RESEARCH

THE C4 SUGAR TEST: AOAC VS SCREENING METHOD

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This article offers some details on the C4 sugar test and two screening methods.

Overview

The C4 sugar test has become increasingly common in recent years, as overseas countries have used it to check for honey that has been adulterated by adding sugar. This article provides some detail on how the test works, and shows why the less expensive screen method is more variable than the traditional (and more expensive) AOAC 998.12 method. Both the AOAC and screen methods have their place, and some examples are given of ways that beekeepers and processors may choose to use them. We also give some explanation about why C4 test results in some mānuka honey are prone to change over time.

What are C4 sugars?

It is now very common for honey to be tested for 'C4 sugars'—an internationally recognised test established by an organisation called AOAC in the late 1990s. The test was developed because honey was being adulterated by adding cheap sugar to expensive honeys as a way of making a 'quick buck'. You could argue that honey is already full of sugar, so there is nothing wrong with doing this.

However, the international standard on honey[1] (called Codex) explicitly states that "Honey sold as such shall not have added to it any food ingredient, including food additives, nor shall any other additions be made other than honey."

If a manufacturer honestly and officially admits that his honey contains certain additives (be that sugars, plant juices and extracts, vitamins, etc.) then it is not a honey anymore, but a honey product. For honey products there are no set limits on C4 sugars, and therefore the C4 sugar test (and many other honey tests) is not required.

The cheapest commercially available sugars (cane sugar and high-fructose corn syrup) are most commonly used in honey adulteration. They contain the same sugars as those found in genuine honey—sucrose, glucose, and fructose—and have the same taste.

Sugar cane and maize (the source of high-fructose corn syrup) are both tropical plants, and they produce their sugars via a slightly different biochemical pathway than that used by nectar-bearing plants. Sugar cane and maize use a process called C4 carbon fixation, whereas nectar-bearing plants use a C3 carbon fixation process. These different carbon fixation processes result in a different carbon isotopic composition in the sugars arising from them (C3 sugars have slightly less of the heavier carbon isotope ^{13}C than C4 sugars).

Modern instrumentation used in the C4 sugar test allows for reliable detection of this change in ^{13}C content, and from that we can estimate the amount of added C4 sugar.

The principles of C4 sugar determination

While it has been common for unscrupulous people to add sugar to honey, it is not common for them to also add pollen or other proteins to the honey. Therefore, the protein found in a honey is a good indication of the level of ^{13}C you would expect to find in a honey, and because of this it is used as an internal standard in the C4 sugar test against which to compare the level of ^{13}C in the whole honey.

For the C4 sugar test we extract the protein from a sub-sample of honey, and then measure the ^{13}C content of both the protein and a sub-sample of the honey itself. The isotopic composition is expressed using a delta (δ) notation, which is the deviation of ^{13}C concentration in the sample from that in the standard material, expressed in per mille (‰). Positive deltas indicate relative enrichment in ^{13}C, whereas negative deltas show depletion in ^{13}C. For example, $\delta^{13}C = -26.02$ means (approximately) that the sample contains 100-2.602 = 97.398% ^{13}C atoms of the standard.

The measured deltas are entered into a formula to estimate the percentage of C4 sugars:

$$\%C4\ sugars = \frac{\delta^{13}C_{protein} - \delta^{13}C_{honey}}{\delta^{13}C_{protein} - (-9.7)} \cdot 100\%$$

The numerator shows the difference in ^{13}C between bulk honey and the protein (the closer to zero the better); number -9.7 in the denominator is the average $\delta^{13}C$ for C4 sugars, as determined in the AOAC standard method[2].

Please note that **this is not a direct measurement of C4 sugars,** but is better referred to as a correlation method. Natural variation in the composition of protein can result in a difference between it and whole honey, which gives rise to an 'apparent' C4 sugar content ranging from -7 to 7%. This is considered acceptable by the AOAC standard. Here, 'apparent' means that the number in this

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Footnotes

1. Codex Standard for Honey. Codex Stan 12-19811. In: *Codex Alimentarius,* FAO, 2001.
2. Method 998.12. In: *Official Methods of Analysis of AOAC INTERNATIONAL,* 1999.

range does not actually show the percent of C4 sugars (effectively, there might be none). It is only when the result is above 7% that the method begins to produce reliable results. In other words, a honey with C4 sugar percent between -7 and 7% as measured using the AOAC C4 sugar method is graded as not being adulterated with C4 sugars.

A common reason for the fluctuations in ^{13}C content of the protein is the presence of pollen from a number of plant species in the honey, especially in multifloral honeys. While the formula can produce negative results, these tend to be reported as zero to the end user (unless, of course, the magnitude of it exceeds 7%).

C4 testing of mānuka honey

Many beekeepers and processors are aware that mānuka honey is prone to increase its 'apparent' C4 sugar test results over time. When we review the actual test results of honey affected by this, we tend to see a negative shift in $\delta^{13}C$ for protein, which must be caused by a reaction of the protein with something else found in the honey.

In contrast, the $\delta^{13}C$ for bulk honey in such 'false positives' does not change over time. Analytica is currently carrying out some research to identify this substance, which may allow the industry to advocate for a modification to the AOAC 998.12 method for mānuka honeys.

The difference between the AOAC and screening method

At Analytica we have two options for C4 sugar testing: one is the AOAC method, and the other is a screening method. There are two major differences between them:

1. the screening test is a lot cheaper; and
2. the screening test produces results which are a lot more variable. This variability is illustrated in Figure 1.

Sample preparation is the same for both methods. The difference is the number of repeats we do for each honey and protein. For the AOAC method, we analyse two repeats of protein and honey for each sample, whereas for screening we do only one of each. This results in a loss of precision for the $\delta^{13}C$ determination (see the two graphs in the upper panel on Figure 1). Also, we lose the opportunity to compare results of repeat tests of the same sample.

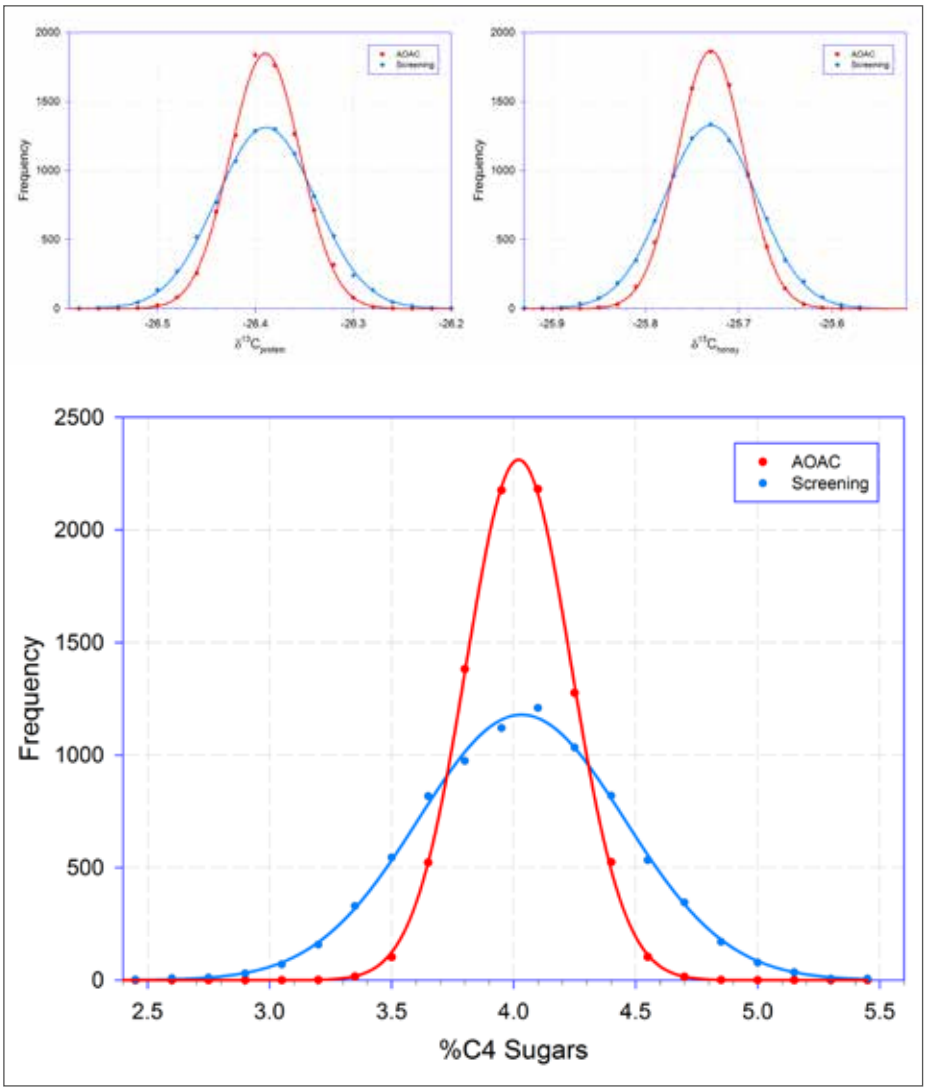

Figure 1. The distribution of $\delta^{13}C$ values for protein and honey (top) and the %C4 sugar (bottom) for AOAC (red) and screening tests.

This loss of precision on the determination of deltas in protein and honey does not seem big; however, because the measurements are independent of each other, any statistical error in them adds together. By combining the protein and honey results in a formula to calculate a C4 sugar value, we see quite an increase in the range of possible C4 sugars results for the same sample (Figure 1, bottom). For the AOAC method the standard deviation is 0.2%, while for the screening method it is 0.4%. This effect is known as 'error propagation' and cannot be avoided, especially in correlation methods like the C4 sugar testing.

Put another way, if a honey has a true result of 4.0% C4 sugars, and is tested many times using the AOAC method, we would expect 95% of results to fall in a range of 3.6 to 4.4%. By comparison, if tested using the screening method, 95% of the results would fall in the range of 3.2 to 4.8%.

When to use the AOAC or screening tests

Despite being more variable, the screening method is useful when a possible error can be tolerated. Examples include:

- estimating the C4 sugar result of multiple drums being considered for purchase or processing (it is better to have some idea about their quality than no idea at all)
- routine processing quality control
- research and development work, if the results are for internal use only and not planned to be published.

The AOAC method is recommended for testing honey where greater precision or accuracy in the test result is important. A good example is testing of final packed retail product, especially if the honey is going to be exported to countries that have a particular interest in C4 sugar levels measured using the AOAC method, like China or the USA.