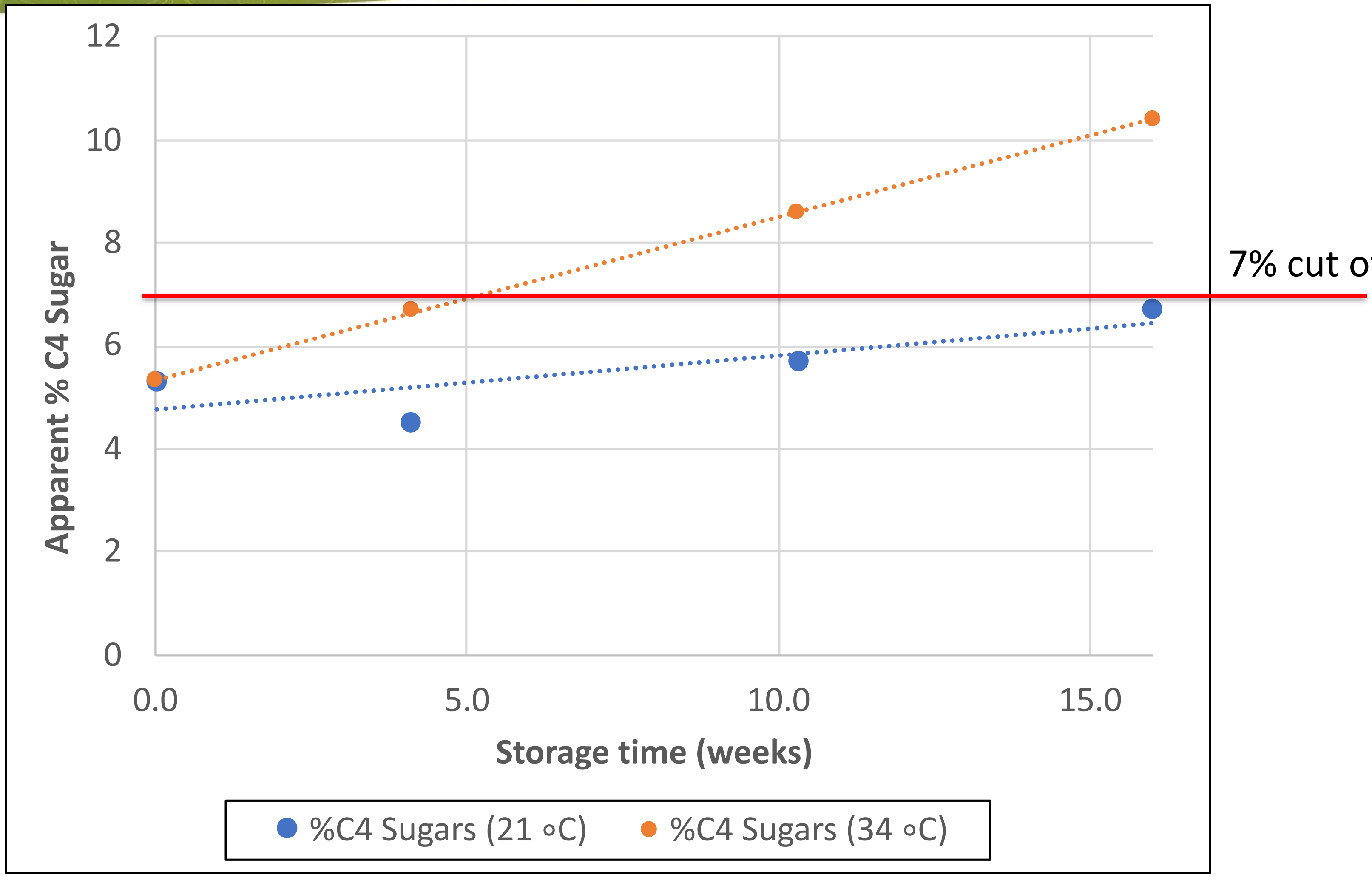


C4 plants: -16 to -10 ‰ e.g. corn, cane sugar

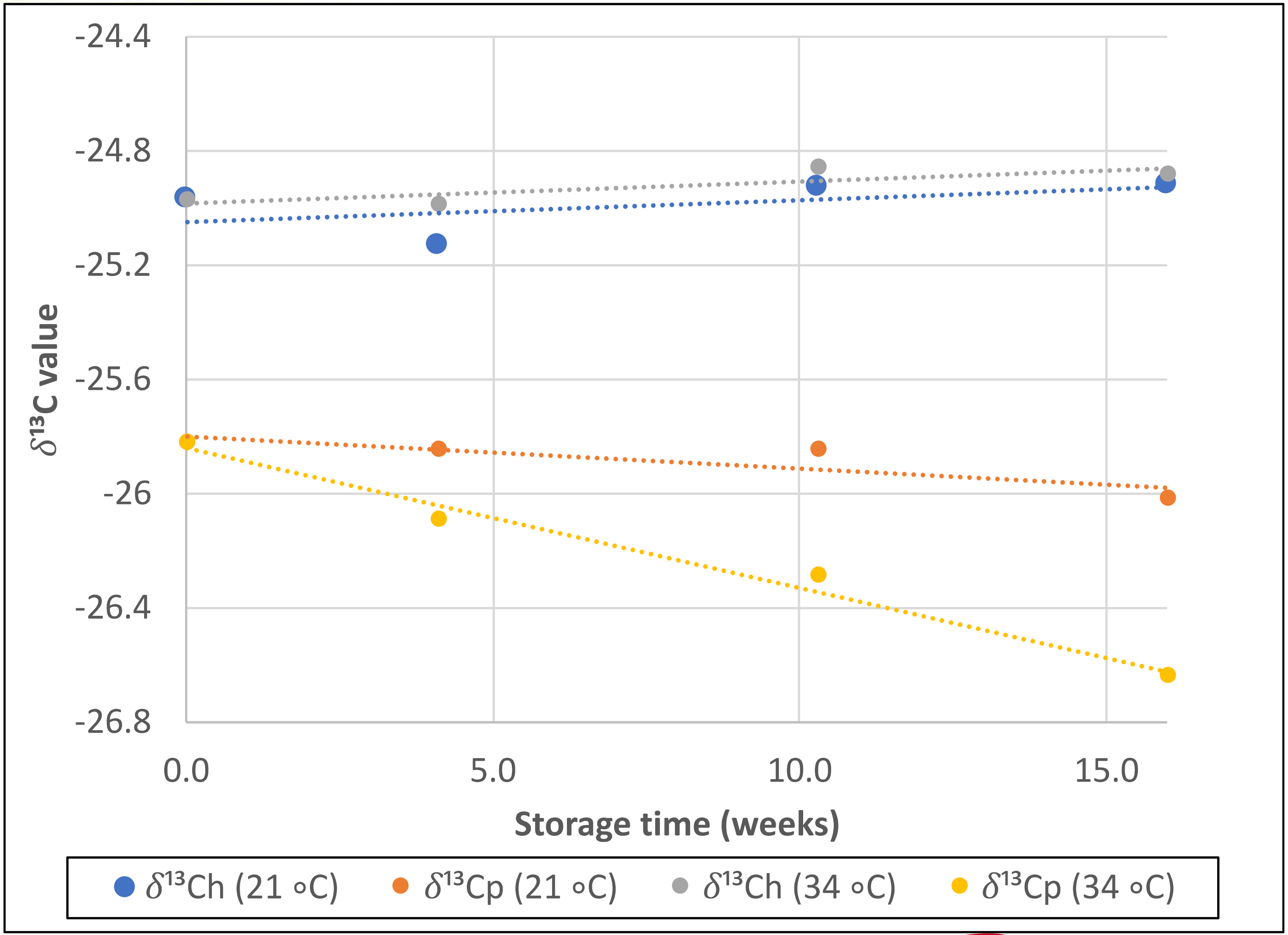
C3 plants: -33 to -24 ‰ e.g. manuka and all other melliferous plants

[2] $\delta^{13}C_{protein}$ shifts negatively
(this is what happens in some mānuka honeys)

Apparent C4 Sugar Levels During Storage



δ13C values, protein and honey



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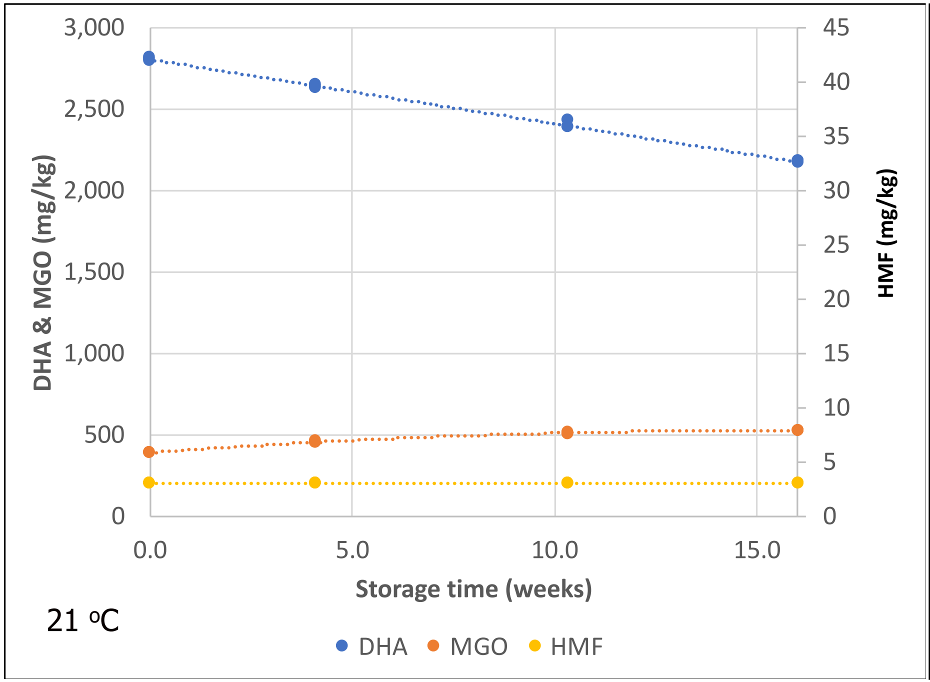
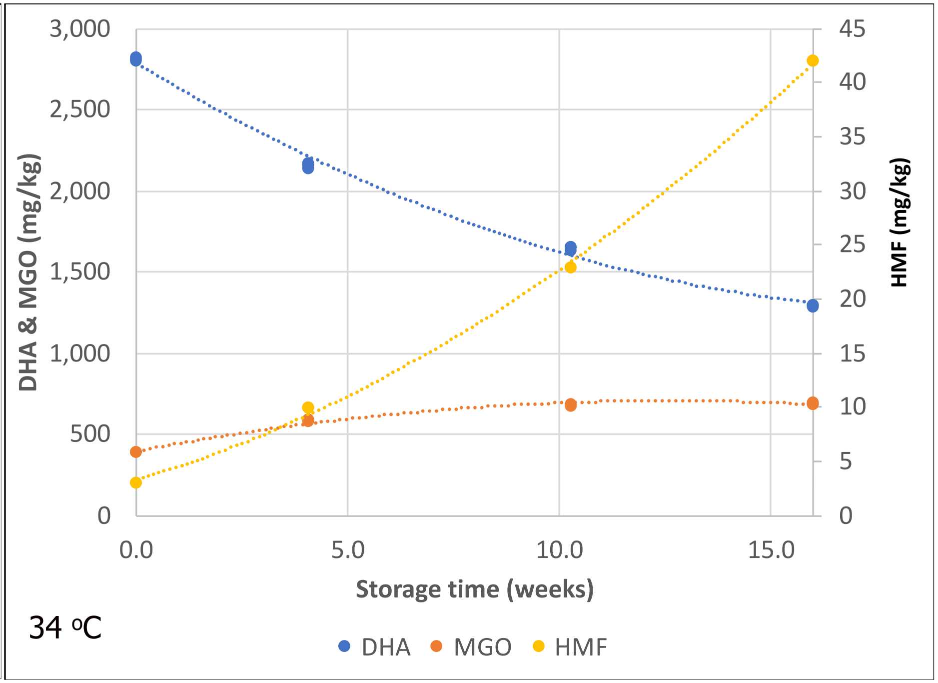
WHAT CAUSES THIS $\delta^{13}C_{PROTEIN}$ DECREASE ?

- Kinetics of methylglyoxal formation in mānuka honey
- Dihydroxyacetone (DHA) → Methylglyoxal (MGO)





There's a substance X, which binds to the protein thereby shifting its $\delta^{13}C$:
X + Protein → XProtein

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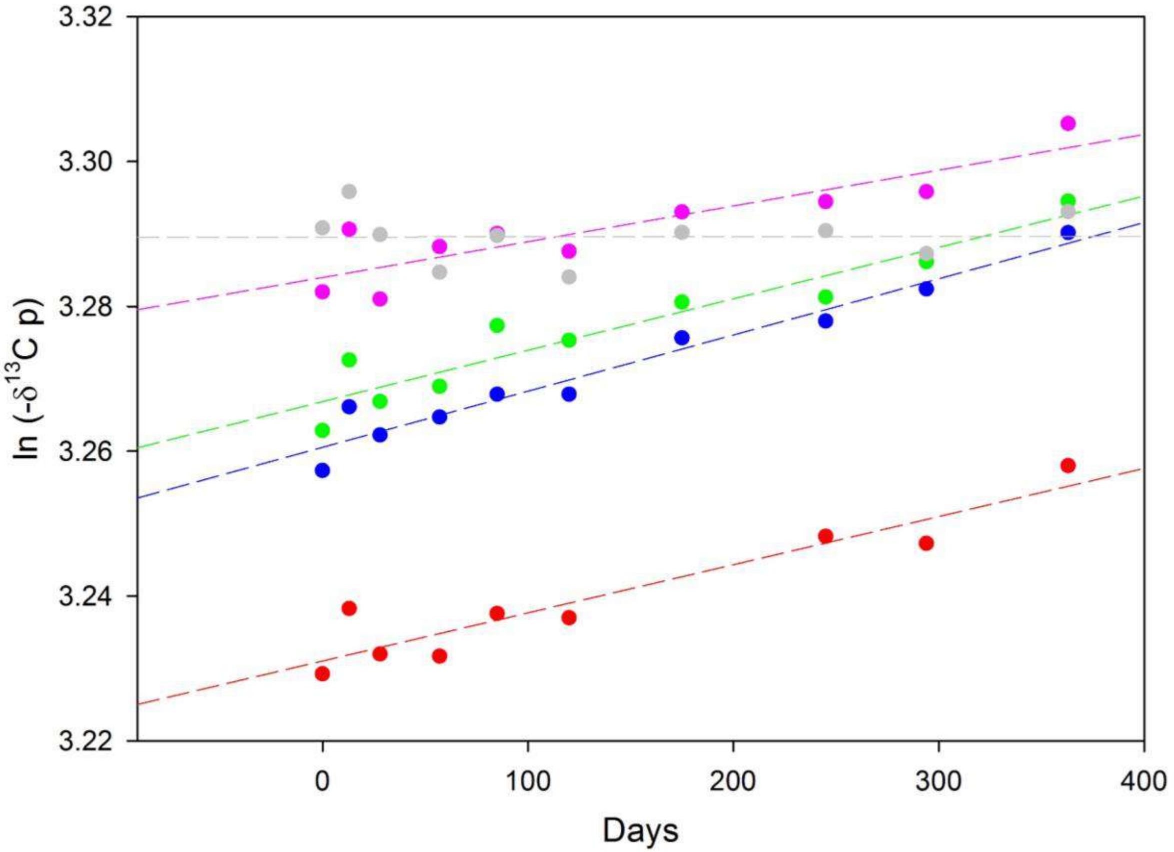
KINETIC MODEL DEVELOPMENT



ln(-δ^{13}C for protein) *vs.* time for four fresh Manuka honeys (coloured lines) and Non-Manuka (grey line).

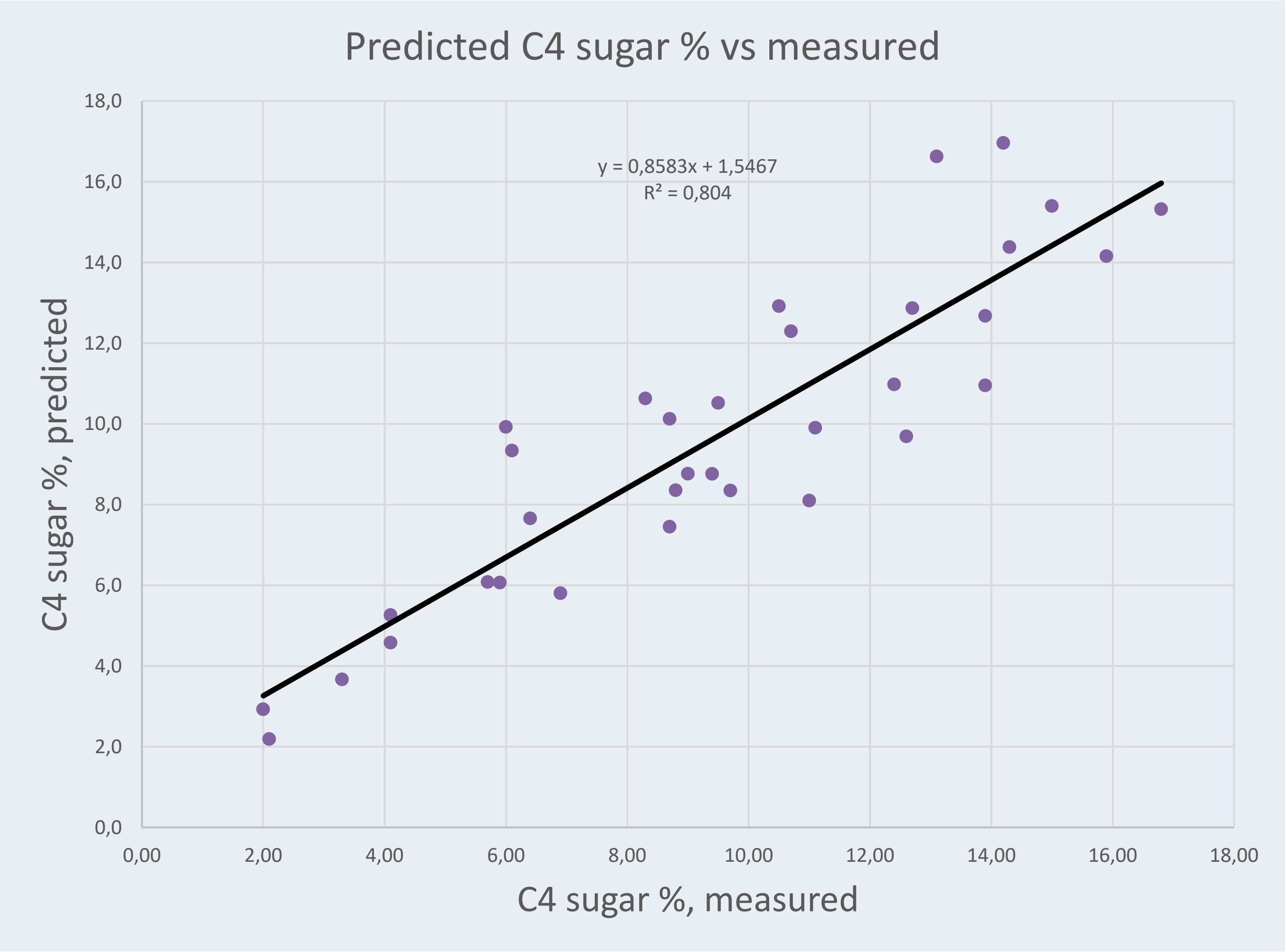
The coloured lines run almost parallel to each other, giving comparable slopes (kinetic constants at 27°C).

$$\delta^{13}C_p(t) = \delta^{13}C_p(0)\exp(-kt)$$

Where $\delta^{13}C_p(t)$ is $\delta^{13}C_p$ at time t; $\delta^{13}C_p(0)$ is the $\delta^{13}C_p$ measured at the beginning (t=0)

k is the kinetic constant for a given temperature.

PREDICTED VS MEASURED C4 SUGAR



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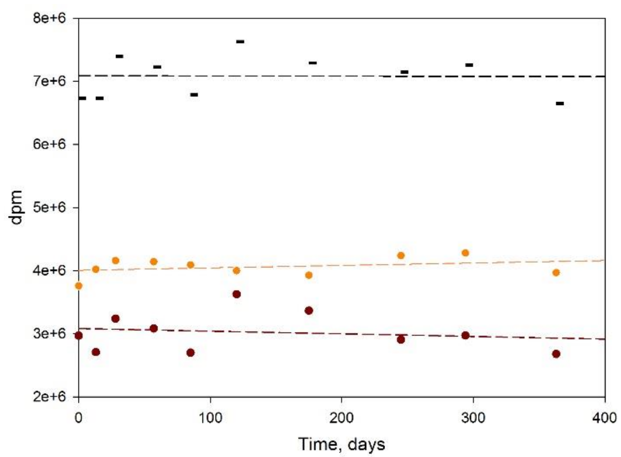
INTERACTION BETWEEN MGO AND PROTEIN

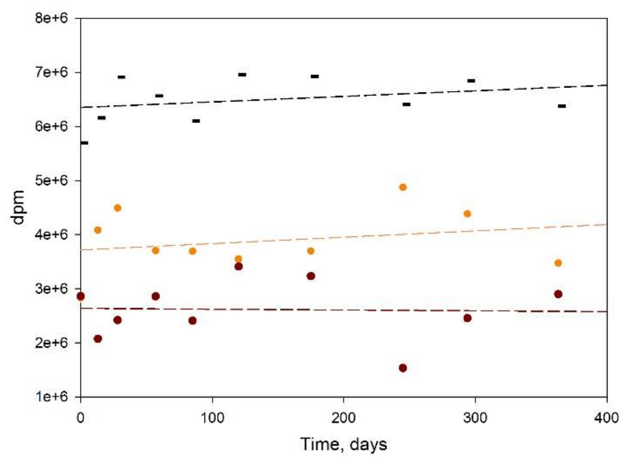
Experiment

- Incubated ^{14}C (radio label) DHA with:
  - Clover honey
  - Fresh mānuka honey
  - Aged mānuka honey
- Measure binding of ^{14}C to protein fraction during incubation

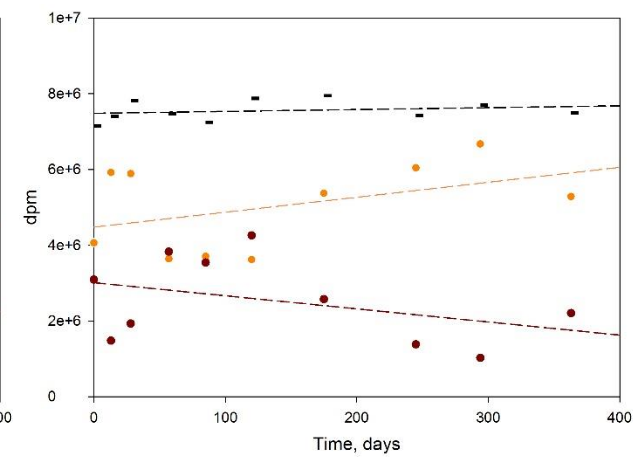
Change in scintillation counts (^{14}C) for honey, protein, and total over time



Clover



Fresh mānuka



Aged mānuka

- - - - - Honey
- - - - - Protein
- - - - - Total

^{14}C binding to protein

- ^{14}C label was incorporated into the protein fraction at high levels (25–43% of total), but its concentration did not change over time. Yet observed a continued decrease in $\delta^{13}C_p$ values (and thus increase in apparent C4 sugar levels) over the same incubation time period.

- If the binding of DHA or MGO was solely responsible for the increase in $\delta^{13}C_p$ values, we would expect the ^{14}C label incorporation into the protein to continue to increase at a similar rate as the change in $\delta^{13}C_p$ values, rather than an initial binding with no further change over time.

Conclusions

- (25-43%) binding of DHA-derived ^{14}C to the precipitated fraction of the AOAC protocol.
- This binding does not change over time.
- It is not clear if it happens in the honey during storage, or during the protein isolation related to the AOAC protocol.
- There is no certainty that the ^{14}C is bound to the protein or to non-proteinaceous organic material that is co-precipitated with protein, or both.

Conclusions

- The change in $\delta^{13}C_p$ to more negative values does actually happen in fresh, high quality mānuka honeys.
  - The rate of change, however, does not depend on the starting concentrations of DHA or MG (either natural, or artificially added).
- The observations above could be explained only by the binding of **yet unknown substance(s)** to the protein fraction in mānuka honeys. This substance must have very negative $\delta^{13}C$, and is gradually exhausted (so that in aged mānuka honeys, $\delta^{13}C_p$ does not change further).
- Using , $\delta^{13}C_{protein}$ as an internal standard is questionable for manuka honeys.

Alternative Test Methodology

Incubated manuka honey tested by the AOAC C4 method and NMR

	46168	46168		KK1893	KK1893		KK1904	KK1904		KK1903	KK1903
Time	2016	2019		2016	2019		2016	2019		2016	2019
Apparent C4 Sugar (%)	8.2	13.6		5.9	10.6		4.1	10.2		3.9	9.9
HMF	7.3	108.1		7.0	80.6		7.1	90.6		6.9	88.5
Pass/Fail ≤ 7.0%	✗	✗		✓	✗		✓	✗		✓	✗
Honey	-24.82	-24.70		-25.62	-25.72		-25.36	-25.16		-25.45	-25.51
Protein	-26.18	-27.06		-26.61	-27.61		-26.03	-26.91		-26.1	-27.24
NMR Analysis											
Variety Manuka	✓	✓		✓	✗		✓	✗		✓	✗
Detection of Sugar Syrups	✗	✗		✗	✗		✗	✓		✗	✗
Multivariate Verification	✓	✗		✓	✓		✓	✓		✓	✗

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Alternative interim solution

Standardizing a value for $\delta^{13}C_{protein}$

	46168	46168	KK1893	KK1893	KK1904	KK1904	KK1903	KK1903
Time	2016	2019	2016	2019	2016	2019	2016	2019
Apparent C4 Sugar (%)	8.2	13.6	5.9	10.6	4.1	10.2	3.9	9.9
HMF	7.3	108.1	7.0	80.6	7.1	90.6	6.9	88.5
Pass/Fail ≤ 7.0%	✗	✗	✓	✗	✓	✗	✓	✗
Honey	-24.82	-24.70	-25.62	-25.72	-25.36	-25.16	-25.45	-25.51
Protein	-26.18	-27.06	-26.61	-27.61	-26.03	-26.91	-26.1	-27.24

Honey	-24.82	-24.7	-25.62	-25.72	-25.36	-25.16	-25.45	-25.51
Protein (Standardised)	-26.2	-26.2	-26.2	-26.2	-26.2	-26.2	-26.2	-26.2
C4 sugar % (Adjusted)	8.4	9.1	3.5	2.9	5.1	6.3	4.5	4.2
Pass/Fail ≤ 7.0%	✗	✗	✓	✓	✓	✓	✓	✓

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- Study the reaction rates of protein binding to MGO and substance X;
- Refine chemical kinetics model to predict the change in C4 sugar levels due to methylglyoxal and "X"-protein binding;
- Understand the influence of pollen source on $\delta^{13}C$ protein values;
- Understand the variation of $\delta^{13}C_{honey}$ in different nectar sources in NZ;
- Modify the AOAC method to account for the influence of protein binding.

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