PHYSICOCHEMICAL AND FLAVOR CHARACTERIZATION OF TUPELO HONEY

BY

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THESIS

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ABSTRACT

Nyssa ogeche (white Tupelo) trees are concentrated mainly in the Appalachicola region in the panhandle of Florida. The honey produced from the nectar of these trees is regarded as a premium honey because its non-granulating tendencies and limited supply due to the small growing region and short bloom time of the Tupelo trees. Unfortunately there are few studies of this unique honey, with only one study done on the physicochemical characteristics and one on the volatiles present. Therefore, it is necessary to conduct an exhaustive physicochemical and flavor characterization of Tupelo honey to confirm and expand upon the limited available research.

Physicochemical characteristics were determined for ten Tupelo honey samples from five different locations collected during two consecutive seasons (2013 and 2014). These included moisture content, ^{0}Brix, water activity (A_w), pH, titratable acidity, ash content, and fructose and glucose contents. On the basis of pollen analyses, nine samples could be considered as authentic Tupelo honeys, with one (designated honey 3 from the 2014 season) containing mainly holly pollen along with appreciable levels of tupelo pollen. This honey also had a noticeably higher pH value and differed significantly in ash content from the other Tupelo honey samples, suggesting ash content to be a good indicator of botanical origin. Sensory screening indicated this sample to be significantly different from the other samples as well. Honey 5 produced during both seasons was the most consistent sampling location with the highest amount of Tupelo pollen content present in each sample. Panelists could not distinguish a difference between the honey 5 samples from both seasons during sensory testing. The characteristic most widely known about Tupelo honey is its relatively high fructose content which was confirmed in

this study. Composition data were comparable to literature values and within the limits set by Codex Alimentarius.

To complete a full flavor characterization, aroma-active compounds in Tupelo honey were identified by gas chromatography-olfactometry (GC-O) and gas chromatography-mass spectrometry-olfactometry (GC-MS-O). Initial analyses were performed on the same ten samples as described above by static headspace solid phase microextraction (H-SPME). Of the 40 compounds detected, the most important compounds based on their perceived odor intensities determined by two assessors were phenylacetaldehye (rosy) and nonanal (citrus). Further analysis was carried out on honey 5 from the 2013 and 2014 seasons due to its consistently high Tupelo pollen content across seasons and the inability of sensory panelists to distinguish between the two samples. The most potent odorants were determined through aroma extract dilution analysis (AEDA) of solvent extracts and sample dilution analysis by H-SPME (SDA-H-SPME). The most potent odorants identified by both dilution analyses techniques were vanillin (vanilla), phenylacetaldehyde (rosy), nonanal (citrus), (*E*)-2-nonenal (dried hay), eugenol (cloves), guaiacol (smoky), 2-phenylethanol (rosy, wine), 2'-aminoacetophenone (grape, corn tortilla), (*E*)-β-damascenone (cooked apple), and an unidentified odorant (RI_{wax}=1731) described as spicy and hay-like. (*E*)-β-Damascenone was determined to be the most potent odorant with extremely high flavor dilution (FD) factors of 59,049 (2013 season) and 19,683 (2014 season). Quantification of (*E*)-β-damascenone using stable isotope dilution analysis combined with H-SPME (SIDA-H-SPME) revealed that the compound had an extremely high concentration and odor-activity value (OAV) compared to other types of honeys and food products. (*E*)-β-Damascenone may be used as a marker compound to distinguish Tupelo honey from other unifloral honeys because of the uniquely high levels present in this honey.

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CHAPTER 1

INTRODUCTION

Thousands of years ago many societies believed bees and honey came from the Gods. For this reason honey hunters would risk their lives climbing trees to obtain this sweet nectar. In most societies around the world honey was used in medicines and religious rituals. Today, honey is a popular sweetener used in several products such as cereals, meats, bakery items, mead and is still used in some medicines and cosmetics (Crane, 1975; Crane, 1980). Worldwide production of honey in 2013 was approximately 3.67 billion pounds according to the Statistics Division of the Food and Agriculture Organization (FAO) of the United Nations (2015). The United States is among the leading producers of honey, which also includes China, Argentina, Turkey, and Ukraine in recent years (FAO, 2015). In 2014 the United States produced 178.3 million pounds of honey (USDA, 2015). Tupelo honey is only a small fraction of this and its production is concentrated mainly in the Appalachicola region in the panhandle of Florida. The honey produced from the nectar of these trees is regarded as a premium honey because of its non-granulating tendencies and limited supply due to the small growing region and short bloom time of the Tupelo trees (Hockersmith, 2004; White et al., 1962).

Honey consists mainly of monosaccharides and water, with small amounts of higher sugars, minerals, vitamins, pigments, and aroma compounds (Maurizio, A., 1975; White et al., 1962). More expensive, premium honeys can become the target of adulteration, with mixing of the finished product with cheap sugar syrups being most common. Identification of adulteration with sugar products can be quite challenging because of the variety of products that can be used as adulterants and the natural variability among different unifloral honeys. In some cases

adulteration will change some of the physicochemical characteristics of a honey (Bogdanov and Martin, 2002). For this reason it is important to identify the typical variations present in these characteristics in the unifloral honeys targeted for adulteration. The only such study on Tupelo honey was done by White and others (1962) involving 6 samples. A higher than average fructose content was reported, which could be very useful in determining adulteration since this is a unique deviation from most unifloral honeys. Pollen analysis can also be used to prevent mislabeling and can be a helpful tool in determining the botanical and geographical origins of a honey (Bogdanov and Martin, 2002). Every technique has its limitations, however, so it is advisable to test several parameters to gain a true understanding of the different unifloral honeys.

Unifloral honeys are also known for their unique flavor profiles and are typically identified as such by the beekeeper based on the flavor, aroma, color and texture of the honey (Molan, P., 1996). Several studies have focused on characterizing the flavor profiles of unifloral honeys and identifying volatile marker compounds (Bianchi et al., 2005; Guyot et al., 1998; Kaskoniene and Venskutonis, 2010). To date the only such study on Tupelo has been carried out by Overton and Manura (1994) to highlight the purge-and-trap technology developed by Scientific Instrument Services, Inc. Thirty-one volatiles were detected and semi-quantified by addition of an internal standard to the adsorbent trap after the sample volatiles had been isolated. This is not a very reliable method since it does not take into account the extraction efficiency, but it does provide semi-quantitative data. Without the use of gas chromatography-olfactometry (GC-O) there is no indication if the compounds identified contributed to the aroma profile of the honey. Since the primary goal of the study was to emphasize the functionality of a new piece of an analytical instrument, further research needs to be done with the use of two column types, multiple extraction methods and GC-O to confirm important volatile compounds. For these

reasons the aim of this study was to provide a full physicochemical and flavor characterization of Tupelo honey.

1.1 REFERENCES

Bianchi, F.; Careri, M.; Musci, M. Volatile Norisoprenoids as Markers of Botanical Origin of Sardinian Strawberry-Tree (Arbutus Unedo L.) Honey: Characterisation of Aroma Compounds by Dynamic Headspace Extraction and Gas Chromatography-Mass Spectrometry. *Food Chem.* **2005**, *89* (4), 527–532.

Bogdanov, S.; Martin, P. Honey Authenticity: A Review. *Swiss Bee Res. Cent.* **2002**, No. 6, 1–22.

Crane, E. *A Book of Honey*; Oxford University Press: Oxford, 1980.

Crane, E. History of honey. In: *Honey: A Comprehensive Survey*; Crane, E., Ed.; Crane, Russak & Company, Inc.: New York, 1975b, 439-488.

Food and Agriculture Organization of the United Nations, Statistics Division. http://faostat3.fao.org/browse/Q/QL/E.

Guyot, C.; Bouseta, a; Scheirman, V.; Collin, S. Floral Origin Markers of Chestnut and Lime Tree Honeys. *J. Agric. Food Chem.* **1998**, *46* (2), 625–633.

Hockersmith, K. S. Apalachicola ' S Gold : Archaeology and History of Tupelo Honey Production in Northwest Florida, Universtiy of South Florida, 2004.

Kaškoniene, V.; Venskutonis, P. R. Floral Markers in Honey of Various Botanical and Geographic Origins: A Review. *Compr. Rev. Food Sci. Food Saf.* **2010**, *9*, 620–634.

Maurizio, A. How bees make honey. In: *Honey: A Comprehensive Survey*; Crane, E., Ed.; Crane, Russak & Company, Inc.: New York, 1975, 77-105.

Molan, P. C. Authenticity of Honey. In: *Food Authentication*; Chapman & Hall, 1996.

Overton, S.; Manura, J. Flavor and Aroma in Commercial Bee Honey – a Purge-and-Trap Thermal-Desorption Technique for the Identification and Quantification of Volatiles and Semivolatiles in Honey. *Am. Lab.* **1994**, *26*, 45–53.

United States Department of Agriculture; National Agricultural Statistics Service; Agricultural Statistics Board. *Honey*; 2015.

White, J. W.; Riethof, M. L.; Subers, M. H.; Kushnir, I. *Composition of American Honeys*; Washington, D.C., 1962.

CHAPTER 2

LITERATURE REVIEW

2.1 HIVE SOCIETY

Honey bees, *Apis mellifera*, produce the majority of the world's honey and have existed for millions of years (Crane, E., 1980; Alqarni et al., 2011). Each hive consists of 1 queen, 20,000 – 30,000 sterile female workers bees, and approximately 500 male drones when mating is necessary (Sudarsan et al., 2011). Queens are produced by feeding selected larvae royal jelly that is produced in the hypopharyngeal glands of a worker bee's head (Crane, E., 1980). When a virgin queen is ready to reproduce she will fly out of the hive and mate with several drones. She will use this sperm to fertilize the 1,000 – 1,500 eggs laid daily during the 2-3 years of her life (Guzman-Novoa et al., 1994; Sudarsan et al., 2011). While the queen is an important part of the social construct of a bee hive, the worker bees fulfill every other role besides reproduction. Worker bees begin their lives within the confines of the hive by helping rear brood through feeding larvae and keeping the brood at an ideal temperature of 34-35^{0}C (93-95^{0}F). They then move on to building and repairing the comb with secretions of wax from their abdomen. The hypopharyngeal glands that produced royal jelly in the early stages of life then gain the ability to produce important enzymes that help convert nectar to honey. The last stage in a worker bee's life is to forage for nectar, pollen, water and propolis outside of the hive (Crane, E., 1980).

The final two stages in a honey bee's life are the most important in honey production. The foragers typically travel 1-2 km to gather nectar from nearby floral sources. The sweetness of the nectar, scent and color of the floral source, quantity of nectar available, and the ease of gathering nectar is what attracts bees to different floral sources. During this process they act as

important pollinators for many types of plants by transferring pollen from the anther to the stigma (Crane, E., 1975a; Crane, E., 1980). Besides nectar, honey bees also gather pollen which is fed to the larvae during development. The nectar, however, is the raw material used to make honey and consists mainly of sugars and water. The forager bee will collect the nectar in her honey sac and transfer it to a bee in the hive for further processing. If the nectar flow is relatively slow this bee will spend 15-20 minutes regurgitating small drops of nectar onto the end of her probiscus, sucking it back up and repeating this process until much of the water is evaporated. To finish the process this half ripe honey is put into combs and fanned with the bee's wings to complete the drying. Once the ripe honey has reached an appropriate moisture level it will be capped to protect it from water uptake (Maurizio, A., 1975). During this process the bees will add enzymes including invertase, diastase, and glucose oxidase. Invertase acts upon the sucrose in the nectar by converting it to glucose and fructose, which are the primary sugars found in most honeys. Diastase breaks down starch and glucose oxidase converts glucose to gluconic acid, the major acid in honey (Crane, E., 1980). As one can see, honey is made by the unique processing machinery found naturally within honey bees.

2.2 BEEKEEPING AND PRODUCTION

The existence of honey bees predates that of humans and the honey they produced was originally stored for their own food usage. Before humans, hives were robbed of their honey by several types of animals, including bears, chimpanzees, honey badgers, and a few types of birds. With the existence of humans, the practice of honey hunting became prevalent. This consisted of the use of ladders or pegs to reach the hives in trees to gather the combs, and a later development involved the use of smoke to subdue the bees to make collection easier (Crane, E., 1975b). The

true art of apiculture began with the use of earthen pots, hollow logs, and straw or wicker skeps to house bees and the eventual use of protective clothing to collect honey (Crane, E., 1980). Modern day beekeeping typically involves the use of Langstroth's hive, which was first created and introduced in 1952. This design consists of several boxes stacked on top of each other. The bottom box has the entrance and contains the queen and the brood. On top of the brood chamber are several supers where excess honey is produced and stored. Between the brood chamber and the supers is a queen excluder that prevents the queen from entering and laying eggs in the supers. Each super is also equipped with several removable frames that have a pre-formed wax foundation where honey bees can create the combs for honey storage (Sudarsan et al., 2011). **Figure 2.1 (A-C)** pictorially shows several of the unique characteristics of the Langstroth hive design. The creation of this multi-level bee box allowed for more advanced beekeeping techniques. Instead of destroying the hive and the bees inside through drowning or suffocation to collect honey, beekeepers are now able to remove individual frames filled with honey comb and replace them with new frames leaving the bees intact. The queen excluder also allows beekeepers to take the supers, but leave the brood. After collection, the beekeeper uncaps the comb and uses a centrifugal extractor to drain the honey from the comb (Crane, E., 1980). Once drained the honey is filtered to rid it of contaminants like wax and bee parts. Often times the pollen is filtered out using micro (80μm) pressure filters, but the International Honey Commission recommends using a filter that is no smaller than 0.2 mm to prevent pollen filtration since pollen is an indicator of botanical and geographical origin (Subramanian et al., 2007; Bogdanov, 1999). The honey can also be heated (pasteurized) to prevent fermentation and granulation (Subramanian et al., 2007).

Worldwide production of honey in 2013 was approximately 3.67 billion pounds according to the Statistics Division of the Food and Agriculture Organization (FAO) of the United Nations (2015). The United States is among the leading producers of honey following China, Argentina, Turkey, and Ukraine in recent years (FAO, 2015). In 2014 the United States produced 178.3 million pounds of honey (USDA, 2015). Honey is a popular sweetener used in several products such as cereals, meats, bakery items, mead and is even used in medicines and cosmetics. Thousands of years ago many societies believed bees and honey came from the Gods and they used honey in medicines and religious rituals. Honey continued to be important throughout history with hives being mentioned in wills and being more valuable than livestock in some instances. Eventually the introduction of cheap sugar replaced the widespread use of honey, but has not been able to completely replace its usage as seen by several products that still contain honey in the marketplace today (Crane, 1975b; Crane, 1980).

2.3 FACTORS INFLUENCING THE COMPOSITION OF HONEY

Honey is defined as "a thick, sweet, syrupy substance that bees make as food from the nectar of flowers and store in honeycombs" in the guidance document for the proper labeling of honey and honey products by the FDA (2014). The Codex Alimentarius standard takes the definition a step further by declaring honey as "…the natural sweet substance produced by honey bees from the nectar of plants or from the secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honey comb to ripen and mature" (2001). This definition is much more comprehensive by including blossom and honeydew honeys and describing how honey is naturally made by the bees, but both

definitions fail to list the typical composition of honey. For example, American honeys are typically comprised of 17.2% moisture, 38.2% fructose, 31.3% glucose, 1.3% sucrose, 7.3% reducing disaccharides, 1.5% higher sugars, 0.57% acids, and 0.169% mineral content (White et al., 1962). The Codex standard goes on to set limits for some components, but an exact standard of identity is difficult to determine because of the various types of unifloral honeys available. According the Codex standard (2001), a unifloral honey may be designated by "…floral or plant source if it comes wholly or mainly from that particular source and has the organoleptic, physicochemical and microscopic properties corresponding with that origin." Moisture content is the most important quality parameter to honey since honeys with higher moisture contents can ferment. Moisture content is affected by environmental conditions, harvest season, maturation of honey in the hive, and handling by beekeepers (Iglesias et al., 2012, Feas et al., 2010). As discussed earlier the bees evaporate the water present in the nectar as part of their natural processing system. In certain locations where humidity is high, however, the beekeeper may need to further dry the honey to obtain a suitable moisture content. Sugar content on the other hand is very much dependent on the nectar source. The quantities of fructose and glucose and the quantities and presence of various di- and oligosaccharides can indicate floral source (Molan, P., 1996). Very small amounts of organic acids, vitamins, pigments, aroma constituents and minerals are also found in the nectar (Maurizio, A., 1975). The mineral content of a honey may be a good indicator of botanical and geographical origin (Viuda-Martos et al., 2010; Bogdanov et al., 2000; Terrab et al., 2004), since the majority of minerals in honey originate from the soil (Pohl, 2009). The mineral content also affects the color of honey as does heating (Vuida-Martos et al., 2010; Molan, P., 1996). Heating honey can cause the Maillard reaction to occur between the amino acids and sugars of honey darkening the color

and creating flavor volatiles (Molan, P., 1996; Castro-Vasquez et al., 2008). Heat can also inactivate the enzymes present in honey and their presence can be an indication of quality (White, J., 1975-5). Many factors affect the composition of honey, including beekeeper practices, bee processing, heating, and floral source; the latter being the most important factor.

2.4 TUPELO HONEY

Tupelo honey is produced from the *Nyssa ogeche* (white Tupelo) tree which is part of the Cornaceae, or dogwood family. The tree is found in southern Georgia and Northern Florida and grows in very moist habitats (Kossuth and Scheer). Two other species of the genera *Nyssa* are also present in eastern North America, including *Nyssa aquatic* and *Nyssa sylvatica* (Wen and Stuessy, 1993). The subject of this research is the honey produced from the nectar of *Nyssa ogeche* flowers and will henceforth be referred to by its common name, Tupelo. Tupelo trees are found in abundance in the panhandle of Florida near the Appalachicola, Chipola and Choctawhatchee River banks and these locations are utilized by beekeepers to produce premium Tupelo honey. The Tupelo season is rather brief, lasting only a few weeks from mid-April to mid-May during normal climatic conditions, causing this honey to be quite rare (Hockersmith, K., 2004).

The annual production of Tupelo honey is difficult to pinpoint because of limited data on the subject. Currently there are 54 beekeepers that are part of the Tupelo Beekeepers Association, but the exact quantity of Tupelo honey produced by each beekeeper can vary drastically between larger operations and small scale backyard beekeepers. The USDA releases monthly honey reports that shed some light on Tupelo honey. The monthly report for June, 2013 describes the Tupelo season to have been short, producing approximately 50% of its normal

crop, but there is no mention of price. The monthly honey report for July, 2014 describes the Tupelo honey crop to be light because of weather conditions, which caused prices to be as high as $3.85-$5.75 per pound. This is significantly higher than the other honeys produced in Florida at that time, with orange blossom honey fetching the highest price at $2.10-$2.25 per pound. The report goes on to say that the gallberry and palmetto honey crops were also affected by weather; however, the cost was not as high as Tupelo with a maximum cost of $2.15 per pound for gallberry and palmetto honeys. Although it is not clear how much Tupelo honey is produced each year it is apparent that Tupelo honey can demand high prices.

Besides being rare and relatively expensive, Tupelo honey has the unique characteristic of being non-granulating due to its higher than average fructose content (White et al., 1962). It is also minimally processed and is not subjected to micro filtration or high heat during extraction or bottling. Reno Plenge, President of the Tupelo Beekeepers Association, briefly described his processing methods over a phone interview. Depending on nectar flow, the supers will be filled in 3-5 days. The combs will be uncapped, extracted and left overnight in a heated tank that is kept between 90-100^{0}F, which is within range of the typical hive temperature. Since the area where Tupelo is produced can be very humid it is usually necessary to dry the honeys further. Mr. Plenge uses dehumidifiers and air conditioners to reduce the moisture content to 18.6% or below since this is the Grade A standard set by the USDA (1985). The honey is strained to rid the final product of bee appendages and other unwanted particulates. This minimal processing technique, without the use of filtration and pasteurization, is common practice among Tupelo producers.

The small growing region of Tupelo trees, short bloom time, and minimal processing increases the price of this premium unifloral honey. These more expensive honeys can become

the target of adulteration. Common adulteration practices include mixing honey with inexpensive sugar products after it has been harvested or feeding bees sugar syrup during the nectar flow to increase yield (Cotte et al., 2003). In order to combat this practice pollen analysis is utilized. Unfortunately there are several limitations of pollen analysis including differing abilities of plants to produce pollen, filtration of pollen granules in the honey sacs of bees, and contamination by pollen that enters the hive through wind currents (Bryant and Jones, 2001). With the ability to micro filter honeys and remove the pollen it is easy to add desired pollen fraudulently to the filtered honey as well (Molan, P., 1996). With the limitations of pollen analysis it is important to confirm the botanical origin of unifloral honeys by testing the physicochemical characteristics. Certain types of unifloral honeys have unique characteristics that can aid in determining the botanical origin (Louveaux et al., 1978; Von Der Ohe et al., 2005). Tupelo honey, for example, has a high fructose content (Louveaux et al., 1978). Research on the physicochemical characteristics of Tupelo honey is limited to a study done by White and others published in 1962. Further research is needed to expand upon and confirm the results obtained in the previous study. Unifloral honeys are also known for their unique flavor profiles and are typically identified as such by the beekeeper based on the flavor, aroma, color and texture of the honey (Molan, P., 1996). Several studies have focused on characterizing the flavor profiles of unifloral honeys and identifying volatile marker compounds (Bianchi et al., 2005; Guyot et al., 1998; Kaskoniene and Venskutonis, 2010). To date the only such study on Tupelo has been carried out by Overton and Manura (1994) to highlight the purge-and-trap technology developed by Scientific Instrument Service, Inc. Since the primary goal of that study was to emphasize the functionality of a new piece of an analytical instrument, further research needs to be done with the use of two column types, multiple extraction methods and gas

chromatography-olfactometry to confirm important volatile compounds. For these reasons the aim of this study was to provide a full physicochemical and flavor characterization of Tupelo honey.

2.5 TABLES AND FIGURES

A

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C

Figure 2.1 Pictorial representations of the Langstroth-type design. **A**: typical bee yard with Langstroth-type design beehives. **B**: view of the brood chamber with the queen excluder and removable frames. **C**: removable frame with a pre-formed wax foundation.

2.6 REFERENCES

Alqarni, A. S.; Hannan, M. a.; Owayss, A. a.; Engel, M. S. The Indigenous Honey Bees of Saudi Arabia (Hymenoptera, Apidae, Apis Mellifera Jemenitica Ruttner): Their Natural History and Role in Beekeeping. *Zookeys* **2011**, *134*, 83–98.

Bianchi, F.; Careri, M.; Musci, M. Volatile Norisoprenoids as Markers of Botanical Origin of Sardinian Strawberry-Tree (Arbutus Unedo L.) Honey: Characterisation of Aroma Compounds by Dynamic Headspace Extraction and Gas Chromatography-Mass Spectrometry. *Food Chem.* **2005**, *89* (4), 527–532.

Bogdanov, S. Honey Quality and International Regulatory Standards: Review by the International Honey Commission. *Bee World* **1999**, *80* (2), 61–69.

Bogdanov, S.; Lüllmann, C.; Martin, P.; von der Ohe, W.; Russmann, H.; Vorwohl, G.; Oddo, L. P.; Sabatini, A.-G.; Marcazzan, G. L.; Piro, R.; et al. Honey Quality, Methods of Analysis and International Regulatory Standards: Review of the Work of the International Honey Commission. *Swiss Bee Res. Cent.* **2000**, No. 8, 1–15.

Bryant, V. M.; Jones, G. D. The R-Values of Honey: Pollen Coefficients. *Palynology* **2001**, *25*, 11–28.

Codex Alimentarius. Codex Standard for Honey. **2001**, 1–8.

Cotte, J. F.; Casabianca, H.; Chardon, S.; Lheritier, J.; Grenier-Loustalot, M. F. Application of Carbohydrate Analysis to Verify Honey Authenticity. *J. Chromatogr. A* **2003**, *1021* (1-2), 145–155.

Crane, E. *A Book of Honey*; Oxford University Press: Oxford, 1980.

Crane, E. The flowers honey comes from. In: *Honey: A Comprehensive Survey*; Crane, E., Ed.; Crane, Russak & Company, Inc.: New York, 1975a, 3-76.

Crane, E. History of honey. In: *Honey: A Comprehensive Survey*; Crane, E., Ed.; Crane, Russak & Company, Inc.: New York, 1975b, 439-488.

Feás, X.; Pires, J.; Iglesias, A.; Estevinho, M. L. Characterization of Artisanal Honey Produced on the Northwest of Portugal by Melissopalynological and Physico-Chemical Data. *Food Chem. Toxicol.* **2010**, *48*, 3462–3470.

Food and Agriculture Organization of the United Nations, Statistics Division. http://faostat3.fao.org/browse/Q/QL/E.

Food and Drug Administration. Guidance for Industry: Proper Labeling of Honey and Honey Products. **2014**, 1–8.

Guzmán-novoa, E.; Page, R. E.; Gary, N. E. Behavioral and Life-History Components of Division of Labor in Honey Bees (Apis Mellifera L .). *Behav Ecol Sociobiol* **1994**, *34*, 409–417.

Guyot, C.; Bouseta, a; Scheirman, V.; Collin, S. Floral Origin Markers of Chestnut and Lime Tree Honeys. *J. Agric. Food Chem.* **1998**, *46* (2), 625–633.

Hockersmith, K. S. Apalachicola ' S Gold : Archaeology and History of Tupelo Honey Production in Northwest Florida, Universtiy of South Florida, 2004.

Iglesias, A.; Feás, X.; Rodrigues, S.; Seijas, J. a.; Vázquez-Tato, M. P.; Dias, L. G.; Estevinho, L. M. Comprehensive Study of Honey with Protected Denomination of Origin and Contribution to the Enhancement of Legal Specifications. *Molecules* **2012**, *17*, 8561–8577.

Kaškoniene, V.; Venskutonis, P. R. Floral Markers in Honey of Various Botanical and Geographic Origins: A Review. *Compr. Rev. Food Sci. Food Saf.* **2010**, *9*, 620–634.

Kossuth, S.; Scheer, R. Ogeechee Tupelo http://www.na.fs.fed.us/pubs/silvics_manual/volume_2/nyssa/ogeche.htm.

Louveaux, J.; Maurizio, A.; Vorwohl, G. Methods of Melissopalynology. *Bee world* **1978**, 139–153.

Maurizio, A. How bees make honey. In: *Honey: A Comprehensive Survey*; Crane, E., Ed.; Crane, Russak & Company, Inc.: New York, 1975, 77-105.

Molan, P. C. Authenticity of Honey. In: *Food Authentication*; Chapman & Hall, 1996.

Overton, S.; Manura, J. Flavor and Aroma in Commercial Bee Honey – a Purge-and-Trap Thermal-Desorption Technique for the Identification and Quantification of Volatiles and Semivolatiles in Honey. *Am. Lab.* **1994**, *26*, 45–53.

Plenge, Reno. Personal communication. March 23, 2015.

Pohl, P. Determination of Metal Content in Honey by Atomic Absorption and Emission Spectrometries. *TrAC - Trends Anal. Chem.* **2009**, *28* (1), 117–128.

Subramanian, R.; Umesh Hebbar, H.; Rastogi, N. K. Processing of Honey: A Review. *Int. J. Food Prop.* **2007**, *10*, 127–143.

Sudarsan, R.; Thompson, C.; Kevan, P. G.; Eberl, H. J. Flow Currents and Ventilation in Langstroth Beehives due to Brood Thermoregulation Efforts of Honeybees. *J. Theor. Biol.* **2012**, *295*, 168–193.

Terrab, A.; Recamales, A. F.; Hernanz, D.; Heredia, F. J. Characterisation of Spanish Thyme Honeys by Their Physicochemical Characteristics and Mineral Contents. *Food Chem.* **2004**, *88*, 537–542.

United States Department of Agriculture; National Agricultural Statistics Service; Agricultural Statistics Board. *Honey*; 2015.

United States Department of Agriculture; Agricultural Marketing Service; Fruit and Vegetable Programs; Market News Division. *National Honey Report*; 2013.

United States Department of Agriculture; Service Agricultural Marketing; Programs Fruit and Vegetable; Division Market News. *National Honey Report*; 2014.

United States Department of Agriculture. United States Standards for Grades of Extracted Honey. *Fed. Regist.* **1985**, *50FR15861*, 1–12.

Viuda-Martos, M.; Ruiz-Navajas, Y.; Zaldivar-Cruz, J. M.; Kuri, V.; Fernández-López, J.; Carbonell-Barrachina, Á. a.; Pérez-Álvarez, J. Á. Aroma Profile and Physico-Chemical Properties of Artisanal Honey from Tabasco, Mexico. *Int. J. Food Sci. Technol.* **2010**, *45*, 1111–1118.

von der Ohe, W.; Oddo, L. P.; Piana, M. L.; Morlot, M.; Martin, P. Harmonized Methods of Melissopalynology. *Apidologie* **2004**, *35*, 18–25.

Wen, J.; Stuessy, T. F. The Phylogeny and Biogeography of Nyssa (Cornaceae). *Syst. Bot.* **1993**, *18* (1), 68–79.

White, J. Composition of honey. In: *Honey: A Comprehensive Survey*; Crane, E., Ed.; Crane, Russak & Company, Inc.: New York, 1975, 157-206.

White, J. W.; Riethof, M. L.; Subers, M. H.; Kushnir, I. *Composition of American Honeys*; Washington, D.C., 1962.

CHAPTER 3

PHYSICOCHEMICAL CHARACTERISTICS OF TUPELO HONEY

3.1 ABSTRACT

Physicochemical characteristics of ten Tupelo honey samples from five different locations collected during two consecutive seasons (2013 and 2014) were determined including moisture content, ^{0}Brix, water activity (A_w), pH, titratable acidity, ash content, and fructose and glucose contents. On the basis of pollen analyses, nine samples could be considered as authentic Tupelo honeys, with one (designated honey 3 from the 2014 season) containing mainly holly pollen along with appreciable levels of tupelo pollen. This sample also had a noticeably higher pH value and differed significantly in ash content from the other Tupelo honey samples, suggesting ash content to be a good indicator of botanical origin. Sensory screening indicated this sample to be significantly different from the other samples as well. Honey 5 produced during both seasons was the most consistent sampling location with the highest amount of Tupelo pollen content present in each sample. Panelists could not distinguish a difference between the honey 5 samples from both seasons during sensory testing. Composition data were comparable to literature values and within the limits set by Codex Alimentarius.

3.2 INTRODUCTION

Currently at the national level in the United States there are no regulations in place regarding a standard of identity for honey; rather there is a guidance document for proper labeling of honey products. The Food and Drug Administration guidance defines honey as a unifloral honey if "the particular plant or blossom is the chief floral source of the honey…"

(2014). On the other hand, the Florida Department of Agriculture and Consumer Services denotes a standard of identity for honey, Florida being the state where the majority of Tupelo honey is produced. This rule states that "honey may be designated according to floral or plant source if it comes predominantly from that particular source and has the organoleptic, physicochemical and microscopic properties corresponding to that origin" (2009), which is nearly identical to the Codex Alimentarius standard (2001).

Pollen analysis is a common technique used to determine the geographical and botanical origin of honey (Louveaux et al., 1978). Although it is a very useful technique there are several limitations to pollen analysis. Since honey is produced from regurgitated nectar, pollen present in the nectar will be found in honey. Some nectar sources produce less pollen than others and this can skew the results. The size of the pollen granules can also have an effect because bees are able to filter the nectar in their honey sacs, with a greater ability to filter larger pollen granules over smaller ones. Pollen from an anemophilous, or wind-pollinated plant can enter the hive through wind currents or on the bodies of bees even though these plants do not produce any nectar. Other pollen types can also become attached to a bee during nectar collection and can become dislodged in the hive, thus contaminating open combs (Bryant and Jones, 2001). Furthermore, the identification of different pollen types is based on the training and experience of the analyst and by comparison to references (Von Der Ohe et al., 2005). Many pollen granules cannot be identified by their genus or species name and can only be listed by family (Louveaux et al., 1978). With the limitations of pollen analysis it is important to confirm the results by testing the physicochemical characteristics of unifloral honey. Certain types of unifloral honeys have unique characteristics that can aid in determining the botanical origin

(Louveaux et al., 1978; Von Der Ohe et al., 2005). Tupelo honey, for example, has a high fructose content (Louveaux et al., 1978).

The physicochemical characteristics are especially important to study because research on Tupelo honey is scarce. The only known study that included Tupelo honey was performed by White and others (1962). The main limitation of that study was the lack of pollen analysis to aid in confirming sample origin. Therefore, the Tupelo samples were labeled as such based on the identification of the beekeepers that offered samples for testing. This can be rather inaccurate considering that Bryant and Jones (2001) discovered that more than 60% of identifications made by the beekeepers as to the nectar sources of honeys were incorrect. The aim of this research was to verify and expand upon some of the physicochemical characteristics of Tupelo honey, including moisture content, ^{0}Brix, water activity (A_w), pH, titratable acidity, ash content, and fructose and glucose contents.

3.3 MATERIALS AND METHODS

Materials

Tupelo honey was obtained from the president of the Tupelo Beekeepers Association (Panama City, Florida). Five different producers provided samples in 2013 and again in 2014. These samples were collected from various locations in the panhandle region of Florida where the majority of Tupelo honey is produced. Locations are indicated on a map (**Figure 3.1**) and in **Table 3.1** at the end of this chapter.

Chemicals

Standardized hydrochloric acid solution (0.05 N) was purchased from Ricca Chemical Company (Arlington, TX) and standardized 0.05 N sodium hydroxide solution was purchased

from Fischer Scientific (Pittsburg, PA). Krystar 300 crystalline fructose was purchased from Tate & Lyle (Decatur, IL) and D-(+)-glucose was purchased from Sigma Chemical Co. (St. Louis, MO).

CO_2-free water was obtained by boiling distilled, deionized water for 5-10 minutes. Deodorized water was made in a similar fashion, except it was boiled to one-third its original volume.

Pollen content

Thirty grams of each sample were sent to Dr. Vaughn Bryant at Texas A&M University for pollen analysis. **Appendix A** contains a summary of the standard procedure used in his laboratory.

Moisture Content and ^{0}Brix

An Atago Hand Refractometer 2520-E02 was utilized to determine the moisture content of each honey sample in triplicate. A small amount of honey was placed on the prism of the refractometer. By viewing through the eyepiece a line between light and dark areas signified the moisture content. A table was used to adjust for temperature since the refractometer is calibrated for 20^{0}C. Another table and calculation were utilized to find ^{0}Brix from the moisture content.

Water Acitivity (A_w)

An Aqua Lab 4TE water activity meter (Decagon Devices, Pullman, WA) was used to determine the water activity at 25^{0}C in triplicate.

pH

The pH of each sample was measured in triplicate according to AOAC Official Method 962.19. Ten grams of honey and 75 mL of CO_2-free distilled, deionized water were mixed

thoroughly and pH values were measured using a digital pH meter (Mettler Toledo, Columbus, OH).

Titratable Acidity

Free, lactone, and total acidity was determined according to AOAC Official Method 962.19. Ten grams of honey and 75 mL of CO_2-free distilled, deionized water were mixed thoroughly using a magnetic stir bar. The electrode of the pH meter was immersed in the solution and each sample was titrated to an endpoint of 8.50 with 0.05 N NaOH solution (free acidity). Without delay, 10 mL of 0.05 N NaOH was pipetted into the solution and immediately back-titrated to an endpoint of 8.30 with 0.05 N HCl solution (lactone acidity). Total acidity was calculated as the sum of the free and lactone acidities. All samples were analyzed in triplicate. Values were expressed as milliequivalents of acids per kg of honey.

Ash Content

Ash content was determined by using a modification of AOAC Official Method 920.181. Five grams of honey were weighed directly into pre-dried 40 mL porcelain crucibles. Each honey was analyzed in triplicate. The samples were placed into a forced air oven at a starting temperature of 60^0C. Over several days the temperature was increased from 60^0C to 80^0C to 100^0C to 120^0C in order to slowly dry the honeys to prevent foaming during the ashing step. Once the honeys were dark and dry they were transferred to a furnace and ashed overnight at 600^0C. All samples were cooled in a desiccator and then weighed to find the ash content.

Sugar Content

Twenty-five milligrams of honey were diluted to 10 mL with Millipore filtered water. Two milliliters of the supernatant were filtered through a 0.45μm PTFE filter (Fischer Scientific, Pittsburg, PA). HPLC analysis was carried out using an Agilent 1200 Series HPLC equipped

with a refractive index detector. The mobile phase was Millipore filtered water with a 300 mm x 7.8 mm i.d. Phenomenex RCM-Monosaccharide column held at 80^0C. The flow rate was 0.6 mL/min with an injection volume of 10 μL. A standard solution consisting of 3 mg/mL of fructose (Tate & Lyle, Decatur, IL) and 1.5 mg/mL of glucose (Sigma, St. Louis, MO) was prepared. Dilutions of this solution of 50%, 30%, and 20% were made to create an external calibration curve.

Sensory Testing

This research was conducted with the approval of the Institutional Review Board (IRB) office of the University of Illinois as IRB protocol number 15305. An R-index by ranking test was used to determine sensory differences from the noise and least significant ranked differences (LSRD) was used to determine if sensory differences existed among samples. The noise was selected based on the sample with the highest Tupelo pollen content, which was honey 5 from the 2013 season. All samples were presented in 125 mL Nalgene PTFE odorless squeeze bottles (Fischer, Pittsburg, PA) that were labeled with three digit codes. Each bottle contained 10 ± 0.1 grams of honey and 5 mL of deodorized water. These amounts were chosen based on preliminary sensory testing and the recommendations of Piana et al (2004). The thirty panelists performed each of the three phases in duplicate with a one minute break between replicates. Panelists were instructed to put the samples in order from most similar to least similar to the noise. The noise was always included as a sample to be ranked as well. A sample ballot for phase 1 can be found in **Appendix D**.

In phase 1, the samples were from the 2013 season. Phase 2 included the 2014 season and honey 5 from the 2013 season as the noise. Phase 3 included honeys 5 and 1 from both seasons since all four of these honeys had the highest Tupelo pollen contents in both seasons.

The sample orders presented to panelists were randomized using Williams Design. A response matrix was constructed for each phase in order to calculate the R-index values. The number of times each sample was placed at each rank was totaled for each phase. The data were then converted to a R-index matrix using O'Mahony's method (1992). In phase 1 for example, samples given a ranking of 1 "most similar to the noise" were designated as noise sure and samples given a ranking of 5 "least similar to the noise" were designated as sample sure. The R-index value for each sample was calculated by O'Mahony's R-index equation (1992) by using honey 5 from the 2013 season as the noise. The critical value (n=55 for phases 1 and 2, n=60 for phase 3) for a two-tailed test at α=0.05 were compared to the calculated R-index values to determine if there were significant differences from the noise. Data was also analyzed by Friedman test of ranked sums analysis with multiple comparison procedure of least significant ranked differences (LSRD) to determine significant differences among samples.

3.4 RESULTS AND DISCUSSION

The results (mean and standard deviation) of the physicochemical characteristics of Tupelo honey can be found in **Table 3.3**. Analysis of variance (ANOVA) and least significant difference (LSD) were carried out for each characteristic. All characteristics were found to be normal with equal variances ($p>0.05$). All samples within a characteristic were not equal and LSD was performed to distinguish differences among samples. Composition of American Honeys (White et al., 1962) will be referred to often because it is the most comprehensive study of American honeys and is the only known publication to include Tupelo honey for testing of physicochemical characteristics. Five hundred and two samples were collected and analyzed, 6 of which were proclaimed as Tupelo honey from the 1957 season (White et al., 1962). **Table 3.4**

displays the experimental ranges and average values for each physicochemical characteristic compared to previously determined values by White and others (1962).

Pollen content

Certain unifloral honeys, like Tupelo, can command a higher price in the market (Bryant, V. and Jones, G., 2001). For this reason, correct identification of these unifloral honeys is important and pollen analysis is a useful tool to determine the geographical and botanical origin of honey (Von Der Ohe et al., 2004). While the Food and Drug Administration only provides a guidance and no regulations for honey, Codex Alimentarius (2001) states that a unifloral honey must "…come wholly or mainly from that particular plant source…". Many melissopalynologists determine unifloral honeys based on frequency classes where the presence of 45% or more of a particular pollen content signifies a predominant pollen (Louveaux et al., 1978; Von Der Ohe et al., 2004; Bryant, V. and Jones, G., 2001). Pollen identification is made by counting pollen grains and using references to determine the plant origin (Louveaux et al., 1978). **Figures 3.2 and 3.3** show the magnification of a Tupelo and holly pollen granule next to a scale for comparison. **Table 3.2** displays the pollen taxa identified in the majority (6 or more) of Tupelo honeys from the 2013 and 2014 seasons. A full list of pollen taxa identified in each sample can be found in **Appendix B** and **C**. Honeys 1, 4, and 5 appear to be the most consistent in terms of Tupelo pollen content observed between seasons, with honey 5 containing the most abundant Tupelo pollen content each year (**Figure 3.4**). **Figure 3.5** visually shows the three pollen taxa identified in all ten Tupelo honey samples. Tupelo and holly are the most prevalent pollen types, with oak being present in minimal amounts. Quercus (oak) is an anemophilous, or wind-pollinated taxa, which means this genus of plant is nectarless (Louveaux et al., 1978). Bees will still forage for pollen from Quercus thereby bringing it into the hive, or pollen of this

genus may enter the hive through the air (Bryant, V. and Jones, G., 2001). Tupelo and holly pollen enter the hive mainly through the collection of their nectar by honey bees. It can also be observed in **Figure 3.5** that honey 3 from the 2014 season is not Tupelo honey, but instead holly honey. This sample is considered holly honey by both the Codex Alimentarius standard, coming mainly from a particular plant source, and the frequency classification, 45% or more of a pollen taxa present signifies a unifloral honey.

Moisture Content and ^{0}Brix

Moisture content is affected by environmental conditions, harvest season, maturation of honey in the hive, and handling by beekeepers (Iglesias et al., 2012, Feas et al., 2010). Fermentation occurs in honeys with high moisture contents, therefore, the Codex Alimentarius standard (2001) states that the moisture content should not exceed 20%. According to the International Honey Commission (1999), this is the only parameter that must be followed in the trade of honey worldwide. The United States Department of Agriculture has set grading standards for honey with a moisture content of 18.6% being given a grade of A or B and a moisture content of 20% defined as grade C (USDA, 1985). Tupelo honey values were found to range from 16.0-18.1% with an average of 17.7%. This is slightly lower than what was characterized by White and others (1962), which ranged from 17.4-18.5% with an average of 18.2%. As mentioned previously, this could be due to climatic differences and beekeeper practices.

Degrees Brix has a linear relationship with moisture content and is commonly calculated based off of moisture content when using a refractometer, as was done in this study. A high correlation of 0.97 was identified by Terrab and others (2004) when using different refractometers to determine moisture content and ^{0}Brix separately. Abnormal ^{0}Brix values can

be a reliable indicator of adulteration (Terrab et al., 2004). Determinations made on Tupelo honey ranged from 80.2-81.9% with an average of 81.2%.

Water Activity (A_w)

Although moisture content is the industry standard used to determine likelihood of fermentation, water activity (a_w) is a more reliable parameter to indicate likelihood of microbial growth (Zamora et al., 2005). Osmotolerant yeast cause fermentation, however, they are unable to grow below a_w value of 0.6 (Viuda-Martos et al., 2010). All tupelo honey samples had a_w values below 0.6, with a range of 0.553-0.585.

pH

Besides water activity, pH is also an important factor that governs and can reduce the growth of microorganisms (Feas et al., 2010). Although pH is a reflection of the presence of hydronium ions in a solution it is not directly related to free acidity. This is due to the buffering action of the minerals and acids present (White et al., 1962). The range of pH values of American honeys is 3.2-4.5 with an average of 3.9 (Crane, 1975). White and others (1962) observed a range of 3.80-4.09 with an average of 3.89 for Tupelo honeys. This study ascertained somewhat similar results with a range of 3.77-4.22 and an average of 3.95. A point of interest is revealed when observing the pH value of the sample that was identified as holly honey through melissopalynological analysis. This sample had a notably higher pH value of 4.22 even though it was not determined to be an outlier by box plot statistical analysis. However, the statistical method of LSD showed that all other samples were significantly different from the holly honey. When the holly honey is removed, the range (3.77-4.03) and average (3.92) correspond to the values determined by White and others.

Titratable Acidity

Gluconic acid in equilibrium with its lactone is the predominant acid in honey, and some other minor organic acids also exist in honey (Viuda-Martos, 2010). Free, lactone and total acidity are identified using a fixed endpoint titration method. This method is rather challenging due to persistent drift of the endpoint because of lactone hydrolysis. The International Honey Commission (2000) noted that this method was not very reproducible. The limit set by Codex Alimentarius (2001) is 50 milliequivalents per kilogram or less for free acidity, but no guideline is given for lactone or total acidity. All samples tested were below this limit for free acidity with a range of 14.03-24.22 meq/kg and an average of 19.18 meq/kg. The lactone content ranged from 1.77-7.77 meq/kg with an average of 4.83 and the total acidity ranged from 15.79-30.72 meq/kg with an average of 23.89 meq/kg. White and others (1962) identified higher values of free, lactone, and total acidity with ranges of 20.41-30.58 meq/kg, 8.03-14.56 meq/kg, 30.27-45.14 meq/kg, and averages of 25.46 meq/kg, 11.12 meq/kg, and 36.59 meq/kg respectively. This could be due to the difficulty and poor precision of the method, or due to the storage methods used by White and others with the majority of Tupelo honey samples stored for 19 months at room temperature before being testing. An increase in free and lactone acidity has been identified in citrus honey with a 12 month storage time at room temperature (Castro-Vasquez et al., 2008). This would also correspond to an increase in total acidity since it is determined as the sum of free and lactone acidity.

Ash Content

The mineral content, or ash, of a honey may be a good indicator of botanical origin (Viuda-Martos et al., 2010; Bogdanov et al., 2000; Terrab et al., 2004). The present study observed a significant difference between the Tupelo honeys analyzed and the odd sample

identified as holly honey. The range for the Tupelo honeys was 0.0649-0.1236% with an average of 0.0924%, while the holly honey value was 0.2332%. This is nearly double the highest Tupelo value and was determined to be an extreme outlier by box plot analysis. The ash content determined by White and others (1962) ranged from 0.108-0.149% with an average of 0.128%. Even with an extensive drying time prior to ashing the complex matrix of honey may have still caused some loss due to foaming, which could account for the slightly lower values reported in this study. A possible decrease in environmental contaminants in the geographic region over the last 53 years could also account for the difference, since the majority of minerals in honey originate from the soil (Pohl, 2009).

Sugar Content

Honey consists mainly of sugars and water, with sugar accounting for more than 95% of dry matter present in honey (Wang and Li, 2011). Fructose and glucose are the most abundant sugars in honey and account for 85-95% of the total carbohydrates present in honey (Crane, 1975). The Codex Alimentarius standard (2001) states that the sum of fructose and glucose should be 60g / 100g or more. All Tupelo honey samples tested were well above this standard. The glucose content of samples analyzed ranged from 25.87-28.58% with an average of 27.28%, while the fructose content was much higher with a range of 41.48-45.46% and an average of 43.57%. White and others (1962) observed similar values with a range of 23.83-29.37% and an average of 25.95% for glucose and a range of 42.25-44.26% with an average of 43.27% for fructose. The fructose content of Tupelo honeys is markedly higher than other American honeys. The average fructose content for American honeys is 38.1 with a standard deviation of 2.1, making Tupelo honey more than two standard deviations above the norm.

Several studies have focused on the contents of specific sugars of honey because of the unfortunate practice of adulteration. Common adulteration techniques include mixing honey with inexpensive sugar products after it has been harvested or feeding bees sugar syrup during the nectar flow to increase yield (Cotte et al., 2003). Adulteration of honey has been identified through differences in isotope ratios, quantities of oligosaccharides present, differences in ratios of sugars such as fructose/glucose, and differences in other physicochemical characteristics from unadulterated samples from the same botanical origin (Bogdanov and Martin, 2002; Cotte et al., 2003). Tupelo honey's higher than average fructose content is noteworthy, making the content of this particular monosaccharide highly representative of Tupelo honey compared to other American honeys. Therefore, the determination of fructose content of Tupelo honey can be a satisfactory initial test for adulteration.

Sensory Testing

The R-index values calculated from the ranking data for phases 1, 2, and 3 are given in **Table 3.5**. The R-index values are compared to the critical value for significance at $p<0.05$, which is 50 ± 12.89 at $n=55$ for phase 1 and 2, and 50 ± 12.36 at $n=60$ for phase 3. Honey 5 from the 2013 season was used as the noise in all three phases since it had the highest amount of Tupelo pollen content. The R-index values for phase 1, which included samples from the 2013 season, indicate that all samples were significantly different from the noise. Phase 2, which included samples from the 2014 season, indicates that honeys 1 and 3 were significantly different from the noise, but honeys 2, 4, and 5 were not significantly different. Phase 3, which included honeys 1 and 5 from both seasons, indicates that honey 1 from both seasons were significantly different from the noise, but honey 5 (2014) was not.

The results from the least significant ranked differences (LSRD) analysis, given in **Table 3.6**, are in agreement with the R-index data. Differences identified by panelists are indicated by superscripts. Data from phase 2 suggests that panelists could distinguish among all authentic Tupelo honey samples and honey 3 (2014), which was identified as holly honey by pollen analysis. Honeys 5 and 1 were chosen to compare between the 2013 and 2014 seasons in phase 3 because they consistently had the highest Tupelo pollen content in both seasons, with honey 5 always having the highest content. For this reason it is interesting that honey 1 in both seasons was determined to be significantly different from the noise in all three phases of testing. In phase 3 honey 1 was identified as significantly different between seasons as well. The exact cause is unknown, but may be due to differences in processing techniques among producers and between years. Results from phase 2 and 3 indicate that panelists could not distinguish a difference between honey 5 from the 2013 season and honey 5 from the 2014 season. The panelists' inability to distinguish differences between seasons for honey 5 indicates a consistent product with carefully controlled processing techniques.

3.5 TABLES AND FIGURES

Table 3.1 Locations of collected samples (panhandle of Florida).

	Location
honey 1	Chipola River, Dalkeith Area
honey 2	South side of Mystic Lake, east side of Appalachicola River
honey 3	Choctawhatchee River, Pine Log and Ebro Area
honey 4	East side of Appalachicola River, southern most area
honey 5	Holmes Creek, Poplar Head Community

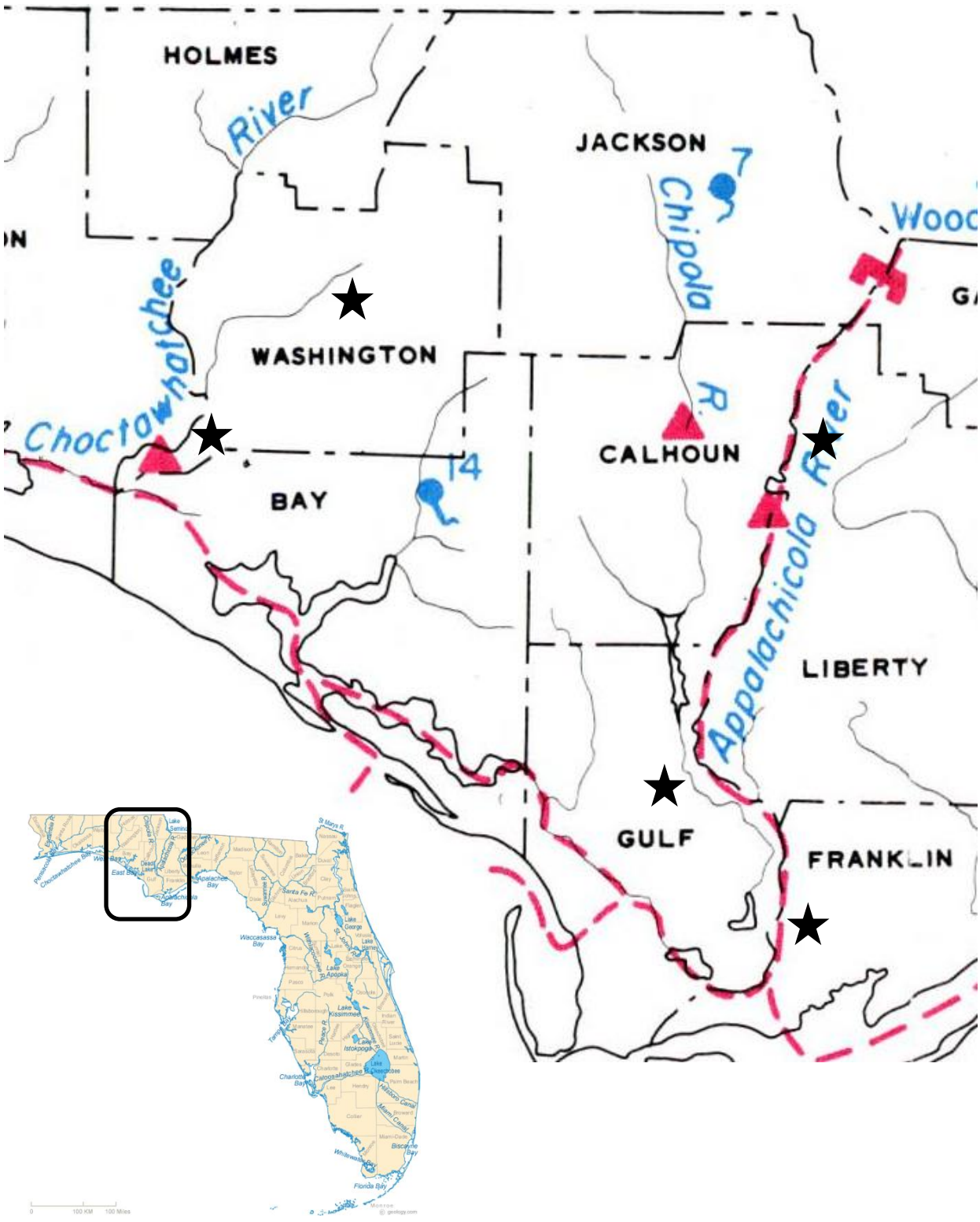

Figure 3.1 Modified map of Florida retrieved from Geology.com. Zoomed in and modified map of select areas in the Florida panhandle courtesy of the Florida Department of Environmental Protection's Florida Geological Survey. Black stars signify Tupelo honey sampling locations.

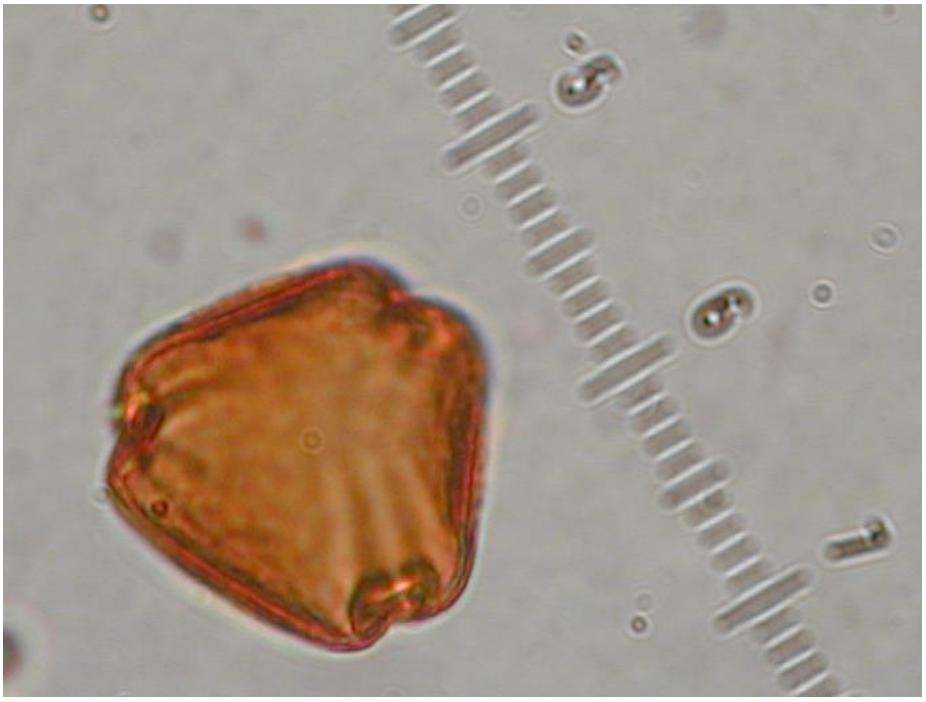

Figure 3.2 Magnification (400X) of a *Nyssa ogeche* (white Tupelo) pollen granule. Scale is 25 μm between numbers.

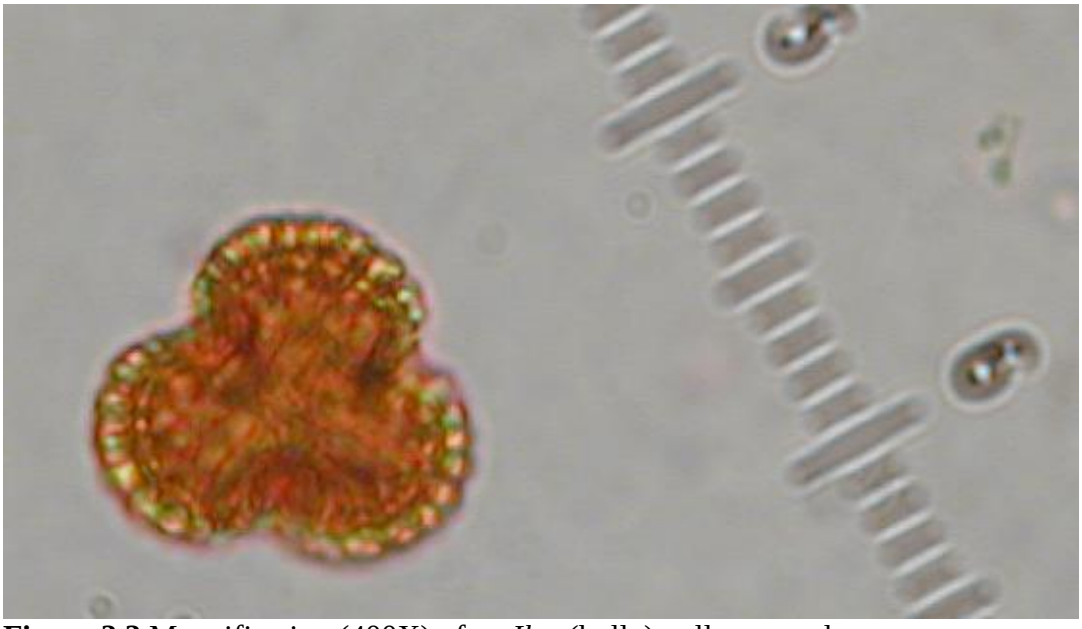

Figure 3.3 Magnification (400X) of an *Ilex* (holly) pollen granule. Scale is 25 μm between numbers.

Table 3.2 Pollen taxa identified in the majority (6 or more) of Tupelo honey samples.

	honey 1		honey 2		honey 3		honey 4		honey 5	
Pollen type	**2013**	**2014**	**2013**	**2014**	**2013**	**2014**	**2013**	**2014**	**2013**	**2014**
***Nyssa ogeche* (tupelo)**	73.3%	69.9%	47.0%	62.6%	60.0%	37.9%	65.1%	68.3%	88.0%	84.7%
***Ilex* (holly)**	18.8%	7.3%	21.5%	26.0%	20.5%	46.3%	27.8%	18.4%	4.1%	7.0%
***Quercus* (oak)**	1.0%	2.4%	3.0%	0.4%	2.0%	1.0%	0.9%	1.7%	0.9%	0.8%
***Gleditsia* (honey locust)**	0.5%	0.5%	1.0%	0.8%	7.8%	9.9%	0.5%	0.7%	0.5%	0.0%
***Rubus* (blackberry, dewberry)**	1.0%	2.4%	0.5%	0.0%	1.0%	0.5%	0.0%	0.7%	0.9%	1.2%
***Vitis* (grape)**	0.0%	0.0%	1.0%	0.4%	0.5%	0.5%	0.5%	2.4%	0.5%	0.4%
ROSACEAE (rose family)	1.5%	2.4%	5.0%	0.0%	0.0%	0.5%	0.9%	1.7%	0.0%	0.4%
***Salix* (willow)**	0.0%	4.9%	6.0%	1.1%	1.5%	0.0%	0.0%	0.7%	0.9%	2.1%

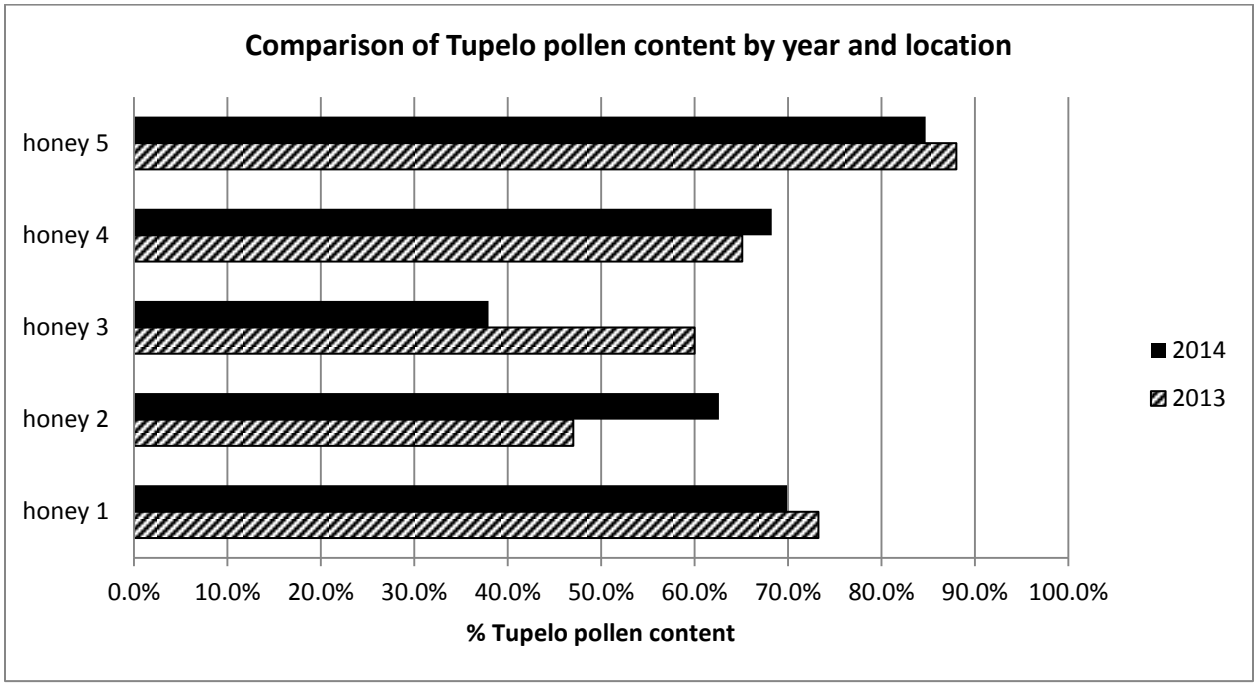

Figure 3.4 Comparison of *Nyssa ogeche* (white Tupelo) pollen content by year and location.

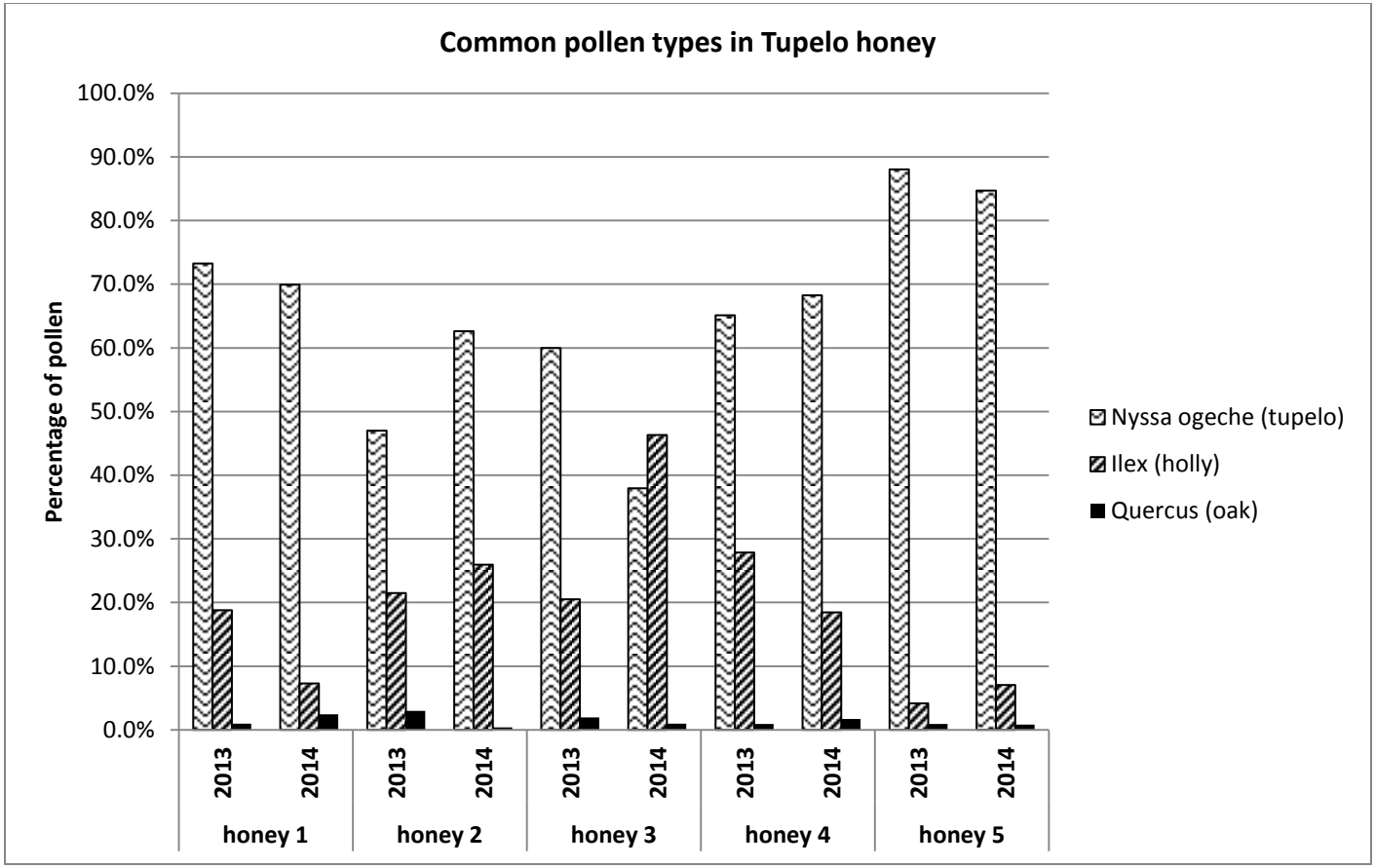

Figure 3.5 Common pollen types identified in all Tupelo honey samples collected.

Table 3.3 Physicochemical characteristics of ten Tupelo honey samples.

Attribute	honey 1		honey 2		honey 3		honey 4		honey 5	
	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014
Aw	0.575 ± 0.0016b	0.555 ± 0.0023d	0.550 ± 0.0008e	0.585 ± 0.0020a	0.556 ± 0.0008d	0.577 ± 0.0005b	0.563 ± 0.0003c	0.553 ± 0.0035de	0.553 ± 0.0035de	0.576 ± 0.0024b
Moisture (% w.b.)	17.7 ± 0.1b	17.0 ± 0.0d	16.4 ± 0.0f	18.1 ± 0.1a	16.7 ± 0.0e	17.5 ± 0.1c	16.9 ± 0.0d	16.4 ± 0.1f	16.0 ± 0.1g	17.7 ± 0.1b
^{0}Brix	80.6 ± 0.1d	81.3 ± 0.0c	81.9 ± 0.0ab	80.2 ± 0.1e	81.6 ± 0.0bc	80.8 ± 0.1d	81.4 ± 0.0c	81.8 ± 0.1ab	81.9 ± 0.6a	80.6 ± 0.1d
Ash (% w.b.)	0.0810 ± 0.0179ef	0.0920 ± 0.0102cde	0.1033 ± 0.0033cd	0.0885 ± 0.0075de	0.1236 ± 0.0076b	0.2332 ± 0.0051a	0.0898 ± 0.0082de	0.1070 ± 0.0133cb	0.0649 ± 0.0109f	0.0816 ± 0.0051e
pH	3.78 ± 0.39a	3.78 ± 0.18a	4.01 ± 0.47cd	3.77 ± 0.31a	4.03 ± 0.37d	4.22 ± 0.49e	4.03 ± 0.60d	3.94 ± 0.19bc	3.89 ± 0.45b	4.03 ± 0.34d
Free Acidity (meq/kg)	21.02 ± 0.59c	24.22 ± 0.26a	18.19 ± 0.66d	21.09 ± 0.74bc	18.62 ± 1.08d	22.44 ± 0.94b	16.78 ± 0.79e	19.44 ± 0.21d	14.03 ± 1.49f	15.99 ± 0.57e
Lactone (meq/kg)	2.94 ± 0.37d	6.50 ± 0.23ab	7.26 ± 3.41a	7.77 ± 2.49a	4.09 ± 0.90bcd	3.72 ± 1.55cd	6.16 ± 0.81abc	4.34 ± 0.89bcd	1.77 ± 0.42d	3.73 ± 0.49cd
Total Aciditiy (meq/kg)	23.96 ± 0.58cd	30.72 ± 0.41a	25.46 ± 2.77bc	28.87 ± 1.75a	22.72 ± 0.55de	26.16 ± 0.63b	21.72 ± 0.08ef	23.78 ± 0.75cd	15.79 ± 1.21g	19.73 ± 0.77f
Glucose (%)	25.87 ± 0.28e	30.69 ± 1.10a	27.57 ± 1.01bcd	27.63 ± 0.32bcd	26.92 ± 0.12cde	27.75 ± 1.17bcd	26.71 ± 1.01cde	27.97 ± 1.20bc	26.50 ± 0.15de	28.58 ± 0.79b
Fructose (%)	44.17 ± 0.66abcd	44.69 ± 0.15ab	43.14 ± 0.23bcd	45.46 ± 0.32a	42.76 ± 0.31de	41.48 ± 1.75e	43.60 ± 1.32bcd	42.94 ± 1.70cde	44.44 ± 0.18abc	43.07 ± 0.56bcd

All values indicate the means ± standard deviations of triplicate readings.
Means within a row that are given the same superscript letter indicate no significant differences ($p<0.05$) between honey samples according to least significant difference (LSD) test.

Table 3.4 Comparison of ranges and averages to previously reported Tupelo honey values.

Physicochemical characteristic	Experimental		White et al. (1962)	
	Range	Average	Range	Average
Aw	0.553-0.585	0.564	-	-
Moisture (% w.b.)	16.0-18.1	17.0	17.4-18.5	18.2
^{0}Brix	80.2-81.9	81.2	-	-
Ash (% w.b.)	0.0649-0.1236*	0.1065*	0.108-0.149	0.128
pH	3.77-4.22	3.95	3.80-4.09	3.89
Free Acidity (meq/kg)	14.03-24.22	19.18	20.41-30.58	25.46
Lactone (meq/kg)	1.77-7.77	4.83	8.03-14.56	11.12
Total Aciditiy (meq/kg)	15.79-30.72	23.89	30.27-45.14	36.59
Glucose (%)	25.9-28.6*	27.28*	23.83-29.37	25.95
Fructose (%)	41.5-45.5	43.57	42.25-44.26	43.27

* indicates that an outlier according to box plot analysis was not included in the range.

Table 3.5 R-index value (percentage) for phases 1-3.

	H1-13	H2-13	H3-13	H4-13	H5-13	H1-14	H2-14	H3-14	H4-14	H5-14
Phase 1$_a$	75.85$^+$	67.31$^+$	65.54$^+$	67.57$^+$	*	-	-	-	-	-
Phase 2$_a$	-	-	-	-	*	75.35$^+$	62.74	90.94$^+$	54.5	42.61
Phase 3$_b$	66.65$^+$	-	-	-	*	78.01$^+$	-	-	-	57

Samples are listed by number and year (ie. H1-13 = honey 1, 2013 season)
$_a$ indicates $p<0.05$ and n=55
$_b$ indicates $p<0.05$ and n=60
*indicates the noise
$^+$ indicates that the R-index for that sample is above 62.89 (n=55) or 62.36 (n=60). This means that the panelists identified this sample as being significantly different from the noise sample.

Table 3.6 Least significant ranked differences (LSRD) multiple comparisons rank superscripts for phases 1-3.

	Ascending order of rank sums from most to least similar compared to the noise					
Phase 1	H5-13 (132)^a	H3-13 (176)^b	H4-13 (180)^{bc}	H2-13 (184)^{bc}	H1-13 (213)^c	
Phase 2	H5-14 (148)^a	H5-13 (161)^{ab}	H4-14 (172)^{ab}	H2-14 (199)^{bc}	H1-14 (236)^c	H3-14 (323)^d
Phase 3	H5-13 (119)^a	H5-14 (129)^a	H1-13 (157)^b	H1-14 (195)^c		

Samples are listed by number and year (ie. H1-13 = honey 1, 2013 season)
Rank sums are presented in parenthesis.
Means within a row that are given the same superscript letter indicate no significant differences ($p<0.05$) between honey samples.

3.6 REFERENCES

Association of Official Analytical Chemists (AOAC). Official Methods of Analysis, 15th ed.; AOAC, Inc.: Arlington, VA, **1990**; 2, 1025-1033.

Acquarone, C.; Buera, P.; Elizalde, B. Pattern of pH and Electrical Conductivity upon Honey Dilution as a Complementary Tool for Discriminating Geographical Origin of Honeys. *Food Chem.* **2007**, *101*, 695–703.

Bogdanov, S. Honey Quality and International Regulatory Standards: Review by the International Honey Commission. *Bee World* **1999**, *80* (2), 61–69.

Bogdanov, S.; Lüllmann, C.; Martin, P.; von der Ohe, W.; Russmann, H.; Vorwohl, G.; Oddo, L. P.; Sabatini, A.-G.; Marcazzan, G. L.; Piro, R.; et al. Honey Quality, Methods of Analysis and International Regulatory Standards: Review of the Work of the International Honey Commission. *Swiss Bee Res. Cent.* **2000**, No. 8, 1–15.

Bogdanov, S.; Martin, P. Honey Authenticity: A Review. *Swiss Bee Res. Cent.* **2002**, No. 6, 1–22.

Bryant, V. M.; Jones, G. D. The R-Values of Honey: Pollen Coefficients. *Palynology* **2001**, *25*, 11–28.

Castro-Vázquez, L.; Díaz-Maroto, M. C.; González-Viñas, M. a.; De La Fuente, E.; Pérez-Coello, M. S. Influence of Storage Conditions on Chemical Composition and Sensory Properties of Citrus Honey. *J. Agric. Food Chem.* **2008**, *56*, 1999–2006.

Codex Alimentarius. Codex Standard for Honey. **2001**, 1–8.

Cotte, J. F.; Casabianca, H.; Chardon, S.; Lheritier, J.; Grenier-Loustalot, M. F. Application of Carbohydrate Analysis to Verify Honey Authenticity. *J. Chromatogr. A* **2003**, *1021* (1-2), 145–155.

Honey: A Comprehensive Survey; Crane, E., Ed.; New York, **1975**.

Feás, X.; Pires, J.; Iglesias, A.; Estevinho, M. L. Characterization of Artisanal Honey Produced on the Northwest of Portugal by Melissopalynological and Physico-Chemical Data. *Food Chem. Toxicol.* **2010**, *48*, 3462–3470.

Food and Drug Administration. Guidance for Industry: Proper Labeling of Honey and Honey Products. **2014**, 1–8.

Florida Department of Agriculture and Consumer Services. Standard of Identity - Honey., 2009.

Florida Lakes, Rivers, and Water Resources. http://geology.com/lakes-rivers-water/florida.shtml. (accessed September 12, 2013).

Iglesias, A.; Feás, X.; Rodrigues, S.; Seijas, J. a.; Vázquez-Tato, M. P.; Dias, L. G.; Estevinho, L. M. Comprehensive Study of Honey with Protected Denomination of Origin and Contribution to the Enhancement of Legal Specifications. *Molecules* **2012**, *17*, 8561–8577.

Louveaux, J.; Maurizio, A.; Vorwohl, G. Methods of Melissopalynology. *Bee world* **1978**, 139–153.

O'Mahony, M. Understanding Discrimination Tests: A User-Friendly Treatment of Response Bias, Rating and Ranking R-Index Tests and Their Relationship to Signal Detection. *J. Sens. Stud.* **1992**, *7* (916), 1–47.

Piana, M.; Oddo, L.; Bentabol, A.; Bruneau, E.; Bogdanov, S.; Declerck, C. Sensory Analysis Applied to Honey: State of the Art. *Apidologie*. **2004**, 17, 26-37.

Pohl, P. Determination of Metal Content in Honey by Atomic Absorption and Emission Spectrometries. *TrAC - Trends Anal. Chem.* **2009**, *28* (1), 117–128.

Snell, L. and Kenner, W. 1974. Surface Water Features of Florida. U.S. Geological Survey and Bureau of Geology, Florida Department of Natural Resources. Tallahassee, FL. Map Series 66. http://purl.fcla.edu/fcla/ic/UF90000309. (accessed October 14, 2013).

Terrab, A.; Recamales, A. F.; Hernanz, D.; Heredia, F. J. Characterisation of Spanish Thyme Honeys by Their Physicochemical Characteristics and Mineral Contents. *Food Chem.* **2004**, *88*, 537–542.

United States Department of Agriculture. United States Standards for Grades of Extracted Honey. *Fed. Regist.* **1985**, *50FR15861*, 1–12.

Viuda-Martos, M.; Ruiz-Navajas, Y.; Zaldivar-Cruz, J. M.; Kuri, V.; Fernández-López, J.; Carbonell-Barrachina, Á. a.; Pérez-Álvarez, J. Á. Aroma Profile and Physico-Chemical Properties of Artisanal Honey from Tabasco, Mexico. *Int. J. Food Sci. Technol.* **2010**, *45*, 1111–1118.

von der Ohe, W.; Oddo, L. P.; Piana, M. L.; Morlot, M.; Martin, P. Harmonized Methods of Melissopalynology. *Apidologie* **2004**, *35*, 18–25.

Wang, J.; Li, Q. X. *Chemical Composition, Characterization, and Differentiation of Honey Botanical and Geographical Origins*, 1st ed.; Elsevier Inc., 2011; Vol. 62.

White, J. W.; Riethof, M. L.; Subers, M. H.; Kushnir, I. *Composition of American Honeys*; Washington, D.C., 1962.

Zamora, M. C.; Chirife, J.; Roldán, D. On the Nature of the Relationship between Water Activity and % Moisture in Honey. *Food Control* **2006**, *17*, 642–647.

CHAPTER 4

IDENTIFICATION OF ODOR IMPORTANT COMPOUNDS IN TUPELO HONEY

4.1 ABSTRACT

Aroma-active compounds in Tupelo honey were identified by gas chromatography-olfactometry (GC-O) and gas chromatography-mass spectrometry-olfactometry (GC-MS-O). Initial analyses were performed on ten samples of Tupelo honey from five different locations collected during two consecutive seasons (2013 and 2014) by static headspace solid phase microextraction (H-SPME). The most important compounds based on perceived odor intensities determined by two assessors were phenylacetaldehye and nonanal. Further analysis was carried out on honey 5 from the 2013 and 2014 seasons due to its consistently high Tupelo pollen content across seasons and the inability of sensory panelists to distinguish between the two samples as discussed in chapter 3. The most potent odorants were determined through aroma extract dilution analysis (AEDA) of solvent extracts and sample dilution analysis by H-SPME (SDA-H-SPME). The most potent odorants identified in both dilution analyses include vanillin, phenylacetaldehyde, nonanal, (*E*)-2-nonenal, eugenol, guaiacol, 2-phenylethanol, 2'-aminoacetophenone, (*E*)-β-damascenone, and an unidentified odorant described as spicy and hay-like. (*E*)-β-Damascenone was determined to be the most potent odorant with extremely high flavor dilution (FD) factors of 59,049 (2013 season) and 19,683 (2014 season). Quantification of (*E*)-β-damascenone using stable isotope dilution analysis by H-SPME (SIDA-H-SPME) revealed that the compound had an extremely high concentration and odor-activity value (OAV) in Tupelo honey compared to other types of honeys and food products.

4.2 INTRODUCTION

Honey bees, *Apis mellifera*, produce the majority of the world's honey and have existed for millions of years (Crane, E., 1980; Alqarni et al., 2011). The nectar from flowers is the raw material collected by worker bees to produce honey. Bees extensively process the nectar by adding enzymes that convert the sucrose present in the nectar to fructose and glucose and others that transforms glucose to gluconic acid, and by evaporating the excess moisture to produce the complex matrix that is honey (Maurizio, A., 1975). Volatiles are present in very small amounts in honey and are affected by the floral source, storage, and the bee physiology. The contribution of volatiles by the floral source is great and is what mainly influences the differing aroma profiles of unifloral honeys (Ruisinger and Schieberle, 2012).

Several studies have been conducted on many different unifloral honeys to determine their volatile compounds and possible marker compounds. Marker compounds should be associated with the floral source of the honey, which include plant-derived compounds like terpenes and norisoprenoids (Manyi-Loh et al., 2011). Much of the research available presently identifies all of the volatiles present, but does not determine which are odor active and contribute to the overall aroma profile of the honey. In order to do this, gas chromatography-olfactometry (GC-O) should be utilized which allows an assessor to smell the compounds as they elute off the column. GC-O is also useful for dilution analyses such as aroma extract dilution analysis (AEDA) and sample dilution analysis (SDA) which involves the determination of odorant potency. This is done by smelling serial dilutions of a sample or aroma extract by GC-O until no odorants are detected. Odorants present in the highest dilution are considered the most potent odorants in the sample (Grosch, 1993).

Currently no exhaustive analysis of the aroma profile of Tupelo honey has been attempted. One study by Overton and Manura (1994) identified 31 volatiles in Tupelo honey in order to showcase purge-and-trap technology. This was an adequate preliminary study, but it needed to be expanded upon with the use of multiple extraction techniques and column types to ensure biases are accounted for in different techniques, and the use of GC-O and dilution analyses to determine the aroma active compounds that contribute to the aroma profile.

In the present study, initial analyses were performed on ten samples of Tupelo honey samples from five different locations collected during two consecutive seasons (2013 and 2014) by static headspace solid phase microextraction (H-SPME). H-SPME-GC-O was utilized to gain an understanding of the basic flavor profile of Tupelo honey. Further analyses were carried out on two samples of honey 5 from the 2013 and 2014 seasons due to the consistently high Tupelo pollen contents for these honeys across seasons and the inability of sensory panelists to distinguish between the two samples as discussed in chapter 3. The most potent odorants were determined through aroma extract dilution analysis (AEDA) of liquid extracts and sample dilution analysis by H-SPME (SDA-H-SPME).

4.3 MATERIALS AND METHODS

Materials

Tupelo honey was obtained from the president of the Tupelo Beekeepers Association (Panama City, Florida). Five different producers provided samples in 2013 and again in 2014. These samples were collected from various locations in the panhandle region of Florida where the majority of Tupelo honey is produced. Locations are indicated in Chapter 3 on a map (**Figure 3.1**) and in **Table 3.1**.

Chemicals

n-Alkane standards, 2-methyl-3-heptanone, 2-ethylbutyric acid, and 6-undecanone were purchased from Sigma-Aldrich Co. (St. Louis, MO). Diethyl ether (anhydrous, 99.9%), methanol (99.9%), sodium carbonate, hydrochloric acid (37.6%), sodium chloride (99%), and sodium sulfate (99%) were obtained from Fischer Scientific (Fairlawn, NJ). Nitrogen, liquid nitrogen, and ultra-high purity (UHP) helium were acquired from S.J. Smith (Davenport, IA). UHP hydrogen was purchased from Specialty Gases of America (Toledo, OH).

Odor-free water was prepared by boiling distilled-deoniozed water in an open flask until the original volume was reduced by one-third.

Reference Standard Compounds

The standard reference compounds used to confirm the odor properties and retention indices of the aroma compounds listed in **Tables 4.1-4.4** were purchased from the companies in parentheses: compound nos. **1-2, 4-7, 11-13, 15-20, 22, 24-25, 28-31, 33, 36, 39-41, 43, 47, 50** (Sigma-Aldrich, St. Louis, MO); **3** (Firmenich, Princeton, NJ); **42** (Avocado Research Chemicals, Lancashire, UK); **51** (Fluka, Bluchs, Switzerland).

Syntheses

cis-1,5-Octadien-3-one was synthesized using the method of Lin and others (1999). 2-acetyl-1-pyrroline was synthesized according to the method of De Kimpe and others (1993) with the first step detailed by Ogawa and others (1982). Hotrienol was synthesized by the method of Yuasa and Kato (2013). $^{2}H_{4}$-β-Damascenone was synthesized using the method of Kotseridis and others (1998).

Isolation of volatile compounds

Liquid extracts

Honey (100 g) was mixed with 550 mL of deodorized water and then spiked with 10 µL of an internal standard mix (5.80 µg/µL of 6-undecanone, 6.42 µg/µL of 2-methyl-3-heptanone, and 6.30 µg/µL of 2-ethyl butyric acid in methanol). The honey solution was extracted with 100 mL of diethyl ether in a liquid-liquid continuous extraction (LLCE) apparatus equipped with a diffusion tube and a 4°C condenser for 18 hours. The volatile compounds of this extract were then isolated from the non-volatile compounds using solvent-assisted flavor evaporation (SAFE) (Engel et. al, 1999), which was operated at high vacuum (~5 x 10^{-5} Torr) with a 40^0C water bath during the 2.5 hour total extraction time. The aroma extract produced by the above LLCE-SAFE procedure was fractionated into acidic (aqueous phase) and neutral-basic (organic phase) fractions by using 5% w/v Na_2CO_3 (3 x 20 mL). The aqueous layer was acidified using 4 N HCl to a pH of approximately 2.0 and then extracted using diethyl ether (3 x 20 mL). Both the acidic and neutral-basic fractions were washed with a saturated NaCl solution (2 x 10 mL for neutral-basic fraction and 2 x 15 mL for acidic fraction). Both fractions were then concentrated to 2 mL by Vigreux column distillation (43°C) and dried over anhydrous Na_2SO_4. The extracts were stored at -20°C and concentrated to 150 µL using a nitrogen stream before analysis. The procedure was followed to obtain acidic and neutral-basic fractions of honey 5 from the 2013 and 2014 season.

Static Headspace Solid Phase Microextraction (H-SPME)

Honey (1g) and 4 mL of an aqueous deodorized saturated NaCl solution were transferred to a glass 22 mL headspace vial equipped with a stir bar and a PTFE-lined silicone cap. The mixture was vortexed until homogenous and placed in a 60^0C water bath. After an incubation

time of 20 minutes, a three-phase (DVB/CAR/PDMS) SPME fiber (Supelco, Bellefonte, PA) was introduced into the headspace for a 30 minute extraction time.

Identification of Aroma Active Compounds and Determination of Potency

H-SPME-Gas Chromatography-Olfactometry (GC-O)

GC-O was performed by two assessors using a 6890 GC (Agilent Technologies, Inc., Palo Alto, CA) equipped with a flame ionization detector (FID) and an olfactory detection port (DATU Technology Transfer, Geneva, NY) to determine the aroma-active compounds in the headspace of Tupelo honeys. SPME injections were made in the hot splitless mode (260°C; 4 min valve-delay time; with final purge flow of 50 mL/min). All ten samples were analyzed using a polar RTX®-Stabilwax column and selected samples were analyzed on a nonpolar RTX®-5 column (both columns 15 m × 0.32 mm i.d. × 0.5 μm df; Restek, Bellefonte, PA). Column effluent was equally split between the olfactory detection port and the FID by the use of deactivated fused silica tubing (both 1 m x 0.25 mm i.d.: Restek). The detector temperatures were both set to 250°C. The oven temperature was programmed from 35°C to 225°C with an initial hold time of 5 minutes, followed by a ramp rate of 10°C/min (6°C/min on the nonpolar column), and a final hold time of 30 minutes. The carrier gas used was helium, at a constant flow rate of 2.0 mL/min.

The perceived odor intensity of each compound was rated during GC-O by two analysts. When the perceived strengths between analysts differed the average value was taken, where a 0 signifies no odor and an 8 indicates a very strong odor. In order to determine which odorants were most potent, the compounds were ranked (rank order) according to their perceived intensities. This was done by first separately arranging the compounds in each sample from

strongest to weakest based on their odor intensities in **Table 4.1**. The strongest odorant was given a ranking of 1 and the next strongest odorant a ranking of 2, and so on. When compounds were equal in strength they were given the same ranking and the next strongest compound was given a ranking as if a tie had not occurred (1 integer higher than the number of compounds ranked above it). For example, in **Table 4.1** nonanal and phenylacetaldehyde were determined to be the most potent odorants in honey 1 (2013) and were both given a rank of 1, while the next most potent odorants were given a rank of 3 and so on. Then, the ranks of each compound were averaged across all ten Tupelo honey samples and were converted to their rank order. This was done by giving the compound with the smallest average a rank of 1 and the compound with the largest average a rank of 40, which is the total number of compounds identified by H-SPME-GC-O. The average rank and rank order data can be found in **Appendix E**.

Aroma Extract Dilution Analysis (AEDA)

Acidic and neutral-basic fractions of honey 5 from both seasons were diluted stepwise at a 1:3 (v/v) ratio with diethyl ether. Each dilution was stored in a 2 mL clear glass vial at -20°C until it was analyzed. GC-O was performed on each dilution using a polar RTX®-wax column with an injection volume of 2 µL to ascertain the potency of the aroma active compounds in Tupelo honeys. To prevent the formation of artifacts due to heating during sample introduction, cool on-column mode (+3 °C temperature tracking mode) injection was utilized. Serial dilutions were sniffed sequentially until no odor active compounds were detectable by the human nose. The GC oven temperature was programmed from 40 to 225^{0}C at a ramp rate of 10 ºC/min, with initial and final holding times of 5 and 20 min, respectively. Helium was used as the carrier gas at a constant flow rate of 2.2 mL/min. Column effluent was split 1:1 between the FID and the sniffing port using deactivated fused silica tubing (each 1 m x 0.25 mm i.d.; Restek). The FID

and sniffing port were maintained at a temperature of 250^{0}C. GCO was conducted by two experienced panelists. The flavor dilution factor (FD factor) for a compound was the highest dilution at which it was detected by both assessors. In instances where the FD factor differed between analysts the average of the two log3FD factors was taken and rounded down to the nearest dilution.

Sample Dilution Analysis (SDA) by H-SPME

Sample dilution analysis by H-SPME was utilized to identify highly volatile compounds that may not be present in the AEDA extracts and their potency (Cadwallader and Heo, 2001). A stir bar and 20 mL of a deodorized saturated NaCl solution were added to honey (5000, 1000, 200 or 40 mg) in a glass vial equipped with a stir bar and a PTFE-lined silicone cap. This solution was mixed until homogenous and placed in a 60^{0}C water bath. After an incubation time of 20 minutes, a three phase (DVB/CAR/PDMS) SPME fiber (Supelco, Bellefonte, PA) was introduced into the headspace for a 30 minute extraction time. Dilutions were sniffed by two assessors on a RTX®-wax column. The method and parameters are the same as what is described previously in the H-SPME-GC-O section of this chapter.

GC-MS-O of liquid extracts and H-SPME

The GC-MS-O system consisted of a 6890 GC/5973N mass selective detector (Agilent Technologies, Inc.) and was used to identify volatile compounds in Tupelo honeys. Injections of neutral-basic and acidic fractions (2 µL) from LLCE-SAFE extracts were made by cold splitless using a CIS4 inlet (Gerstel, Germany; initial temperature -50^{0}C, held 0.10 min, ramped at 12^{0}C/s to 260^{0}C with a final hold time of 10 min; value-delay 1.10 min, with final inlet purge flow of 50 mL/min). H-SPME injections were in the hot splitless mode (260 °C; 4 min valve-delay with final inlet purge flow of 50 mL/min). Separations were performed on both a polar Stabilwax®-

DA column (30 m x 0.25 mm id x 0.25 µm film; Restek) and a non-polar HP-5MS column (30 m x 0.25 mm id x 0.5 µm film; Hewlett Packard, Palo Alto, CA). The carrier gas was helium at a constant flow rate of 1.0 mL/min. The sniff port line temperature was 250°C. The oven temperature was programmed from 35°C to 250°C with a ramp rate of 6°C/min and a hold time of 45 minutes. The H-SPME analysis was carried out on selected samples on both column types. MSD conditions were: capillary direct interface temperature, 250 ºC; ionization energy, 70 eV; mass range, 35-350 amu; electron multiplier voltage (Autotune +200V); scan rate, 5.2 scans/s.

As described by van den Dool and Kratz (1963), the retention index (RI) for each compound was calculated using its retention time (RT) compared to the RTs for a series of standard *n*-alkanes. Mass spectra were compared against the NIST2008 mass spectral database, and retention indices along with aroma descriptors were compared to literature values in order to tentatively identity each aroma active compound. Tentative identification was confirmed by comparing mass spectra, retention indices and aromas of authentic reference standards under the same GC-MS-O analyses used to analyze samples (Molyneux and Schieberle, 2007).

Quantitation by Stable Isotope Dilution Analysis (SIDA)

The deuterium labeled isotope of (*E*)-β-damascenone ($^{2}H_{4}$-(*E*)-β-damascenone) was prepared in methanol to dilute the isotope to a concentration of 85 µg/mL. The isotope solution (5 µL) was spiked into a mixture of 0.10 g honey with 1 mL saturated NaCl solution in a glass SPME vial. Each honey was analyzed in triplicate. Samples were analyzed on a nonpolar SAC-5 column (30 m x 0.25 mm id x 0.25 µm df; Supelco, Bellefonte, PA) in splitless mode. The GC-MS system consisted of a 6890A GC/5973N mass selective detector (Agilent Technologies, Inc.) equipped with a Gerstel MPS2 autosampler. Each sample was held at 60^{0}C during the

incubation step (20 min) and extraction step (30 min). The inlet was held at 260^{0}C. The oven temperature was programmed from 35°C to 250°C with a ramp rate of 4°C/min and a hold time of 30 minutes. The carrier gas used was helium with a constant flow rate of 0.7 mL/min. The integrated areas of the isotope and (*E*)-β-damascenone were found using Enhanced Data Analysis Software (Agilent Technologies, Inc.). The following equation was used to calculate the concentration of (*E*)-β-damascenone in each run and averaged to determine the final concentration in each Tupelo honey sample.

$$\frac{\frac{\text{area}_{\text{target ion}}}{\text{area}_{\text{isotope ion}}} \text{x } R_f \text{ x mass}_{\text{isotope added}}}{\text{honey weight}} = \text{mass}_{\text{target}}$$

The response factor (R_f) was determined to be 1.04 by a previous colleague (Lorjaroenphon and Cadwallader, 2015) and the target ion and the isotope ion were 69 and 73, respectively.

4.4 RESULTS AND DISCUSSION

Initial analyses were performed on ten samples of Tupelo honey from five different locations collected during two consecutive seasons (2013 and 2014). H-SPME-GC-O was utilized to gain an understanding of the basic flavor profile of Tupelo honey. Further analyses were carried out on two samples of honey 5 from the 2013 and 2014 seasons due to the consistently high Tupelo pollen contents for these honeys across seasons and the inability of sensory panelists to distinguish between the two samples as discussed in chapter 3. The most potent odorants were determined through SDA-H-SPME and AEDA of extracts prepared by LLCE-SAFE and were found to be fairly consistent in terms of the identification of the most potent odorants across both analysis methods and between samples. A schematic detailing the

isolation, identification and quantification techniques utilized in this study can be found in **Figure 4.1**.

H-SPME-Gas Chromatography-Olfactometry (GC-O)

Initial analyses of ten Tupelo honey samples by H-SPME-GC-O indicated a total of 40 odorants, of which 9 were unidentified (**Table 4.1**). The perceived odor intensity of each compound was rated during GC-O by two analysts. When the perceived strengths between analysts differed the average value was taken, where a 0 signifies no odor and an 8 indicates a very strong odor. Most compounds were rated with weak (2) to medium (5) odor intensities as seen in **Table 4.1**. These values are rather arbitrary since they were dependent on the odor perception by analysts, but can be helpful when determining common aroma compounds among the Tupelo honeys. When rank order of the different compounds are determined, trends begin to emerge that can help identify which compounds are the most common and important in Tupelo honey aroma. For example, phenylacetaldehyde, which has a rose aroma, and nonanal, which has a citrus aroma, were ranked the highest for odor intensities (**Appendix E**). Other important odorants identified included (*Z*)- and (*E*)-β-damascenone (cooked apple), guaiacol (smoky), 2'-aminoacetophenone (grape, corn tortilla), (*E*)-2-nonenal (dried hay), hotrienol (floral), vanillin (vanilla), and an unknown (RI_{wax}=1731) described as spicy and hay-like. It is clear that Tupelo honey has a complex flavor profile with varied aroma compounds. While this information provides an understanding of the aroma-active compounds present in Tupelo honey it does not truly distinguish which compounds are the most potent and important odorants. In order to accomplish this, dilution analyses need to be utilized to determine which compounds impact the flavor profile the most. Two such complimentary analyses techniques, SDA-H-SPME-GC-O and AEDA-GC-O, were implemented in this study and are discussed below.

Sample Dilution Analysis (SDA) by H-SPME

Sample Dilution Analysis (SDA) involves the stepwise decrease in sample volume or mass combined with GC-O to determine the potency of volatile compounds. More potent compounds will continue to be detected in smaller sample volumes and this allows for the determination of flavor dilution (FD) factors. A higher FD factor signifies a more potent odorant in the overall flavor profile of a sample (Cadwallader and Heo, 2001). Static headspace solid phase microextraction (H-SPME) is a common extraction technique because it does not involve the use of toxic solvents, requires minimal sample preparation, and allows for the extraction of highly volatile compounds found in the headspace without interference from the sample matrix (Manyi-Loh et al., 2011).

SDA-H-SPME was carried out on the 2013 and 2014 honey 5 samples (**Table 4.2**). This allowed for the determination of FD factors for each of the 40 odorants detected to determine the most potent odorants in the headspace of the honeys. The most important compounds detected at the highest dilution (least amount of sample required to detect odorant) were nonanal (citrus), (*Z*)- and (*E*)-β-damascenone (cooked apple), eugenol (cloves), and an unidentified (RI_{wax}=1731) compound (spicy, hay). The next most important compounds, being detected in at least one sample at the highest dilution (FD=125), were phenylacetaldehyde (rosy) and vanillin (vanilla). Compounds having an FD factor of 25 in at least one sample were octanal (orange), (*E*)-2-nonenal (dried hay), guaiacol (smoky), 2-phenylethanol (rose, wine), 2'-aminoacetophenone (grape, corn tortilla), isoeugenol (cloves, woody), and several unknowns (nos. 8, 32, 34, 37). These results for SDA-H-SPME were fairly consistent with data obtained by H-SPME analysis done on all ten samples. Some of the differences between SDA-H-SPME and perceived intensities determined by H-SPME were hotrienol (floral) and heptanal (orange) being perceived

as more intense by H-SPME, while eugenol (cloves), isoeugenol (cloves, woody), and an unknown (RI_{wax}=2034) compound (peachy, cloves) were perceived as less intense by H-SPME than by SDA-H-SPME. SDA-H-SPME determined that the importance of different compounds was consistent between samples with deviations of one FD factor on occasion. The main limitation of this method was the inability to complete the dilution analysis until no odorants were detected because of the difficulty in accurately weighing out miniscule amounts of samples.

Aroma Extract Dilution Analysis (AEDA)

Although H-SPME provides several benefits it also has its own limitations. The extraction of volatiles can depend on the affinity they have for the SPME fiber and on the addition of sodium chloride, heat, and agitation to release volatiles from the matrix (Cuevas-Glory et al., 2007). Since every extraction method seems to have its own challenges it is important to utilize at least two extraction techniques to ascertain the full aroma profile of a sample. For this reason honey 5 samples from both seasons were extracted by liquid-liquid continuous extraction (LLCE) in addition to SPME. This technique is a gentle and slow process that relies on the solubility of compounds in organic solvents and has the ability to extract compounds of varying volatilities (Andujar-Ortiz et al., 2009). Solvent-assisted flavor evaporation (SAFE) is then utilized to clean-up the extract and separate out any nonvolatile components extracted by LLCE (Engel et. al, 1999). The extracts are then separated into their acidic and neutral-basic fractions for easier GC-O analysis. Aroma extract dilution analysis (AEDA) can then be performed on sequential dilutions of these extracts to identify the most potent odorants. As in SDA, FD factors can be determined for each volatile based on the last (or highest) dilution in which each compound is detected.

In the basic-neutral fraction, 33 compounds were detected, 7 of which were unidentified (**Table 4.3**). In the acid fraction, 12 compounds were detected, 5 of which were unidentified (**Table 4.4**). (*E*)-β-Damascenone was determined to be the most potent odorant with extremely high FD factors of 59,049 in the 2013 season and 19,683 in the 2014 season. The next most potent odorants had FD factors of 2,187 and 729, which are three to four dilutions lower than (*E*)-β-damascenone. These compounds include eugenol, vanillin, and a spicy, hay-like unidentified odorant (RI_{wax}=1722). Several other compounds had FD factors of 729 or 243 depending on the sample analyzed, including 2'-aminoacetophenone and several unidentified compounds with floral or woody descriptors (nos. 44-46).

Comparison of Extraction Methods and Compound Potency

Table 4.5 includes selected compounds to compare the most potent odorants across extraction methods. Odorants were included if at least one sample had an FD factor of 25 or greater by SDA-H-SPME or a FD factor of 81 or greater by AEDA. Only compounds identified by both extraction methods were included, along with their rank order. When comparing the AEDA and SDA data there were several similarities, including the high potency of (*E*)-β-damascenone, eugenol, vanillin, and the unidentified spicy, hay-like compound (no. 27). There were also several differences, including compounds such as nonanal, (*E*)-2-nonenal, phenylacetaldehyde, and (*Z*)-β-damascenone being determined to be less potent by AEDA than by H-SPME. When comparing the calculated rank order of compounds from H-SPME and the FD factors from SDA-H-SPME, the most potent odorants identified by SDA-H-SPME (FD factor of 125 in at least one sample) were given rankings of ten or greater, meaning that they

were perceived to be in the top ten most potent odorants of the 40 total odorants detected. The only difference was eugenol being given a much lower ranking in comparison to its FD factor.

Another difference between extraction methods was the absence of compounds from one of the extraction methods all together. Nine compounds only found by H-SPME included several aldehydes, hexanal, heptanal, octanal, and decanal; alcohols such as hotrienol and isoeugenol; and several unknown compounds (nos. 8, 14, and 23). Compounds found only in the basic-neutral fraction included, acetal, 2-acetyl-1-pyrroline, 1-octen-3-ol, and several unknown compounds (nos. 44-46). Compounds found only in the acid fraction included Furaneol, sotolon, and several unknown compounds (nos. 37, 48, and 49). It is also interesting to note that guaiacol, vanillin and the spicy, hay-like compound (no. 27) were found in both the acid and basic-neutral fractions. This characteristic may be helpful in determining the identity of this unknown compound (no. 27) in the future.

GC-MS-O of liquid extracts and H-SPME

While GC-O is a useful method to determine odor active compounds and their potency, GC-MS is necessary to identify these compounds. The use of GC-MS-O enables an analyst to match the odor properties with the corresponding peak and mass specta easily since these data are simultaneously collected. The GC-MS-O was mainly used to acquire mass spectra for the purpose of positively identifying volatiles in this study. Of the 51 odorants detected, 28 were positively identified by the comparison of retention indices (RIs), odor properties and mass spectra of detected compounds with authentic reference compounds. Ten compounds were tentatively identified because useful mass spectra could not be acquired due to low abundance or

interference (co-elution) by more abundant substances. The remaining 13 compounds are indicated as unknown (unidentifiable).

Comparison to other honeys

The only known research involving the volatile analysis of Tupelo honey identified 31 volatile compounds. Since GC-O was not utilized, however, it is unlikely that many of the compounds identified are odor-active. Common compounds identified in both the present study and the previous research include diacetyl (2,3-butanedione), 3-methylbutanal, caproic acid (hexanal), heptanal, phenylacetaldehyde, 2-phenylalcohol, isoeugenol, and β-damascenone (Overton and Manura, 1994). Several important compounds identified in this study are absent from the Overton and Manura results, including eugenol, nonanal, vanillin, and many more. Since the previous research was primarily done to highlight a new purge-and-trap technology for a company, it did not represent an in depth thorough analysis of Tupelo honey and should be approached with caution. Furthermore, no information about the sample used indicated whether or not pollen analysis was conducted to validate its authenticity.

Many studies have analyzed the volatiles of different unifloral honeys and some have even attempted to identify marker compounds that are specific to that type of honey, whether it be in the unique combination of certain volatiles present or the large quantity of a specific compound. A simple comparison between important odorants identified in this study and volatiles determined in other honeys will be attempted. The criteria set for comparison includes any compound that received a FD factor of 25 or above for SDA or 729 and above for AEDA in either sample. In addition, each compound must have been detected by both SDA and AEDA.

By this criteria, vanillin, phenylacetaldehyde, nonanal, (*E*)-2-nonenal, eugenol, guaiacol, 2-phenylethanol, 2'-aminoacetophenone, and (*E*)-β-damascenone will be discussed.

Aldehydes. Vanillin was identified in raspberry, rape, heather, alder buckthorn, corontillo and buckwheat honeys (Montenegro et al., 2009; Zhou et al., 2002; Ruising and Schieberle, 2012; Seisonen et al., 2015; Kaskoniene et al., 2010). Phenylacetaldehyde was detected in most honeys including Tupelo, lavender, haze, chestnut, eucalyptus, raspberry, rape, heather, alder buckthorn, buckwheat, star thistle, blueberry, wildflower, clover, cranberry, thyme, citrus, rosemary, black mangrove, lime tree, alfalfa, and apple honeys (Kaskoniene et al., 2010; Seisonen et al., 2015; Ruising and Schieberle, 2012; Agila and Barringer, 2012; Zhou et al., 2002; Alissandrakis et al., 2007; Castro-Vasquez et al., 2008; Castro-Vasquez et al., 2009; Pino, 2012; Overton and Manura, 1994; Guyot et al., 1998). Nonanal was found to be present in strawberry tree, black mangrove, citrus, rosemary, lavender, thyme, eucalyptus, heather, star thistle, blueberry, wildflower, clover, cranberry, rhododendrum, and Christ's thorn honeys (Kaskoniene et al., 2010; Agila and Barringer, 2012; Alissandrakis et al., 2007; Castro-Vasquez et al., 2008; Castro-Vasquez et al., 2009; Pino, 2012; Bianchi et al., 2004). In a study conducted to determine the effect of the bees and comb to the volatiles of honey by feeding them only a saccharose solution, nonanal and decanal were detected in very small amounts due to contribution by the bees and comb wax (Jerkovic et al., 2010). (*E*)-2-Nonenal has not been identified in the present literature search and may be unique to Tupelo honey, but further combing of the literature needs to take place to be certain.

Alcohols. Eugenol has been identified in cashew, thyme, lavender, heather, corontillo, raspberry, rape, and alder buckthorn honeys (Ruising and Schieberle, 2012; Seisonen et al., 2015; Montenegro et al., 2009; Pino, 2012; Castro-Vasquez et al., 2009; Alissandrakis et al.,

2007; Kaskoniene et al., 2010). In a sensory study conducted for the National Honey Board, panelists ranked Tupelo honey as having a high spicy, cinnamon aroma and flavor (Rtech laboratories, 2001). This could be due to the high potency (determined by SDA and AEDA) of eugenol which has a spicy, clove aroma. Guaiacol is present in lime tree, citrus, heather, and quillay honeys (Montenegro et al., 2009; Pino, 2012; Castro-Vasquez et al., 2009; Kaskoniene et al., 2010; Castro-Vasquez et al., 2008; Guyot et al., 1998). Guaiacol may also be a marker compound for Tupelo honey in combination with others because of its uncommon presence in other honeys. 2-phenylethanol was detected in Tupelo, wildflower, clover, chestnut, lime tree, citrus, heather, eucalyptus, haze, cashew, cambara, rosemary, lavender, thyme, black mangrove, raspberry, rape, alder buckthorn, and buckwheat honeys (Overton and Manura, 1994; Zhou et al., 2002; Alissandrakis et al., 2007; Kaskoniene et al., 2010; Seisonen et al., 2015; Pino, 2012; Castro-Vasquez et al., 2009; Castro-Vasquez et al., 2008; Guyot et al., 1998).

Ketones. 2'-aminoacetophenone has been identified in chestnut, lime tree, rhododendrum, and heather honeys (Castro-Vasquez et al., 2009; Kaskoniene et al., 2010; Guyot et al., 1998). This may also be a marker compound for Tupelo honey. (*E*)-β-Damascenone has been found in Tupelo, wildflower, clover, alfalfa, citrus, rosemary, lavender, thyme, eucalyptus, heather, buckwheat, thyme, raspberry, rape, alder buckthorn, black mangrove, ulmo, corontillo, and quillay honeys (Overton and Manura, 1994; Ruising and Schieberle, 2012; Montenegro et al., 2009; Pino, 2012; Seisonen et al., 2015; Alissandrakis et al., 2007; ; Zhou et al., 2002; ; Castro-Vasquez et al., 2009).

Quantitation by Stable Isotope Dilution Analysis (SIDA)

Stable Isotope Dilution Analysis (SIDA) is a highly accurate quantification technique because it utilizes a stable, isotopically-labeled version of the compound of interest as an internal standard. (*E*)-β-Damascenone was chosen to quantify because of its extremely high FD factors determined during AEDA. A large peak was also observed on chromatograms which is surprising since it is uncommon to detect this compound with a peak due to its extremely low odor threshold of 0.002 parts per billion in water (Buttery et al., 1990). The structures for $^{2}H_{4}$-(*E*)-β-damascenone and (*E*)-β-damascenone can be found in **Figure 4.2**.

The concentration of (*E*)-β-damascenone in Tupelo honey samples (**Table 4.6**) ranged from 3.30-13.42 μg/g (ppm) with an average of 7.88 μg/g (ppm). It can be observed that the sample previously identified by pollen analysis to be holly honey is much lower than the other samples. The full effect of these concentration values cannot be understood until they are compared to other honeys and food products, including the calculated odor activity values (OAV) which are determined by dividing the concentration of the compound by the odor threshold. Ruisinger and Schieberle (2012) determined the odor threshold of (*E*)-β-damascenone to be 0.01 ppb in an aqueous fructose-glucose solution that was formulated to mimic honey. OAVs are important because a value greater than 1 signifies that a compound should contribute to the odor of a food product (Manyi-Loh et al., 2011). **Table 4.7** compares the concentrations and OAVs of the experimental data to several types of honeys and other food products. It is obvious that (*E*)-β-damascenone is an extremely important odorant in Tupelo honey. With its extremely high concentration it may also be considered a marker compound to distinguish Tupelo from other honey types. (*E*)-β-damascenone is formed through carotenoid degradation and is commonly found in fruits, wine, and honey (Kus et al., 2013), but it is uncertain as to the

source of the uniquely high level found in Tupelo honey. Further research needs to be conducted on the flowers of the Tupelo tree to determine their carotenoid content and provide an explanation for this phenomenon.

4.5 TABLES AND FIGURES

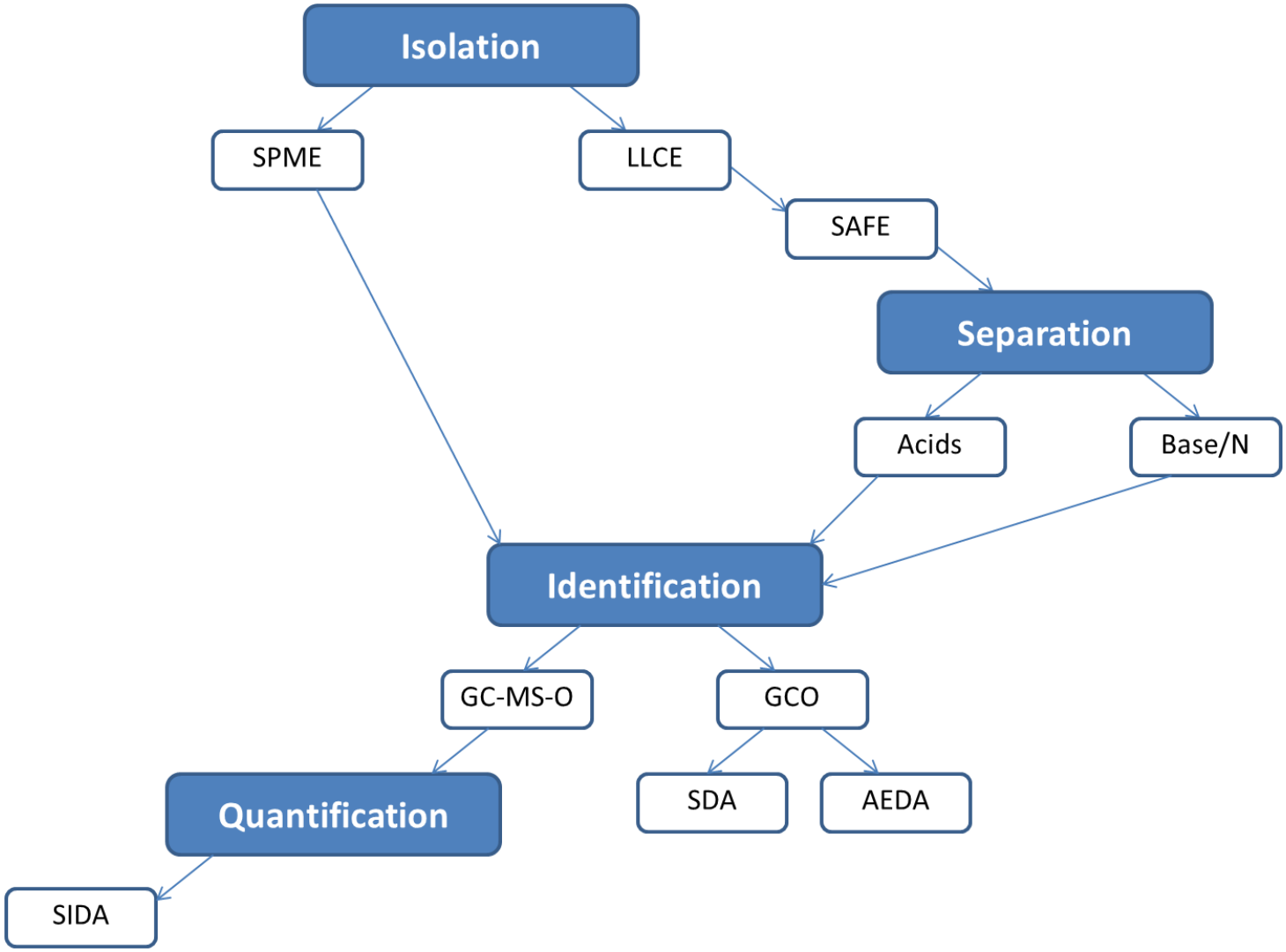

Figure 4.1 Schematic of the isolation, identification, and quantification techniques utilized.

Table 4.1 Aroma-Active Compounds Determined by Static Headspace Solid-Phase Microextraction/Gas Chromatography-Olfactometry (H-SPME/GC-O) Analysis of Tupelo Honeys from Five Different Locations (2013 and 2014).

no.[a]	compound	odor description [b]	RI [c]		odor intensity [d]									
					location 1		location 2		location 3		location 4		location 5	
			WAX	HP-5	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014
2	2-/3-methylbutanal	dark chocolate	926	636	2	3	2.5	3.5	2.5	2.5	1,5	3	2.5	2
3	2,3-butanedione [e]	buttery	984	556	1	3.5	2.5	2	2	4	2.5	4	3	2
4	hexanal	green, cut-grass	1082	800	2	3	3	2	0	3.5	2	3.5	0	2
5	heptanal	pungent, orange	1210	904	4	5.5	3	4	3	4.5	0	4	2	3
6	octanal	pungent, orange	1299	1005	2.5	4.5	4	3	3	4.5	2	4.5	2	3.5
7	1-octen-3-one [e]	mushroom	1307	981	2	3.5	3	2.5	0	4	2	2.5	2	4
8	unknown (MW = 152)	piney, pine oil	1326	1077	2.5	3.5	3	3	2	3	2.5	3	2	2
10	(*Z*)-1,5-octadien-3-one [e]	metallic, geranium	1378	986	4	2.5	2	2.5	2	2.5	3	3	2	2.5
11	dimethyl trisulfide [e]	sulfurous, cabbage	1382	970	3	2.5	1.5	3	2	3	2	2.5	4	2.5
12	nonanal	pungent, citrus	1395	1105	5.5	6	3.5	5	4	5.5	4	5.5	4	5
14	unknown	pungent, fresh	1442	1103	3	3.5	2.5	3	2.5	3.5	1.5	3.5	3	3
15	3-(methylthio)propanal (methional) [e]	boiled potato	1459	911	0	2	2	2	2	2	0	3	2	2
16	decanal	green, soapy	1497	1207	2.5	3.5	2.5	3	2	4	2	4	2.5	3
17	(*Z*)-2-nonenal [e]	stale, dried hay	1506	1154	3	2	2	3	2	3	2	4	2	2
18	(*E*)-2-nonenal [e]	stale, dried hay	1533	1163	3.5	3.5	3.5	4	4	4	3	4	3	3.5
19	linalool	floral, lavender	1544	1103	0	2	3	4	2	2	2	4	2	3
20	(*E*,*Z*)-2,6-nodienenal [e]	cucumber	1584	1157	3	3	3	4	3	3	2	3.5	4	3
21	hotrienol	floral, perfume	1607	1113	5	2.5	4	4.5	4	3	4	3	3	3
22	butanoic acid	cheesy, fecal	1629	807	3	3	0	3	0	3	4	2	0	3
23	unknown	saffron, hay	1632	-- [f]	3	0	3	0	3	0	2.5	0	3	0

Table 4.1 continued

no.[a]	compound	odor description [b]	RI [c]		odor intensity [d]									
					location 1		location 2		location 3		location 4		location 5	
			WAX	HP-5	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014
24	phenylacetaldehyde	floral, dried rose	1644	1049	5.5	6	5.5	5.5	4	5	4.5	5.5	5	5
25	3-methylbutanoic acid	cheesy, dried fruit	1670	850	3	0	3	3	3	4.5	2	3.5	3	3
26	unknown (phellandral?)	metallic, hay	1718	1287	4	0	4	0	4	3	0	3	4.5	3
27	unknown	spicy, saffron , hay	1731	1170	3	4	3	4	3	5	5	5	4	4
28	(*Z*)-β-damascenone	floral, cooked apple	1773	1372	3.5	4	4	4.5	3	4.5	3.5	3	4	3.5
29	(*E*)-β-damascenone	floral, cooked apple	1818	1394	5	4.5	4.5	4	3.5	4	4.5	5	4	4.5
30	2-methoxyphenol (guaiacol)	smoky	1862	1097	4.5	5	4	2.5	4.5	4.5	3	4	5	5
31	2-phenylethanol	floral, rosy, wine	1911	1120	3	3.5	3	3.5	3	3.5	4	3.5	3	3.5
32	unknown	floral, saffron	1966	--	5	3	3	4	2.5	3	3.5	3	2	3
34	unknown	fruity, peachy, cloves	2034	--	2	3	0	3	5	3	1.5	3	4.5	2.5
35	unknown	sweaty, body odor	2048	--	5	3	3	4	3	3	3	3	3	2
36	4-methylphenol (*p*-cresol)	dung, animal stable	2090	1089	3	3.5	3	0	3.5	3.5	2	4	4	3
37	unknown	sweet, grape, candy	2134	--	3	3	3	3	3	3	2	1	3	4
39	4-allyl-2-methoxyphenol (eugenol)	spicy, cloves	2169	1366	3.5	3	3	3	3	2	3	3	4	3
40	thymol	thyme, woody	2185	1297	2.5	3	0	3	3	2.5	0	3	5	3
42	4-vinyl-2-methoxyphenol (*p*-vinylguaiacol)	spicy, cloves, woody	2199	1326	4	3	0	2.5	3	2	3	3	4	3
43	2'-aminoacetophenone	grape, musky, corn tortilla	2225	1313	3.5	4	4	4	4.5	4.5	4	3	4.5	4.5

Table 4.1 continued

no.[a]	compound	odor description [b]	RI [c]		odor intensity [d]									
					location 1		location 2		location 3		location 4		location 5	
			WAX	HP-5	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014
47	(*E*)-4-propenyl-2-methoxyphenol (isoeugenol)	spicy, cloves, woody	2360	1465	2	3	2	3	3	3	0	3	3	3
50	phenylacetic acid	rosy	2563	1264	4	0	3	0	3.5	0	2.5	0	3	0
51	4-hydroxy-3-methoxybenaldehyde (vanillin)	vanilla	2567	1417	3	4	3	4	2.5	4	3	4.5	4.5	4.5

[a] Numbers corresponds to those in **Tables 4.2** – **4.5**. [b] Odor property determined by GC-O. [c] Retention indices determined on two different stationary phases by H-SPME-GC-MS-O [RTX-Stabilwax (WAX)] and HP5-MS (HP5)]. [d] Average post peak odor intensity of two panelists determined by GC-O on RTX-Stabilwax column (0 = no odor and 8 = very strong odor). [e] Mass spectrum unavailable, compound tentatively identified by comparison of its retention indices and odor properties with those of reference standard. [f] Not determined.

Table 4.2 Aroma-Active Compounds Determined by Sample Dilution Analysis - Static Headspace Solid-Phase Microextraction/Gas Chromatography-Olfactometry (SDA-H-SPME/GC-O) of Tupelo Honeys from Location 5 (2013 and 2014).

no.[a]	compound	odor description [b]	RI [c]		FD Factor [d]	
			WAX	HP-5	2013	2014
2	2-/3-methylbutanal	dark chocolate	926	636	5	5
3	2,3-butanedione [e]	buttery	984	556	1	1
4	hexanal	green, cut-grass	1082	800	5	5
5	heptanal	pungent, orange	1210	904	1	5
6	octanal	pungent, orange	1299	1005	25	25
7	1-octen-3-one [e]	mushroom	1307	981	5	5
8	unknown (MW = 152)	piney, pine oil	1326	1077	5	25
10	(*Z*)-1,5-octadien-3-one [e]	metallic, geranium	1378	986	1	5
11	dimethyl trisulfide [e]	sulfurous, cabbage	1382	970	1	1
12	nonanal	pungent, citrus	1395	1105	125	125
14	unknown	pungent, fresh	1442	1103	5	25
15	3-(methylthio)propanal (methional) [e]	boiled potato	1459	911	1	1
16	decanal	green, soapy	1497	1207	1	5
17	(*Z*)-2-nonenal [e]	stale, dried hay	1506	1154	5	5
18	(*E*)-2-nonenal [e]	stale, dried hay	1533	1163	25	25
19	linalool	floral, lavender	1544	1103	1	5
20	(*E*,*Z*)-2,6-nodienenal [e]	cucumber	1584	1157	5	5
21	hotrienol	floral, perfume	1607	1113	5	5
22	butanoic acid	cheesy, fecal	1629	807	1	1
23	unknown	saffron, hay	1632	-- [f]	1	5
24	phenylacetaldehyde	floral, dried rose	1644	1049	25	125
25	3-methylbutanoic acid	cheesy, dried fruit	1670	850	5	5

Table 4.2 continued

no.[a]	compound	odor description [b]	RI [c]		FD Factor [d]	
			WAX	HP-5	2013	2014
26	unknown (phellandral?)	metallic, hay	1718	1287	5	5
27	unknown	spicy, saffron, hay	1731	1170	125	125
28	(*Z*)-β-damascenone	floral, cooked apple	1773	1372	125	125
29	(*E*)-β-damascenone	floral, cooked apple	1818	1394	125	125
30	2-methoxyphenol (guaiacol)	smoky	1862	1097	25	25
31	2-phenylethanol	floral, rosy, wine	1911	1120	25	5
32	unknown	floral, saffron	1966	--	25	25
34	unknown	fruity, peachy, cloves	2034	--	25	25
35	unknown	sweaty, body odor	2048	--	5	5
36	4-methylphenol (*p*-cresol)	dung, animal stable	2090	1089	5	5
37	unknown	sweet, grape, candy	2134	--	5	25
39	4-allyl-2-methoxyphenol (eugenol)	spicy, cloves	2169	1366	125	125
40	thymol	thyme, woody	2185	1297	5	5
42	4-vinyl-2-methoxyphenol (*p*-vinylguaiacol)	spicy, cloves, woody	2199	1326	5	5
43	2'-aminoacetophenone	grape, musky, corn tortilla	2225	1313	25	25
47	(*E*)-4-propenyl-2-methoxyphenol (isoeugenol)	spicy, cloves, woody	2360	1465	25	25
50	phenylacetic acid	rosy	2563	1264	1	1
51	4-hydroxy-3-methoxybenaldehyde (vanillin)	vanilla	2567	1417	125	25

[a] Numbers corresponds to those in **Tables 4.1** and **4.3 - 4.5**. [b] Odor property determined by GC-O. [c] Retention indices determined by GC-MS-O on two stationary phases [RTX-Stabilwax (WAX)] and [HP5-MS (HP-5)]. [d] Flavor dilution (FD) factor = greatest sample mass analyzed (5000 mg) divided by smallest sample mass (40, 200, 1000, 5000 mg) at which an odorant could be detected by GC-O (RTX-Stabilwax column). [e] Mass spectrum unavailable, compound tentatively identified by comparison of its retention indices and odor properties with those of reference standard. [f] Not determined.

Table 4.3 Neutral/Basic Aroma-Active Compounds Determined by Aroma Extract Dilution Analysis of Tupelo Honeys from Location 5 (2013 and 2014).

No. [a]	Compound	Odor description [b]	RI [c]		FD factor [d]	
			WAX	HP-5	2013	2014
1	1,1-diethoxyethane (acetal)	Fruity	903	734	< 3	< 3
2	2-/3-methylbutanal	dark chocolate	923	<700	< 3	< 3
3	2,3-butanedione	buttery	981	<600	< 3	< 3
7	1-octen-3-one [e]	mushroom	1300	980	< 3	< 3
9	2-acetyl-1-pyrroline [e]	roasty, popcorn	1334	928	9	3
10	(*Z*)-1,5-octadien-3-one [e]	metallic, geranium	1369	985	3	< 3
11	dimethyl trisulfide [e]	sulfurous, cabbage	1372	974	< 3	< 3
12	nonanal	pungent, citrus	1396	1105	9	9
13	1-octen-3-ol	mushroom	1411	982	--	< 3
15	3-(methylthio)propanal (methional)	boiled potato	1450	902	3	< 3
17	(*Z*)-2-nonenal [e]	stale, dried hay	1502	1154	< 3	3
18	(*E*)-2-nonenal [e]	Stale, dried hay	1531	1163	3	3
19	linalool	floral, lavender	1545	1099	< 3	< 3
20	(*E*,*Z*)-2,6-nodienenal [e]	cucumber	1580	1158	< 3	< 3
24	phenylacetaldehyde	floral, dried rose	1636	1050	27	3
26	unknown	metallic, hay	1703	-- [f]	27	81
27	unknown	spicy, saffron, hay	1722	--	729	729
28	(*Z*)-β-damascenone	floral, cooked apple	1768	1372	27	3
29	(*E*)-β-damascenone	floral, cooked apple	1821	1394	59049	19683
30	2-methoxyphenol (guaiacol)	smoky	1852	1096	81	81
31	2-phenylethanol	floral, rosy, wine	1906	1120	81	27
32	unknown	floral, saffron	1967	--	27	9

Table 4.3 continued

No. [a]	Compound	Odor description [b]	RI [c]		FD factor [d]	
			WAX	HP-5	2013	2014
34	unknown	fruity, peachy, cloves	2025	--	81	81
36	4-methylphenol (*p*-cresol)	dung, animal stable	2029	1089	27	9
37	unknown	sweet, grape, candy	2125	--	81	81
39	4-allyl-2-methoxyphenol (eugenol)	spicy, cloves	2165	1365	2187	729
40	thymol	thyme, woody	2175	1296	9	3
42	4-vinyl-2-methoxyphenol (*p*-vinylguaiacol)	spicy, cloves, woody	2187	1323	9	27
43	2'-aminoacetophenone	grape, musky, corn tortilla	2207	1313	243	729
44	unknown	floral, rosy	2265	--	729	243
45	unknown	woody, incense, peppery	2275	--	243	243
46	unknown	floral, jasmine	2335	--	729	81
51	4-hydroxy-3-methoxybenaldehyde (vanillin)	vanilla	2533	1411	9	9

[a] Numbers corresponds to those in **Tables 4.1 - 4.2** and **4.4 - 4.5**. [b] Odor property determined by GC-O. [c] Retention indices determined on two different stationary phases by GC-O [RTX-Stabilwax (WAX)] and GC-MS-O [HP5-MS (HP-5)]. [d] Flavor dilution factor determined by GC-O on RTX-Stabilwax column. [e] Mass spectrum unavailable, compound tentatively identified by comparison of its retention indices and odor properties with those of reference standard. [f] Not determined.

Table 4.4 Acidic Aroma-Active Compounds Determined by Aroma Extract Dilution Analysis of Tupelo Honeys from Location 5 (2013 and 2014).

No. [a]	Compound	Odor description [b]	RI [c]		FD factor [d]	
			WAX	HP-5	2013	2014
22	butanoic acid	cheese, fecal	1625	813	9	27
25	3-methylbutanoic acid	cheesy, dried fruit	1671	852	27	27
27	unknown	spicy, saffron, hay	1728	-- [f]	27	81
30	2-methoxyphenol (guaiacol)	smoky	1859	1096	9	3
33	4-hydroxy-2,5-dimethyl-3(*2H*)-furanone (Furaneol™)	burnt sugar	2031	--	9	27
35	unknown	sweaty, body odor	2052	--	27	9
38	unknown	waxy, candle, paraffin	2161	--	9	9
41	3-hydroxy-4,5-dimethyl-2(*5H*)-furanone (sotolon) [e]	curry, spicy	2186	--	81	27
48	unknown	sweaty, body odor	2362	--	--	9
49	unknown	body odor	2455	--	9	9
50	phenylacetic acid	rosy	2526	1260	81	81
51	4-hydroxy-3-methoxybenaldehyde (vanillin)	vanilla	2531	1411	2187	2187

[a] Numbers corresponds to those in **Tables 4.1** – **4.3** and **4.5**. [b] Odor property determined by GC-O. [c] Retention indices determined on two different stationary phases by GC-O [RTX-Stabilwax (WAX)] and GC-MS-O [HP5-MS (HP-5)]. [d] Flavor dilution factor determined by GC-O on RTX-Stabilwax column. [e] Mass spectrum unavailable, compound tentatively identified by comparison of its retention indices and odor properties with those of reference standard. [f] Not determined.

Table 4.5 Comparison of odor important compounds based on results shown in **Tables 4.1-4.4**.

no.[a]	Compound	Odor description [b]	SPME	SDA-H-SPME[d]		AEDA[d]	
			Rank order[c]	Honey 5		Honey 5	
				2013	2014	2013	2014
12	nonanal	pungent, citrus	2	125	125	9	9
18	(*E*)-2-nonenal [e]	stale, dried hay	8	25	25	3	3
24	phenylacetaldehyde	floral, dried rose	1	25	125	27	3
26	unknown (phellandral?)	metallic, hay	21	5	5	27	81
27	unknown	spicy, saffron , hay	6	125	125	729	729
28	(*Z*)-β-damascenone	floral, cooked apple	7	125	125	27	3
29	(*E*)-β-damascenone	floral, cooked apple	3	125	125	59049	19683
30	2-methoxyphenol (guaiacol)	smoky	5	25	25	81	81
31	2-phenylethanol	floral, rosy, wine	13	25	5	81	27
32	unknown	floral, saffron	17	25	25	27	9
34	unknown	fruity, peachy, cloves	26	25	25	81	81
37	unknown	sweet, grape, candy	20	5	25	81	81
39	4-allyl-2-methoxyphenol (eugenol)	spicy, cloves	18	125	125	2187	729
43	2'-aminoacetophenone	grape, musky, corn tortilla	4	25	25	243	729
50	phenylacetic acid	rosy	36	1	1	81	81
51	4-hydroxy-3-methoxybenaldehyde (vanillin)	vanilla	9	125	25	2187	2187

[a] Numbers correspond to those in **Tables 4.1** – **4.4**. [b] Odor property determined by GC-O. [c] Calculated as discussed on pages 45-46 and found in **Appendix E**. [d] Flavor dilution factor determined by GC-O on RTX-Stabilwax column. [e] Mass spectrum unavailable, compound tentatively identified by comparison of its retention indices and odor properties with those of reference standard.

$^{2}H_4$-(*E*)-β-damascenone (*E*)-β-damascenone

Figure 4.2 Chemical structures of $^{2}H_4$-(*E*)-β-damascenone and (*E*)-β-damascenone.

Table 4.6 Concentration of (*E*)-β-damascenone in ppm (μg/g) of ten Tupelo honey samples.

	Year	
Sample	**2013**	**2014**
honey 1	10.35	7.66
honey 2	6.46	13.42
honey 3	8.69	3.30
honey 4	4.61	9.16
honey 5	7.67	7.46

Table 4.7 (*E*)-β-damascenone concentration (ppb) in several honeys and various other products.

Honey type	Concentration (ppb)	OAV[c]
Threshold$_1$	0.01[a]	
Tupelo*	7,880	788,000
Tupelo$_2$	74.2	7,420
Black mangrove$_3$	332	33,200
Rape$_1$	7.6	760
Buckwheat$_4$	6.5[b]	650
Acacia$_5$	3.2	320
Linden$_5$	7.8	780
Citrus$_6$	5.3	530
Rosemary$_6$	5.5	550
Lavender$_6$	6.6	660
Thyme$_6$	11.5	1,150
Eucalyptus$_6$	7.4	740
Heather$_6$	10.5	1,050
Ulmo$_7$	1,090	109,000
Corontillo$_7$	3,720	372,000
Quillay$_7$	190	19,000
Other products		
Threshold (in water)$_8$	0.002	
Black tea$_5$	1.7	850
Concord juice$_9$	4.92	2,460
Riesling wine$_9$	0.85	425
Bourbon whiskey$_{10}$	10.0	5,000
Apple brandy$_{11}$	198.0	99,000

*Signifies average concentration determined in this study. [a]Odor threshold determined in an aqueous fructose-glucose solution. [b]Average of H-SPME data was used. [c]Odor acitivity value. Subscripts signify the following references: 1=Ruisinger and Schieberle, 2012; 2=Overton and Manura, 1994; 3=Pino, 2012; 4=Zhou, et al., 2002; 5=Sen, et al., 1991; 6=Castro-Vazquez et al., 2009; 7=Montenegro et al., 2009; 8=Buttery et al., 1990; 9=Acree, et al., 1981; 10=Masuda and Nishimura, 1980; 11=Schreier, et al., 1978.

4.6 REFERENCES

Acree, T.; Braell, P.; Butts, R. The Presence of Damascenone in Cultivars of Vitis Vinifera (Linneaus), Rotundifolia (Michaux), and Labruscana (Baily). *J. Agric. Food Chem.* **1981**, *29* (3), 688–690.

Agila, A.; Barringer, S. Application of Selected Ion Flow Tube Mass Spectrometry Coupled with Chemometrics to Study the Effect of Location and Botanical Origin on Volatile Profile of Unifloral American Honeys. *J. Food Sci.* **2012**, *77* (10), 1103–1108.

Alissandrakis, E.; Tarantilis, P. a.; Harizanis, P. C.; Polissiou, M. Comparison of the Volatile Composition in Thyme Honeys from Several Origins in Greece. *J. Agric. Food Chem.* **2007**, *55*, 8152–8157.

Alqarni, A. S.; Hannan, M. a.; Owayss, A. a.; Engel, M. S. The Indigenous Honey Bees of Saudi Arabia (Hymenoptera, Apidae, Apis Mellifera Jemenitica Ruttner): Their Natural History and Role in Beekeeping. *Zookeys* **2011**, *134*, 83–98.

Andujar-Ortiz, I.; Moreno-Arribas, M. V; Martín-Alvarez, P. J.; Pozo-Bayón, M. a. Analytical Performance of Three Commonly Used Extraction Methods for the Gas Chromatography-Mass Spectrometry Analysis of Wine Volatile Compounds. *J. Chromatogr. A* **2009**, *1216*, 7351–7357.

Bianchi, F.; Careri, M.; Musci, M. Volatile Norisoprenoids as Markers of Botanical Origin of Sardinian Strawberry-Tree (Arbutus Unedo L.) Honey: Characterisation of Aroma Compounds by Dynamic Headspace Extraction and Gas Chromatography-Mass Spectrometry. *Food Chem.* **2005**, *89*, 527–532.

Buttery, R. G.; Teranishi, R.; Ling, L. C.; Turnbaugh, J. G. Quantitative and Sensory Studies on Tomato Paste Volatiles. *J. Agric. Food Chem.* **1990**, No. 38, 336–340.

Cadwallader, K. R.; Heo, J. Aroma of Roasted Sesame Oil: Characterization by Direct Thermal Desorption-Gas Chromatography-Olfactometry and Sample Dilution Analysis. *ACS Symp. Ser.* **2001**, *782* (2), 187–202.

Castro-Vázquez, L.; Díaz-Maroto, M. C.; González-Viñas, M. a.; De La Fuente, E.; Pérez-Coello, M. S. Influence of Storage Conditions on Chemical Composition and Sensory Properties of Citrus Honey. *J. Agric. Food Chem.* **2008**, *56*, 1999–2006.

Castro-Vázquez, L.; Díaz-Maroto, M. C.; González-Viñas, M. a.; Pérez-Coello, M. S. Differentiation of Monofloral Citrus, Rosemary, Eucalyptus, Lavender, Thyme and Heather Honeys Based on Volatile Composition and Sensory Descriptive Analysis. *Food Chem.* **2009**, *112*, 1022–1030.

Crane, E. *A Book of Honey*; Oxford University Press: Oxford, 1980.

Cuevas-Glory, L. F.; Pino, J. a.; Santiago, L. S.; Sauri-Duch, E. A Review of Volatile Analytical Methods for Determining the Botanical Origin of Honey. *Food Chem.* **2007**, *103*, 1032–1043.

De Kimpe, N. G.; Stenens, C. V.; Keppens, M. A. Synthesis of 2-acetyl-1-pyrroline, the principal rice flavor component. *J. Agric. Food Chem.* **1993**, *41*, 1458-1461.

Engel, W.; Bahr, W.; Schieberle, P. Solvent Assisted Flavour Evaporation - a New and Versatile Technique for the Careful and Direct Isolation of Aroma Compounds from Complex Food Matrices. *Eur. Food Res. Technol.* **1999**, *209*, 237–241.

Grosch, W. Detection of Potent Odorants in Food by Aroma Extract Dilution Analysis. *Trends Food Sci. Technol.* **1993**, *4*, 68–73.

Guyot, C.; Bouseta, A.; Scheirman, V.; Collin, S. Floral Origin Markers of Chestnut and Lime Tree Honeys. *J. Agric. Food Chem.* **1998**, *46*, 625–633.

Jerković, I.; Marijanović, Z.; Ljubičić, I.; Gugić, M. Contribution of the Bees and Combs to Honey Volatiles: Blank-Trial Probe for Chemical Profiling of Honey Biodiversity. *Chem. Biodivers.* **2010**, *7*, 1217–1230.

Kaškoniene, V.; Venskutonis, P. R. Floral Markers in Honey of Various Botanical and Geographic Origins: A Review. *Compr. Rev. Food Sci. Food Saf.* **2010**, *9*, 620–634.

Kotseridis, Y.; Baumes, R.; Skouroumounis, G.K. Synthesis of labelled [2H4]ß-damascenone, [2H2]2-methoxy-3- isobutylpyrazine, [2H3]a-ionone, and [2H3]ß-ionone, for quantification in grapes, juices and wines. *J. Chromatography A* **1998**, *824*, 71-78.

Kuś, P. M.; Jerkovič, I.; Tuberoso, C. I. G.; Šarolič, M. The Volatile Profiles of a Rare Apple (Malus Domestica Borkh.) Honey: Shikimic Acid-Pathway Derivatives, Terpenes, and Others. *Chem. Biodivers.* **2013**, *10*, 1638–1652.

Lorjaroenphon, Y.; Cadwallader, K.R. Typical odorants of cola-flavored carbonated beverages - Part II. Characterization of potent odorants by quantitative analysis and sensory studies of aroma reconstitution and omission models. *J. Agric. Food Chem*. **2015**, *63*, 776-786.

Lin, J.; Welti, D.H.; Vera, F.A.; Fay, L.B.; Blank, I. Synthesis of deuterated volatile lipid degradation products to be used as internal standards in isotope dilution assays. 2. Vinyl ketones. *J. Agric. Food Chem.* **1999,** *47*, 2822-2829.

Manyi-Loh, C. E.; Ndip, R. N.; Clarke, A. M. Volatile Compounds in Honey: A Review on Their Involvement in Aroma, Botanical Origin Determination and Potential Biomedical Activities. *Int. J. Mol. Sci.* **2011**, *12*, 9514–9532.

Masuda, M.; Nishimura, K. Occurrence of Formation of Damascenone, Trans-2,6,6-Trimethyl-1-Crotonyl-Cyclohexa-1,3-Diene, in Alcoholic Beverages. *J. Food Sci.* **1980**, *45* (396-397).

Maurizio, A. How bees make honey. In: *Honey: A Comprehensive Survey*; Crane, E., Ed.; Crane, Russak & Company, Inc.: New York, 1975, 77-105.

Molyneux, R.; Schieberlie, P. Compound Identification : A Journal of Agricultural and Food Chemistry Perspective. *J. Agric. Food Chem.* **2007**, No. 55, 4625–4629.

Montenegro, G.; Gómez, M.; Casaubon, G.; Belancic, A.; Mujica, a. M.; Peña, R. C. Analysis of Volatile Compounds in Three Unifloral Native Chilean Honeys. *Phyton (B. Aires).* **2009**, *78*, 61–65.

Ogawa, K.; Nomure, Y.; Takeuchi, Y.; Tomoda, S. Silver(I)-catalyzed oxidation of cyclic secondary amines with peroxodisulphate. *J. Chem. Soc. Perkin trans I.* **1982**, 3031-3035.

Overton, S. and Manura, J. Flavor and aroma in commercial bee honey – a purge-and-trap thermal-desorption technique for the identification and quantification of volatiles and semivolatiles in honey. *American Laboratory*. **1994**, *26*, 45-53.

Pino, J. a. Analysis of Odour-Active Compounds of Black Mangrove (Avicennia Germinans L.) Honey by Solid-Phase Microextraction Combined with Gas Chromatography-Mass Spectrometry and Gas Chromatography-Olfactometry. *Int. J. Food Sci. Technol.* **2012**, *47*, 1688–1694.

Rtech laboratories. *Sensory Attributes of Honey*; 2001.

Ruisinger, B.; Schieberle, P. Characterization of the Key Aroma Compounds in Rape Honey by Means of the Molecular Sensory Science Concept. *J. Agric. Food Chem.* **2012**, *60*, 4186–4194.

Schreier, P.; Drawert, F.; Schmid, M. Changes in the Composition of Neutral Volatile Componets during the Production of Apple Brandy. *J Sci Food Agric* **1978**, *29*, 728–736.

Seisonen, S.; Kivima, E.; Vene, K. Characterisation of the Aroma Profiles of Different Honeys and Corresponding Flowers Using Solid-Phase Microextraction and Gas Chromatography–mass Spectrometry/olfactometry. *Food Chem.* **2015**, *169*, 34–40.

Sen, A.; Laskawy, G.; Schieberle, P.; Grosch, W. Quantitative Determination of Beta-Damascenone in Foods Using a Stable Isotope Dilution Assay. *J. Agric. Food Chem.* **1991**, *39*, 757–759.

van den Dool, H.; Dec. Kratz, P. A Generalization of the Retention Index System Including Linear Temperature Programmed Gas-Liquid Partition Chromatography. *J. Chromatogr.* **1963**, No. 11, 463–471.

Viuda-Martos, M.; Ruiz-Navajas, Y.; Zaldivar-Cruz, J. M.; Kuri, V.; Fernández-López, J.; Carbonell-Barrachina, Á. a.; Pérez-Álvarez, J. Á. Aroma Profile and Physico-Chemical Properties of Artisanal Honey from Tabasco, Mexico. *Int. J. Food Sci. Technol.* **2010**, *45*, 1111–1118.

Yuasa, Y.; Kato, Y. A practical and convenient synthesis of hotrienol, an excellent fruity smelling compound. *J. Agric. Food Chem.* **2013,** *51,* 4036-4039.

Zhou, Q.; Wintersteen, C. L.; Cadwallader, K. R. Identification and Quantification of Aroma-Active Components That Contribute to the Distinct Malty Flavor of Buckwheat Honey. *J. Agric. Food Chem.* **2002**, *50*, 2016–2021.

CHAPTER 5

SUMMARY AND CONCLUSIONS

Nyssa ogeche (white Tupelo) trees are concentrated mainly in the Appalachicola region in the panhandle of Florida. The honey produced from the nectar of these trees is regarded as a premium honey because of its non-granulating tendencies and limited supply due to the small growing region and short bloom time of the Tupelo trees. Unfortunately there are few studies of this unique honey, with only one study conducted on the physicochemical characteristics and one on the volatile components, each study having its own limitations. The study involving the physicochemical characteristics did not confirm the botanical origin of the honeys with pollen analysis and the majority of the samples were stored for 19 months before analysis, which can affect some of the characteristics being tested. The research conducted on the volatiles of Tupelo honey was essentially done to demonstrate the capabilities of a new piece of analytical equipment and was not an exhaustive determination. It became clear that with the limited amount of research available on Tupelo honey a full physicochemical and flavor characterization was needed.

Physicochemical characteristics were determined for ten Tupelo honey samples from five different locations collected during two consecutive seasons (2013 and 2014). These included moisture content, ^{0}Brix, water activity (A_w), pH, titratable acidity, ash content, and fructose and glucose contents. On the basis of pollen analyses, nine samples could be considered as authentic Tupelo honeys, with one (designated honey 3 from the 2014 season) containing mainly holly pollen along with appreciable levels of tupelo pollen. This sample also had a noticeably higher pH value and differed significantly in ash content from the other Tupelo honey samples,

suggesting ash content to be a good indicator of botanical origin. Sensory screening indicated this sample to be significantly different from the other samples as well. Honey 5 produced during both seasons was the most consistent sampling location with the highest amount of Tupelo pollen content present in each sample. Panelists could not distinguish a difference between the honey 5 samples from both seasons during sensory testing. The characteristic most widely known about Tupelo honey is its relatively high fructose content which was confirmed in this study. Composition data were comparable to literature values and within the limits set by Codex Alimentarius.

To complete a full flavor characterization, aroma-active compounds in Tupelo honey were identified by gas chromatography-olfactometry (GC-O) and gas chromatography-mass spectrometry-olfactometry (GC-MS-O). Initial analyses were performed on the same ten samples as described above by static headspace solid phase microextraction (H-SPME). Of the 40 compounds detected, the most important compounds based on the perceived odor intensities determined by two assessors were phenylacetaldehye (rosy) and nonanal (citrus). Further analysis was carried out on honey 5 from the 2013 and 2014 seasons due to its consistently high Tupelo pollen content across seasons and the inability of sensory panelists to distinguish between the two samples. The most potent odorants were determined through aroma extract dilution analysis (AEDA) of liquid extracts and sample dilution analysis by H-SPME (SDA-H-SPME). The most potent odorants identified by both dilution analyses techniques were vanillin (vanilla), phenylacetaldehyde (rosy), nonanal (citrus), (*E*)-2-nonenal (dried hay), eugenol (cloves), guaiacol (smoky), 2-phenylethanol (rosy, wine), 2'-aminoacetophenone (grape, corn tortilla), (*E*)-β-damascenone (cooked apple), and an unidentified odorant (RI_{wax}=1731) described as spicy and hay-like. (*E*)-β-Damascenone was determined to be the most potent odorant with extremely

high flavor dilution (FD) factors of 59,049 (2013 season) and 19,683 (2014 season) from AEDA analysis.

Comparisons to literature of these nine potent odorants detected by AEDA and SDA-H-SPME reveal possible marker compounds. The potency of guaiacol and 2'-aminoacetophenone determined could point to these compounds being marker compounds of Tupelo because of the small number of identifications of these compounds in other honeys in the present literature. (*E*)-2-Nonenal could also be an important marker compound since it was not found in the literature on honey. A majority of the research done on honey, however, does not use GC-O in their analyses. (*E*)-2-Nonenal was one the compounds that was only tentatively identified in this study due to the lack of mass spectra data available, so it is possible that (*E*)-2-nonenal is present in many honeys but remains unknown to most researchers because of the lack of GC-O analysis. Analyses of more unifloral honeys need to be conducted with the use of both GC-O and GC-MS techniques to truly determine the odor active compounds in various honeys.

Quantification of odor-active compounds and the calculation of their OAVs are essential to understanding the flavor profile of a matrix. OAVs are determined by the concentration of a compound divided by its odor threshold. Compounds with the highest OAVs contribute the most to the aroma profile of a matrix. Quantification of (*E*)-β-damascenone using stable isotope dilution analysis by H-SPME (SIDA-H-SPME) revealed that the compound had an extremely high concentration and odor-activity value (OAV) in Tupelo honey compared to other types of honeys and selected food products. (*E*)-β-Damascenone may be used as a marker compound to distinguish Tupelo honey from other unifloral honeys because of the uniquely high levels present in this honey. The mechanism known to be responsible for the formation of (*E*)-β-damascenone is carotenoid degradation. Further research needs to be conducted on the Tupelo flowers to gain

a full understanding of the source and mechanism behind the unusually high level of (*E*)-β-damascenone present in Tupelo honeys.

APPENDIX A

POLLEN ANALYSIS PROCEDURE

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October 1, 2013

Dear Samantha,

Specific details about the extraction and analysis procedures I used for the samples you sent are mentioned below and these are identical to those I normally use on other such samples. I also have included a pollen summary of the contents of the sample.

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 10 g sample of raw honey for the analysis. The sample of raw honey is diluted with 10 ml of distilled water and 150 ml of alcohol (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 or more 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.

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], **Lamiaceae** [mints], **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. Thus, unless you know exactly which potential genera in each of these families might have been utilized by your honeybees, trying to narrow down the potential types in any one of these families becomes extremely time consuming. In some other large plant families, such as the **Fabaceae** (legumes), we are often able to identify some of the major 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 a knowledge of the potential

types that might exist within the range of the beehive that produced the honey being examined. Often for precise identifications of these types we need to conduct studies at levels of higher resolution using scanning electron microscopy (SEM).

A pollen concentration value (PC) of pollen grains per 10 g 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 during winter 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

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

I hope this summary gives you a better idea about the technique we used. Should you have any questions or desire additional clarification of this report please let me know.

If we can assist you in the future, please let us know. We will invoice you later.

Sincerely,

Vaughn M. Bryant, Jr.

Professor and Director

APPENDIX B

POLLEN ANALYSIS RESULTS FOR THE 2013 SEASON

Pollen Taxa	Honey 1	%	Honey 2	%	Honey 3	%	Honey 4	%	Honey 5	%
***Acer* (maple)**	0	0.0%	1	0.5%	2	1.0%	1	0.5%	0	0.0%
ASTERACEAE (sunflower-type)	0	0.0%	1	0.5%	0	0.0%	0	0.0%	0	0.0%
BRASSICACEAE (mustard family)	0	0.0%	3	1.5%	0	0.0%	0	0.0%	6	2.8%
***Carya* (pecan, hickory)**	3	1.5%	0	0.0%	1	0.5%	2	0.9%	0	0.0%
***Castanea* (chestnut, chinquapin)**	1	0.5%	0	0.0%	0	0.0%	0	0.0%	0	0.0%
Eucalyptus (gum)	0	0.0%	0	0.0%	0	0.0%	1	0.5%	0	0.0%
***Gleditsia* (honey locust)**	1	0.5%	2	1.0%	16	7.8%	1	0.5%	1	0.5%
***Ilex* (holly)**	38	18.8%	43	21.5%	42	20.5%	59	27.8%	9	4.1%
Ligustrum (privet)	0	0.0%	0	0.0%	2	1.0%	0	0.0%	0	0.0%
***Liquidambar* (sweet gum)**	0	0.0%	0	0.0%	1	0.5%	0	0.0%	0	0.0%
***Magnolia* (magnolia)**	2	1.0%	5	2.5%	1	0.5%	0	0.0%	0	0.0%
***Melilotus* (sweet clover)**	0	0.0%	1	0.5%	0	0.0%	0	0.0%	2	0.9%
***Nyssa ogeche* (tupelo)**	148	73.3%	94	47.0%	123	60.0%	138	65.1%	191	88.0%
POACEAE (grass family)	0	0.0%	1	0.5%	0	0.0%	0	0.0%	0	0.0%
Phyla (frogfruit)	0	0.0%	0	0.0%	0	0.0%	1	0.5%	0	0.0%
Pinus (pine)	0	0.0%	0	0.0%	1	0.5%	0	0.0%	0	0.0%
Platanus (sycamore)	0	0.0%	0	0.0%	0	0.0%	0	0.0%	1	0.5%
***Prunus* (plum, peach, cherry)**	0	0.0%	0	0.0%	0	0.0%	3	1.4%	0	0.0%
***Quercus* (oak)**	2	1.0%	6	3.0%	4	2.0%	2	0.9%	2	0.9%
***Rhus* (sumac)**	0	0.0%	12	6.0%	0	0.0%	0	0.0%	0	0.0%
ROSACEAE (rose family)	3	1.5%	10	5.0%	0	0.0%	2	0.9%	0	0.0%
***Rubus* (blackberry, dewberry)**	2	1.0%	1	0.5%	2	1.0%	0	0.0%	2	0.9%
***Salix* (willow)**	0	0.0%	12	6.0%	3	1.5%	0	0.0%	2	0.9%
Sapium (Chinese tallow tree)	0	0.0%	0	0.0%	5	2.4%	0	0.0%	0	0.0%
SCROPHULARIACEAE	0	0.0%	4	2.0%	1	0.5%	0	0.0%	0	0.0%
***Vitis* (grape)**	0	0.0%	2	1.0%	1	0.5%	1	0.5%	1	0.5%
Unknown pollen	2	1.0%	2	1.0%	0	0.0%	1	0.5%	0	0.0%
Totals (# of pollen granules and %)	202	100.0%	200	100.0%	205	100.0%	212	100.0%	217	100.0%
Lycopodium spores counted	63		52		103		49		71	
Pollen conc. per 10 g of honey	59,583		71,473		36,985		80,400		56,795	

APPENDIX C

POLLEN ANALYSIS RESULTS FOR THE 2014 SEASON

Pollen Taxa	Honey 1	%	Honey 2	%	Honey 3	%	Honey 4	%	Honey 5	%
Acer (maple)	0	0.0%	10	3.8%	0	0.0%	1	0.3%	0	0.0%
ASTERACEAE (sunflower-type)	0	0.0%	0	0.0%	1	0.5%	0	0.0%	0	0.0%
Berchemia (rattan vine)	0	0.0%	0	0.0%	1	0.5%	0	0.0%	1	0.4%
Carya (pecan, hickory)	0	0.0%	0	0.0%	0	0.0%	2	0.7%	0	0.0%
Castanea (chestnut, chinquapin)	1	0.5%	1	0.4%	0	0.0%	0	0.0%	0	0.0%
Cyrilla (black titi)	14	6.8%	3	1.1%	0	0.0%	4	1.4%	3	1.2%
Diospyros (persimmon)	0	0.0%	0	0.0%	1	0.5%	1	0.3%	0	0.0%
Fraxinus (ash)	0	0.0%	0	0.0%	1	0.5%	1	0.3%	0	0.0%
Gleditsia (honey locust)	1	0.5%	2	0.8%	20	9.9%	2	0.7%	0	0.0%
Ilex (holly)	15	7.3%	68	26.0%	94	46.3%	54	18.4%	17	7.0%
Liquidambar (sweet gum)	0	0.0%	1	0.4%	0	0.0%	1	0.3%	0	0.0%
Magnolia (magnolia)	0	0.0%	1	0.4%	0	0.0%	0	0.0%	0	0.0%
Myrica (wax myrtle)	0	0.0%	0	0.0%	0	0.0%	1	0.3%	0	0.0%
Nyssa ogeche (tupelo)	144	69.9%	164	62.6%	77	37.9%	200	68.3%	205	84.7%
Prunus (plum, peach, cherry)	0	0.0%	4	1.5%	0	0.0%	0	0.0%	1	0.4%
Quercus (oak)	5	2.4%	1	0.4%	2	1.0%	5	1.7%	2	0.8%
RANUNCULACEAE (buttercups)	2	1.0%	0	0.0%	0	0.0%	5	1.7%	0	0.0%
Rhus (sumac)	0	0.0%	0	0.0%	1	0.5%	0	0.0%	1	0.4%
ROSACEAE (rose family)	5	2.4%	0	0.0%	1	0.5%	5	1.7%	1	0.4%
Rubus (blackberry, dewberry)	5	2.4%	0	0.0%	1	0.5%	2	0.7%	3	1.2%
Sabal/ Serenoa (palmetto)	0	0.0%	1	0.4%	0	0.0%	0	0.0%	0	0.0%
Salix (willow)	10	4.9%	3	1.1%	0	0.0%	2	0.7%	5	2.1%
Trifolium (clover)	1	0.5%	0	0.0%	0	0.0%	0	0.0%	0	0.0%
Vitis (grape)	0	0.0%	1	0.4%	1	0.5%	7	2.4%	1	0.4%
Unknown pollen	3	1.5%	1	0.4%	2	1.0%	0	0.0%	2	0.8%
Totals (# of pollen granules and %)	206	100.0%	262	100.0%	203	100.0%	293	100.0%	242	100.0%
Lycopodium spores counted	85		48		220		184		106	
Pollen conc. per 10 g of honey	45,036		101,432		17,147		29,591		42,425	

APPENDIX D

SENSORY BALLOT FOR PHASE 1 TESTING

Code:_____

Tupelo Honey Difference Test

Instructions:

1. First sniff the noise sample by squeezing the bottle and taking short, shallow sniffs ("bunny sniffs").
2. Then sniff the rest of the samples labeled with three digit codes in the same manner, in the order listed below. *Check to* ***ensure that you are sniffing the samples in the order listed below.***
3. Put the three digit coded samples in order from most similar to the noise to least similar to the noise below.

Noise	**522**	**706**	**466**	**914**	**258**
	Most similar				Least similar
Noise	_____	_____	_____	_____	_____

----------**Please take a one minute break**----------

After the one minute break, raise your hand to receive the next set of samples.

Instructions:

1. First sniff the noise sample by squeezing the bottle and taking short, shallow sniffs ("bunny sniffs").
2. Then sniff the rest of the samples labeled with three digit codes in the same manner, in the order listed below. *Check to* ***ensure that you are sniffing the samples in the order listed below.***
3. Put the three digit coded samples in order from most similar to the noise to least similar to the noise below.

Noise	**671**	**842**	**594**	**393**	**480**
	Most similar				Least similar
Noise	_____	_____	_____	_____	_____

APPENDIX E

DATA FROM TABLE 4.1 CONVERTED TO AVERAGE RANK AND RANK ORDER

no.[a]	compound	odor description [b]	Average rank [c]	Rank order [c]
2	2-/3-methylbutanal	dark chocolate	26.6	38
3	2,3-butanedione [d]	buttery	22.5	29
4	hexanal	green, cut-grass	25.9	37
5	heptanal	pungent, orange	13.0	11
6	octanal	pungent, orange	13.7	12
7	1-octen-3-one [d]	mushroom	22.7	30
8	unknown (MW = 152)	piney, pine oil	21.8	28
10	(*Z*)-1,5-octadien-3-one [d]	metallic, geranium	25.3	35
11	dimethyl trisulfide [d]	sulfurous, cabbage	24.5	34
12	nonanal	pungent, citrus	3.2	2
14	unknown	pungent, fresh	19.4	22
15	3-(methylthio)propanal (methional) [d]	boiled potato	32.2	40
16	decanal	green, soapy	19.6	25
17	(*Z*)-2-nonenal [d]	stale, dried hay	24.1	32
18	(*E*)-2-nonenal [d]	stale, dried hay	9.7	8
19	linalool	floral, lavender	22.7	31
20	(*E*,*Z*)-2,6-nodienenal [d]	cucumber	14.3	14
21	hotrienol	floral, perfume	11.9	10
22	butanoic acid	cheesy, fecal	24.2	33
23	unknown	saffron, hay	26.6	39
24	phenylacetaldehyde	floral, dried rose	1.5	1
25	3-methylbutanoic acid	cheesy, dried fruit	16.6	19
26	unknown (phellandral?)	metallic, hay	18.3	21
27	unknown	spicy, saffron , hay	7.3	6
28	(*Z*)-β-damascenone	floral, cooked apple	8.9	7
29	(*E*)-β-damascenone	floral, cooked apple	5.1	3

Appendix E continued

no.[a]	compound	odor description [b]	Average rank [c]	Rank order [c]
30	2-methoxyphenol (guaiacol)	smoky	7.0	5
31	2-phenylethanol	floral, rosy, wine	14.2	13
32	unknown	floral, saffron	15.7	17
34	unknown	fruity, peachy, cloves	21.3	26
35	unknown	sweaty, body odor	15.0	15
36	4-methylphenol (*p*-cresol)	dung, animal stable	15.2	16
37	unknown	sweet, grape, candy	18.0	20
39	4-allyl-2-methoxyphenol (eugenol)	spicy, cloves	16.0	18
40	thymol	thyme, woody	21.7	27
42	4-vinyl-2-methoxyphenol (*p*-vinylguaiacol)	spicy, cloves, woody	19.4	23
43	2'-aminoacetophenone	grape, musky, corn tortilla	6.6	4
47	(*E*)-4-propenyl-2-methoxyphenol (isoeugenol)	spicy, cloves, woody	19.5	24
50	phenylacetic acid	rosy	25.4	36
51	4-hydroxy-3-methoxybenaldehyde (vanillin)	vanilla	10.0	9

[a] Numbers correspond to those in **Tables 4.1** – **4.5**. [b] Odor property determined by GC-O. [c] Calculated as discussed on pages 45-46. [d] Mass spectrum unavailable, compound tentatively identified by comparison of its retention indices and odor properties with those of reference standard.