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Decoding the Flavor of Kentucky's Native Pawpaw Fruit

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BY

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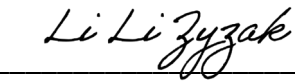

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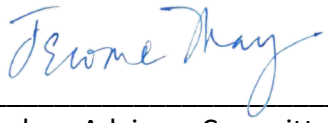

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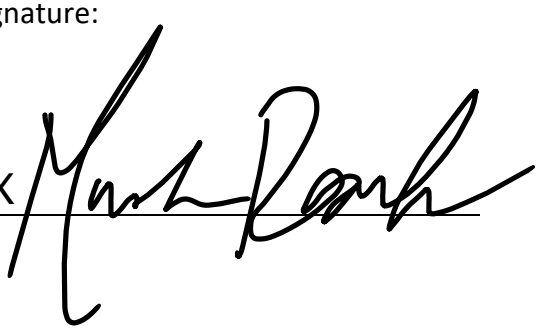
X 

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ABSTRACT

The objective of this research was to decode the flavor of *Asimina triloba*, or the pawpaw fruit, to identify and quantitate the aroma-active compounds that are present. Gas chromatography – olfactometry (GC-O) was applied on capillary GC columns with various means of extraction. The volatile compounds present were extracted using both headspace solid phase micro-extraction (HS-SPME) for 30 minutes at 23°C and 50°C, and solvent extraction using methylene chloride. The sample extracts were analyzed with both gas chromatography – mass spectrometry (GC-MS) and gas chromatography – olfactometry (GC-O). To eliminate potential artifacts that were observed when using HS-SPME at 50°C, the study focused on analyzing the SPME samples performed at 23°C and solvent extraction for characterization of the aroma compounds within the pawpaw fruit. Throughout this study, forty-four aroma-active compounds were observed. Of the forty-four compounds, fifteen were identified for the first time within the pawpaw fruit. Acetaldehyde, diacetyl, eugenol, homofuraneol, delta-octalactone, gamma-octalactone, and vanillin are a few of the recently identified odor-active compounds. It was observed that these odor-active compounds had high flavor dilution (FD) factors. The esters within the pawpaw alongside these high intensity aroma-active compounds contribute to the unique aroma associated with the pawpaw fruit. The odor has often been described as tropical, creamy, sweet, and a mixture of banana, mango, and pineapple-like. In addition, some of these flavor compounds were quantitated and comparisons were made to different cultivars as well as during the ripening stage. These results identified one marker compound (3-hydroxy ethyl butyrate) as a potential way to

distinguish Mango pawpaw cultivar from others. Overall, this work provides a good foundation for future pawpaw researchers who may try to understand the flavor differences of various pawpaw cultivars.

Keywords

Gas Chromatography – Olfactometry (GC-O), Solid Phase Micro Extraction (SPME), Gas Chromatography – Mass Spectrometry (GC-MS), Aroma Extract Dilution Analysis (AEDA), Aroma compounds, Flavor, pawpaw

Abbreviations

(ppm) parts per million; (ppb) parts per billion; (GC-FID) Gas Chromatography – Flame Ionization Detector; Gas Chromatography – Olfactometry (GC-O); Solid Phase Micro Extraction (SPME); Gas Chromatography – Mass Spectrometry (GC-MS); Aroma Extract Dilution Analysis (AEDA); Flavor Dilution (FD)

Table of Contents

List of Tables

List of Figures

I. Introduction

1.1 History of the Pawpaw Fruit

In the realm of chemistry, there are many different types of work, research, and opportunities that lie beyond what is learned in the classroom. For this project, the focus was fragrance and flavor chemistry. Flavor and fragrance chemistry are often grouped together because the two senses are complimentary to each other. Flavor chemistry is the science behind the foods and beverages that are enjoyed daily. It is the innovative mixing of various food-safe chemicals, botanical oils, and extracts to reconstitute flavors that are marketable and widely adored. Oftentimes, these chemically-created flavors will mimic or enhance flavors that occur naturally. Flavor is a crucial part of the whole eating or drinking experience. Flavor is experienced when a complex mixture of high molecular weight compounds and volatile compounds stimulate chemical senses, like taste, simultaneously[18].

Fragrance chemistry is similar to flavor chemistry except that fragrance chemistry focuses on the stimulation of the chemical sense smell rather than taste. Fragrance chemistry includes the creation of products like perfumes, scented makeup, and all the smells that are encountered on a day-to-day basis, but not really taken into consideration all the time. There are thousands of compounds that stimulate the chemical responses for how smell is perceived. These compounds are also known as aroma compounds, aroma-active compounds, or odor-active compounds. The analysis of various aromas and flavors allows these fragrance and flavor chemists to reconstitute flavors and odors for industrial uses, thus making this a highly viable field of chemistry.

The aim of this study was to decode the pawpaw fruit to identify and quantitate the aroma-active compounds. The pawpaw fruit are a point of interest in fragrance and flavor chemistry due to the unique and pleasant flavor and aroma. *Asimina triloba*, or the pawpaw fruit, are the largest fruit indigenous to North America[16]. Specifically, these fruits are native to environments with temperate climates. Within the United States, the pawpaw fruit can be found in Kentucky, the Ohio Valley, and regions along the eastern coast. These fruits are a part of the pantropical *Annonaceae* family. Within this family, there are over 2,400 species in over 100 genera of trees, shrubs, and lianas[7] with the *Asimina* being the only mild climate genus in the family.

The pawpaw fruit is a unique fruit in appearance, fragrance, and taste. The pawpaw fruit grows in clusters on trees, and they have a green outward appearance, but on the inside, they have a yellow/orange flesh with several large seeds along the length of the fruit. The flavor of the pawpaw has often been described as a tropical-like fruit with notes resembling a cross between bananas, pineapples, and mangoes[16]. The pawpaw fruit has a very short shelf-life meaning that it can sometimes be hard to obtain. The pawpaw fruit generally begins ripening in August and peaks in the latter half of September to early October. Therefore, the season in which the pawpaw fruit is available and ready to eat is extremely short. Not only that, but once the fruit is ripe and picked, they deteriorate quickly. Generally, once the fruit reaches maturity, it decomposes in less than two weeks[19]. Because of this issue, the desire for a pawpaw flavor profile is high. With the creation of the flavor profile, a synthetic blend of

chemicals could be created to replicate the pawpaw fruit flavor and aroma for human consumption at a larger scale.

In the 1990's, McGrath and Karahadian[17, 19] analyzed the volatile compounds within the pawpaw fruit. These scientists used Tenax^{GR} GC traps to capture the volatile compounds and eluted them using diethyl ether before performing GC analyses on a packed GC column. Using gas chromatography – olfactometry (GC-O), these scientists were able to identify 14 different odorants that were composed of acetoin, gamma-hexalactone, and various esters, predominantly ethyl butyrate, ethyl hexanoate, and ethyl octanoate. Low resolution was observed and therefore, it was hypothesized that some odor-active compounds may not have been detected or analyzed. Since then, there has been minimal research performed on the compounds that reside within the pawpaw fruit. Seeing as the pawpaw fruit is widely inaccessible, the fruit has not been studied like many other food and beverage products. Fragrance and flavor chemistry is a widely evolving field of chemistry in which scientists do and learn more every day. However, the pawpaw fruit gets overlooked during these studies, so there is more to be learned about these fruits. This conundrum sparked the interest of this Kentucky native fruit, which has allowed for the project to be pursued.

The approach being applied is to use a capillary GC column which significantly increases theoretical plates compared to a pack GC column. In addition, the use of SPME and liquid extraction is more exhaustive than dynamic headspace technique used by McGrath and Karahadian. These approaches will enable the capture of the whole boiling point spectrum of compounds present in pawpaw. Therefore, the breath of compounds

captured with these techniques plus the use of capillary over pack GC columns will provide a more exhaustive approach to identifying the aroma compounds in pawpaw fruit.

1.2 Preparing the Pawpaw

There are several components to this experiment including the use of HS-SPME and solvent extractions, gas chromatography – olfactometry (GC-O), gas chromatography – mass spectrometry (GC-MS), aroma extract dilution analysis (AEDA), and gas chromatography – flame ionization detection (GC-FID). Each component has an important role in the identification and quantitation of the aroma-active compounds in the pawpaw.

To prepare the pawpaw fruit sample, the sample is crushed up and placed into a vial with a septum screw cap. After preparing the pawpaw sample, the volatile compounds are extracted. Throughout this experiment, two different methods of volatile extraction have been used: head space solid phase micro-extraction (HS-SPME) and solvent extraction.

A SPME assembly consists of the SPME fiber, which is a thin fiber that is coated with adsorbent material that the volatiles will adsorb during extraction—the SPME fiber acts as the stationary phase. When using HS-SPME, organic analytes are transferred from a matrix to a stationary phase. Typically, SPME is used for gaseous samples, but it can also be used for solid and liquid analytes. The most common method of SPME extraction, and the one used throughout this experiment is HS-SPME. This involves extraction of the analytes in the headspace above the sample. Once the sample is in a

capped and sealed vial, HS-SPME can be performed. The first step of using SPME is to pierce the vial's septum with the SPME fiber's casing. The SPME fiber is held in a device that resembles a syringe. So once the casing has pierced the septum and is in the headspace region of the vial, the syringe plunger is depressed to expose the coated stationary phase of the SPME fiber. The fiber is then exposed to the headspace for a specific amount of time. During this time, analytes that have an affinity for the stationary phase will absorb. Increasing the time, temperature and/or agitation of the sample may drive analytes to the HS to adsorb to the stationary phase. After the allotted exposure time, the fiber is then retracted back into the needle casing to protect the target analytes from outside forces or contamination. After the SPME fiber has been retracted into its casing, the syringe is removed from the vial and directly injected into the heated GC inlet. The syringe plunger is once again depressed, but this time it is for analyte desorption. The heated inlet of the GC will allow the analytes to thermally desorb, thus allowing them to move through the GC column for analysis (Figure 1).

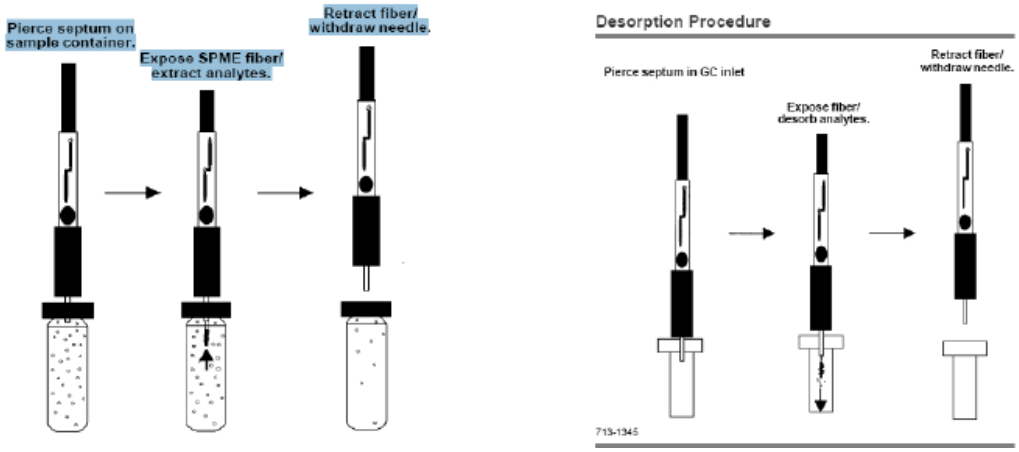


Figure 1. Diagram showing the principles of SPME.

After HS-SPME was conducted, solvent extraction was also used to extract any volatile compounds that may be trapped in the sample. Using solvent extraction after HS-SPME enables all the volatile compounds to be extracted with no selective evaporation issues. When using solvent extraction, the sample is fully submerged in a solvent that has a low boiling point such as dichloromethane (DCM) which has a boiling point of 39.6°C. A low boiling point is a necessity so that the solvent will evaporate before the aroma compounds. Ethyl acetate and acetoin have boiling points of 77°C and 148°C respectively. However, compounds such as acetaldehyde and diacetyl have boiling points of 20.8°C and 88°C. When DCM is used for extracting these two compounds, any concentration step to remove DCM solvent will also result in loss of both acetaldehyde and diacetyl completely as they are more volatile than the solvent. The sample solid is then removed from the solvent and the solvent is then filtered to remove any excess debris. This filtrate is then analyzed using GC.

1.3 Instrumental Analysis

The identification and analysis of aroma compounds was almost impossible before the creation of gas chromatography (GC)[17, 19]. However, since the invention of GC, the identification and analysis of these odor-active compounds has surged tremendously. Generally, the samples that are analyzed using GC are volatile compounds, thus making odor-active compounds prime substances for this type of analysis because many of them are volatile. Volatile compounds are substances that evaporate readily, so when heated, they become gaseous. Within the pawpaw fruit,

there are many volatile compounds that make up the flavor and aroma, although the pawpaw has not been analyzed as often as other types of foods and beverages.

Chromatography itself refers to the separation of a mixture or a vapor through a medium where the components will move at different rates. When gas chromatography is used, the samples are separated while in a gaseous phase. Whenever a sample is injected into the GC inlet, the sample travels to a heated GC column that resides in the column oven. The column oven heats the column to a specified temperature, which heats the samples to keep them in a gaseous phase. Within this heated GC column, the volatile compounds within a sample will begin to separate (Figure 2). The separation that occurs within the column and the time that it takes, or the retention time, is dependent on a few factors. Of these is the compounds' affinity for the stationary phase, or the inside of the column. If the compound has a high affinity for the stationary phase, it will be more attracted to it; therefore, sticking to the stationary phase for a longer period. Another factor that affects the separation time is the boiling point of the compound. If the boiling point of the volatile is higher, the probability of it being in the gas phase is lower than a molecule with a lower boiling point, thus, it will take a longer time to move through the column and to the detector. Also, molecular weight and boiling point are often directly proportional to each other. Generally, compounds with higher molecular weights result in higher boiling points. There are also other factors to consider when talking about boiling point including the intermolecular forces that the compound experiences. In turn, this will also increase the retention time of the compound within the GC column. The retention time of compounds eluting from the

column (termed the eluate) are monitored by a detector. The detectors used in this work are olfactometry, MS, and FID.

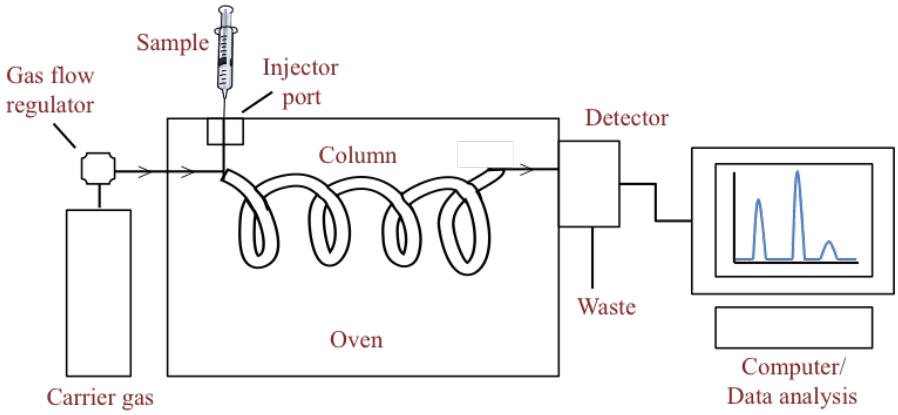


Figure 2. Diagram of gas chromatograph (GC).

Olfactometry was commonly used throughout this project. Olfactometry determines the degree of sensitivity to odorants. Generally, olfactometry is used in conjunction with GC, making the instrumentation a hyphenated technique called gas chromatography – olfactometry (GC-O). GC-O comprises techniques that use the human nose to detect and assess volatile compounds that elute from a GC separation. The assessor sniffs the eluate and records the presence of odor compounds through an odor port. Predominantly, there are three functions of olfactometry: to detect the frequency of a particular odor in a series of experiments, to determine the lowest concentration at which a molecule can be detected, and finally, to determine the intensity of an odor[11]. The third function is usually on a scale of 0-9 or by a mark on a line that is labelled 'least intense' to 'most intense'. When using GC-O in assessment of food and beverage products, the goal is to identify the components that have odorous properties.

Identifying components with odorous properties allows scientists to determine which compounds contribute most to the smell and tase of the food or beverage.

While the samples are running, an assessor must be present to act as the detector for the olfactometer. The assessor sits at the olfactory detection port and holds their nose up to what is called a nose cone. This nose cone funnels and directs all aromas present straight into the assessor's nose so that the detection can be noted. To note the presence of an aroma-active compound, the assessor generally has a remote and a headset with a microphone. The remote will indicate intensities of various aromas either on a numbered scale or with a "least to most intense" rating. As the assessor indicates the intensity at which they experience the aroma, it is also the assessor's job to distinguish what the aroma is or where they have experienced it before. The assessor should speak clearly and concisely into the microphone with the identity of the fragrance. While the assessor is running the olfactometry portion, the mass spectrometer is also ionizing the volatile compounds, separating them based on their mass-to-charge ratio and detecting them to observe the molecular mass and any fragmentation patterns. The molecule fragments with some parts possessing a positively charged species which can pass through the mass analyzer and be detected once it has reached the electron multiplier detector.

Mass spectrometry (MS) is used to analyze the components within the pawpaw fruit. The MS measures the mass-to-charge ratio (m/z) of an ion to determine the mass of its respective molecule. When using MS, the ions that are analyzed are usually generated within the instrument. This occurs by inducing the gain or loss of a charge

from an uncharged species. The ion source produces ions in a variety of ways including electron ejection, electron capture, cationization, deprotonation, or the transfer of a charged molecule from the condensed phase to a gaseous phase. Ionization can be achieved in different ways including electron ionization (EI), chemical ionization (CI), and using desorption techniques.

In this experiment, the compounds are ionized using electron ionization. The vaporized sample enters the ionization chamber via the sample inlet. Then, there is a metal filament that is heated by an electric current to emit electrons from the sample. This happens because the analytes are bombarded with a stream of electrons that will knock one or more electrons off the analyte creating a positively charged ion. The ejected electrons are then captured by a trap electrode. The produced ions are electrically pushed out from the ion source by the positive voltage on the repeller. From the ionization source, the cationic sample is pushed to the quadrupole mass analyzer where the ions are separated based on their m/z ratio. The quadrupole mass analyzer uses a direct current voltage and a radio-frequency voltage to filter the sample based on the mass-to-charge ratio[14]. The mass analyzer filters the ions based on the stability of their paths in the oscillating electric fields. After passing through the quadrupole, the ions are detected by the high energy dynode (HED) electron multiplier detector. The outcome of the ionizations, ion separation, and detection is that the resulting mass spectrum provides beneficial information such as the molecular weight and structural information.

Gas chromatography, olfactometry, and mass spectrometry, although three separate forms of instrumentation, are used in conjunction with each other to form gas chromatography – olfactometry – mass spectrometry (GC-O-MS) (Figure 3). This instrumentation is used on each pawpaw sample resulting in gas chromatograms, olfactograms, and mass spectra. The samples first enter the GC column through the SPME fiber. As mentioned previously, the components within the pawpaw fruit will separate based on multiple factors while in the heated column. Once the volatile compounds in the pawpaw fruit are separated and detected, these compounds travel simultaneously to the olfactometer and mass spectrometer.

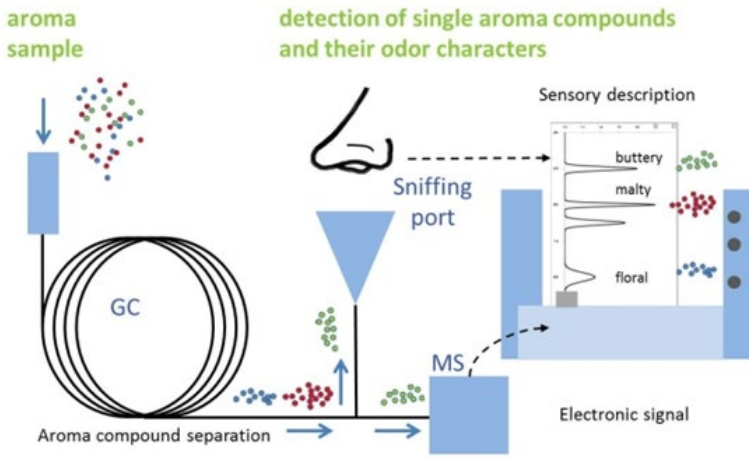


Figure 3. Diagram showing the Gas Chromatograph-Mass Spectrometer-Olfactory (GC-MS-O) instrument.

1.4 Classifying Aroma Compounds

Once the initial instrumental analysis has been completed, the next step is to classify the aroma active compounds based on their intensities. To do that, it is

necessary to deem which of the aroma-active compounds play more crucial roles in the overall flavor composition of the pawpaw fruit. This is made possible using aroma extract dilution analysis, or AEDA. The AEDA process begins with a concentrated extract of the pawpaw fruit. This sample serves as the base for the dilution analysis seeing as each dilution will then be compared to the original, undiluted, fragrant pawpaw sample. Using the pawpaw sample, a series of serial dilutions will be completed. After the serial dilutions are made, the samples are run through the GC-O to obtain new chromatograms for the odor-active compounds that are present in the diluted samples. As the samples become increasingly diluted, the present aroma-active compounds are also diminishing slowly. These steps will be repeated until all the original aroma compounds are no longer detected by the assessor. The number of dilutions that it takes to remove the scent helps to determine the relative odor strength. Compounds that more strongly contribute to the fragrance and flavor of the pawpaw fruit require more dilutions to fully rid the sample of the aroma. On the other hand, compounds that do not play as strong of a role in the flavor composition of the pawpaw fruit require fewer dilutions to remove the aroma. These compounds are assigned a flavor dilution (FD) factor based on dilution from the starting aroma extract to group them together. The FD factor is assigned depending on the last dilution step that the odor compound was detectable. When a compound has a higher FD factor, it plays a stronger role in the fragrance and flavor composition of the fruit whereas low FD factors have little control over the fragrance and flavor.

II. Gas Chromatography – Olfactometry of Pawpaw Fruit

The invention of gas chromatography was a tremendous leap forward in the development of identifying the volatile compounds responsible for aroma. The importance of aroma in society dates back thousands of years; there are mentions of aroma and fragrance in ancient Babylon[1]. In 1952, Martin and James were credited with inventing the modern gas chromatograph which is one of the most used analytical instruments[1]. The early gas chromatographs used packed columns which resulted in insufficient resolution of compounds. However, in the early 1970's, technology enabled the development of capillary columns for GC. Walt Jennings was a leader in the development of glass capillary columns for GC application[21]. In 1974, Jennings founded J&W Scientific and began manufacturing capillary GC columns from a garage with a graduate student. These capillary columns dramatically increased theoretical plates and what were typically 30 peaks on a packed GC column became more than a hundred on these new capillary columns. The invention of a benchtop mass spectrometer that could be connected as a detector to the GC was another milestone in flavor research as scientists could now more definitively identify compounds based on their mass spectra. Another major achievement in the field of flavor research was the introduction of an olfactory, or sniff, port at the end of a GC column[9]. This olfactory port enabled the flavor researcher to identify the regions of a GC chromatogram that were responsible for important odors of a product. These achievements have now become standardized approaches in flavor research and most labs are equipped with a Gas Chromatograph – Mass Spectrometer – Olfactory detector system (Figure 3 and Figure 4).

Figure 4. Using the laboratory GC-MS-O instrument for pawpaw flavor research. The headset allows us to record aroma descriptors each time an odor is detected.

The perception of aroma is derived from the interaction of volatile compounds with olfactory receptors at a level above their odor detection threshold[11]. Studies have shown that not all volatile compounds contribute to the aroma. For example, there have been more than 500 volatile compounds identified in coffee; however, the aroma of coffee has been replicated with only 27 compounds[9]. The invention of an olfactory port connected to a gas chromatograph enabled the identification of which volatile compounds are most likely responsible for the aroma of the sample[2]. Charm analysis, aroma extract dilution analysis (AEDA) and odor activity value (OAV), were techniques developed to process the GC-O data into relevant responses for the aroma of food

products[2, 12]. Soon afterwards, validation of this technique was shown by the process of reconstitution and omission experiments using these identified odor-active compounds in the food product[11]. Therefore, the tools and approaches exist to identify the odor important compounds in food products and the aim of this study was to apply some of these techniques to pawpaw fruit.

The pawpaw fruit is native to the eastern part of the United States and grows wild in the forest canopy[23]. It has broad leaves like tropical plants and produces fruits which have a resemblance of other tropical fruits such as mango, pineapple, and banana[5]. The fruit has a green exterior skin and a yellowish-orange flesh with large seeds and resembles a mango; however, the flesh is slightly softer (Figure 5). The aroma is quite intense and attractive. One attribute which has prevented the distribution of this fruit is its rapid deterioration upon reaching its maturity, typically less than two weeks[19]. Therefore, the most common environment to experience this fruit is at a local farmers market, pawpaw festival, or a restaurant which may prepare special desserts around the pawpaw fruit. One of the more desirable desserts for this fruit is ice cream.

Figure 5. Picture of pawpaw fruit growing as a cluster and a cross-section of the fruit (KSU-Chappell). Photo credit: Jonathan Palmer, Kentucky State University.

Previous work on the volatiles of pawpaw fruit were performed by McGrath and Karahadian[16, 17]. In this study, McGrath and Karahadian captured the pawpaw volatiles on Tenax GC^{R} traps and eluted them with diethyl ether prior to gas chromatographic analysis. McGrath and Karahadian identified many ester volatiles, with ethyl hexanoate, ethyl octanoate, and ethyl butyrate being the predominant compounds based on area. In addition, they identified acetoin and gamma-hexalactone in their extracts. The scientists experimented with gas-chromatography – olfactometry (GC-O) on a packed 3 m x 2 mm i.d. silane-deactivated glass column containing 10% SE-54 and were able to detect approximately 14 odorants which included various esters, acetoin, and gamma-hexalactone. Due to the low resolution with a packed column, it was hypothesized that additional odor-relevant compounds could have been missed in this early research.

Therefore, the aim of this study was to perform a more in-depth analysis of the aroma-active compounds (GC-O) in pawpaw fruit using a variety of approaches such as Solid Phase Micro-Extraction (SPME) and solvent extraction. Additional insights into the odor active compounds of pawpaw may provide a better understanding for the tropical character of this odiferous fruit.

It was decided to use SPME as a first approach while analyzing the aroma of the pawpaw fruit. This technique uses a solid fiber adsorbent to collect volatiles above a sample. The adsorbed volatiles are then desorbed into the GC inlet and transported onto the GC column (Figure 1). This technique is relatively simple and can be automated with an auto sampler.

Using SPME – Gas Chromatography – Mass Spectrometry – Olfactometry (SPME-GC-MS-O), many odor-active compounds in a Susquehanna pawpaw sample were detected. Figure 6 shows the chromatogram (top trace) and olfactogram (bottom trace) of SPME-GC-MS-O of Susquehanna pawpaw sample. Notice that not all the peaks that are present are odor active. For instance, the peak labeled 16 is the odor active compound homofuraneol; however, it is on the shoulder of a very large peak which is not odor-active. The large peak mentioned was identified as ethyl dodecanoate which is a C-12 ester compound. The ability to detect homofuraneol, but not ethyl dodecanoate, as a major aroma compound in pawpaw fruit flavor shows the importance of using GC-O for determining which compounds are aroma active in food products.

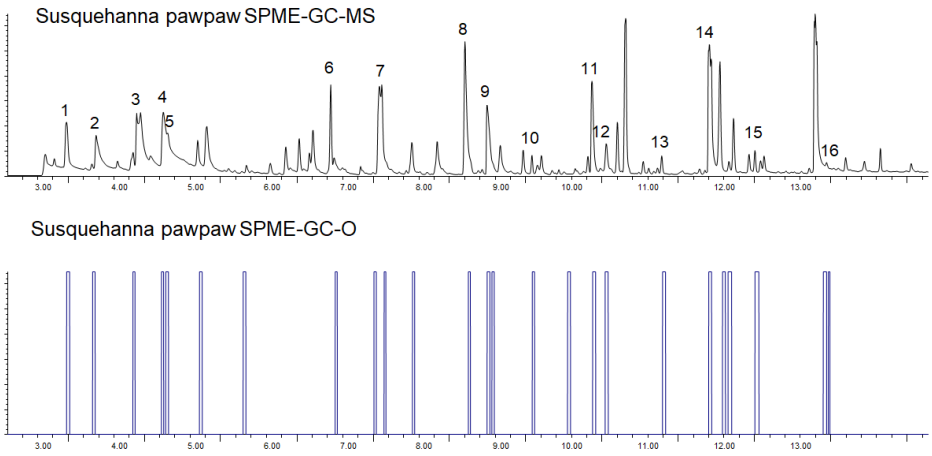


Figure 6. SPME-GC-MS-O of Susquehanna pawpaw fruit at 50ºC for 20 min. (1) acetaldehyde, (2) 2-methylpropanal, (3) 3-methylbutanal, (4) diacetyl, (5) ethyl butyrate, (6) ethyl hexanoate, (7) acetoin, (8) ethyl octanoate, (9) acetic acid, (10) linalool, (11) butyric acid, (12) phenylacetaldehyde, (13) citronellol, (14) hexanoic acid, (15) gamma-octalactone, (16) homofuraneol

Overall, SPME was a good approach in the initial evaluation of pawpaw fruit aroma. Through the project, it was observed that SPME is reproducible as similar results are seen in repetitive analysis. In addition, the Gerstel MPS autosampler provides consistent sampling procedure which helps with reproducibility. Sixteen odor compounds were initially identified with five of these compounds being newly reported to be present in the pawpaw fruit (diacetyl, phenylacetaldehyde, citronellol, gamma-octalactone, and homofuraneol). SPME is a headspace analysis technique, and it was decided that a liquid-liquid extraction technique should be applied as well to see if there are other aroma active compounds that may have missed in the initial analysis. For the liquid-liquid extraction technique, dichloromethane was chosen as the solvent because it has commonly been used in extraction of flavor compounds from food products[13]. The liquid-liquid extraction methodology was followed as described in the methods section. This analysis was repeated five more times to determine consistency of detecting the odors eluting at the olfactory port. Odors that were detected at least three times were considered to be repeatable and recorded in Table 1.

Table 1. List of odor active compounds detected in liquid-liquid extraction of Atwood pawpaw with dichloromethane and analyzed by GC-MS-O.

Odor Description	Compound ID
Sweet	Acetaldehyde
Buttery (S)	Diacetyl
Lactone (M)	Gamma-Octalactone
Cotton Candy (S)	Homofuraneol
Fruity (M)	Ethyl Butyrate
Fruity (M)	Ethyl Hexanoate
Potato (M)	*Methional*
Cheesy (L)	Butyric Acid
Stinky Acid (M)	Hexanoic Acid
Spicy Notes / Cinnamon (M)	*Eugenol*
Soapy / Perfume (L)	*Coumarin*
Vanillin (S)	*Vanillin*
Roasted 2-ap (M)	*2-Acetyl-1-Pyrroline*
Floral (L)	*Geraniol*
Lactone (L)	*Delta-Octalactone*
Clove-like (L)	?
Floral / Sweet (L)	Citronellol
Spicy / Licorice (M)	?
Waxy (S)	*Octanoic Acid*
Ester / Tequila notes	Ethyl Octanoate

The compounds highlighted in italicized font within Table 1 were not detected in the SPME-GC-MS-O analysis, showing that the liquid-liquid extraction approach enabled the identification of some new odor active compounds in the Atwood pawpaw fruit. These new compounds had a wide range of odor qualities. For instance, methional

smelled like a cooked potato and 2-acetyl-1-pyrroline has a characteristic roasted note which is responsible for the aroma of fragrant rice. In addition to these cooked notes, there was vanillin, eugenol, coumarin, and delta-octalactone compounds which exhibited the characteristic vanilla, creamy, coconut, and spicy cinnamon aroma qualities. These aroma compounds give the pawpaw fruit a distinct sweet, creamy, spicy character which contrasts with the fruity esters. The complexity of odor compounds may be a reason why the flavor of pawpaw is sometimes hard to describe for many people. The esters will give a fruity, pineapple character but then the lactones and vanillin will give creamy, dairy notes.

In summary, the liquid-liquid extraction approach enabled the detection of more odor active compounds in the pawpaw fruit. The next step was to extract a larger amount (100 g) of the pawpaw fruit and perform aroma extract dilution analysis (AEDA) as described in the methods section. AEDA is a common approach in flavor analysis and is based on the principle that the concentrated extract is diluted in a series of dilutions (3-fold as shown in Figure 7 below) and then analyzed on the GC-O instrument. With each dilution, there is the chance that one or more compounds are no longer detected at the olfactory port. As shown in Figure 7, one compound is detected at the 1/3 dilution but not 1/9 dilution. This compound would have a flavor dilution value of 3.

AEDA is an excellent way to characterize the aroma-active compounds based on their intensities and the role that they play in the overall flavor profile of the sample. AEDA begins with a concentrated extract of the sample – meaning a solvent extraction is performed on the pawpaw samples, then the extracts are pooled together and

concentrated to make a “concentrate extract”. This concentrated extract is the most odiferous sample and will result in the highest number of odorants detected. Then, serial dilutions are performed on this concentrated extract. After the serial dilutions, GC-O is run to obtain new chromatograms and olfactograms for the then present odor-active compounds. As seen in figure 7, as the samples become more diluted, the aroma compounds are slowly diminishing. These steps are repeated until all the original aroma-active compounds are no longer sensed. The number of dilutions that it takes to get rid of the scent allows for the determination of relative odor strength. The compounds that strongly contribute to the flavor will still be detected with sequential dilutions of the aroma extract. Therefore, compounds with higher flavor dilution (FD) values play a strong role in the overall flavor.

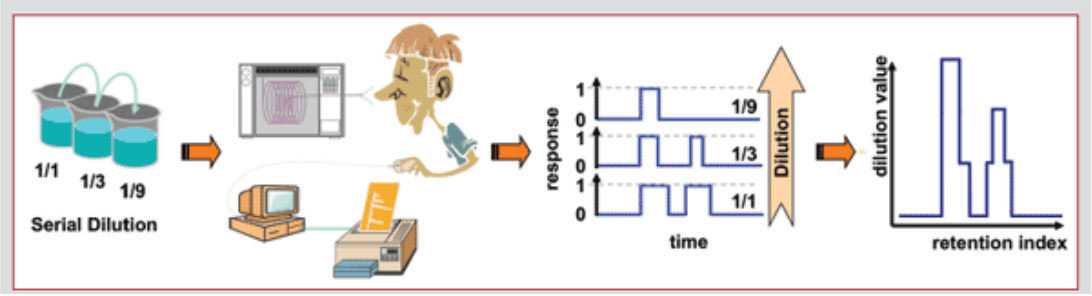


Figure 7. Example of an Aroma Extract Dilution Analysis (AEDA) for determining the flavor dilution value of each aroma active compound found in the extract.

The AEDA analysis of the Atwood pawpaw resulted in the detection of 44 distinct odor active compounds (Table 2-4). 30 of these aroma active compounds were able to be identified and 14 were unknown compounds due to sensitivity issues and/or co-elution issues. Further work would be needed to identify these additional 12

compounds. Fractionation techniques across solid phase extraction cartridges such as C-18 or silica can be used to potentially remove co-eluting compounds on a GC. In other cases, labs have used 2-D GC techniques to successfully identify odor active compounds in complex matrixes[8].

Table 2. AEDA of Atwood pawpaw fruit showing the 18 initially identified odor compounds detected with flavor dilution (FD) values ranging from 8 to 1024.

Compound ID	Odor Description	FD Factors
Homofuraneol	Cotton Candy	1024
Diacetyl	Buttery	1024
Gamma-Octalactone	Lactone, Coconut	512
Acetaldehyde	Sweet, Fruity, Fresh	512
Ethyl Butyrate	Fruity	512
Butyric Acid	Cheesy	512
Methional	Potato	256
Ethyl Hexanoate	Fruity	256
Hexanoic Acid	Stinky Acid	256
Eugenol	Spicy Notes	256
Coumarin	Soapy / Perfume	256
Vanillin	Vanillin	256
2-Acetyl-1-Pyrroline	Roasted. Corn Chip	128
Geraniol	Floral	128
Delta-Octalactone	Lactone, Creamy	128
Citronellol	Floral / Sweet	32
Octanoic Acid	Waxy	32
Ethyl Octanoate	Ester / Tequila notes	16

Table 3. AEDA of Atwood pawpaw fruit showing the 12 subsequent identified odor compounds detected with flavor dilution (FD) values ranging from 8 to 1024.

Compound ID	Odor Description	FD Factors
Acetic Acid	Solvent-like, Glue	16
3-Hyrdoxy Ethyl Butyrate	Fruity, Buttery, Cheesy	16
2-Isobutyl-3-Methoxy Pyrazine	Woody, Earthy	16
Linalool	Sweet, Floral	16
3-Hydroxy Ethyl Hexanoate	Watermelon Notes	16
Methyl Cinnamate	Strawberry	16
Delta-Nonalactone	Sweet, Creamy	16
Decanoic Acid	Waxy	16
Phenylacetic Acid	Woody, Floral	16
Dihydrocinnamic Acid	Woody	16
p-Cresol	Stinky, Indole-like	8
Gamma-Hexalactone	Caramelized Sugar Notes	8

Table 4. AEDA of Atwood pawpaw fruit showing the 14 unidentified odor compounds detected with flavor dilution (FD) values ranging from 8 to 1024.

Compound ID	Odor Description	FD Factors
? (m/z = 99)	Cloves, Spice, Sweet	64
?	Spicy, Licorice-like	32
?	Indole-like	16
?	Caramelized Note	16
?	Caramelized Note	16
?	Lactone, Creamy	16
?	Woody	16
?	Plastic	16
?	Woody, Stinky	16
?	Spicy, Eugenol-like	16
?	Creamy, Lactone, Caramelized	16
?	Earthy	8
?	Fruity	8
?	Phenolic, Waxy	8

III. Materials and Methods

3.1 Samples and Chemicals

Pawpaw samples (fresh and frozen puree) were provided by Sheri B. Crabtree from the Kentucky State University (KSU) pawpaw research program (Frankfort, KY). The fresh fruits were picked at ripe stage and immediately analyzed or stored frozen at -20°C until time of analysis. Fresh pawpaw samples of the cultivars Atwood™, Mango, Susquehanna, and Sunflower pawpaw were provided. In addition, a frozen puree of the 2019 Susquehanna fruit was also provided and used for initial GC-O experiments and method development.

Dichloromethane and sodium hydroxide were purchased from Fisher Scientific (Hanover Park, IL, USA) and ethanol was from Greenfield Global (Shelbyville, KY). Acetaldehyde, 2,3-butanedione, 3-hydroxy-2-butanone (acetoin), gamma-octalactone, delta-octalactone, delta-nonalactone, gamma-hexalactone, 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone (homofuraneol), ethyl butyrate, ethyl hexanoate, ethyl heptanoate (internal standard), ethyl octanoate, methional, acetic acid, butyric acid, hexanoic acid, octanoic acid, decanoic acid, phenylacetic acid, 2-isobutyl-3-methoxypyrazine, methyl cinnamate, eugenol, coumarin, vanillin, geraniol, citronellol, methyl octanoate, ammonium sulfate, and alkane standard (C7-C30) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultra-pure water was sourced from a Milli-Q system (Millipore, Bedford, MA, USA). Acetic acid-d_4, acetaldehyde-d_4, 2,3-butanedion-d_6, decanoic acid-d3, octanoic acid-d_{15}, hexanoic acid –d_{11}, and butyric acid-d_7 were purchased from CDN Isotopes (Quebec, Canada).

3.2 SPME Fiber and Extraction Conditions for Pawpaw

The 3-phase SPME fiber (DVB/CAR/PDMS; DVB – divinylbenzene is aromatic and nonpolar; CAR – carboxen is polar; PDMS – polydimethylsiloxane is nonpolar. The 3-phase fiber comprises nonpolar, polar, and aromatic properties) 2 cm was chosen for the headspace extraction of pawpaw volatiles as this fiber has been shown to extract the widest polarity range of volatiles and for its proven capability of extracting flavor molecules from various fruit samples such as strawberries and raspberries[11, 14]. Fresh pawpaw fruit and frozen pawpaw puree (2.5 g) was placed in a clear 20 mL screw-cap vial with PTFE septa (Pal Parts, Raleigh, NC). The DVB/CAR/PDMS fiber (Supelco, Bellefonte, PA, USA) was used for all SPME analyses and the extractions were carried out using a Gerstel MPS SPME auto sampler (Gerstel, Linthicum, MD, USA). SPME headspace extraction was carried out for 20 minutes at both 23°C and 50°C. Then fiber was desorbed into Agilent split/splitless GC inlet operated at 250°C in splitless mode for 6 minutes.

3.3 Sample Preparation for Aroma Extract Dilution Analysis (AEDA)

To prepare an extract, 100 g of pawpaw fruit and 200 g of deionized water were homogenized in a blender at low speed for 1-minute intervals until the sample was homogenous (approximately 3-4 intervals were required). The solution was then centrifuged in an Eppendorf 5804R centrifuge at 3000 rpm for 20 minutes to remove any solids present. The clear solution was transferred to 50 mL glass conical centrifuge vials. To 30 g of pawpaw supernatant, 10 g of ammonium sulfate and 6 g of dichloromethane were added. The sample was inversion mixed by hand for 5 minutes

followed by 1 minute on the vortex mixer. The sample was centrifuged at 1500 rpm for 10 minutes and the dichloromethane layer was removed with a glass Pasteur pipet and transferred to a separate glass vial. A second and third extraction of the pawpaw supernatant was performed identical to the first and the dichloromethane extracts were combined (14 g recovered). GC-O was performed on this initial extract prior to concentration to identify potential volatile compounds that could be lost during the concentration step. Both acetaldehyde and diacetyl, along with homofuraneol were detected in the initial extract prior to concentration. Aroma Extract Dilution Analysis (AEDA) was performed on the sample using the Agilent GC-MS coupled to a Gerstel Olfactory Detection Port (ODP) 3. The combined extracts (14 g) were concentrated to ~500 µL using a Biotage TurboVap LV and 1µl was injected splitless into the GC for olfactory analysis. GC-O was performed on this concentrated extract and then the volume was increased two-fold, successively: 1mL, 2 mL, 4 mL, 8 mL, 16 mL, 32 mL [at 32 mL, the sample was split into 8-4 mL aliquots each for further dilutions to minimize solvent usage]. Therefore, the next dilution was 4 mL to 8 mL, then 16 mL, 32 mL, and 64 mL. These dilutions resulted in 11 samples which corresponded to 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, and 1024x dilutions. Odor active compounds were given a flavor dilution factor (FD) based on dilution step that the odor was last detected by GC-O. For example, if an odor compound was detected at the 4^{th} dilution step, then it would have an FD factor of 16. Identification of odor active compounds was based on a mass spectra matching to the NIST 14 library, retention index on DB-5 and DB-Wax GC columns, odor description, and comparison with injection of standards.

3.4 Quantitation

Quantitation of the aroma compounds in the pawpaw was achieved through Gas Chromatography – Flame Ionization Detection (GC-FID) using relative response factors according to Cachet *et al.* 2014[19]. The Agilent 7820a gas chromatograph column was split 2:1 (FID: MS) between an FID and Agilent 5977 mass spectrometer (MS) using the Gerstel UFlowManager®. This setup enabled us to determine that compounds were well separated on the column and had no interfering compounds as determined by evaluation of the mass spectral data. Quantitation was achieved using calibration curves generated as a plot of ratio of concentration of analyte to the concentration of internal standard (ethyl heptanoate) vs. ratio of peak area response of analyte to internal standard. All calibration curves achieved correlation coefficients of $R^2 > 0.98$. These techniques were used to quantitate the concentration of volatile compounds in the pawpaw sample. Quantitation analysis was performed in triplicate and data is reported with standard deviations. The pawpaw sample preparation was identical to the AEDA approach above. To 100g of pawpaw and 200 g of deionized water was added 200 µL of 1000 ppm ethyl heptanoate (in ethanol). The pawpaw sample was then homogenized in the blender and extraction carried out according to procedure above. This would account for sample compound loss during centrifugation steps to remove solids. Calibration curves were calculated and plotted versus area counts of the internal standard by GC-FID.

3.5 Gas Chromatography – Mass Spectrometry – Olfactometry (GC-MS-O)

The analysis of aroma volatiles extracted by HS-SPME, and liquid injection was performed using a Model 7820A gas chromatograph (GC) equipped with a 5977 mass spectrometer detector (MSD) and Flame Ionization Detector (FID) from Agilent (Agilent Technologies, Santa Clara, CA, USA). Olfactometry was performed using the Gerstel Olfactory Detection Port (ODP3) which was connected to the Agilent 7820a gas chromatograph (GC) with the Agilent 5977 mass spectrometer (MS). The spilt ratio was 2:1 (olfactory port: MS) using the Gerstel UFlowManager®. The GC was coupled with a Gerstel Multipurpose Sampler (MPS) with SPME capability (Linthicum, MD, USA). The injector port had a 0.754 mm deactivated GC liner, and the inlet was kept at a constant temperature of 250°C. A fused silica HP-5ms-UI column (30 m x 0.25 mm ID x 0.25 µm thick film) and J&W DB-Wax (30 m x 0.25 mm ID x 0.25 µm film thickness) Agilent Technologies (Santa Clara, CA, USA) were used for analysis. Helium was used as the carrier gas with a flow rate of 1 mL/min. The initial oven temperature was 50°C with a hold time of 1 minute. Then the temperature rose to 240°C at 15°C/min then held for 5 minutes. The MSD operated in electron ionization mode at 70 eV. The MSD transfer line was set at 280°C. The ion source was heated at 230°C and the MS quads were both heated at 150°C. SPME was performed without solvent delay. Liquid extract samples were injected in split mode (10:1) and a 3.5-minute solvent delay. The mass acquisition range was 35 to 250 m/z.

3.6 Olfactometry

Four olfactory panelists, who were trained in GC-O and odor recognition, each performed 3 replications. Intensity of odor compounds was rated on a 9-point scale (low, medium, strong; – and +). For example, medium can be medium -, medium, or medium +. An aroma peak was determined to be aroma active if it was detected with at least half of the analyses. For the AEDA analysis, FD factors were based on an average response for the last dilution that an odor was detected. In GC-O analysis, it is best practice to compare and combine data from at least two scientists to ensure there is no anosmia—the inability to smell some or all compounds—for certain compounds within the analyst. The data from two analysts were combined to compensate for any sensitivity differences that might exist between the panelists. In the data comparison, there was no apparent anosmia for the odor active compounds in pawpaw.

3.7 Compound Identification

Aroma active compounds were identified by a combination of retention indices, mass spectra comparison with libraries (NIST 14, FFNSC3), odor description, and confirmation by injecting authentic standards on the same columns. Alkane linear retention indices were obtained using a (C_7-C_{30}) alkane standard mixture (Sigma-Aldrich, St. Louis, MO, USA). Linear retention indices of aroma compounds were calculated on both a DB-5 and DB-Wax GC column.

3.8 Data Analysis

Aroma volatile compound identification and quantitation were performed using Agilent Technologies' ChemStation software (version F.01.03). Microsoft Excel 2016

(Redmond, WA, USA) was used for the calculation of means and for graphing of calibration curves.

IV. Quantitation of Flavor Compounds in Pawpaw Fruit

Next, some quantitation of the odor active compounds in the pawpaw fruit was performed. Quantitation was achieved using gas chromatography – flame ionization detection (GC-FID). Figure 8 shows the separation of the compounds acetoin, butyric acid, ethyl butyrate, hexanoic acid, ethyl hexanoate, octanoic acid and ethyl octanoate in the 16-fold diluted sample. With this good peak resolution, GC-FID can be used for quantitation of these aroma compounds.

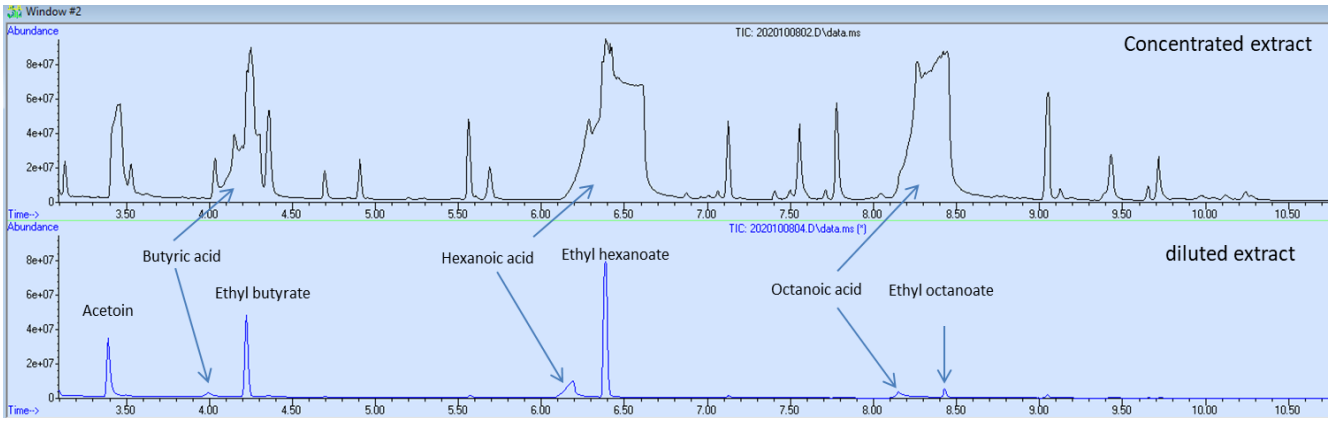


Figure 8. Chromatogram of the liquid-liquid extract of Susquehanna pawpaw concentrated (top) and diluted (bottom) on a DB-5 column.

The pawpaw extract was also analyzed on a wax GC column (Figure 9). The organic acids (butyric, hexanoic, and octanoic) interact much more with the stationary phase on the wax column which results in their longer retention time. This is apparent when comparing ethyl hexanoate with hexanoic acid. Figure 8, which is the DB-5 column, has ethyl hexanoate and hexanoic acid almost coeluting, whereas, on the wax column, ethyl hexanoate and hexanoic acid are separated by approximately 4.5 minutes.

In addition, the chromatography for the acids results in better peak shape on the wax column. The internal standard used for quantitation, ethyl heptanoate, is shown in Figure 9.

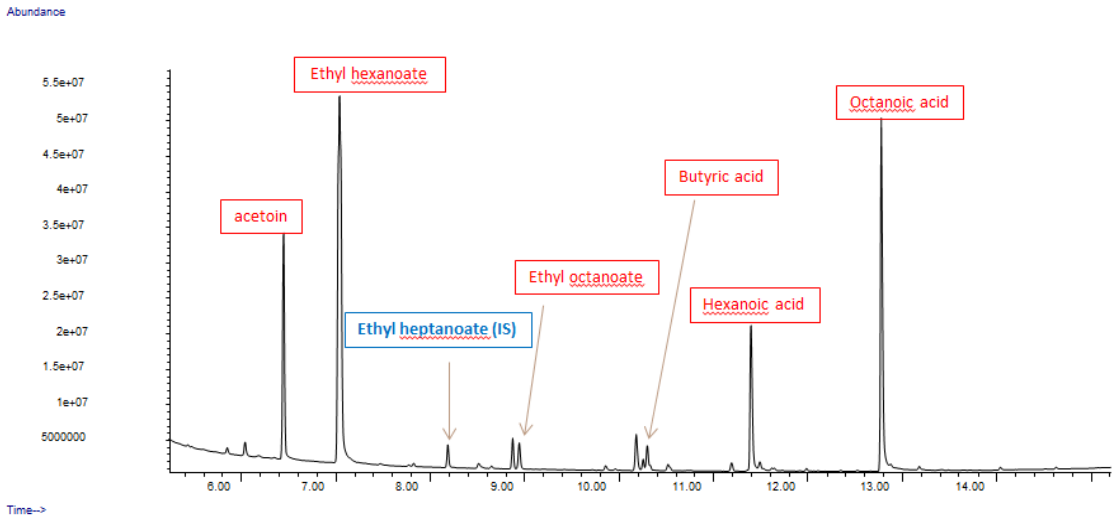


Figure 9. Chromatogram of the liquid-liquid extract of Susquehanna pawpaw analyzed on a wax column.

For quantitation, the end of the GC column was attached to a disk splitter (Gerstel) which enabled the flow to split between two detectors (MS and FID). The configuration was set up to split 2:1 between the flame ionization and mass spectrometer detector. Therefore, either FID or MS could be used for quantitation, depending on the presence of interfering compounds in the GC run. The mass spectrum for the internal standard, ethyl heptanoate, is shown in Figure 10. And the mass spectra for ethyl butyrate, ethyl hexanoate, ethyl octanoate, ethyl acetate, and acetoin are shown in Figures 111-15. The ion m/z = 88 is a characteristic ion for ethyl esters, except

for ethyl acetate which gives a strong m/z = 43 ion. Acetoin is similar and the ions m/z = 43, 45 or 88 are suitable for quantitation by MS. Figure 16 shows the mass spectrum and proposed fragmentation pattern for ethyl acetate which has a molecular weight of 88 g/mol. The longer chain ethyl esters generate a m/z = 88 by alpha cleavage to the carbonyl, which is possible esters of C-2 and higher carbon chains. However, with ethyl acetate, a m/z = 88 would be the molecular ion.

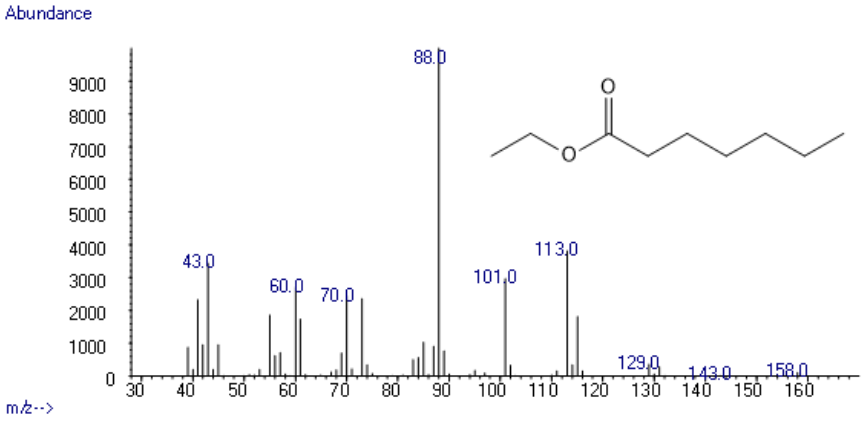


Figure 10. Mass spectrum and structure for ethyl heptanoate which was used as the internal standard.

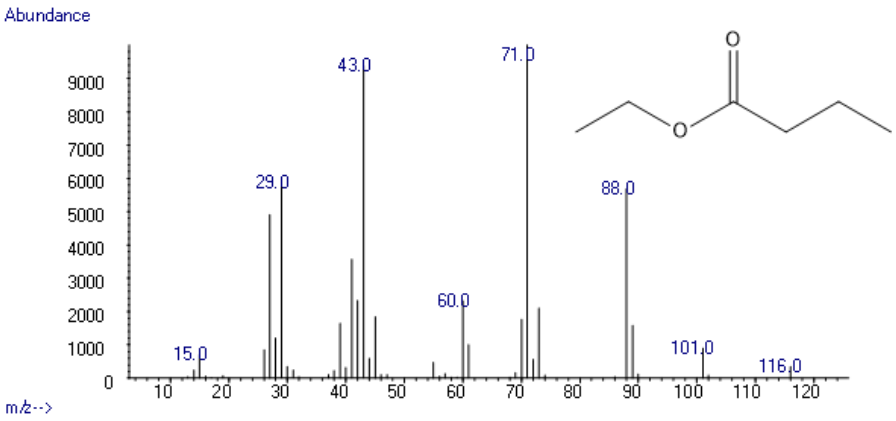


Figure 11. Mass spectrum and structure for ethyl butyrate.

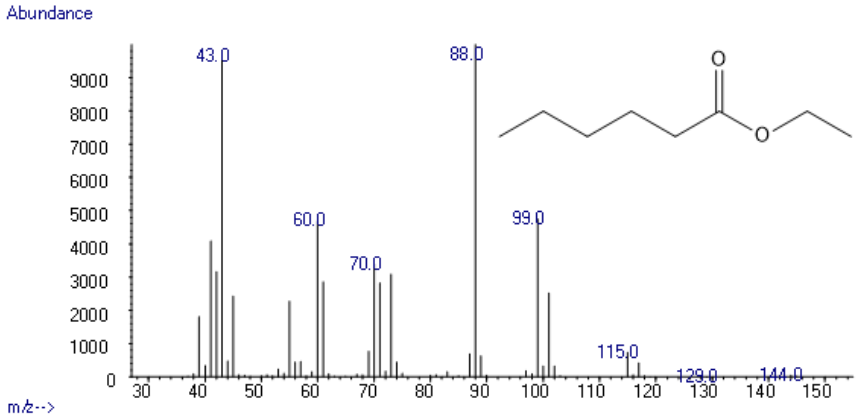


Figure 12. Mass spectrum and structure for ethyl hexanoate.

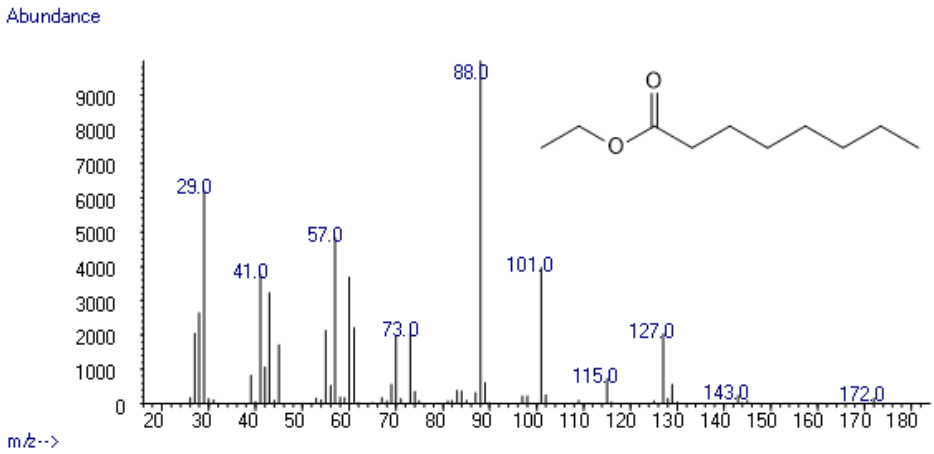


Figure 13. Mass spectrum and structure for ethyl octanoate.

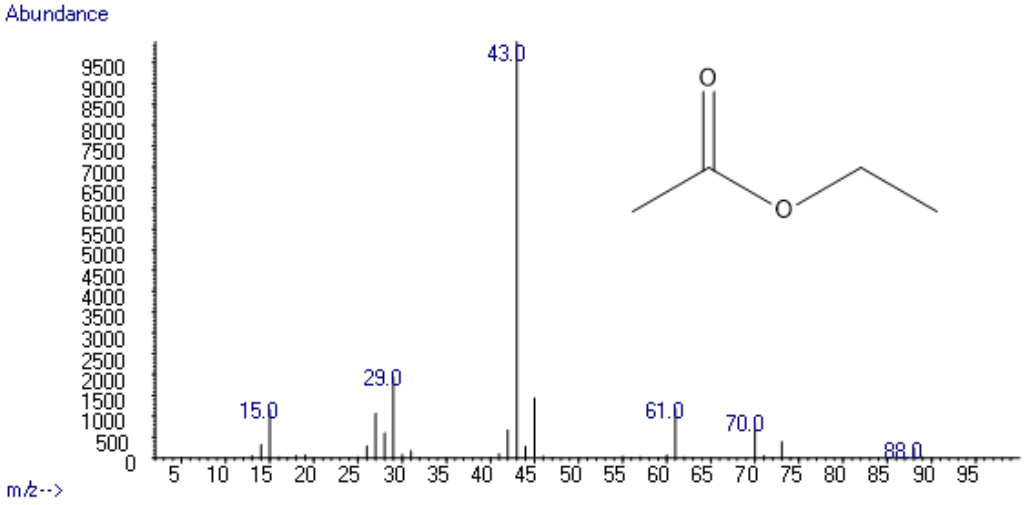


Figure 14. Mass spectrum and structure for ethyl acetate.

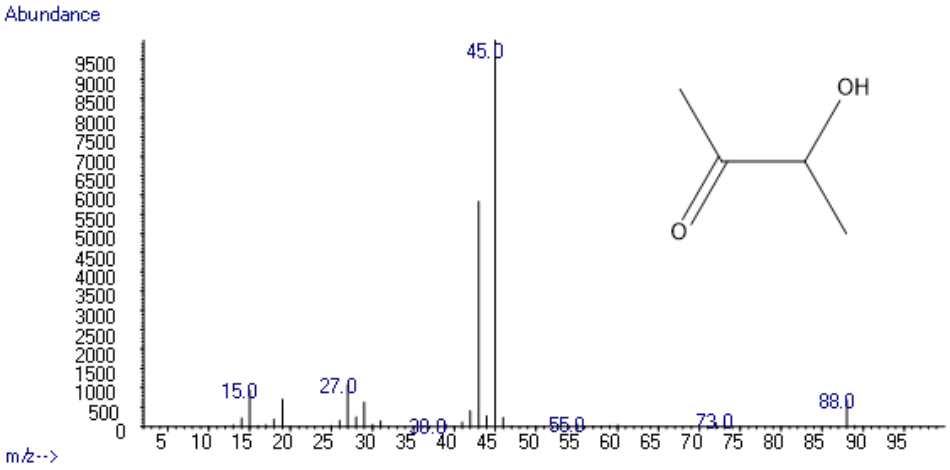
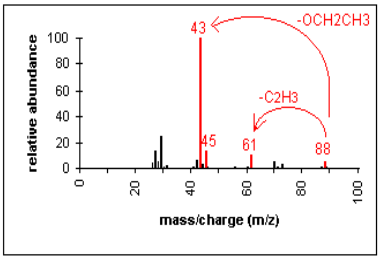


Figure 15. Mass spectrum and structure for acetoin (3-hydroxy-2-butanone).

Ester

Fragments appear due to bond cleavage next to C=O (alkoxy group loss, -OR) and hydrogen rearrangements.

Ethyl acetate ($C_4H_8O_2$) with MW = 88.11



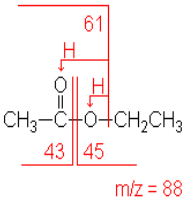


Figure 16. Proposed fragmentation pattern for ethyl acetate. Mass Spectrometry - Fragmentation Patterns - Chemistry LibreTexts.

To determine the levels of compounds in pawpaw, standard calibration curves relative to the internal standard added to the extract are needed. In this work, standard calibration curves were created against the internal standard, ethyl heptanoate. The

calibration curve for acetoin is shown in Figure 17. This curve was developed to cover the range of acetoin from 0 to 4000 ppm. Based on the initial work, it was confirmed that this range would cover the levels of acetoin in the samples. This standard curve shows a correlation of $R^2 = 0.9965$, which is fairly linear and would work well for calculations.

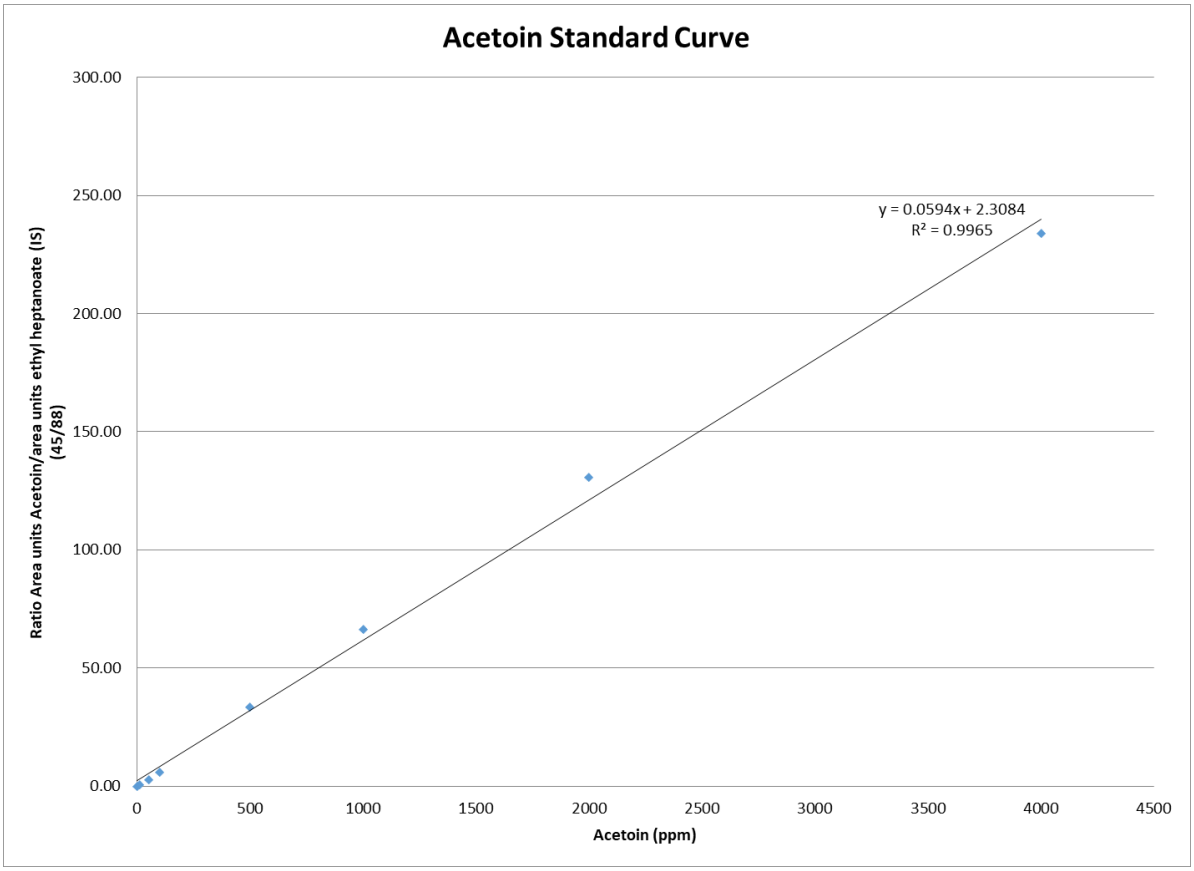


Figure 17. Calibration curve for acetoin.

A standard calibration curve for ethyl hexanoate is shown in Figure 18. The 6-point standard curve shows a correlation of $R^2 = 0.9977$ and spans 0 to 100 ppm for

ethyl hexanoate. Previous[16] research had reported values of 15-60 ppm for ethyl hexanoate in pawpaw fruits.

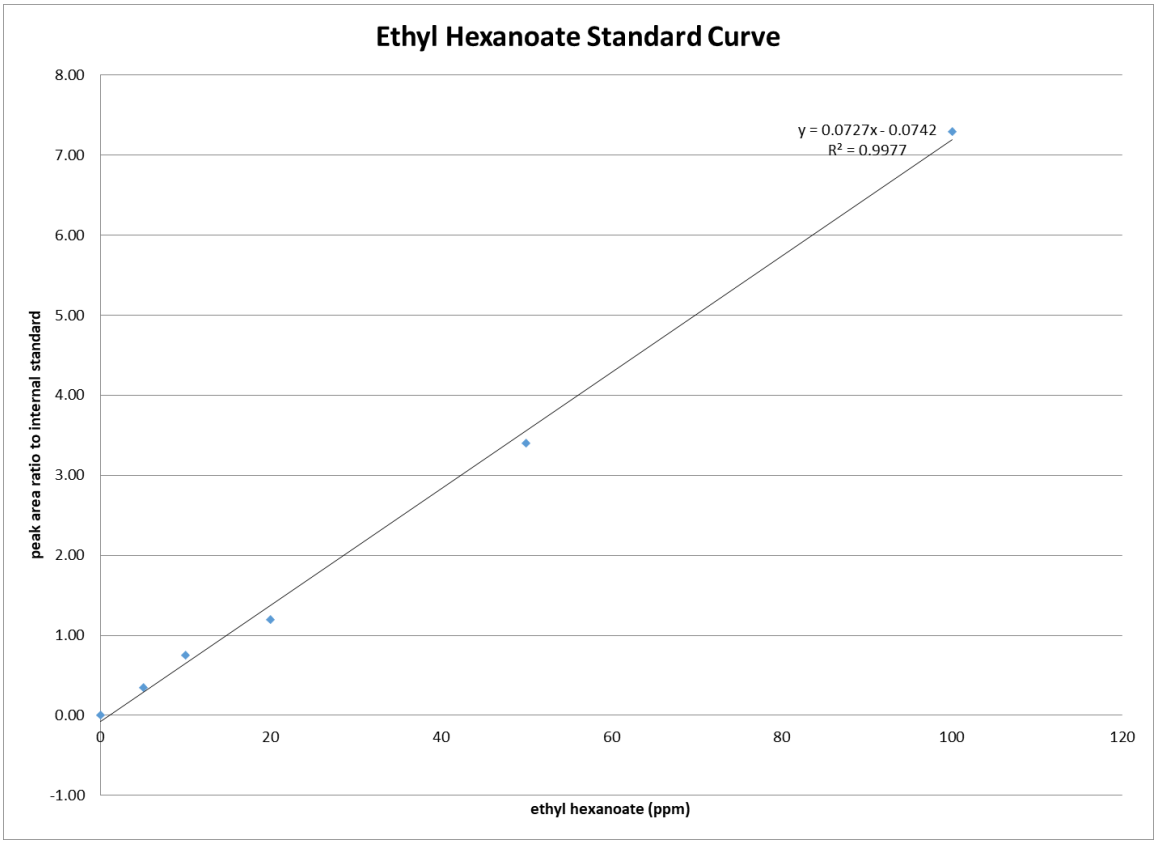


Figure 18. Calibration curve for ethyl hexanoate.

For the first quantitation experiment, the differences in the esters between an unripe, ripe, and overripe Susquehanna pawpaw fruit were compared. An unripe pawpaw fruit is very green in color and the flesh is very firm. As it ripens, the flesh softens, and the color begins to darken. When it is overripe, the flesh is extremely soft, and the color turns brown/black. The picture below (Figure 19) shows a Susquehanna fruit at different stages of ripeness, from unripe (left) to overripe (far right).

Figure 19. Susquehanna pawpaw fruit at different stages of ripening. From left to right the fruit is unripe to ripe and then overripe on the far right.

Quantitation was performed of this Susquehanna at different stages of ripeness. The unripe (far left) was compared with the ripe (two center samples combined) and the overripe (far right). Using the GC-FID method, acetoin, ethyl acetate, ethyl butyrate, ethyl hexanoate, and ethyl octanoate were reliably quantitated. The results of this analysis are shown in Table 5 and Figures 20 and 21. The unripe fruit has almost undetectable levels of acetoin and the ethyl esters. However, the ripe fruit has a 1000-fold increase in acetoin and easily detectable levels of ethyl esters, with ethyl hexanoate having the highest level at 47 ppm. It was surprising to see that the overripe sample had even higher levels of acetoin, but the ethyl esters began to decrease. It is possible that hydrolysis of the esters back into the free acid and ethanol could be a reason for this observation (Figure 22).

Table 5. Changes in concentration of esters and acetoin during the ripening of a Susquehanna pawpaw.

	Concentration (ppm)				
Cultivar	**Acetoin**	**Ethyl Acetate**	**Ethyl Butyrate**	**Ethyl Hexanoate**	**Ethyl Octanoate**
Susquehanna (Under Ripe)	1.18 +/- 0.08	0.009 +/- 0.0015	0	0	0
Susquehanna (Ripe)	1354 +/- 81	0.50 +/- 0.07	3.61 +/- 0.16	469. +/- 2.9	0.80 +/- 0.06
Susquehanna (Over Ripe)	2544 +/- 159	1.22 +/- 0.14	2.44 +/- 0.37	17.9 +/- 0.62	0.50 +/- 0.02

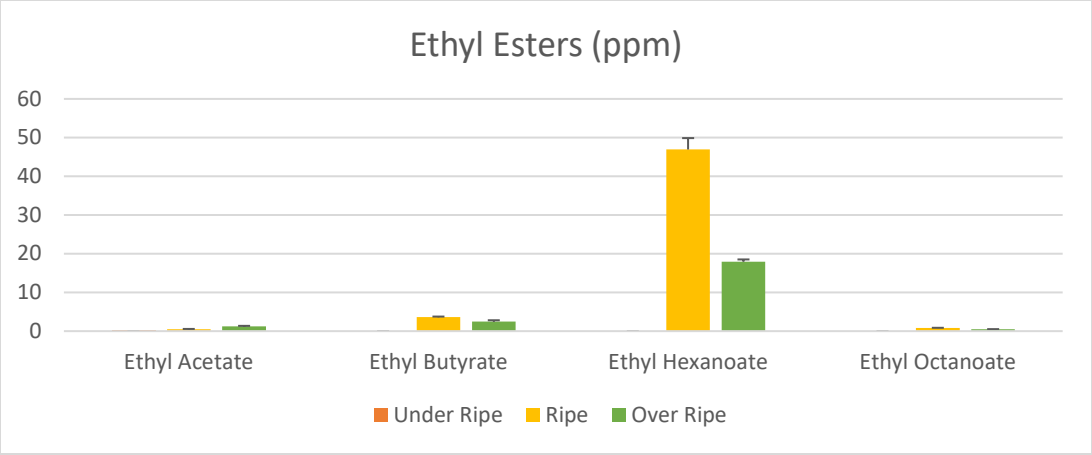


Figure 20. Bar graph representing the concentrations of the ethyl esters in the Susquehanna pawpaw fruit at different ripening stages with standard deviation.

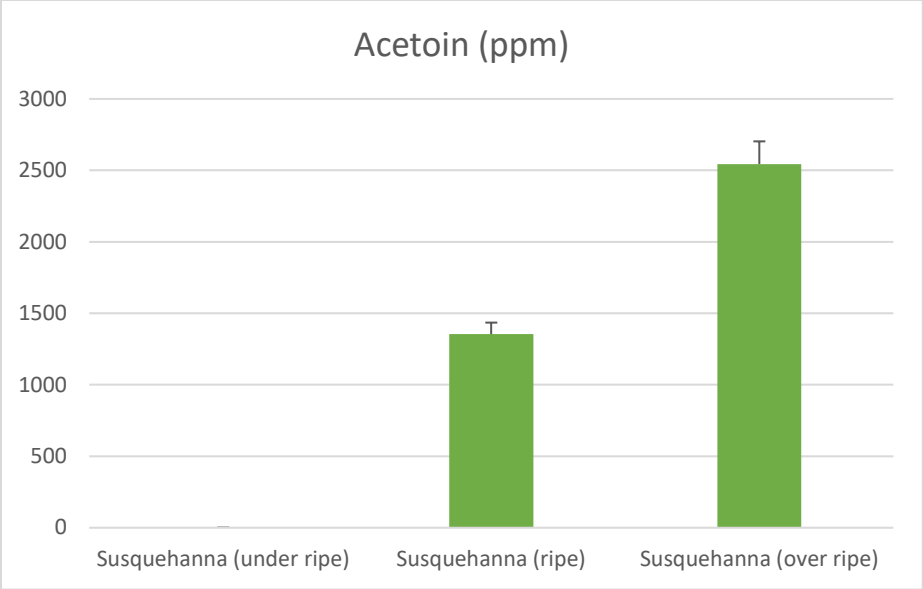


Figure 21. Bar graph representing the concentrations of acetoin in the Susquehanna pawpaw fruit at different ripening stages with standard deviation.

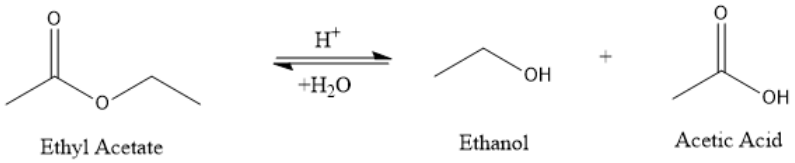


Figure 22. Hydrolysis of ethyl acetate into ethanol and its free acid.

In addition to the Susquehanna variety, samples of both the Atwood and Mango pawpaw were obtained. The flavor of these three cultivars had noticeable differences. Sensory descriptor data was collected on these three cultivars and the results are shown in Table 6. From the observations, the Susquehanna was perceived to be fruitier, ester-like and similar to pineapple aroma. The Atwood had a fruity character but also was

described as buttery, creamy and banana as well. Then the Mango was closer to the Susquehanna with pineapple, fruity and tropical notes. Overall, the odors that were detected were buttery, fruity, floral, pineapple, maple syrup, coconut, and cheesy. These odors are consistent with descriptors used to describe the aroma of pawpaw fruit.

Table 6. Sensory differences between three different cultivars of pawpaw fruits (Susquehanna, Atwood, and Mango).

Cultivar	Sensory Descriptions
Susquehanna	Fruity, Pineapple, Ester-like
Atwood	Fruity, Banana, Creamy, Buttery
Mango	Tropical, Fruity, Mango, Pineapple

Next, the level of esters and acetoin in these three cultivars of pawpaw fruit was quantitated. The results are shown in Table 7 and Figures 23 and 24. The Susquehanna is mostly influenced by the elevated levels of ethyl hexanoate and acetoin which can influence the pineapple and ester-like aroma. The Atwood was described as fruity but also buttery and creamy. The higher acetoin level, which has a sweet, buttery, dairy and creamy odor could be responsible for that attribute being detected in the sensory descriptor. The Mango variety was noticeably more tropical in aroma, and this may be driven by the high levels of ethyl hexanoate, ethyl butyrate, ethyl acetate, and ethyl

octanoate in this variety. These high levels of esters are known to contribute fruity and tropical flavor character to fruits.

Table 7. Levels of ethyl esters and acetoin in different cultivars of pawpaw fruits (Susquehanna, Atwood, and Mango).

	Concentration (ppm)				
Cultivar	**Acetoin**	**Ethyl Acetate**	**Ethyl Butyrate**	**Ethyl Hexanoate**	**Ethyl Octanoate**
Susquehanna	1354 +/- 81	0.5 +/- 0.07	3.61 +/- 0.16	47 +/- 2.9	0.80 +/- 0.06
Atwood	3054 +/- 224	0.10 +/- 0.008	2.64 +/- 0.14	30 +/- 1.50	0.40 +/- 0.06
Mango	1544 +/- 235	1.19 +/- 0.016	6.44 +/- 0.38	59.8 +/- 4.1	1.46 +/- 0.18

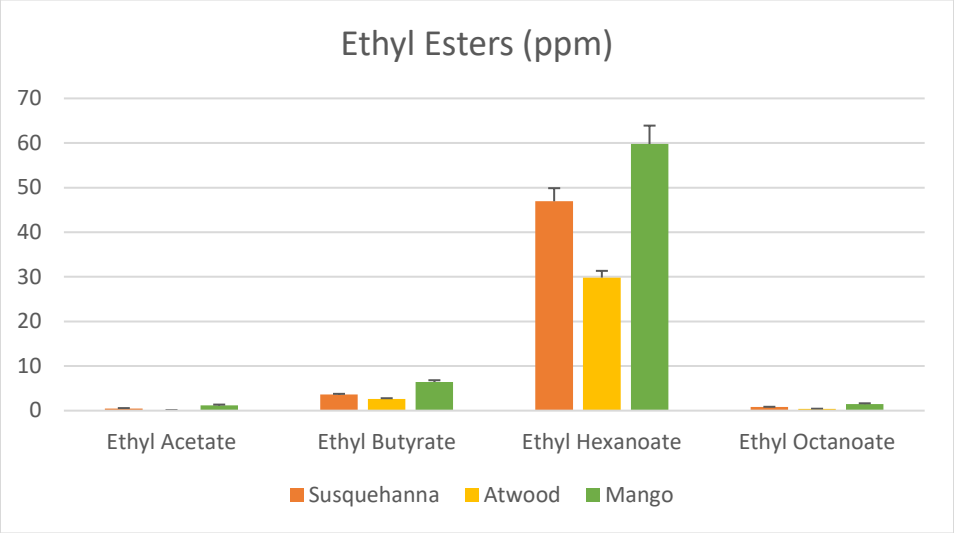


Figure 23. Bar graphs representing the concentrations of ethyl esters in different pawpaw cultivars with standard deviation.

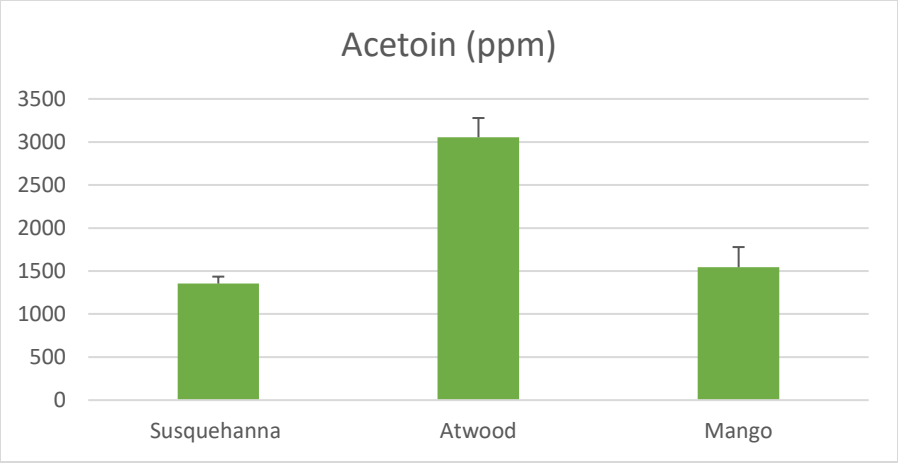


Figure 24. Bar graphs representing the concentrations of acetoin in different pawpaw cultivars with standard deviation.

An interesting aspect of pawpaw aroma compounds is the abundance of hydroxyl ethyl ester compounds. For example, the compound ethyl butyrate is present in just about all fruits. This compound can be formed by the condensation of butyric acid and ethanol with the loss of water. In pawpaw fruits, the presence of 3-hydroxy ethyl butyrate and 3-hydroxy ethyl hexanoate compounds have been observed. These hydroxyl ethyl esters are not commonly found in fruits and only mentioned as minor trace components at best. However, as you can see in Figure 25, the compounds 3-hydroxy ethyl butyrate and 3-hydroxyl ethyl hexanoate are both significant peak heights in the chromatograms. On repetitive analysis, the mango variety was always much lower in 3-hydroxy ethyl butyrate compared to the Atwood variety.

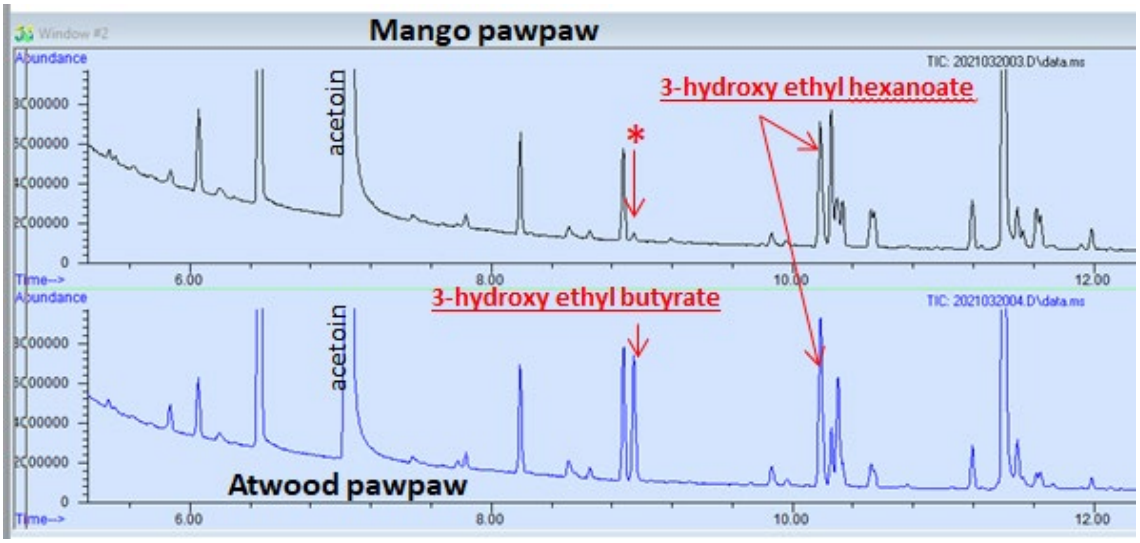


Figure 25. GC-MS profile of a liquid-liquid extract of Mango (top) and Atwood (bottom) pawpaw volatiles. The 3-hydroxy ethyl butyrate and 3-hydroxy ethyl hexanoate are highlighted with arrows. Notice the lack of 3-hydroxy ethyl butyrate in the Mango pawpaw extract.

V. Summary and Conclusions

In summary, this project has led to new insights into the chemical compounds responsible for the aroma and flavor of pawpaw fruit. Fifteen aroma active compounds that have not been reported previously in pawpaw fruit were identified here. These new discoveries give a better understanding of the sensory properties of pawpaw, which has been described as a cross between a banana, mango, and pineapple. Previous research on pawpaw fruit had summarized that ethyl esters were responsible for its flavor. However, this work has identified additional compounds such as diacetyl, acetaldehyde, lactones, acids, furanones, floral alcohol compounds, and vanillin as key compounds contributing to the flavor of pawpaw. This new knowledge will provide a pathway to recreate the flavor of pawpaw from individual compounds. These compounds together with the ethyl esters identified, provide a basis for the fundamental flavor of pawpaw fruit.

It is interesting to note that pawpaw shares many of the aroma active compounds found in dairy products[8]. These similarities could help explain the popularity of pawpaw ice cream as one of the main applications for this fruit. There are at least 55 different pawpaw cultivars growing in the United States and sensory analysis have described some as having significantly different flavors[10]. In my analysis, I showed how three cultivars (Susquehanna, Atwood, and Mango) differed in both sensory attributes and in the levels of five important odor compounds. These differences may help explain some of the sensory differences for those cultivars. Further studies could include

evaluation of the most extreme flavor variants by GC-O and quantitation. Sensory analysis of the different cultivars has shown significant differences in flavor profiles[5], and this may be explained by variations in the levels of the important aroma compounds presented in this work. This work provides foundational insights for further flavor research of the pawpaw cultivars. Additional future work will focus on completing identification of additional compounds and quantitation of these important aroma compounds across multiple cultivars.

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APPENDICES

[Appendix A: Gas Chromatography – Olfactometry (raw data) on Aroma Extract Dilution Analysis analysis]

[Appendix A: Gas Chromatography – Olfactometry (raw data) on Aroma Extract Dilution Analysis analysis]

	FD = 0 (1)	FD = 2	FD = 4	FD = 8	FD = 16	FD = 32	FD = 64	FD = 128	FD = 256	FD = 512	FD = 1024	FD = 2048
Retention	Odor Descriptor	Odor Descriptor	Odor Descriptor	Odor Descriptor	Odor Descriptor	Odor Descriptor	Odor Descriptor	Odor Descriptor	Odor Descriptor	Odor Descriptor	Odor Descriptor	Odor Descriptor
==========	==========	==========	==========	==========	==========	==========	==========	==========	==========	==========	==========	==========
		01.34 buttery S	01.40 creamy buttery	01.35 buttery M	01.37 buttery M	01.39 buttery L	01.43 buttery L	01.41 buttery L	01.41 buttery L	01.43 buttery L-		detected a hint of buttery here at 2048x
		01.53 sweet syrupy, creamy nice M	(acetoi	01.53 sweet fruity M	01.55 fruity M	01.61 fruitty L+	01.61 fruitty L					
			01.60 sweete fruitty S									
			01.77 buttery L				02.77 maybe a sweet, lactone note L-					
2.08	buttery S	02.03 plastic L										
2.91	fruity M	02.91 floral fruity M	02.93 fruity M-	02.93 fruity M	02.95 fruity L	02.94 fruity L+	02.96 fruitty L	02.97 fruity L	02.96 fruitty L-	02.96 fruitty L-		
3.64	mushroom L+	03.64 earthy mushro	03.64 earthy, musty	03.63 mushroom, earthy L-		03.68 mushroom L-						
						04.01 dead animal L-						
4.38	green L-											
4.59	fruity pineapple M	04.60 fruity pineapp	04.61 fruity pineapple L+				04.69 maybe something there L-					
4.81	earthy M	04.79 something L										
4.95	solvent M to S	04.97 solvent S	05.02 solvent M	05.01 solvent S	05.03 solvent M	05.04 solvent L+	05.06 solvent plastic L		05.06 maybe something L			
5.06	roasted note L	05.09 meaty methion	05.08 meaty L-	05.13 solvent plastic L+	05.14 plastic M	05.14 plastic L	05.16 plastic L-		05.15 plastic L-			
5.11	vinyl, plastic S	05.14 vinyl plastic S	05.14 solvent M									
5.48	something L											
6.22	cotton candy L											
6.3	sweaty acid S	06.32 sweaty acid ch	06.32 sweaty cheesy	06.34 sweaty cheesy a	06.33 cheesy S	06.35 sweaty cheesy acid	06.35 stinky cheese M	06.37 sweaty acid M	06.35 cheesy L	06.38 cheesy L		
6.55	floral PA like L				06.57 maybe cheesy solvent L-							
6.66	sweaty acid L	06.61 dead animal L	06.61 dead animal L									
7.27	floral nice M	07.28 floral note M	07.31 maybe a floral	07.26 floral L-	07.31 maybe something L			07.38 vinyl L-				
			07.39 maybe a sweaty note L-									
7.83	floral L		07.88 floral stinky M									
7.87	sweaty acid stinky M	07.86 stinky acid M	07.88 floral stinky M	07.88 dead animal L+	07.87 floral, then s	07.88 floral note L	07.90 sweaty dead animal L					
7.96	still the acid or a fru	07.92 fruity note L										
8.54	lactone M	08.56 creamy lactone	08.55 lactone creamy	08.56 lactone coconut	08.57 lactone M-	08.58 lactone L-	08.59 lactone L	08.59 lactone L-	08.60 lactone L			
8.99	lactone L	08.98 lactone L+	08.97 lactone L	08.99 lactone L	09.02 lactone L-	08.99 lactone L-	09.00 lactone L-					
9.25	something I smelle	09.28 strong plasti	or 09.25 waxy M	09.27 something L								
9.34	creamy carmelized	09.37 carmelized note L										
9.44	cotton candy nice S	09.43 cotton candy S	09.43 cotton candy M	09.45 cotton candy S	09.44 creamy carme	09.45 carmelized cotton	09.46 carmelized suga	09.45 carmelized su	09.46 carmelized su	09.48 carmelized su	09.49 carmelized sug	09.49 carmelized sugar note M
9.94	stinky note L											
10.02	something L	10.07 maybe some	10.08 spicy sweet L-	10.09 maybe somethin	10.08 something L	10.09 spicy note L-	10.11 spice note L					
10.33	floral plastic L											
10.41	maple syrup S	10.41 maple syrup S	10.42 maple syrup S	10.44 maple syrup M-	10.44 maple syrup	10.44 carmelize dsugar n	10.46 maple syrup L					
11.45	lactone L	11.51 maybe something L										
		11.70 floral L-		11.77 maybe a waxy odor L								
12.34	something M	12.37 de ad animal M	12.39 dead animal M-	12.45 dead animal L		12.48 dead animal L						
12.4	floral M	12.42 floral L										
12.56	vanillin M	12.57 vanillin M	12.60 vanillin L									
12.64	dead animal M	12.66 dead animal M	12.70 dead animal L	12.74 dead animal L	12.78 dead animal	12.80 dead animal L		12.83 dead animal	12.83 dead animal L	12.85 dead animal L		
					13.05 vanillin L- ?							

AEDA analysis of Atwood pawpaw

[Appendix B: GC Hydrocarbon standards analyzed to determine retention index]

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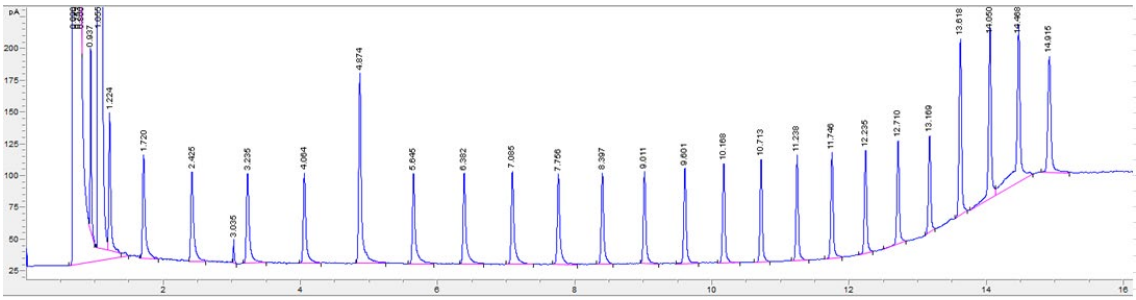
2. Calculation of Retention Indexes with Hydrocarbon Standards

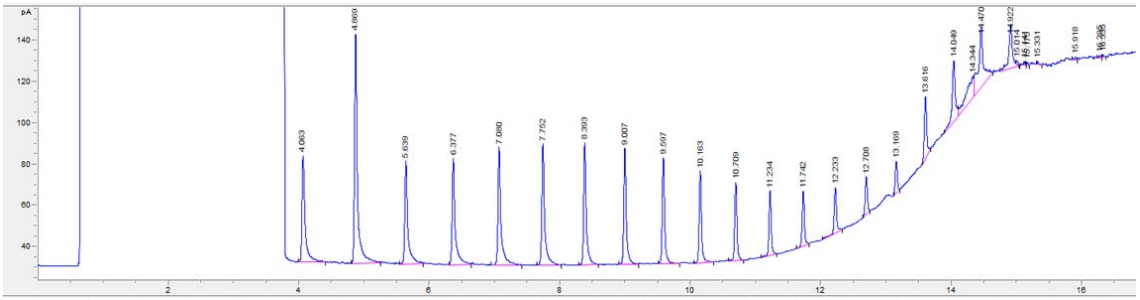


HC std	time (min)	RI
C14	4.063	1400
C15	4.869	1500
C16	5.639	1600
C17	6.377	1700
C18	7.08	1800
C19	7.752	1900
C20	8.393	2000
C21	9.007	2100
C22	9.597	2200
C23	10.163	2300
C24	10.709	2400
C25	11.234	2500
C26	11.742	2600
C27	12.233	2700

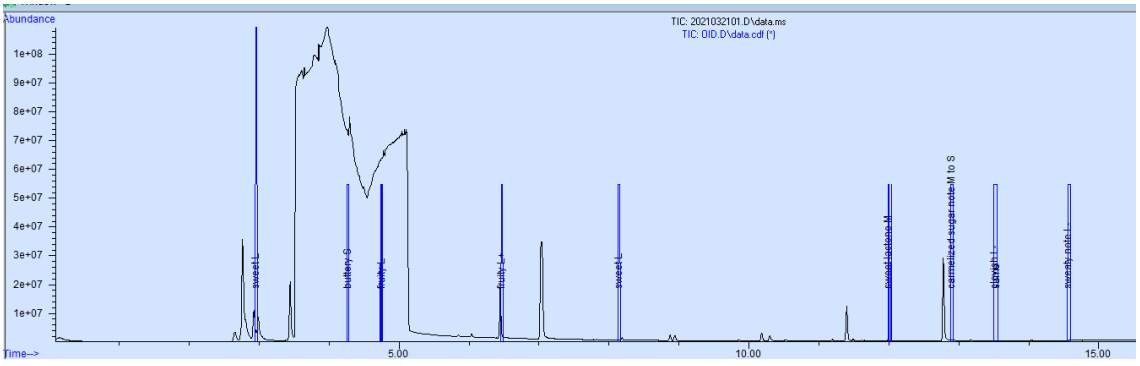
C28	12.708	2800
C29	13.169	2900
C30	13.616	3000
C31	14.049	3100
C32	14.47	3200
C33	14.922	3300

[Appendix C: GC-O Atwood Pawpaw]

[Appendix C: GC-O Atwood Pawpaw]



Gas Chromatogram – Olfactometry of Atwood pawpaw initial extract – shows acetaldehyde as an important aroma compound eluting before the solvent peak between 3-5 min. Also, diacetyl and ethyl butyrate elute under the solvent peak. In Aroma Extract Dilution Analysis, these compounds disappear during concentration of the sample by removing the solvent (they are more volatile than solvent). That is why it is important to smell the initial extract because these compounds are no longer detected in the concentrated sample, as they are removed during concentration.



SAMPLE-DESCRIPTION: P-fruit Atwood large extract unconc GCO1 3-20

Peak Start Peak End Intensity Comment

02.95	02.97	2	sweet L	acetaldehyde
04.26	04.28	1	buttery S	diacetyl
04.75	04.76	1	fruity L-	ethyl butyrate
06.46	06.48	1	fruity L+	ethyl hexanoate
08.13	08.16	1	sweet L	gamma
11.99	12.00	1	sweet lactone M	octalactone
12.89	12.92	1	caramelized sugar note M to S	homofuraneol
13.50	13.53	1	clovish L-	isoeugenol?
13.53	13.56	1	same	?
14.56	14.60	1	sweaty note L-	?

[Appendix D: GC-FID Quantitation data]

[Appendix D: GC-FID Quantitation data]

Initial Quantitation Experiments for volatiles in pawpaw

Compound	FEMA #	TIC peak area	~ratio		m/z peak area	odor threshold in water (ppb) Leffingwell OT table
ethyl acetate	2414					5000
ethyl butyrate	2427					1
ethyl hexanoate	2439	25024810	**1.000**			1
ethyl octanoate	2449	11779077	0.471			15
acetic acid	2006					?
butyric acid	2221					240
hexanoic acid	2559					3000
octanoic acid	2799	188816340	7.545			3000
decanoic acid	2364	424715452	16.972			10000
gamma hexalactone	2556	491799897	**1.000**	1.786		1600
gamma octalactone	2796	275295920	0.560	**1.000**		7
diacetyl	2370					2.3-6.5
acetoin	2008	883719169	35.314			800
acetaldehyde	2003	231630719	9.256			15-120
vanillin	3107	30314562	0.062	0.110		20-200
homofuraneol	3623					?
furaneol	3174					31
sotolone	3634					0.001
delta octalactone		96775962	0.197	0.352		400
isoeugenol		69114422	0.141	0.251		?

purple font uses datafile 2021032106.d
green font uses datafile 2021032101.d

*Assume density of 1.0 and 1ug/ul

Odor Detection Thresholds & References (leffingwell.com)

*** ethyl butyrate** *m/z = 43 is about same height (peak area) as diacetyl m/z=43; 43 is smaller in ethyl butyrate therefore estimate ethyl butyrate to be ~5-10 times more in concentration.*

	min	m/z =43	m/z =43	m/z =43
	2.93	***acetaldehyde***	56525763	**1.000**
	4.24	***diacetyl***	1732523	**0.031**

***Therefore, estimate diacetyl to be ~3% or less of the acetaldehyde concentration based on equal intensity m/z = 43 fragment*

RT (min)	compound	TIC peak area	ppm
2.907	acetaldehyde	655838797	57.70
6.05	methyl hexanoate	52886092	0.27
6.46	ethyl hexanoate	674297005	59.89
8.19	ethyl octanoate	73170029	1.47
9.8	butyric acid	17310502	2.05
10.52	gamma hexalactone	18501176	2.19
7.26	IS-ethyl heptanoate undeca	37730213	4.47
11.99	gamma octalactone	8211927	0.97
	100g pawpaw juice made from 126g Atwood flesh + 350g dd H2O		
	To 100g, add 1000 ul of 1.69ug/ul IS (ethyl heptanoate in ethanol)		
	** IS = 1690ug/100g = 16.9ug/g or 16.9 ppm		
	However, juice is 126g pawpaw + 350 g dd H2O = 26.47% pawpaw		
	Therefore, estimate IS (ethyl heptanoate) at 16.9 x .2647 = 4.47 ppm ethyl heptanoate		

[Appendix E: SPME-GC-O on wax column]

[Appendix E: SPME-GC-O on wax column]

SPME-GC-O: Susquehanna pawpaw experiments (runs 1+2) on wax GC column.

MS-ODP_SPMEfast New with back column split2.M		
Peak St	Comment	Compound
---	---	
4.08	buttery S	diacetyl or acetoin
4.61	fruity chewing gum M	ethyl butyrate?
4.7	cheesy note M	?
5.83	green like hexanal L	
6.68	fruity M	ethyl hexanoate
7.5	earthy note L-	
9.12	glue M	acetic acid
10.75	stinky acid M	butyric acid
10.95	kind like a sweet vanillin character note L	
11.82	floral like phenylacetaldehyde L	citronellol
12.54	stinky like dead animal M	hexanoic acid
12.88	waxy or lactone M	delta octalactone??
13.29	coconut lactone M+	gamma octalactone
13.75	coconut lactone L	delta octalactone or delta nonalactone? m/z = 99
14.16	waxy M	
14.35	sweet carmelized sugar note M	
15.08	something there L	
15.2	sweet buttery creamy note L-	
15.73	waxy L	
17.02	lactone M	
17.66	floral note L	
18.71	vanillin M	vanillin
19.06	dead animal smell L	

MS-ODP_SPMEfast New with back column split2.M			
Peak Start	Peak End	Intensity	Comment

04.15	04.17	2	buttery S diacetyl or acetoin
04.66	04.71	2	fruit chewing gum M ethyl butyrate?
06.69	06.72	2	fruity M
09.07	09.11	2	waxy L-
09.14	09.18	2	glue acetic acid like M
10.74	10.79	2	stinky like butyric acid S
12.56	12.60	2	stinky like dead animal hexanoic acid? M
12.90	12.94	2	waxy L+
13.30	13.33	2	coconut lactone M
13.76	13.79	2	lactone M
14.16	14.20	2	waxy S
14.40	14.44	2	sweet like carmelized sugar M
15.10	15.13	2	something there L
15.14	15.15	2	?
17.02	17.04	2	?
17.06	17.07	2	?
18.73	18.77	2	vanillin M+
19.12	19.16	2	dead animal smell L

[Appendix F: 2020 Harvest of pawpaw (frozen samples) Quantitation data]

[Appendix F: 2020 Harvest of pawpaw (frozen samples) Quantitation data]

Quantitation data from previous samples (Dr. Zyzak) for comparison

Sample Name		KSU Under Ripe Atwood	KSU Ripe Atwood	KSU Over Ripe Atwood	Sunflower Under Ripe	Sunflower Ripe	Sunflower Over Ripe
RT	Constituent	Approx. Conc. (ppm)					
8.51	ethyl acetate	0.020	0.455	2.832	0.028	0.248	1.570
12.83	acetoin	7.24	375.00	1560.00	1.95	893.00	2570.00
13.49	methyl butyrate		0.967	0.447	0.004		
17.31	ethyl butyrate	0.086	18.600	42.100		2.840	0.395
16.71	butyric acid	0.149	2.330	20.600	0.125	5.640	8.460
19.46	ethyl 2-butenoate		1.940	5.040		0.654	1.510
20.13	methyl 3-hydroxybutyrate		0.239	0.692			16.600
23.25	methyl hexanoate		1.120	1.820	0.020	0.733	
23.75	ethyl 3-hydroxybutyrate		4.110	19.900		0.373	1.540
26.55	ethyl hexanoate		49.600	39.369		43.000	0.908
31.16	2,3-butandiol 2-butyrate		2.313	12.800		0.573	0.985
31.82	methyl octanoate	0.012	0.732	1.190		0.223	
32.41	homofuraneol peak1 (5-ethyl-4-hydroxy-2-methylfuran-3(2H)-one)		1.800	4.400		2.140	6.690
32.81	homofuraneol		6.220	12.800		9.020	14.200
34.00	octanoic acid	2.13	150.00	264.00	0.14	211.00	70.80
34.60	ethyl octanoate		30.758	21.361		1.340	0.870