FOOD COMPOSITION AND ADDITIVES

Modification of AOAC *Official Method*℠ 998.12 to Add Filtration and/or Centrifugation: Interlaboratory Comparison Exercise

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An international ring test was undertaken in 2012 among 10 international honey testing laboratories to examine the effects of filtration and/or centrifugation addition to AOAC 998.12 method (C4 sugar detection in honey). During protein extraction, when using the repetitive washing method, any insoluble material (i.e., pollen, dust) is coextracted along with protein which may result in contamination of the protein isotope value and result in a false-positive test. A modification step involving filtration and/or centrifugation to remove insoluble material before protein flocculation was proposed. Results were compared across 10 laboratories internationally and were found to be an excellent assessment of interlaboratory variability with the standard variances between laboratories better than ±0.2‰ for honey and ±0.3‰ for protein.

An interlaboratory ring test exercise was organized to evaluate the issue of false-positive failures with New Zealand Manuka honey. The ring test examined the suitability of the internationally applied AOAC Method **998.12** (1) and proposed modifications of this method. AOAC Method **998.12** determines the presence of C4 (corn syrup or cane sugars) in honey by comparing the carbon isotopic value of whole honey with its internal protein. Most analytical laboratories use the repetitive washing option of this AOAC method, developed by White (2). However, it has been found that this method may falsely indicate the presence of C4 sugars in certain honey, such as New Zealand Manuka, due to high pollen content and/or other insoluble material that is coextracted with the protein (3, 4). Filtration and/or centrifugation are proposed as modifications to the method to assure accurate determination of C4 sugars by removing insoluble material before protein precipitation. The ring test was announced in December 2011, and 10 laboratories were invited to participate by the study coordinator Jo Marie Cook. All 10 laboratories agreed to take part (*see* Appendix 5 for participants, on *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac). Study codirectors Dana Krueger, Karyne Rogers, and Klaus Beckmann, provided five honey samples for the study, and these were distributed to participants in January 2012, along with written instructions to ensure consistent methodologies among laboratories. By the end of April 2012, all laboratories had reported their results, which were analyzed statistically, and AOAC Method **998.12** performance on Manuka and other honeys was investigated, as well as the significance of filtration and centrifugation modifications to the method. The results are described below.

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Sample Selection

Five honey samples were acquired for the exercise. Two samples obtained from New Zealand were Manuka honey. Two were obtained from the United States, one of which was a pure commercial clover honey while the other was a blend of this honey with 15% corn syrup (HFCS 55) to provide an adulterated sample. One further sample was from Germany and was a pure monofloral Romanian rape honey (Table 1). Honeys 1 and 2 (Manuka) were selected because they had previously failed AOAC Method **998.12**, although they were purported to be genuine. Honeys 4 and 5 were selected as genuine honey, and had previously passed AOAC Method **998.12**. Honey 3 was intentionally adulterated and failed this method, as expected.

Sample Analysis

The five honey samples were received by Cook in December 2011 and were treated in a double blind scenario. Each sample was split into six subsamples and assigned a random code corresponding to a letter of the alphabet (Table 2). Duplicate subsamples were sent to each laboratory in three groups, one for each of the intended treatment, according to the methods outlined in the analytical instructions (*see* Appendixes 2–4 and 7). The data key was only released to participants once all samples had been reported in full from all laboratories.

Method 1 consisted of treatment via the official AOAC Method **998.12**, and laboratories could use either the repetitive

Table 1. Samples used in the interlaboratory ring test exercise

Honey sample	Type	Location	Information
1	Manuka 1	New Zealand	B17094683, MFG: 2011-02-22
2	Manuka 2	New Zealand	B17119575, MFG: 2011-05-11
3	Honey with 15% C4 sugar	U.S.	Honey No. 4 blended with 85:15 high-fructose corn sweetener (C4)
4	Clover honey	U.S.	North American commercial clover honey
5	Romanian rape	Germany	More than 80% rape pollen, pure monofloral honey

wash or dialysis method. Method 2 used a filtration pretreatment before protein precipitation, which involved filtering the diluted sample through a 10 µm filter prior to analysis via AOAC Method **998.12** to remove fine insoluble material, such as pollen. Method 3 had an additional pretreatment step of a 5 min centrifugation at 3500 rpm prior to filtration and subsequent analysis via AOAC Method **998.12**. The centrifuged solution was decanted off onto a filter paper leaving any extracted solid sludge remaining at the base of the centrifuge tube.

Evaluation of the Reported Data

Four data points were reported by each laboratory for each sample: (*1*) average carbon isotope ($\delta^{13}C$) value (‰) whole honey; (*2*) average $\delta^{13}C$ value (‰) protein extract; (*3*) difference (honey–protein extract, ‰; and (*4*) C4 sugars (%).

Collaborators were asked to supply any modification made to AOAC Method **998.12** other than those specified in Appendixes 2–4. Excel data reporting sheets were e-mailed to the collaborators. Data were reported to Cook by each laboratory, who in turn released the final results to the three codirectors of the study (Krueger, Rogers, and Beckmann). A summary showing each laboratory's results for the five honey samples and three test methods, including their corresponding duplicate results, can be found in Table 3. Results annotated with superscripts in Table 3 represent critical outliers, where a represents critical values for the Grubbs test and b represents critical values for the Cochran test.

With modern instrumentation and careful analytical handling, laboratories are capable of reporting SDs of $\delta^{13}C$ values to within ±0.2‰. Laboratories did not show any significant critical values for Cochran or Grubbs tests for Method 1 (AOAC Method **998.12**), suggesting that all laboratories are performing this test competently. Fourteen significant Cochran or Grubbs test variances did occur for Methods 2 and 3 (out of 200 data pairs) for Laboratories 2, 3, 5, 6, and 8. This suggests that some of the laboratories had analytical accuracy or precision issues with some of the honey and/or protein samples. The protein variance could be explained due to technical variation in performing the new steps (filtration and centrifugation) added in Methods 2 and 3 (Appendixes 3 and 4).

Basic statistics calculated from the 10 participating laboratories are shown in Table 4, where all data (including outliers) have been analyzed. In general, all of the whole honeys had SD values within the expected ±0.2‰ range. Honey 5, Sample A, Method 3 and Honey 3, Sample B, Method 3 had higher-than-expected SD values (0.4 and 0.3‰) due to single outliers outside the expected range for these honeys. The outliers arose from either sample handling errors, including data reporting errors or a one-off poor analytical sample precision and accuracy of the specific sample. The statistical method performance of the three methods is summarized in Table 5.

Averages and SDs of the protein isotope ratios measured by the 10 laboratories are shown in Table 6, where all data (including outliers) have been analyzed. In general, the protein had larger SDs than the whole honey, and ranged between 0.2 and 0.7‰. The Manuka honey proteins (Proteins 1A, 1B, 2A, and 2B) and Romanian rape honey proteins (Proteins 5A and 5B) had higher SDs for Methods 2 and 3 than U.S. honey Proteins 3 and 4. Manuka honey Protein 2B, Method 2 had the highest SD of 0.7‰ due to a single reported value lying significantly outside the expected range, while Proteins 1A and B, Methods 2 and 3, Protein 2A, Method 3, and Proteins 5A and B, Method 3 also had higher-than-expected values (up to 0.6‰) due to outliers.

The five honeys were analyzed by 10 laboratories in duplicate (A and B) according to three methods outlined in the instructions supplied to each laboratory (Appendixes 2–4). Honey 1, Samples 2 and 3, when analyzed using the official AOAC Method **998.12** (Method 1), failed this test, yielding apparent C4 (corn syrup or cane) sugars between 8.6 and 11.2% (Table 7). The highest level of C4 sugars was found for Honey 3 (11.2 and 10.7%, respectively), which had been intentionally adulterated with 15% corn syrup. The C4 sugar level of Honey 3 calculated in Method 1 was not as high as the actual adulteration, but this is compensated by its addition to a honey with C4 sugar values of around –3%, which would compensate the 15% adulteration to be around 12% C4 sugar (the value determined by its analysis in Method 1). Honeys 4 and 5 had C4 sugar values that are negative due to the whole honey having a more negative value than the protein, suggesting the protein content of the bee food source at the time of nectar collection was isotopically more positive than the whole honey. Such results are reported as 0% C4 sugars in AOAC method.

When filtration or filtration and centrifugation (Methods 2 or 3) are applied to each honey, the protein isotope values become more positive than in Method 1. Method 3 has more positive protein values than Method 2, suggesting that the modifications of Method 3 (filtration and centrifuge) had a larger effect on each honey than Method 2:

$$\Delta(\text{protein–whole honey})\ \text{Method 1} > \text{Method 2} > \text{Method 3}$$

Table 2. Data key of the 30 samples provided to each laboratory

Method	Honey	1	2	3	4	5
1	Untreated A	C	X	A	Y	Q
1	Untreated B	CC	D	J	T	P
2	Filtered A	N	Z	H	V	O
2	Filtered B	K	I	F	AA	M
3	Centrifuged A	R	W	U	B	G
3	Centrifuged B	E	S	L	DD	BB

Table 3. Data reported by 10 laboratories

		Method 1				Method 2				Method 3			
	Fraction	Honey		Protein		Honey		Protein		Honey		Protein	
Lab	Split	A	B	A	B	A	B	A	B	A	B	A	B
							Sample 1						
1	1	–24.90	–25.00	–26.40	–26.40	–25.00	–24.90	–26.50	–26.30	–24.90	–25.00	–26.00	–25.90
2	1	–25.09	–25.07	–26.37	–26.73	–25.06	–25.10	–27.83[a]	–27.92[a]	–25.10	–25.12	–27.75[a]	–27.90[a]
3	1	–24.78	–24.75	–26.23	–26.26	–24.97	–24.77	–25.98	–26.22	–24.89	–24.90	–26.02	–26.02
4	1	–25.06	–25.04	–26.45	–27.24	–25.05	–25.10	–26.36	–26.25	–25.16	–25.14	–26.09	–26.11
5	1	–25.18	–25.30	–26.63	–26.41	–25.04	–24.94	–26.25	–26.22	–25.35	–24.96	–26.44	–26.29
6	1	–25.20	–25.20	–26.40	–26.50	–25.40[a]	–25.50[a]	–26.00	–26.10	–24.90	–25.20	–26.30[b]	–25.80[b]
7	1	–24.71	–24.95	–26.54	–26.37	–24.90	–24.95	–25.90	–26.19	–25.11	–24.97	–26.04	–25.85
8	1	–24.88	–24.77	–25.92	–26.24	–24.84	–24.86	–25.69	–25.68	–24.77	–24.82	–25.76	–25.73
9	1	–25.00	–25.00	–26.70	–26.80	–25.00	–25.10	–26.40	–26.30	–25.00	–24.90	–26.30	–26.30
10	1	–25.00	–25.00	–26.60	–26.70	–25.00	–24.90	–26.10	–26.10	–25.10	–24.90	–26.30	–26.20
							Sample 2						
1	2	–25.00	–25.00	–26.40	–26.40	–25.00	–24.90	–26.20	–26.30	–24.80	–25.00	–26.00	–26.30
2	2	–24.95	–24.93	–26.40	–26.41	–24.94	–25.10	–26.20[b]	–28.09[b]	–25.00	–25.00	–27.47[a]	–26.62[a]
3	2	–24.74	–24.86	–26.15	–26.01	–24.76	–24.70	–26.00	–26.05	–24.78	–24.80	–25.95	–25.96
4	2	–24.92	–24.93	–26.26	–26.36	–24.92	–25.03	–26.24	–26.22	–25.01	–24.99	–26.07	–26.05
5	2	–25.02	–25.02	–26.64	–26.50	–24.88	–24.78	–26.36	–26.10	–24.84	–24.89	–26.01	–25.80
6	2	–25.00	–25.00	–26.50	–26.00	–25.10	–25.30	–26.20	–26.60	–25.20	–25.20	–26.00	–26.20
7	2	–24.79	–24.81	–26.49	–26.30	–24.82	–24.83	–26.14	–26.46	–24.86	–24.82	–25.75	–25.90
8	2	–24.74	–24.69	–26.16	–25.87	–24.67	–24.63	–25.51[a]	–25.54[a]	–24.66	–24.68	–25.69	–25.71
9	2	–24.83	–24.96	–26.80	–26.80	–25.00	–25.00	–26.60	–26.30	–24.90	–24.80	–26.20	–26.10
10	2	–24.90	–24.90	–26.40	–26.50	–24.80	–24.80	–25.90	–26.20	–24.80	–24.90	–26.20	–26.20
							Sample 3						
1	3	–24.30	–24.20	–26.20	–26.10	–24.40	–24.40	–26.40	–26.10	–24.30	–24.30	–25.20	–25.00
2	3	–24.29	–24.27	–26.31	–26.07	–24.41	–24.37	–25.91	–25.60	–24.43[b]	–25.13[b]	–25.63	–25.64
3	3	–24.09	–24.15	–25.71	–25.68	–24.25	–24.30	–25.70	–25.66	–24.19	–24.22	–25.43	–25.43
4	3	–24.35	–24.36	–26.07	–25.94	–24.34	–24.37	–25.97	–26.00	–24.45	–24.40	–25.39	–25.48
5	3	–24.17	–24.51	–26.17	–26.50	–24.16	–24.16	–26.03	–26.05	–24.34	–24.15	–25.75	–25.59
6	3	–24.50	–24.60	–26.20	–25.90	–24.50	–24.50	–25.60	–25.70	–25.00[b]	–24.60[b]	–25.50	–25.80
7	3	–24.11	–24.01	–26.02	–26.01	–24.24	–24.26	–25.82	–25.49	–24.32	–24.31	–24.94	–24.95
8	3	–24.12	–24.12	–25.82	–25.85	–24.12	–24.17	–25.35	–25.31	–24.17	–24.08	–25.35	–25.25
9	3	–24.20	–24.40	–26.20	–26.30	–24.30	–24.30	–26.00	–26.10	–24.20	–24.30	–26.00	–26.10
10	3	–24.30	–24.30	–26.00	–26.10	–24.20	–24.30	–25.60	–25.70	–24.20	–24.30	–25.80	–25.80
							Sample 4						
1	4	–26.40	–26.50	–26.00	–26.00	–26.40	–26.50	–26.00	–26.50	–26.30	–26.40	–25.30	–24.90
2	4	–26.56	–26.54	–26.25	–26.16	–26.48	–26.51	–25.49	–25.47	–26.44	–26.52	–25.65	–25.58
3	4	–26.36	–26.23	–25.63	–25.55	–26.22	–26.24	–25.67	–25.84	–26.18	–26.26	–24.59[b]	–25.30[b]
4	4	–26.55	–26.55	–25.93	–25.91	–26.58	–26.60	–25.89	–25.95	–26.67	–26.59	–25.32	–25.30
5	4	–26.45	–26.26	–26.26	–26.29	–26.38	–26.36	–25.72	–25.59	–26.40	–26.34	–25.33	–25.39
6	4	–26.70	–26.60	–25.80	–26.20	–26.90[a]	–27.10[a]	–25.80	–26.10	–26.80	–26.70	–25.60	–25.50
7	4	–26.29	–26.36	–25.64	–25.91	–26.42	–26.40	–25.91	–25.67	–26.41	–26.38	–24.96	–24.84
8	4	–26.30	–26.22	–25.83	–25.77	–26.24	–26.26	–24.93	–24.86	–26.22	–26.22	–24.91	–24.99
9	4	–26.40	–26.30	–26.30	–26.40	–26.50	–26.40	–25.90	–26.00	–26.40	–26.40	–25.80	–26.00
10	4	–26.50	–26.50	–26.10	–26.10	–26.50	–26.50	–25.90	–25.80	–26.60	–26.60	–25.90	–25.80

Table 3. (*continued*)

		Method 1				Method 2				Method 3			
	Fraction	Honey		Protein		Honey		Protein		Honey		Protein	
Lab	Split	A	B	A	B	A	B	A	B	A	B	A	B
						Sample 5							
1	5	–26.50	–26.50	–26.20	–26.20	–26.50	–26.70	–26.20	–26.10	–26.40	–26.40	–24.80	–24.70
2	5	–26.61	–26.58	–26.43	–26.37	–26.42	–26.56	–25.43	–25.35	–26.59	–26.51	–25.27	–25.32
3	5	–26.34	–26.39	–25.60	–25.70	–26.40	–26.20	–25.79	–25.63	–26.41	–26.36	–24.43	–24.60
4	5	–26.61	–26.59	–26.20	–26.29	–26.65	–26.64	–25.85	–26.01	–26.76	–26.65	–25.21	–25.15
5	5	–26.28	–26.33	–26.50	–26.25	–26.37	–26.35	–25.31	–25.26	–25.52[b]	–26.32[b]	–25.62	–25.30
6	5	–26.60	–26.70	–26.40	–26.10	–26.20[b]	–26.90[b]	–26.10	–26.10	–26.70	–26.70	–25.70[b]	–24.80[b]
7	5	–26.43	–26.37	–26.04	–26.22	–26.48	–26.50	–26.00	–25.96	–26.50	–26.45	–24.94	–24.82
8	5	–26.35	–26.39	–26.08	–25.95	–26.39	–26.36	–25.24	–25.21	–26.35	–26.33	–24.91	–25.06
9	5	–26.60	–26.60	–26.50	–26.60	–26.60	–26.60	–26.00	–26.10	–26.40	–26.60	–26.10	–26.10
10	5	–26.70	–26.60	–26.20	–26.00	–26.50	–26.70	–25.90	–25.80	–26.60	–26.60	–25.80	–25.90

[a] Significant Grubbs test for interlaboratory repeatability.

[b] Significant Cochran test for within-laboratory repeatability.

Overall, it is noted that the relative protein difference between Method 1 C4 sugar values and Method 3 values are smaller for Honeys 1 and 2 (between 0.3 and 0.6%) than for Honeys 3, 4, and 5 (between 0.5 and 1.1%), suggesting that filtration and/or centrifugation had a larger effect on the resultant C4 sugar values of the non-Manuka samples.

One of the goals of this study was to investigate the contribution of insoluble material such as pollen that could be coextracted with protein. To achieve this goal, filtration and/or centrifugation were proposed. To remove pollen it is necessary to use suitable filter paper with the correct retention pore size. Many pollens commonly found in honey have a grain diameter greater than 30 µm; however, Manuka pollen is much smaller and ranges from 10 to 15 µm in diameter. We tested the use of 10 µm filter paper as specified in Method 2, which has a retention pore size compatible with Manuka pollen. We do note that it might be possible that the triangular-shaped Manuka grain could deform under filtration and pass through 10 µm filter pores. Method 2 specified a 10 µm filter paper, and Method 3 a 1 µm filter paper (which would provide improved retention of Manuka pollen).

An assessment of filter papers used by each laboratory was undertaken (Table 8). The filter papers used for Method 2 have a pore retention size ranging between 0.7 to >11 µm. We note that six laboratories used filter papers with retention pores <8 µm for Method 2, while nine used around 1 µm filter papers for Method 3. Protein-honey differences reported in Table 7 show there is only a small decrease seen between Methods 1, 2, and 3 for Manuka samples (Honeys 1 and 2). Larger differences are seen for Honeys 3, 4, and 5.

Laboratories 1, 3, 4, 6, 7, and 10 used filter papers with larger (>6 µm) pore retention in Method 2 than in Method 3. Laboratories 1, 3, 4, and 7 reported that protein from Honeys 3, 4, and 5 (clover and rape honey) were more negative in Method 2 than Method 3, suggesting that more insoluble material (primarily pollen) was removed when smaller pore retention

Table 4. Summary of averages and SDs of raw honey data and method

		Method 1 AOAC **998.12**		Method 2 Filtered AOAC **998.12**			Method 3 Filtered + centrifuged AOAC **998.12**		
	Sample	Average	SD	Average	Difference compared to Method 1	SD	Average	Difference compared to Method 2	SD
1	Manuka 1A	–25.0	0.2	–25.0	0.0	0.2	–25.0	0.0	0.2
	Manuka 1B	–25.0	0.2	–25.0	0.0	0.2	–25.0	0.0	0.1
2	Manuka 2A	–24.9	0.1	–24.9	0.0	0.1	–24.9	0.0	0.2
	Manuka 2B	–24.9	0.1	–24.9	0.0	0.2	–24.9	0.0	0.2
3	U.S. with 15% C4A	–24.2	0.1	–24.3	–0.1	0.1	–24.4	–0.1	0.3
	U.S. with 15% C4B	–24.3	0.2	–24.3	0.0	0.1	–24.4	–0.1	0.3
4	U.S. pure A	–26.5	0.1	–26.5	0.1	0.2	–26.4	0.0	0.2
	U.S. pure B	–26.4	0.2	–26.4	–0.1	0.2	–26.4	0.0	0.2
5	Romanian rape honey A	–26.5	0.1	–26.5	0.0	0.1	–26.4	0.0	0.4
	Romanian rape honey B	–26.5	0.1	–26.6	–0.1	0.2	–26.5	0.0	0.1

Table 5. Statistical method performance

	All data				With outliers removed			
Sample	Mean	s_r[a]	s_R[b]	N[c]	Mean	s_r	s_R	N
				Method 1				
Total SIRA[d]								
1	–24.99	0.069	0.165	10	–24.99	0.069	0.165	10
2	–24.90	0.042	0.105	10	–24.90	0.042	0.105	10
3	–24.27	0.097	0.158	10	–24.27	0.097	0.158	10
4	–26.42	0.069	0.139	10	–26.42	0.069	0.139	10
5	–26.50	0.040	0.135	10	–26.50	0.040	0.135	10
Protein SIRA								
1	–26.49	0.22	0.275	10	–26.49	0.22	0.275	10
2	–26.37	0.146	0.249	10	–26.37	0.146	0.249	10
3	–26.06	0.124	0.209	10	–26.06	0.124	0.209	10
4	–26.00	0.114	0.253	10	–26.00	0.114	0.253	10
5	–26.19	0.117	0.260	10	–26.19	0.117	0.260	10
				Method 2				
Total SIRA								
1	–25.02	0.070	0.178	10	–24.97	0.070	0.098	9
2	–24.90	0.072	0.169	10	–24.90	0.072	0.169	10
3	–24.30	0.030	0.113	10	–24.30	0.030	0.113	10
4	–26.47	0.056	0.219	10	–26.42	0.036	0.120	9(6)
5	–26.50	0.178	0.178	10	–26.50	0.178	0.178	10
Protein SIRA								
1	–26.31	0.106	0.590	10	–26.14	0.109	0.230	9(2)
2	–26.26	0.452	0.517	10	–26.24	0.181	0.196	8(2,8)
3	–25.80	0.128	0.286	10	–25.80	0.128	0.286	10
4	–25.75	0.154	0.379	10	–25.75	0.154	0.379	10
5	–25.77	0.068	0.351	10	–25.77	0.068	0.351	10
				Method 3				
Total SIRA								
1	–25.01	0.127	0.146	10	–25.01	0.127	0.146	10
2	–24.90	0.057	0.149	10	–24.90	0.057	0.149	10
3	–24.37	0.189	0.207	10	–24.26	0.065	0.099	8(2,6)
4	–26.44	0.047	0.177	10	–26.44	0.047	0.177	10
5	–26.46	0.188	0.263	10	–26.52	0.060	0.139	9(5)
Protein SIRA								
1	–26.26	0.133	0.590	10	–26.08	0.071	0.216	8(2,6)
2	–26.11	0.216	0.396	10	–26.00	0.107	0.186	9(2)
3	–25.50	0.096	0.337	10	–25.50	0.096	0.337	10
4	–25.35	0.194	0.398	10	–25.39	0.118	0.376	9(3)
5	–25.23	0.224	0.511	10	–25.22	0.104	0.521	9(6)

[a] s_r = Standard deviation of repeatability (uncertainty under repeatable conditions) or minimum uncertainty in the measurement

[b] s_R = Standard deviation of reproducibility (uncertainty under reproducible conditions) or maximum uncertainty in the measurement

[c] N = Number of samples

[d] SIRA = Stable Isotope Ratio Analysis .

Table 6. Summary of averages and SDs of raw protein data and method

		Method 1 AOAC **998.12**		Method 2 Filtered AOAC **998.12**			Method 3 Filtered + centrifuged AOAC **998.12**		
	Sample	Average	SD	Average	Difference compared to Method 1	SD	Average	Difference compared to Method 2	SD
1	Manuka 1A	–26.4	0.2	–26.3	0.1	0.6	–26.3	0.0	0.6
	Manuka 1B	–26.6	0.3	–26.3	0.3	0.6	–26.2	0.1	0.6
2	Manuka 2A	–26.4	0.2	–26.1	0.3	0.3	–26.1	0.0	0.5
	Manuka 2B	–26.3	0.3	–26.4	–0.1	0.7	–26.1	0.3	0.3
3	U.S. with 15% C4A	–26.1	0.2	–25.8	0.3	0.3	–25.5	0.3	0.3
	U.S. with 15% C4B	–26.1	0.2	–25.8	0.3	0.3	–25.5	0.3	0.4
4	U.S. pure A	–26.0	0.3	–25.7	0.3	0.3	–25.3	0.4	0.4
	U.S. pure B	–26.0	0.3	–25.8	0.2	0.4	–25.4	0.4	0.4
5	Romanian rape honey A	–26.2	0.3	–25.8	0.4	0.3	–25.3	0.5	0.5
	Romanian rape honey B	–26.2	0.3	–25.8	0.4	0.4	–25.2	0.6	0.5

filter paper is used in Method 3. Although Laboratories 6 and 10 reported that they also used a filter paper with larger retention size in Method 2, there was no significant difference between the protein isotope values of Methods 2 and 3. Laboratory 10 used the same larger pore retention size (6 μm) for Methods 2 and 3. As would be expected, there was very little change in protein isotope values for all honey samples. Although Laboratory 6 used an 11 μm pore retention filter paper for Method 2 and a Whatman 934-AH glass fiber filter comparable to Whatman GF/C (around 1.5 μm) for Method 3, there was no significant difference between protein isotope values from Honeys 3, 4, and 5 using Methods 2 or 3, as previously seen in Laboratories 1, 3, 4, and 7. Laboratories 2, 5, 8, and 9 used filter papers with small (around 1 μm) pore retention sizes for both Methods 2 and 3, and no significant difference was found between proteins from Methods 2 and 3.

It is interesting to note that Honeys 1 and 2 (Manuka) showed only small variations in protein isotope values between Methods 2 and 3 regardless of the filter pore retention size used. Difference in protein isotope values for Honeys 3, 4, and 5 show that in Method 2 (contrary to Method 3) there must be larger insoluble particles (including pollen) that affect the protein composition isotopic value. This effect is understandable given the discrimination between most non-Manuka pollen (around 30–100 μm) versus Manuka pollen, which is quite small (around 7–15 μm). Under vacuum filtration, it is possible that Manuka pollen would pass through filter papers with a larger retention pore size, explaining the minimal changes between Methods 2 and 3 found by all laboratories.

Discussion of the Effects of Method Modifications on Pass/Fail Results

We compared the effectiveness of the three methods to authenticate the ring test honeys. Three honeys (1, 2, and 3) failed Method 1 (AOAC Method **998.12**), while Honeys 4 and 5 passed this test. When adding filtration in Method 2, Honeys 1, 2, and 3 still failed. We note that using the Method 2 modification, Honey 3 (the adulterated sample) had C4 sugars

Table 7. Averages of whole honey, protein, Δ (protein-whole honey), and C4 sugars for Methods 1, 2, and 3, excluding outliers

		Method 1 AOAC **998.12**				Method 2 Filtered AOAC **998.12**				Method 3 Filtered + centrifuged AOAC **998.12**			
		Mean											
Honey sample	Split	Honey	Protein	Difference	C4 sugar, %	Honey	Protein	Difference	C4 sugar, %	Honey	Protein	Difference	C4 sugar, %
1	A	–25.0	–26.4	–1.4	8.6	–25.0	–26.1	–1.2	7.0	–25.0	–26.1	–1.1	6.8
1	B	–25.0	–26.6	–1.6	9.2	–25.0	–26.2	–1.2	7.3	–25.0	–26.0	–1.0	6.3
2	A	–24.9	–26.4	–1.5	9.2	–24.9	–26.1	–1.3	7.6	–24.9	–26.0	–1.1	6.8
2	B	–24.9	–26.3	–1.4	8.5	–24.9	–26.2	–1.3	7.8	–24.9	–26.0	–1.1	6.8
3	A	–24.2	–26.1	–1.8	11.2	–24.3	–25.8	–1.6	9.6	–24.4	–25.5	–1.1	7.2
3	B	–24.3	–26.1	–1.8	10.7	–24.3	–25.8	–1.5	9.1	–24.3	–25.5	–1.2	7.7
4	A	–26.5	–26.0	0.5	–2.9	–26.4	–25.7	0.7	–4.3	–26.4	–25.4	1.0	–6.5
4	B	–26.4	–26.0	0.4	–2.3	–26.4	–25.8	0.6	–4.0	–26.4	–25.4	1.1	–6.9
5	A	–26.5	–26.2	0.3	–1.7	–26.5	–25.8	0.7	–4.3	–26.5	–25.1	1.4	–9.1
5	B	–26.5	–26.2	0.3	–2.1	–26.6	–25.8	0.8	–4.7	–26.5	–25.2	1.3	–8.2

Table 8. Filter paper used by each laboratory in the study

	Method 2		Method 3	
Lab	Filter type	Pore retention size, µm	Filter type	Pore retention size, µm
1	Whatman 598 1/2	8–10	Whatman GF/C	1.2
2	Whatman GF/C	1.2	Whatman GF/C	1.2
3	MN 617 1/4	7–12	Chromafil RC-45/25	0.45
4	Whatman Grade 3	6	VWR Glass fiber 696	1.2
5	Whatman GF/C	1.2	Whatman GF/C	1.2
6	Whatman Grade 1	>11	Whatman 934-AH glass fiber	>1.5
7	Whatman Grade 40	8	Whatman GF/C	1.2
8	Whatman GF/C	1.2	Whatman GF/C	1.2
9	Whatman GF-F	0.7	Whatman GF-F	0.7
10	Whatman Grade 3	6	Whatman Grade 3	6

of 9.6 and 9.1%, respectively. These levels are still classified to be exceeding the failure threshold of 7% C4 sugar.

Interlaboratory sample precision and accuracy of data without outlier removal is compared between 10 laboratories and for AOAC Method **998.12** (Method 1); the standard errors (SEs) between laboratories is better than ±0.2‰ for honey and ±0.3‰ for protein (Tables 4 and 6). Once the filtration step for the protein is included (Methods 2 and 3), SEs for protein increase up to ±0.7‰ due to analytical variation among laboratories. These errors would be a concern for honey exporters who test their honey in the country of origin before export, then undergo testing in the country of import. If the exported honey had C4 sugar values between 3 and 7%, this level of uncertainty would add a risk of failure when tested in another laboratory.

Method 3 modification appeared to have a bigger effect on reducing C4 sugar levels of all samples, in part due to the extra centrifugation step and that the laboratories were instructed to use 1 µm filter paper instead of 10 µm as in Method 2. The modification effects were sufficient enough so that both Manuka honeys (1 and 2) passed using Method 3 modifications. However, we note that Honey 3 had C4 sugar values of 7.2 and 7.7%, respectively, which indicates they were close to passing the AOAC test (threshold limit of 7% C4 sugar). Under these circumstances, the modifications of Method 3 were not fit-for-purpose, as they significantly masked the extent of sugar adulteration of Honey 3.

Removal of pollen and other insoluble material will affect the final protein $\delta^{13}C$ values, especially if the pollen and/or solids have different isotopic signatures to the internal protein. We must then ask if filtration and removal of pollens to mask honey type or country of origin, which is becoming more commonly used, could be artificially affecting the outcome of AOAC Method **998.12** by removing pollen?

Honey is rarely monofloral due to the concurrent flowering of different species. However, AOAC Method **998.12** is dependent on the whole honey (primarily the nectar component) having a close relationship to its corresponding protein source (primarily the pollen that is consumed at the same time as nectar collection). It does not take into account that nonrelated pollen is often packed or consumed and stored as protein into frames during nectar collection, nor the isotopic variation of different pollen or nectar species acquired during the honey season.

Moreover, the results of this study show that in the case of Honeys 4 and 5, where the C4 sugar values are negative, there may arise opportunities for blending of genuine honey with sugar-adulterated honey to achieve test results that still fall within the test's 7% C4 sugar threshold. Without concurrent testing of other homologs, we see that AOAC Method **998.12** has limitations.

There are still specific areas where there is key knowledge missing, such as the pollen count (grains/10 g) and pollen types found in each honey. We do not know if these honeys have low or high levels of insoluble material relative to the internal protein, which may explain why they showed smaller or larger protein shifts upon filtration and/or centrifugation. We also do not know if there is a contribution of protein derived from nectar and the cumulative isotopic effect of isotopically different floral sources (both pollen and nectar) that flower concurrently. It is possible that certain honeys might be more prone to fail because of these key knowledge gaps, and further multivariate analyses with larger data sets would elucidate these differences.

This study shows that based on this sample set, filtration of insoluble pollen is not recommended to avoid false-positive results. However, a more serious outcome shows the limitations of AOAC Method **998.12** as a measure of C4 sugar addition, especially when accounting for protein derived from other sources that are isotopically different from the whole honey or honey that has protein isotope values more negative than its corresponding honey. To resolve this issue, the method should be superseded by an indicative or qualitative method that provides evidence of the absence of C4 contribution to the key sugar components in honey. AOAC INTERNATIONAL should urgently consider the applicability of AOAC Method **998.12** and investigate suitable alternatives. Future work should consider adapting methods to analyze $\delta^{13}C$ individual sugars (LC/isotope ratio MS) that are more useful for detecting and quantifying genuine C4 adulteration, especially as some countries (i.e., New Zealand) only use nonhydrolyzed C4 cane sugar syrups without enzymatic conversion of the sucrose. Other markers should also be developed to include organic acids (5), such as amino acids, and more intensive microscopy tests to determine pollen type, quantity, and their isotope values that could affect protein isotope values.

Conclusions

The results of this study show that Methods 2 and 3 (filtration and/or centrifuging) yield protein $\delta^{13}C$ values that are more positive than Method 1. This effect is larger for Method 3 than for Method 2 due to the added centrifugation step and smaller retention pore size of the filter paper. This effect is larger for Honeys 3, 4, and 5 (non-Manuka honeys) than for Honeys 1 and 2 (Manuka honey). Method repeatability is similar for all three methods. The reproducibility is slightly larger for Methods 2 and 3 than for Method 1 due to the added filtration and/or centrifugation step. Methods 2 and 3 both had an increased occurrence of outlying results.

Method 3 modifications, which included prior centrifuging and filtration of Honey 3 (an adulterated honey with 15% added corn syrup), yielded proteins that approached acceptance criteria for AOAC Method **998.12** (false-negative results).

Negative C4 sugar values, such as those for Honeys 3, 4, and 5, mask the true extent of adulteration during AOAC tests modifications.

The proposed method modifications did not reliably predict the absence/presence of C4 sugars for some honeys, such as Manuka.

References

(1) *Official Methods of Analysis*, (1999) 16th Ed., 5th Rev., AOAC INTERNATIONAL, Gaithersburg, MD, Methods **995.17**, **998.12**,

(2) White, J.W. (1992) *J. AOAC Int.* **75**, 543–548

(3) Cotte, J.F., Casabianca, H., Lhéritier, J., Perrucchietti, C., Sanglar, C., Waton, H., & Grenier-Loustalot, M.F. (2007) *Anal. Chim. Acta* **582**, 125–136. http://dx.doi.org/10.1016/j.aca.2006.08.039

(4) Rogers, K.M., Somerton, K., Rogers, P., & Cox, J. (2010) *Rapid Commun. Mass Spectrom.* **24**, 2370–2374. http://dx.doi.org/10.1002/rcm.4642

(5) Daniele, G., Maitre, D., & Casabianca, H. (2012) *Identification, Quantification, and Carbon Stable Isotopes Determinations of Organic Acids in Monofloral Honeys, A Powerful Tool for Botanical and Authenticity Control*. doi: 10.1002/rcm.6310, 1993–1998.