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Dear Sean,

I have completed the pollen study of the two honey samples you submitted for analysis. Specific details about the extraction and analysis procedures I used for this sample is mentioned below and are identical to those I normally use on other such samples. These procedures are outlined below.

EXTRACTION PROCEDURE:

To conduct a pollen study of raw honey we first must dilute it before the pollen can be removed for analysis. For our study, we use a 10g sample of raw honey for the analysis. The sample of raw honey is diluted with 10 ml of distilled water and 100 ml of ETOH, and then heated to 100^{o} F to ensure a complete mixture. This is a technique that we developed and has now been adopted by most others (Jones and Bryant, 2004, **The use of ETOH for the dilution of honey** *Grana* 43: 174–182).

Next, we add one tablet containing a total of 18,583 ***Lycopodium*** spores to enable us to conduct a pollen concentration study for each sample. We use these lycopod spores because they are not utilized by bees for any purpose and thus we do not have to worry about these being found in natural honey sources. Once these initial stages are complete, the pollen sample is dehydrated with glacial acetic acid and then heated in a mixture of a sulfuric acid and acetic anhydride. This chemical treatment, called *acetolysis*, is designed to remove lipids, waxes, and cytoplasm thereby making the pollen easier to identify.

Once the acetolysis process is complete, each sample is again dehydrated in glacial acetic acid and treated with a series of distilled water rinses. The resulting pollen residue is stained to create contrast for microscopic analysis and photography. Finally, we mix a few drops of glycerin into the sample and mount one drop of it on each microscope slide for analysis. To ensure an accurate representation of the overall sample we stir the sample for one minute on a Vortex stirrer before removing each drop for analysis. Our laboratory experiments and published results have demonstrated that this technique ensures that each drop is a true reflection of the original sample.

Analysis of a honey sample follows a two-step procedure. First, the sample is scanned at 400x under a microscope, initial identifications are made of each pollen type, and key photographic images are taken of each pollen type. During this procedure if a pollen grain is not one we are familiar with, we will compare it with our extensive modern pollen reference

samples on file in our laboratory in hopes of finding a match. Second, a quantitative pollen count is conducted for each sample to determine the pollen types present and the frequency of each taxon.

A statistically valid quantitative pollen count of 200+ pollen grains is conducted for each sample as originally recommended for honey specimens in 1978, by Louveaux, Maurizio, & Vorwohl (***Bee World*, Vol. 59:139-157**). Quantitative counts are used because testing has shown that these offer an accuracy of greater than 95% as to the actual composition of pollen taxa within a given honey sample. The result of our pollen count for your sample is included below (Table 1).

We have followed the reporting system recommended by Louveaux *et al.* (op. cit.) and others who stress that pollen results should be listed according to percentage classes rather than actual percentages when counts of between 200-1200 grains per sample are conducted. We show the actual percentage counts for general reference but these are not deemed totally accurate for honey samples until a total count in excess of 1,200 pollen grains per sample is reached. We rarely count this many pollen grains for a honey sample because in most cases it is not needed and because larger counts add cost and time considerations.

The recognized pollen percentage's classes used for honey analysis are:

- A= >45%, called predominant pollen types
- B= 16-45%, called secondary pollen types
- C= 3-15%, called important minor pollen types
- D= <3%, called a minor pollen types

In making quantitative counts, each pollen type is identified to the family, genus, or in some cases species level. Sometimes the pollen types within one plant family (such as the **Asteraceae** [composites]; **Liliaceae** [lilies], **Myrtaceae** [gum family], **Poaceae** [grasses], **Rhamnaceae** [buckthorns], **Rosaceae** [rose family] and **Ericaceae** [ericades]) are diagnostic at the family level yet often many of their genera are not easily separated into specific types or species because of their morphological similarity with one another. In some other large plant families, such as the **Fabaceae** (legumes), we are able to identify some taxa to the generic level yet others in this family produce pollen types that are too similar to one another to distinguish at the genus level without extensive reference collections and studies at levels of higher resolution using scanning electron microscopy (SEM).

A pollen concentration value (PC) of pollen grains per 10g of honey was calculated for your sample. This value usually ranges from a few thousand pollen grains to more than one million. As Maurizio (1975) has noted, the number of pollen grains in individual honey samples can vary greatly, therefore, she recommends using a set of concentration categories. Honey pollen counts in **Category I:** contain less than 20,000 grains/l0 g. Often, honey in this category represents samples that have been pressure-filtered, honey from floral sources that produce little pollen, honeys that were partly produced by sugar-feeding bees, or honey that has been adulterated by adding high-fructose syrup or adding highly-filtered honey with no pollen. Usually, honeydew honey samples also fall into this first category. Pollen concentration counts in **Category II:** contain between 20,000-100,000 grains/10 g, which includes the majority of honey produced in the world from most floral sources. **Category III:** pollen concentration

values range from 100,000-500,000 grains/10 g and represent floral sources that are high pollen producers or indicate that some of the comb storage cells containing pure pollen may have been mixed with the extracted honey. **Category IV:** includes pollen concentrations between 500,000-1,000,000 grains/10 g. That category along with honey in **Category V:** (containing pollen concentrations of more than 1,000,000 grains/10 g) indicate honey that is produced from a few floral sources that are extremely rich in pollen (i.e., *Myosotis sylvatica, Cynoglossum officinale*, etc.).

Pollen concentration values are very important and useful because they give us a general idea of the amount of pollen present and also suggest the geographical location where the honey was produced. In some cases, adulterated honey samples that have been mixed with highly-filtered honey or with quantities of other sugars (i.e., cane sugar or corn syrup) will contain low pollen concentration values. Nevertheless, without chemical isotope testing for possible adulteration, pollen concentration values alone are generally not sufficient to warrant such a claim for added sugar adulteration.

We calculated our pollen concentration value using the formula

$$PC = \frac{(\#\text{ of } \textbf{Lycopodium} \text{ spores added}) \times (\#\text{ of pollen grains counted})}{(\#\text{ of } \textbf{Lycopodium} \text{ spores counted}) \times (\text{amount of honey (grams) processed})}$$

The complete pollen count for your samples is listed below. A summary of the pollen types found and the pollen concentration values is also noted.

ANALYSIS

Sample 1 Honey:

This honey sample would also appear to be a Mixed Floral Honey under normal pollen analysis procedures. According to the International Bee Commission (IBC), to be a unifloral honey it should contain at least 45% pollen from a single, primary nectar source (Table 1). Therefore, according to the IBC rules, your honey sample should be called a mixed floral honey because it is not dominated by any one pollen type. However, as I point out in the attached article that discusses the importance of using pollen coefficient values, pollen percentages need to be considered in terms of their true importance in honey samples. As I point out in that article, not all flowers or pollen types are "created equal." Therefore, for some pollen types that goal of 45% is nearly impossible, and sourwood is one of those types. As you can see in Table 1, the primary pollen, and the apparent primary nectar sources in this sample are coming from a variety of plants including sourwood. There are a number of other pollen and nectar types represented in minor amounts, as you can see in the list shown in Table 1.

If we use pollen coefficient values to determine the true nectar value (TNV) in your sample 1, it reveals that it is a good sourwood sample and the pollen concentration value is less than 10,000 pollen grains/10 grams of honey (Table 1). As you may know, the lower the pollen concentration value the better the purity of the sourwood honey because sourwood pollen is very underrepresented in honey. For good sourwood honey, the expected pollen

concentration value should be close to, or below 10,000 pollen grains/10 grams of honey; the lower the better. Sample 1 has a low pollen concentration value of just over 5,000 pollen grains per 10 grams of honey. The True Nectar Value (TNV) of sample 1 is about 84.5% nectar from sourwood flowers (Table 1). Therefore, in reality this honey is a good example of sourwood honey.

Sample 2 Honey:

Your Sample 2 honey is similar to Sample 1. It is also a good sourwood honey, but it has a slightly lower percentage of sourwood nectar at 83% (Table 1). You can see that Sample 2 also has a lower pollen concentration value that is just over 3,000 pollen grains per 10 grams of honey.

These data suggest that Sample 1 has more "corrected" sourwood nectar but it has a slightly higher pollen concentration value. It is hard to say which of the two samples is better. They are both good examples.

Relative Pollen Counts of the 2016 Honey Samples
Table 1

Collinsworth Honey 2016

Pollen Taxa	1	%	TNV	2	%	TNV
Acer **(maple)**	0	0.0%		0	0.0%	
AMARANTHACEAE (amaranth & goosefoot)	0	0.0%		1	0.6%	
ASTERACEAE (dandelion-type)	10	4.8%		3	1.7%	
ASTERACEAE (sunflower-type)	4	1.9%		0	0.0%	
BRASSICACEAE (mustard family)	0	0.0%		2	1.1%	
Castanea **(chestnut, chinquapin)**	0	0.0%		0	0.0%	
Cephalanthus **(buttonbush)**	1	0.5%		0	0.0%	
Chenopodium **(goosefoot)**	0	0.0%		0	0.0%	
Cornus **(dogwood)**	0	0.0%		0	0.0%	
CYPERACEAE (sedge)	0	0.0%		3	1.7%	
Gleditsia **(honey locust)**	0	0.0%		0	0.0%	
Ilex **(holly, yaupon)**	14	6.7%		0	0.0%	
Lagerstroemia **(crepe myrtle)**	0	0.0%		0	0.0%	
Liriodendron **(tulip tree)**	20	9.5%	3.5%	0	0.0%	
Lonicera **(honeysuckle)**	0	0.0%		0	0.0%	
Magnolia **(magnolia)**	0	0.0%		0	0.0%	
Melilotus **(clover)**	0	0.0%		0	0.0%	

Mimosa **(various species)**	**0**	**0.0%**		**0**	**0.0%**	
Nyssa **(tupelo)**	**6**	**2.9%**		**0**	**0.0%**	
ONAGRACEAE	**0**	**0.0%**		**0**	**0.0%**	
Oxydendrum arboreum **(sourwood)**	**24**	**11.4%**	**84.5%**	**16**	**9.0%**	**83.0%**
Parthenocissus **(Virginia creeper)**	**1**	**0.5%**		**10**	**5.6%**	
Pinus **(pine)**	**0**	**0.0%**		**0**	**0.0%**	
Plantago **(plantain)**	**13**	**6.2%**		**29**	**16.4%**	
POACEAE (grass family)	**0**	**0.0%**		**0**	**0.0%**	
Prunus **(plum, peach, cherry)**	**0**	**0.0%**		**0**	**0.0%**	
Quercus **(oak)**	**6**	**2.9%**		**0**	**0.0%**	
RANUNCULACEAE (buttercups)	**0**	**0.0%**		**0**	**0.0%**	
Rhododendron/Kalmia **(laurel)**	**9**	**4.3%**		**8**	**4.5%**	
Rhus /Toxicodendron **(sumac, poison ivy)**	**13**	**6.2%**	**1.0%**	**5**	**2.8%**	**0.50%**
ROSACEAE (rose family)	**12**	**5.7%**		**6**	**3.4%**	
Rubus **(blackberry, dewberry)**	**11**	**5.2%**		**0**	**0.0%**	
Rumex **(dock)**	**1**	**0.5%**		**0**	**0.0%**	
SCROPHULARIACEAE	**0**	**0.0%**		**11**	**6.2%**	
Tilia **(basswood)**	**0**	**0.0%**		**0**	**0.0%**	
Trifolium **(clover)**	**59**	**28.1%**	**4.0%**	**74**	**41.8%**	**7.50%**
Vicia **(vetch)**	**0**	**0.0%**		**1**	**0.6%**	
Vitis **(grape)**	**3**	**1.4%**		**0**	**0.0%**	
Zanthoxylum **(prickly ash)**	**1**	**0.5%**		**0**	**0.0%**	
Zea mays **(maize)**	**0**	**0.0%**		**3**	**1.7%**	
All other nectar sources combined			**7.0%**			**9.00%**
Unknown pollen	**2**	**1.0%**		**6**	**3.4%**	
Totals	**210**	**100.0%**	**100.0%**	**177**	**100.0%**	**100%**
Lycopodium spores counted	**740**			**1,021**		
Pollen concentration per 10 grams of honey		**5,273**			**3,221**	

Honey Pollen Categories

A= >45% predominant pollen type
B= 16-45% secondary pollen type
C= 3-15% important minor pollen type
D= <3% minor pollen type

Honey Pollen Concentration Categories

Category I	0-20,000/10 g
Category II	20,000-100,000/10 g
Category III	100,000-500,000/10 g
Category IV	500,000-1,000,000/10 g
Category V	over 1,000,000/10 g

Should you desire additional clarification of this report please let me know. If we can

assist you in the future, please let us know. We did receive your check, thank you.

Sincerely,

Vaughn M. Bryant, Jr.
Professor and Director