Characterizing and Manipulating Sorghum Kernel Wax as a Possible Alternative for Carnauba Wax

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ABSTRACT

Natural waxes are industrial products with many applications. Carnauba wax, from South American palm trees, is the most widely used wax due to high melting point and hardness. There is no domestic source for a natural wax with these characteristics. I characterized and manipulated waxes from domestic *Sorghum bicolor* kernels to determine if sorghum kernel waxes could be a viable alternative to carnauba. Carnauba's wax composition is mostly long chain alkyl esters while sorghum is mostly acids and alcohols. To test the hypothesis that long chain wax esters are responsible for carnauba's high melting point, we fractionated carnauba wax resulting in an alkyl ester-rich fraction and mixed it with sorghum kernel wax to simulate an alkyl ester-rich sorghum kernel wax. Ester-enriched sorghum kernel wax had a melting point of 81.0°C compared to carnauba at 84.9°C, showing that increasing the ester content in sorghum kernel wax results in a substantial increase in melting point.

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List of Abbreviations

TLC - Thin layer chromatography

GC-EI-MS - Gas chromatography - mass spectrometry

HPLC-MS - High performance liquid chromatography - mass spectrometry

DSC - Differential scanning calorimetry

FTIR - Fourier transform infrared spectroscopy

DDGS - dried grains with solubles

BSTFA - N,O-Bis(trimethylsilyl)trifluoroacetamide

PCA - Principal component analysis

LAH - Lithium aluminum hydride

ATR- Attenuated Total Reflection

Chapter 1. Literature Review

1.1 Overview of Natural Waxes

Natural waxes and natural wax polymers are important industrial products used in many applications, such as car polish, cosmetics, pharmaceuticals and edible food coatings. These natural waxes have varying chemical and physical properties that make them more suitable for specific applications. Edible food coatings are one application that has become increasingly popular as they have the ability to reduce food waste (Paidari et al., 2021).

These edible coatings are made of natural polymers that can be an alternate source for plastic packaging. With a growing concern for the environment, alternatives to plastic have been increasingly demanded. These natural polymers come from a variety of sources and have different properties for various purposes. All of these natural polymers have a few things in common, a few of those are: protecting food against loss of nutrients, safe to eat, convenient to use, and reduces packaging waste (Paidari et al., 2021; Shit and Shah, 2014; Xie et al., 2020). The sources of these edible polymers range from plants such as sorghum or palm trees, to beeswax and chitosan, which is derived from shrimp or prawn. One of the most prevalent natural waxes is carnauba wax (de Freitas et al., 2019).

Carnauba wax is the most used natural wax as its chemical and physical properties make it the perfect candidate for edible food coatings and industrial applications (de Freitas et al., 2019). The first property is its high melting point. Carnauba wax has the highest melting point of any other natural wax product at about 83°C-86°C (Journal of the American College of Toxicology, 1984). Another property that carnauba wax is the best in, is hardness. This wax is said to be harder than concrete (Onyechi and Okafo, 2016), which may seem to be a problem in the food industry, but is actually an advantage. Carnauba wax is applied as a very thin film on the surface of foods or pills, so it is still edible and does not cause any damage to the consumer. The last physical property that sets carnauba wax aside, is its low solubility. Carnauba wax is nearly insoluble in water, which makes it a perfect candidate for edible food or pill coatings. Since the wax is quite hydrophobic, it will repel any moisture that could cause degradation of the product, as well as keep moisture in the system that was originally there (de Freitas et al., 2019).

There are widely used natural polymers that are not plant based, particularly chitosan. Chitosan is derived from chitin, which is a highly abundant natural polymer. Chitin is found in the exoskeletons of crustaceans and insects, as well as the cell walls of fungi (Younes and Rinaudo, 2015). The most common source of chitin is from crab shells and shrimp exoskeletons, which can be from marine waste. The chitin must go through deproteinization, then deacetylation, then acid dissolution to get to the chitosan film form (Aider, 2010; Younes and Rinaudo, 2015). The chitosan is then joined with other

supporting natural polymers to form the final form product. This film has a few desirable properties that are similar to carnauba wax. A few of these are: superhydrophobicity, high mechanical strength, biodegradability and good barrier properties (Aider, 2010; Younes and Rinaudo, 2015). A property that chitosan has that carnauba does not, is antifungal and antioxidant activity (Aider 2010). Chitosan has been shown to reduce or inhibit the spread of a few different fungi, as well as acting as a hydrogen donor to prevent oxidation (Younes and Rinaudo, 2015). One downfall of chitin films is how brittle they are. This brittleness is lowered by adding plasticizers, which can have negative effects such as decreased film cohesion (Aider 2010). Carnauba wax may be a better option for an edible coating as it is all natural, plant based, and does not require any additives to have all of the desired properties.

A more common edible coating that is quite a bit different from chitosan and carnauba wax, is beeswax. Beeswax is a lipid based substance that can be an edible food coating due to its low polarity that blocks the transfer of moisture. Beeswax is in the same family of waxes as carnauba, since carnauba is also a lipid based wax (Xie et al., 2020). Unlike carnauba wax which has a melting point of above 80°C, beeswax has a melting point of 62°C-65°C (Journal of the American College of Toxicology, 1984), which is too low to be used for the same applications as carnauba wax. Another negative of pure waxes, such as beeswax, is the poor mechanical strength. These natural waxes tend to be mixed with other materials to increase their strength and resistance to wear (Velickova et al. 2015;

Xie et al., 2020). One application of this is by combining beeswax with chitosan. This combination of chitosan with beeswax improves the mechanical properties of the wax, like hardness (Velickova et al. 2015; Xie et al., 2020). Beeswax has several positive properties, but is not widely used in industry and still needs to be optimized for it to be as widely used as carnauba wax.

Another wax type that would need to be optimized in order to be an alternative for carnauba wax is sorghum kernel wax. Sorghum wax is derived from the kernels of *Sorghum bicolor,* a close relative of the traditional corn plant. Sorghum is a drought-tolerant crop and grown in the United States. The kernels can be processed in different ways for purposes such as bioethanol production or animal feed, and the wax is typically a byproduct of these processes. However, sorghum kernel wax has been shown to have similar properties to carnauba wax. The melting point and hardness of sorghum kernel wax are lower than that of carnauba wax, but the composition is similar (Hwang et al., 2002). The composition of sorghum and carnauba are thought to be responsible for the difference in melting temperatures.

In conclusion, natural waxes are important in many industries. These various types of natural waxes, carnauba, chitosan, beeswax, and sorghum kernel wax, all have desirable properties that would be best fit for different applications. Carnauba is the most industrially used of any other natural wax as its high melting point and hardness make it

ideal for many applications. There is a high demand for natural waxes for food preservation and coatings, pharmaceutical applications, cosmetics industry and many more. These natural waxes require further research and development so we can continue to improve their sustainability and usage. This review will now focus on carnauba and sorghum kernel wax as carnauba cannot be produced in the United States and there is a need for a more sustainable domestic source of wax with its properties.

1.2 Importance of an Alternative and Issues with Carnauba

Carnauba wax is a widely used plant wax that is harvested from the Brazilian palm tree, *Copernicia prunifera*. The Brainy Insights reports that the carnauba wax market is projected to increase by about 4.32% in the next ten years, and the restraining factor of the market is the high cost of the wax. Carnauba wax is the only wax on the market with a high melting point, high hardness, and low solubility. These physical properties along with the fact that it is only produced in a small region of the world, makes it a sort of exclusive product. Carnauba wax is used in industry in edible coatings, food ingredients, cosmetics, and pharmaceuticals, and other products (de Freitas et al., 2019; Journal of the American College of Toxicology, 1984).

However, the harvesting and production of carnauba wax have a few ethical issues such as the harsh heat conditions that workers endure to harvest the wax (Initiative for

Responsible Carnauba, 2020). Carnauba wax production also presents several environmental issues, such as deforestation and the use of pesticides to repel the insects that host a parasite responsible for Chagas disease in humans (Abad-Franch et al., 2015). These palm trees are native to Brazil and since they require moist soil and full sun, they are very difficult to be grown anywhere else. Brazil is also one of the only carnauba wax exporting countries, with the main importers of carnauba wax being Japan, the United States and Europe.

These various types of edible food coatings and natural waxes, carnauba, chitosan, beeswax, and sorghum kernel wax, all have desirable properties that would be better for different applications. Carnauba is the most used of any other edible wax coating and dominates the natural wax industry. There is a high demand for plastic alternatives for food preservation, and thus a high demand for natural waxes. These edible food coatings and natural waxes require further research and development so we can continue to reduce the amount of plastic used, as well as find more sustainable waxes.

1.3 Sorghum as the Leading Alternative

Carnauba wax has a characteristic high melting point and hardness that makes it suitable for industrial applications. Its chemical composition is unique due to the large presence of esters, with smaller amounts of alcohols, acids, and hydrocarbons (Bianchi et al., 1978;

Harron et al., 2017; Hwang et al., 2002; Hwang et al., 2018). The main type of esters in carnauba wax are aliphatic esters, which make up roughly 38-40% of the wax, followed by 20-23% hydroxycinnamic aliphatic diesters, and 12-14% ω-hydroxy aliphatic esters (Vandenburg and Wilder, 1979). Aliphatic, or wax, esters contain the ester functional group and long-chain aliphatic tails on both sides of it. These esters make up around 80% of the total composition of carnauba wax (de Freitas et al., 2019) and are thought to be responsible for the high melting point and hardness that carnauba wax exhibits.

A possible domestic source for a carnauba wax alternative and thus a potential solution to the environmental problems associated with carnauba wax production is sorghum kernel wax. Sorghum can be grown in dry regions since it is a drought tolerant crop. This allows for it to be grown around the US and not have to be imported from another country. The use of sorghum also allows for farmers to use less water on their fields, since sorghum requires 30% less water than corn (Getachew et al., 2016). Sorghum also has a grain component that allows for its use in bioethanol production, as well as flour and other food products (Hums et al., 2018). It has a high nutritional value that is comparable to corn, but with a slightly higher protein content (Douglas et al., 1990). Since sorghum kernel wax can be a byproduct of bioethanol production, improving its physical properties will allow for reduction of waste. Sorghum wax has been reported to contain hydrocarbons, aldehydes, free fatty alcohols, and free fatty acids, with minimal presence of wax esters (Hwang et al., 2002). In terms of the chemical composition of sorghum kernel wax, there

is some disagreement in the literature. Harron et. al. reported that sorghum kernel wax contains minimal amounts of wax esters, but high amounts of fatty acids, aldehydes, and alcohols. Bunger and Kummerow reported that sorghum kernel wax contained 48% hydrocarbons, 19% fatty alcohols, and 16% fatty acids. However, Dalton and Mitchell reported that sorghum kernel wax contained 5% hydrocarbons, 49% wax esters, and 46% free fatty alcohols. These conflicting reports show a need to further study the chemical composition of sorghum kernel wax to understand how it could be a possible alternative to carnauba wax. There is also a difference in reported melting points for sorghum kernel wax that range from 56-85°C (Cifti et al., 2022; Hwang et al., 2004). This range of melting point temperatures could be due to extraction or processing differences, but it is not confirmed.

There is a critical need to thoroughly understand the chemical composition of sorghum kernel wax and how that affects its physical properties in order to mimic the desirable traits of carnauba wax. This critical need leads to the overall objective of this research, which is to show the difference between the chemical and physical properties of sorghum and carnauba waxes, as well as develop a method to increase the melting point of the sorghum kernel wax to above 80°C to serve as an alternative for carnauba wax.

1.4 Means for Characterization and Melting Point Determination of Natural Waxes

The analysis of natural waxes has engaged chemists for many years. Natural waxes are nonpolar, which causes solubility issues in polar solvents. They also have the potential to be quite large (>800 m/z), which can make volatilization difficult. Their composition is a mix of compounds such as alkanes, fatty acids, fatty alcohols, wax esters, ketones and sterols (Doan et al., 2017; Harron et al., 2017; Moreau et al., 2018; Vrkoslav et al., 2010). These compound classes also vary in chain length, which can affect the physical properties of the waxes. The combination of compound class and varying chain length requires some sort of separation, such as chromatography, to be able to analyze the composition of the waxes accurately (Harron et al., 2017; Moreau et al., 2018; Vrkoslav et al., 2010).

There are many techniques that can be used to determine the chemical composition of a sample. These can include: chromatography mass spectrometry, spectroscopy, NMR, or electrochemistry. Chromatography mass spectrometry is ideal for characterizing natural waxes since there is a large precedent of this method in literature and it is able to distinguish between compound classes and chain lengths (Harron et al., 2017; Hums and Moreau, 2019; Hwang et al., 2002; Moreau et al., 2018; Vrkoslav et al., 2010). Spectroscopy can be used to give compound class information based on functional groups, but is unable to tell chain length distribution. NMR can be difficult for the characterization of natural waxes due to needing quite a bit of material and it is unable to

differentiate between isomers of ester and secondary alcohol compounds, which are common in waxes. The use of electrochemistry has no precedent for the analysis of natural wax mixtures. This review of analytical techniques will cover gas chromatography mass spectrometry, liquid chromatography mass spectrometry, Fourier Transform infrared spectroscopy, and differential scanning calorimetry.

Gas chromatography mass spectrometry (GC-EI-MS) is a commonly used analytical chemistry instrument that can give qualitative information about the composition of a sample. These samples are volatilized and injected into the column where they are separated based on their boiling point polarity, and size. Compound classes with different properties can interact more, or less, with the stationary phase (column) and cause separation. Once compounds elute from the column, they enter into the mass spectrometer, where they are ionized and fragmented by either chemical or electrical ionization. These ions are then accelerated by an oppositely charged plate into the mass analyzer, typically a quadrupole or time-of-flight, and separated by their mass-to-charge ratios. These compounds and fragments are then recognized by the detector which results in peaks and spectra that can be analyzed and identified. The size of the peak is relative to the amount of that compound present in the sample. The amount of that compound can be quantified by comparing the peak size and abundance to that of a standard. This technique is called mass balance and can be used to quantify compounds present and determine if the GC-EI-MS is unable to detect any mass.

GC-EI-MS sample preparation and run time is longer than that of LC-MS samples. Since the compounds need to be volatilized, part of the sample preparation includes derivatization. Derivatization is a chemical modification to the native compounds that allows them to have improved analysis. This typically involves adding a trimethylsilyl (TMS) group to functional groups that contain an active hydrogen, such as hydroxyl (-OH) or carboxyl (-COOH). The effect of derivatization on analysis is increased volatility, increased thermal stability, increased ionization efficiency, and increased separation. This derivatization step in the sample preparation process takes around an hour and the running of the samples takes about an hour and a half per sample. The total time needed for sample preparation and running the sample is about three days.

GC-EI-MS has quite a few applications and is the typical method for analysis of plant samples (Harron et al., 2017; Hums and Moreau, 2019; Hwang et al., 2002; Moreau et al., 2018; Vrkoslav et al., 2010). They are used for volatile and semi-volatile organic compounds such as hydrocarbons, fatty acids and alcohols, aldehydes, and esters. GC-EI-MS is also ideal for complex mixtures, since the sample is separated in both the column and the mass analyzer before the compounds are detected. Due to the GC-EI-MS's ability to fragment the compounds, it is possible to hypothesize a structure based on the fragmentation pattern and molecular ion peak. GC-EI-MS also does not require the sample to be fully soluble in a solvent, as the sample simply must be volatile. There are techniques such as an atmospheric solids analysis probe (ASAP), where the

solid sample is placed in a capillary tube and applied with a voltage and heated gas in order to ionize and volatilize the solid sample. There are some limitations to the use of GC-EI-MS for natural waxes. Since the compounds must be volatile, GC-EI-MS is not suitable for high molecular weight compounds, which can be present in some wax mixtures in the form of very-long-chain alkyl esters(Bianchi et al., 1978; Harron et al., 2017; Hwang et al., 2002; Hwang et al., 2018).

Liquid chromatography-mass spectrometry (LC-MS) is another popular method for analyzing the composition of wax samples. LC-MS samples are dissolved in solvent and injected into the column, which unlike GC-EI-MS, is not heated. The compounds are separated by how they interact with the stationary phase due to their polarity, size, and charge. The end of the column feeds into the ionization source and the compounds are converted into gas-phase ions. This ionization process can happen in a few different ways. The two most common ionization techniques are atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI). In ESI, the solution is passed through a capillary nozzle at a high voltage, which results in a fine spray of ions. These ions are still in solvent, but the solvent is evaporated by the heat and electric field which repels solvent from the charged particles. These ions are then analyzed by the mass spectrometer. APCI is different from ESI in the way that the solution is still made into a fine spray, but is not ionized until it reaches the Corona needle which is supplied with a voltage. The solvent is evaporated shortly after the fine spray is created. These two

ionization techniques have different applications and uses, despite their similarities. ESI is typically used for polar and moderately polar molecules, such as peptides, proteins, and other biomolecules (Banerjee and Mazumdar, 2012). It is widely used in proteomics and other biological applications. APCI is better for nonpolar molecules, such as hydrocarbons and natural products (Chen et al., 2015; Saliu et al., 2011). Due to these reasons and literature, it was decided that APCI would be a better fit in this project for the analysis of natural waxes.

Liquid samples can also be analyzed directly with a mass spectrometer, without the use of a column for separation. This technique is called direct-infusion mass spectrometry. This was the technique used in this project as the LC-MS available, did not have a column installed. The disadvantages of this technique is that all compounds are detected at one time, since they are not being separated by a column. This makes analysis a bit more difficult since the waxes are a complex mixture of compounds. This direct-infusion APCI MS also does not detect fatty alcohols (Bernabé-Zafón et al., 2006; Cassani et al., 2004), which is a large component of the wax composition according to literature and GC-EI-MS results. The direct-infusion APCI MS also may not be able to ionize alkanes due to their lack of a functional group, which could lead to undetectable mass according to mass balance (Eljarrat and Barceló, 2006). Despite these potential issues, the advantages of this technique are limited sample preparation since these do not need to be

derivatized, ability to detect high molecular weight and nonvolatile compounds such as esters.

Fourier Transform Infrared Spectroscopy (FTIR) is a technique that can analyze the infrared absorption or emission of a sample. It helps identify the chemical composition of the sample by analyzing how it interacts with infrared light. FTIR results can help identify presence of certain functional groups due to their bond type. Each bond type, such as C-H, O-H, or C=O, have characteristic absorbance wavelengths which allows for identification of compound classes present in the sample. One downside of FTIR is its inability to determine chain length of the different compound classes. This leads to the use of FTIR only to determine which compound classes are present in the sample.

This technique can be done on solid samples with no sample preparation using an Attenuated Total Reflection (ATR) cell. The ATR cell is made of crystal which allows for the infrared light to undergo multiple internal reflections in the crystal. The now evanescent wave passes through the refractive crystal and onto the sample. The benefit for using the ATR cell in FTIR is the ability to place the crude sample, in this project wax or a kernel, onto the ATR cell for analysis. Since waxes are typically found on the surface of the kernels (Hums et al., 2018), this reduces the need for extraction of wax to determine the compound class presence. This technique also allows for a targeted analysis by looking at certain wavelengths emitted by certain functional groups.

Differential scanning calorimetry is a technique used to study the thermal properties of materials, such as melting point, crystallinity, and enthalpy. The instrument works by using a reference pan, which is typically an empty pan, and measuring the difference in heat flow between the reference pan and the sample pan. The sample preparation involves weighing the pan and lid, along with the filled sample pan to determine the precise weight of the sample. Weights of the sample can range from a few milligrams to as high as several tens of milligrams. This sample should be homogenous so the heat flow will be evenly distributed throughout the sample and results can be reproducible. The results can describe glass transition temperatures, melting point, crystallization, purity, enthalpy, and onset of melting temperature.

The GC-EI-MS is able to detect and quantify the compounds aside from the long-chain esters, and the direct-infusion MS is able to detect and quantify the long chain-esters. The combination of GC-EI-MS and direct-infusion MS, allows for a complete picture of the chemical composition of the natural waxes. FTIR-ATR is also a reasonable technique to attain compound class information with little to no sample preparation.

From the review of techniques above, it was decided that for the complete chemical characterization of the natural waxes, sorghum and carnauba, that both GC-EI-MS and direct-infusion MS would be utilized. The direct-infusion MS would be used in APCI

mode, as it is better for nonpolar compounds, such as those present in the waxes, and ESI would not give the best results.

Aim 1: Analyze the chemical and physical properties of sorghum kernel wax, as well as carnauba wax. Develop a thorough understanding of the various chemical and physical properties of each wax type using: (1) GC-EI-MS, (2) HPLC-MS, (3) DSC, (4) FTIR.

Aim 2: Modify the sorghum kernel wax to give it similar properties as carnauba wax. Determine which chemical characteristics are the main driving force of the high melting point of carnauba wax and test the effect of adding an ester-rich fraction derived from carnauba wax to sorghum kernel wax.

CHAPTER 2.

2.1 Introduction

Plant waxes are high-value industrial plant products for which the United States has no domestic source. Carnauba wax is a widely used plant wax that is harvested from the Brazilian palm tree *Copernicia prunifera.* This wax is used in many industries, such as pharmaceuticals and edible food coatings, due to its high melting point and hardness. Although carnauba has many desirable qualities, it is associated with several environmental and ethical issues such as worker welfare, preserving biodiversity, and loss of forests, which were investigated in 2018 by the Initiative for Responsible Carnauba (IRC). Fortunately, carnauba is not the only crop that produces wax. *Sorghum bicolor* (sorghum), a drought tolerant crop that is grown in the United States, also produces a wax with properties similar to those of carnauba wax. Unfortunately, though sorghum is grown on large scales, the melting point and hardness of sorghum kernel wax are currently not high enough for it to be an alternative for imported carnauba wax (Hwang et al., 2002). If there is not a viable alternative to carnauba wax, the U.S. will have to continue to increase imports of carnauba wax from Brazil, which are already at around $40 million per year, to account for the growing demand for plant waxes.

A key reason for the widespread use of carnauba wax is its high melting point, low solubility in water, and high hardness, which is due to its unique chemistry (de Freitas et al., 2019; Journal of the American College of Toxicology, 1984; Onyechi and Okafo,

2016). Plant waxes are composed of a mixture of very-long-chain fatty acid-derived chemicals that vary in two major dimensions: (i) head group oxidation state ("compound class"), and (ii) aliphatic tail chain length ("chain length"). The main compound classes of plant waxes are fatty alcohols, free fatty acids, alkanes, fatty aldehydes, and wax esters (Kolattukudy 1969). These compound classes range in chain length from free fatty acids as short as 16 carbons in length to wax esters with more than 60 carbons (Kolattukudy 1969). The composition of carnauba wax is mostly wax esters of C56 or above (Harron et al., 2017) and carnauba wax has a melting point of 83-86°C (Journal of the American College of Toxicology, 1984). Sorghum kernel wax is made up of mostly C28 and C30 fatty acids (Hwang et al., 2002). The melting point of sorghum kernel wax seems to depend on a variety of factors including, for example, extraction methods and solvents, as well as the exact variety of sorghum used. There are many possible means by which to prepare a sorghum kernel wax isolate, but two common sources of sorghum kernel wax are wax from sorghum distillers dried grains with solubles (DDGS) and sorghum bran wax, which is extracted from the outer layer of the kernel using hexane reflux. Since there are multiple ways to isolate sorghum kernel wax, there is a high amount of variability in reports on the melting point of sorghum kernel waxes: 56-85°C (Cifti et al., 2022; Hwang et al., 2004) depending on the study. All together, the existing data suggest that sorghum has the potential to be a viable alternative to carnauba wax, and that the difference in chemical composition of sorghum (mainly C28/C30 fatty acids) and

carnauba wax (mainly C56+ wax esters) may be the barrier to sorghum being a viable alternative for carnauba wax.

The objective of this study was to verify the chemical composition of the sorghum kernel waxes using multiple analytical approaches and to explore methods for increasing its melting point so it can be a viable alternative for carnauba wax. This objective was achieved using mass spectrometry, FTIR spectroscopy, differential scanning calorimetry, and synthetic organic chemistry reactions to manipulate wax composition. There is currently no other wax with the same physical properties as carnauba, and this research contributes important knowledge that may help develop sorghum kernel waxes as a possible alternative for carnauba that can be grown in a wider variety of conditions and produced on a commercial scale.

2.2 METHODS

2.2.1 Characterization of Waxes and Melting Point Determination

Differential scanning calorimetry (DSC) measurements were completed on a TA Instruments DSC 250+ calorimeter (New Castle, DE, USA). Each sample was run in triplicate using between 5 and 20 mg of the sample in Tzero pans. Samples were run using a heat–cool–heat cycle, with temperatures ranging from 0 to 110 °C and a ramp rate

of 10 °C/min. DSC data was analyzed using the TRIOS software. Both heat cycles were used in analysis and the two peaks were integrated to give melting point, onset melting temperature, and enthalpy values. Each sample was run 3 times, giving 6 different values for each measurement. All data points were averaged and the standard deviation was calculated.

Attenuated total reflectance─Fourier transform infrared (ATR–FTIR) spectroscopy was performed on the samples to look for ester presence. A Nicolet iS50 (Thermo Fisher Scientific; Waltham, MA) with a diamond ATR cell was used. Each spectrum was an average 128 scans with a 4 cm^{-1} resolution over all wavelengths from 500-4000 wavenumbers. Samples were placed directly on the ATR cell and sorghum kernels were positioned to give a good signal. In order to identify if esters are present, the 1170 (C-O) and 1735 (C=O) peaks were monitored. The analysis was done in triplicate for all samples and standards. FTIR data was analyzed by monitoring the absorbance of 1170 (C-O) and 1735 (C=O) peaks. The absorbance was normalized to the absorbance at 1715, a region where there were no peaks, to compare values across all sample types.

GC-EI-MS analyses were conducted by dissolving wax in a 1 mg/mL concentration in chloroform and 50 µL was transferred into a GC vial insert, then spiked with 2.5 µg of tetracosane, octadecanol, and stearic acid (alkane, alcohol, acid) internal standards. Standard solutions were made by dissolving 1 mg of pure compound (Sigma Aldrich) in 100 ml of chloroform in a volumetric flask. 2.5 µls of this 1mg/mL solution were added

for each of the standards. Materials were weighed out using OHAUS PR Series analytical balance. The chloroform was allowed to evaporate overnight and it was then derivatized in 50 µl of 1:1 N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine, and incubated (70 °C for 45 min). Samples were analyzed on a 7890B Network GC (Agilent) equipped with an 7693A Autosampler (Agilent) equipped with a split/splitless injector and an HP-5 capillary column (Agilent, length 30 m x 0.250 mm x 0.25 µm film thickness); 2 µl of sample was injected on-column with He as the carrier gas with a flow rate of 1 mL/min. The initial temperature of the GC oven was 50°C and held for 2 min, followed by the first ramp at a rate of 40°C/min until it reached 200°C and was held for 2 min, then ramped at a rate of 3 °C/min until 320 °C and held for 30 min. The total run time for each sample was 77.75 min. with a solvent delay of 8 min. The analytes were detected using an Agilent 5977B (GC/MSD) Mass Selective Detector (EI 70 eV; m/z 40–800, 2 scans/s). Peaks were identified by comparing mass spectra against those of authentic standards, previously published spectra for authentic standards, or commercial mass spectral libraries. Analysis of samples was done using a mass spectral library for peak identification and mass balance calculations for quantification of each compound. The area of the peak of each compound was compared to the peak area of the corresponding standard (alcohols to octadecanol, alkanes to tetracosane, acids to stearic acid, and esters to propyl stearate). I took 2.5 µl (amount of standard added), divided it by the area of the corresponding standard, and then multiplied by the area of the peak of interest to calculate the ug of that compound. The area of the peaks were the average of 3

independent samples. These amounts were then totaled and subtracted from the total amount added to start, resulting in an amount of undetected mass. Unknown peak amounts were calculated using an average area for the three standards. These methods were also used for monitoring the products of the LAH and H_2SO_4 reactions and wax extraction from the kernels.

Direct-infusion APCI-MS analyses were conducted by dissolving 1 mg of wax in 600ul of chloroform and 600ul of methanol to mimic methods used in "Analysis of sorghum kernel wax and carnauba wax by reversed phase liquid chromatography mass spectrometry" by Andew Harron et. al 2017. 10 ul of propyl stearate, and 1 mg of tetracosane, octadecanol, and stearic acid were added for mass balance calculations. 1 ul of TFA was added to assist in ionization of samples. Sample was then heated and vortexed to ensure it was completely mixed and injected into a Bruker MicroTOF-III mass spectrometer using atmospheric pressure chemical ionization (APCI) in positive ion mode. End plate offset was 500 V, capillary was 4000 V, corona was 4500nA, nebulizer pressure was 4.1 Bar, dry gas was 6.0 l/min, drying gas temperature was 350°C, and vaporizer temperature was 400°C. The mass spectrometer was using a "wide tune" method. Analysis was done by referencing a mass spectral library to identify peaks, as well as using literature (Harron et al., 2017; Vrkoslav et al., 2011). "Counts" of each peak were compared to counts of corresponding standard peaks to get an amount of that peaks compound. The difference between total compound and starting material was larger than

GC-EI-MS as all peaks were not identified since LC-MS was only used for ester characterization.

2.2.2 Organic Reactions

A transesterification reaction was performed on carnauba wax in order to determine if esters are present using GC-EI-MS. Due to the high molecular weight of the esters, the reaction was performed so the transesterified products could be analyzed since they have higher volatility. The wax was dissolved in 200 ul of methanol. Slight heat and vortexing was needed to allow wax to fully dissolve. 150 ul of 1% H_2SO_4 in methanol was added and the mixture was then heated at 90°C for 90 minutes. Solution was taken off heat, and 400 ul of hexane was added. The mixture was vortexed and the top layer was removed and placed in a different vial. It was allowed to evaporate overnight. The solid was then resuspended in 50 ul of a 1:1 solution of BSTFA and pyridine for derivatization and heated at 70°C for 45 minutes. The derivatized product was then run on the GC-EI-MS to confirm the reaction worked.

An LAH reduction was done by adding 200 mg of sorghum DDGS wax to a suspension of 50 mg LAH in dry THF. This was stirred for 24 hours and TLC was done to show consumption of the starting material. A Feiser workup was done to remove remaining

aluminum. Product was then analyzed using GC-EI-MS. This methodology was derived from Busta and Jetter 2017.

2.2.3 Extraction and Fractionation of Waxes

Wax was extracted from sorghum kernels by placing them in a scintillation vial and adding chloroform. They were then heated at 45°C for 30 minutes. The chloroform was removed and placed into a GC vial and allowed to evaporate overnight. Following evaporation, 50ul of a 1:1 solution of BSTFA and pyridine for derivatization and heated at 70°C for 45 minutes. The derivatized product was then run on the GC-EI-MS.

In order to fractionate carnauba wax, one gram of wax was placed in a 250ml flask followed by 75ml of ethyl acetate and 0.5ml of chloroform. The mixture was covered with aluminum foil and heated at 100°C with vigorous stirring for 2-3 hours, the process was deemed completed when all yellow wax was dissolved in the heated organic solution and the solution was soluble and clear. The solution was cooled to room temperature which resulted in the precipitation of a white crystalline solid that was further filtered using a Buckner funnel under negative pressure washing with a 95% ethyl acetate 5% chloroform premade mixture. The process was performed three times. The white solid was dried and weighed (0.64 grams) and the collection and wash solution were transferred to a 250ml flask and evaporated. Fifty milliliters of hexane were added to the

evaporated beaker and heated and stirred at 80°C for one hour. The hot hexane was decanted and evaporated resulting in yellow oil. The 250ml also contained yellow oil and was transferred and dried using chloroform. The extraction process resulted in two additional yellow oil-like isolates of which MS spectra were obtained.

The data was collected from the native instrument software and wrangled using in-house R scripts designed by Dr. Busta. All R code used in this project is available at [URL].

Data was merged from multiple runs on the same instrument and signal to noise was reduced by subtracting the background.

2.3 Results and Discussion

The goal of this study was to conduct a detailed characterization of sorghum kernel wax in comparison with carnauba wax. Multiple analytical techniques and instruments were used to better understand the chemical composition and physical properties of the waxes, as well as the products of reactions used to transform the waxes in various ways, the better to understand their properties. I used DSC to characterize the waxes' melting points and mass balance EI and APCI MS, as well as FTIR to characterize the chemical composition of carnauba and sorghum kernel wax samples (section 2.3.1). I also used multiple different synthetic organic chemistry techniques to manipulate and fractionate

the crude waxes in a series of experiments aimed at understanding how to increase the melting point of sorghum kernel wax isolates (sections 2.3.2-2.3.4).

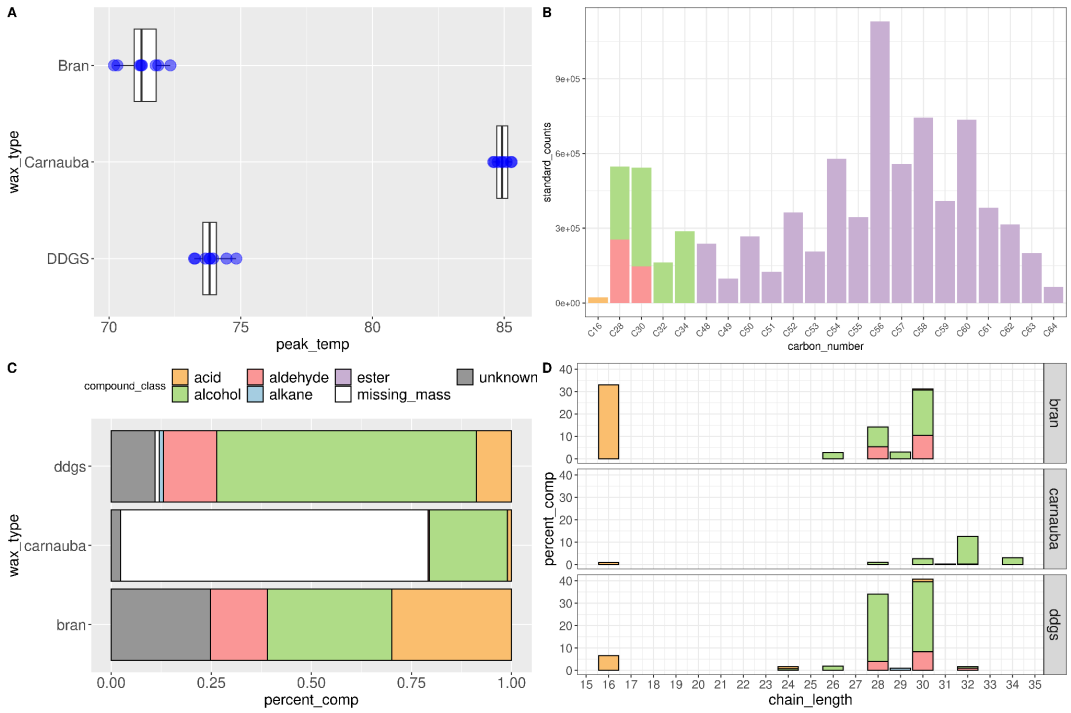

Figure 1. The chemical composition and melting point analysis of carnauba, sorghum bran and sorghum DDGS wax. A) *The boxplot shows the peak melting temperature of each wax type on the x-axis in degrees Celsius and the wax type on the y-axis. Each point represents one sample and the interquartile range is shown by the box.* ***B)*** *A bar chart showing the counts of each compound class in carnauba wax on the y-axis, along with the carbon chain length on the x-axis. Data was collected from the native software on the APCI-MS instrument.* ***C)*** *A stacked bar chart showing the percent of each compound class in each wax type, along with the undetected mass and unknown peaks on the x-axis. The y-axis shows the four different wax types and the different colors correspond to the different compound classes.* ***D)*** *A bar chart showing the chain length on the x-axis and the percent composition of each on the y-axis. The wax type is faceted and the compound class is identified by color. Plots A-C were created using an average of 3 individual samples. Legend in plot A is applicable for all plots. All data was collected and analyzed using the native software on the respective instruments. Further analysis and plotting was done using R studio.*

2.3.1 General Characterization of Waxes

In order to determine the melting point and chemical composition of crude waxes from sorghum bran, sorghum DDGS, and carnauba, differential scanning calorimetry, mass spectrometry, and spectroscopy were used. Using differential scanning calorimetry, the melting point of each wax isolate was determined to be the following: carnauba wax has the highest melting point of 84.9°C, followed by sorghum DDGS wax at 73.9°C, and sorghum bran wax at 71.3°C (Fig. 1A). Specifically, GC-EI-MS and APCI MS were used as they are ideal for determining chain lengths and position of secondary functional groups. Since chain length is an important part of wax composition and function, NMR was not used as it struggles to distinguish between chain lengths and mixtures of chain lengths. GC-EI-MS was used for mass balance calculations by using a known mass of sample and known masses of standards (acid, alcohol, alkane; previously reported as major wax components of sorghum and carnauba wax isolates) to quantify the amount of each compound present. It was found that the GC-EI-MS was unable to detect a significant amount of mass from the carnauba wax from the mass balance calculations (Fig. 1C), so further chemical characterization is needed using direct-infusion APCI-MS following literature precedent. Direct-infusion APCI-MS was utilized in order to detect the high molecular weight esters that literature reports to be in major abundance in carnauba wax. The results show that alkyl esters are indeed present in carnauba wax in high abundance (Fig. 1B). The highest abundance alkyl ester is C56 which is in agreement with previous reports (Harron et al., 2017). Thus, via APCI-MS, carnauba wax

contains 1% acid, 20% alcohol, >1% aldehyde, and ~80% esters. Using GC-EI-MS, it was determined that sorghum DDGS wax contains 9% acid, 65% alcohol, 13% aldehyde, 1% alkane, and 11% unidentified peaks. Sorghum bran wax contains 33% acid, 35% alcohol, 16% aldehyde, and 16% unidentified peaks. According to mass balance calculations, sorghum DDGS wax contained 1% mass that was undetected and sorghum bran wax had all mass accounted for.

Sorghum bran and DDGS waxes have similar chain lengths, but different amounts of each. Sorghum bran wax has a higher amount of C16 acid, while DDGS wax has higher amounts of C28 and C30 alcohols. Carnauba has the longest chain alcohols of any of the wax types (C32). Figure 1C shows the different ratios of the compound classes in each wax type, regardless of chain length. The chain length distribution was quite different between the three wax types (Fig. 1D). In conclusion, via a general characterization of sorghum and carnauba wax, I was able to confirm that carnauba's melting point was higher than that of either sorghum DDGS wax or sorghum bran wax. This melting temperature gives the goal of creating a sorghum kernel wax product with a melting point of greater than 80°C. The difference in melting point between sorghum kernel wax and carnauba wax seems likely to be due to either (i) sorghum has acids and aldehydes and carnauba does not, and (ii) carnauba has esters and sorghum does not. These two possibilities will be explored in sections 2.3.2.

2.3.2 Esters are the main driving force of carnaubas high melting point

My characterization of sorghum kernel and carnauba wax found two main differences between the two waxes, one of which was the lower abundance of fatty acids in sorghum, suggesting that if I reduce fatty acids in sorghum wax it would become more carnauba wax-like. This was done using an LAH reduction reaction to convert carboxylic acids and aldehydes to primary alcohols. This was done by adding sorghum DDGS wax to a suspension of LAH in dry THF and stirring for 24 hours and reaction products were analyzed using GC-EI-MS.

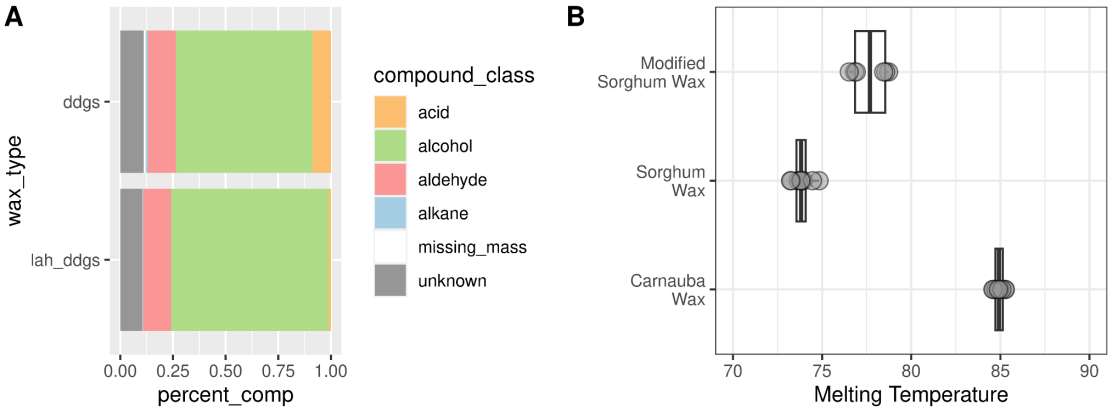

Figure 2: The chemical composition and melting point of crude sorghum DDGS and the LAH reaction product. A. *A stacked bar chart showing the percent of each compound class in DDGS wax before and after the reaction, along with the undetected mass and unknown peaks on the x-axis. The y-axis shows the two different wax types and the different colors correspond to the different compound classes.* ***B.*** *The boxplot shows the peak melting temperature of each wax type on the x-axis in degrees Celsius and the wax type on the y-axis. Each point represents one sample and the interquartile range is shown by the box. All data was collected and analyzed using the native software on the respective instruments. Further analysis and plotting was done using R studio.*

The results of this reaction showed an increase in alcohol content, from 64.84% to 73.85% (Fig. 2A), but the aldehydes did not fully convert to alcohols. This could be due

to the reaction not reaching completion. However, the reaction product did have a higher melting point (77.67 °C) than the crude sorghum DDGS (73.88 °C) (Fig. 2B). This temperature increase suggests that the absence of fatty acids are not solely responsible for the high melting point of carnauba wax, however, if this reaction were to go to completion, it could increase the melting point further. This reaction showed that increasing alcohol content increases the melting point of the wax, however it seems unlikely that the absence of fatty acids are solely responsible for the high melting point of carnauba wax.

Based on my characterization of the waxes in the previous section, the second major difference between sorghum kernel wax and carnauba wax was that carnauba wax had large amounts of alkyl esters, while sorghum did not. In order to determine if esters are the driving force of the high melting point of carnauba wax, an H_2SO_4 transesterification reaction was done to break the alkyl esters into the alcohol and acid portions. This was done by heating the wax in 1% sulfuric acid in methanol for 90 minutes at 90°C.

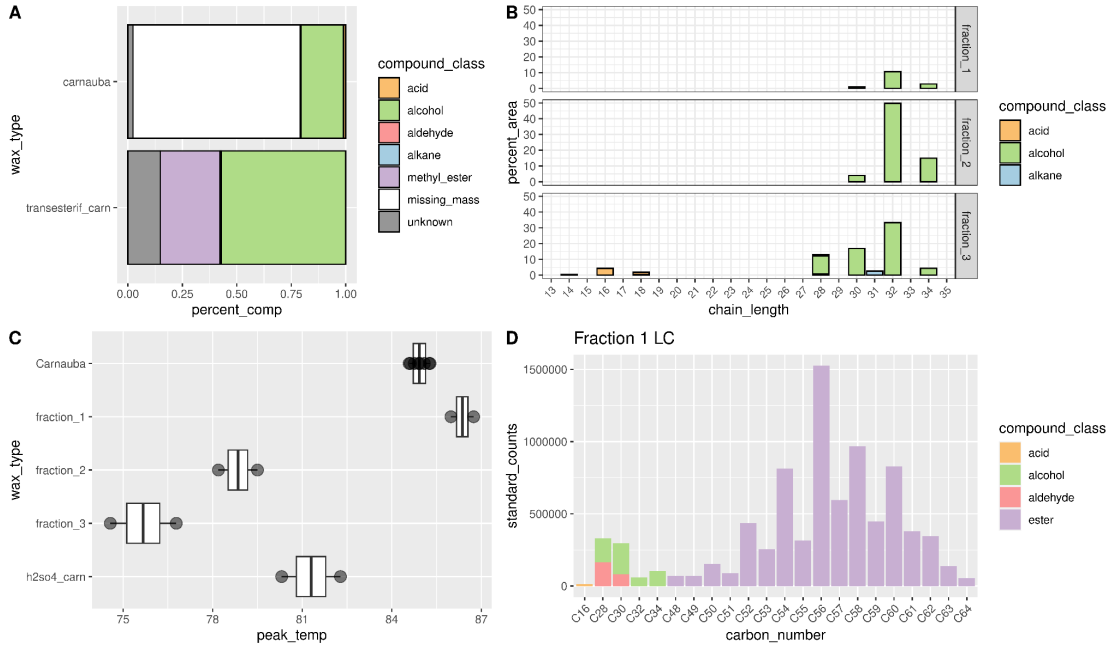

Figure 3: The chemical composition and melting point of fractionated and transesterified carnauba wax. ***A.*** *A stacked bar chart showing the percent of each compound class in carnauba wax before and after the H_2SO_4 reaction, along with the undetected mass and unknown peaks on the x-axis. The y-axis shows the two different wax types and the different colors correspond to the different compound classes. All data was collected and analyzed using the native software on the respective instruments. Further analysis and plotting was done using R studio.* ***B.*** *A faceted bar chart showing the chain length and compound class composition of fractionated carnauba wax is shown. The x-axis shows chain length and y-axis shows the relative percent area of the compound and is faceted by fraction number. The different colors correspond to the different compound classes.* ***C.*** *The boxplot shows the peak melting temperature of each wax type on the x-axis in degrees Celsius and the sample name on the y-axis. Each point represents one sample and the interquartile range is shown by the box.* ***D.*** *A bar chart showing the chain length distribution and compound class assignments for the ester-enriched fraction. The x-axis shows the chain length and the y-axis shows the counts resulting from the direct-infusion APCI-MS.*

The results of the H_2SO_4 transesterification reaction showed that the reaction was successful due to the increase in alcohol content (19.53% to 57.17%) and the presence of methyl esters (27.43%). This shows that the long-chain alkyl esters are breaking down into their component parts (Fig. 3A). The reaction product was also run on the DSC to determine how breaking the alkyl esters would affect the melting point of the carnauba wax. The melting point decreased from ~85°C to ~81°C (Fig. 3C). This decrease in

melting point supports the hypothesis that the alkyl esters are playing an important role in the desirable physical properties of carnauba wax, specifically its high melting point.

To further explore the notion that esters are the driving force for the high melting point of carnauba wax, fractionation of carnauba wax was also done to create ester-enriched fractions (Fig. 3B&D) to determine the effect of increasing the ester content of the wax. This was done by colleague Mike Williams using a recrystallization method. This also resulted in two acid and alcohol rich fractions (Fig. 3B). The melting points of these fractions followed the trend with the ester-rich fraction having the highest melting point at ~86°C, fraction 2 at ~78°C, and fraction 3 at ~76°C (Fig. 3C, Supplemental Figure 1).

To determine if adding esters to sorghum kernel wax would increase the melting point to a comparable temperature of carnauba, the ester-enriched fraction (Fraction #1, derived from carnauba wax) was added to the sorghum kernel wax in different ratios and the melting point was tested using DSC.

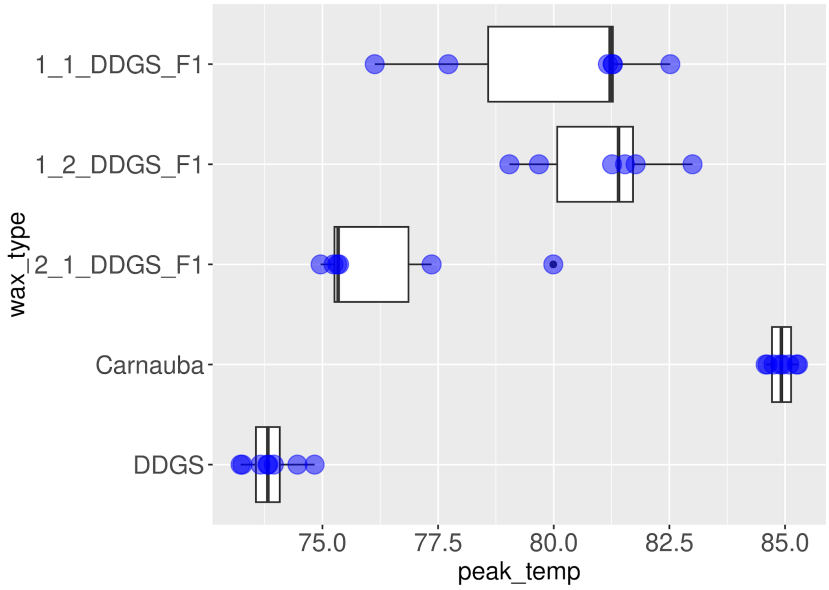

Figure 4: The melting point of native sorghum DDGS and carnauba wax, along with the 3 different ratios of fraction 1 and DDGS wax. *The boxplot shows the melting temperature (°C) on the x-axis and the sample type on the y-axis. Each point represents one heating cycle of the sample and the interquartile range is shown by the box.*

The results of adding fraction 1 to sorghum DDGS wax lead to a substantial increase in melting point. The mixtures ranged from ~75°C to ~83°C (Fig. 4). This is an increase of at least one degree and the highest melting point is within a degree of carnauba wax. These results show that adding an ester-rich product to sorghum kernel wax increases its melting point enough to above 80°C to compete with carnauba wax.

In conclusion, since increasing the alcohol content only slightly increased the melting point, breaking the esters decreased the melting point, and increasing ester content significantly raised the melting point, alkyl esters seem to be the major contributor to the high melting point of carnauba wax. Since the alkyl esters are composed of a very-long-chain fatty acid condensed with a very-long-chain fatty alcohol, it could be

possible to join those two compound classes that are already present in sorghum kernel wax to create the alkyl esters and increase sorghum's melting point.

2.3.3 Sorghum can produce esters

In light of the importance of alkyl esters in creating a high melting point for carnauba wax, it is interesting to consider a report from 2000 that describes the discovery of alkyl esters in a sorghum wax isolate (Weller et al., 2000). So far, in the work presented here, we had been using sorghum wax isolates from an unknown variety or from a mixture of kernels of unknown varieties. In the 2000 report, the authors described obtaining their isolate from a variety of sorghum called “Midland”, which suggests that there could be significant differences in composition of sorghum varieties. Accordingly, we next turned to exploring whether certain sorghum varieties can produce esters and if that ability differs over sorghum varieties.

We began by investigating a wax isolate from BTx623, a common research variety of sorghum. A transmethylation reaction was performed in order to break any potential alkyl esters apart for better detection, followed by subsequent analysis with GC-EI-MS. GC-EI-MS was utilized due to its ability to determine which chain length fatty acids and alcohols were present in the alkyl esters. This chain length information is useful for

comparison to potential sorghum kernel wax esters to determine how similar or different they may be.

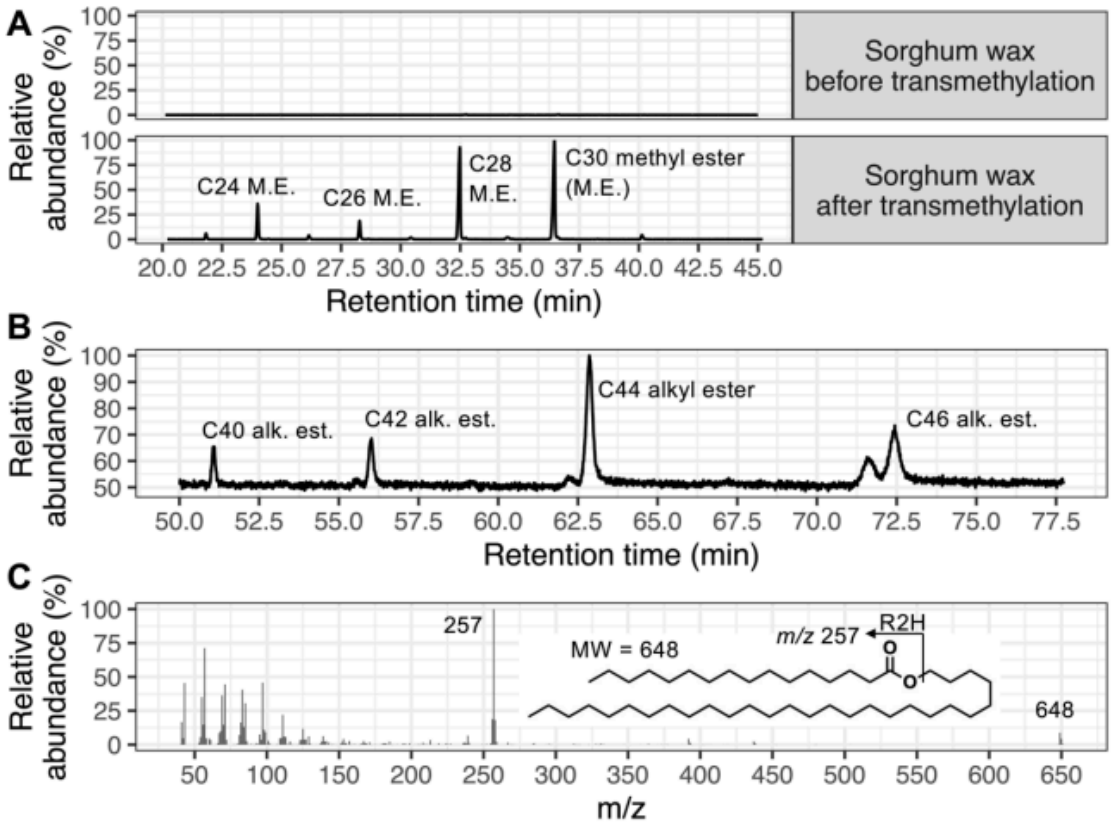

Figure 5. Sorghum waxes contain carnauba wax-like wax esters. A. *GC-EI-MS single ion chromatograms (m/z 84, specific to methyl esters) of sorghum kernel wax extract (top) and after (bottom) transmethylation. Transmethylation released large amounts of C28 and C30 methyl esters, suggesting that sorghum kernels make wax esters using C28 and C30 fatty acids.* ***B.*** *GC-EI-MS total ion chromatogram of a >60 minute run showing small amount of intact wax esters, dominated by the C44 homolog.* ***C.*** *Mass spectrum of the C44 wax ester from panel B. The molecular ion (m/z 648) and the typical rearrangement and double proton transfer fragment (m/z 257) are labeled.*

The results of this reaction showed presence of methyl esters (Fig. 5 A), which supports that there are esters present in crude sorghum kernel wax from at least some sorghum varieties. There were also some intact alkyl esters detected in an extended GC-EI-MS run

in small amounts, but with the high molecular weight of these compounds it is difficult to detect them in a quantitative fashion. As shown in the mass balance data for carnauba wax (Fig. 1C), GC-EI-MS is not a reliable instrument for detection of very-long-chain alkyl esters.

As shown in Fig. 5, at least some sorghum varieties can produce esters, but it is unknown if there is a variation in the abundance of alkyl esters between these varieties. Testing the abundance of esters across many varieties can be quite laborious since waxes would need to be extracted from kernels and run on APCI-MS. In order to screen many varieties for esters, FTIR was utilized since esters are present on the surface of the kernels. I did this by placing the kernels on the ATR cell of the FTIR instrument and looking for a signal at 1735 (C=O stretch).

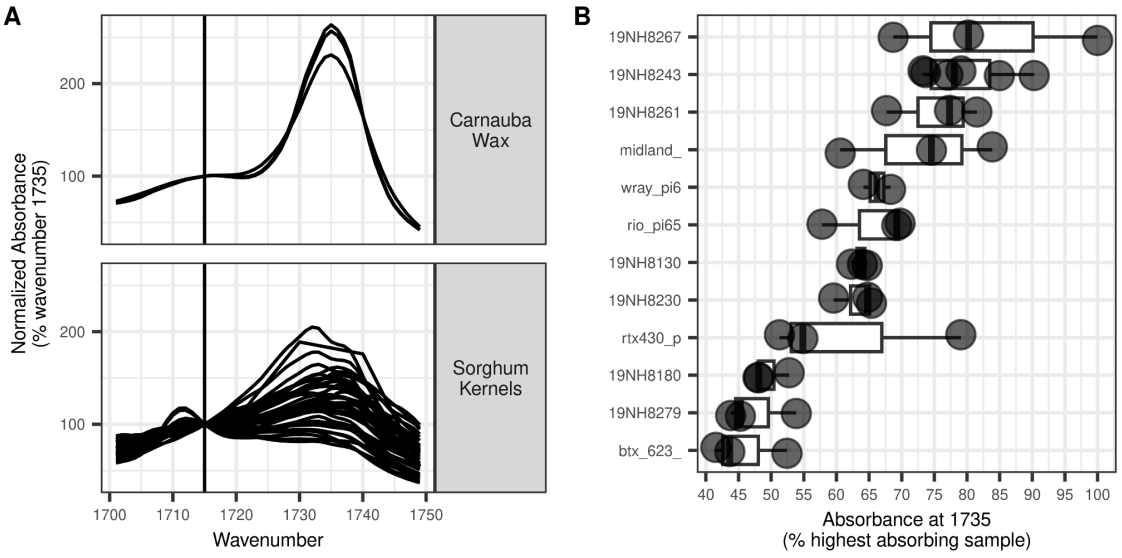

Figure 6: FTIR enables rapid assessment of ester abundance and reveals diverse ester content in sorghum varieties. A. *Absorbance of carnauba wax and sorghum kernel wax determined using FTIR. The left panel shows the absorbance of carnauba wax, which is rich in wax esters, at that wave number. Each line represents the analysis of a separate piece of carnauba wax. In the left lower panel, each line shows the absorbance of a different sorghum kernel using the same measurement technique.* ***B.*** *Absorbance at 1735 wavenumbers (a proxy for ester content) of select sorghum varieties.*

Figure 6A shows the FTIR absorbance of carnauba wax, which is known to have high ester abundance (Bianchi et al., 1978; Harron et al., 2017; Hwang et al., 2002; Hwang et al., 2018), and the absorbance of various sorghum varieties kernels. Since we know that carnauba contains mostly esters (~80%), this confirms that monitoring 1735 using FTIR can detect ester presence in a crude sample. This confirms that there is a high ester presence in carnauba wax and suggests varying ester presence in the different sorghum varieties. The sorghum varieties (Fig. 6B) were sourced from collaborators and show promising results for naturally occurring esters in some sorghum varieties. For example, the BTx623 showed half the absorbance at 1735 than the 19NH8267 variety. Also, the Midland variety seems to be ester-rich, which agrees with the 2000 report (Weller et al., 2000).

2.4 Conclusion and Future Directions

Sorghum kernel wax and carnauba wax have quite different compositions and melting points. Sorghum bran and DDGS waxes have similar chain lengths, but different amounts of each. Sorghum bran wax has a higher amount of C16 acid, while DDGS wax has higher amounts of C28 and C30 alcohols. Carnauba has the longest chain alcohols of any of the wax types (C32). The melting points are also quite different with carnaubas being the highest of any wax type. This difference in melting point seems to be mainly due to the high abundance of alkyl esters and absence of fatty acids in carnauba wax. By decreasing the fatty acid presence in sorghum kernel wax, there was an increase in melting temperature, but not to the threshold of 80°C. Due to reducing the fatty acids not creating enough of an increase in temperature, we moved to increase ester presence in sorghum kernel wax. This was done by fractionating carnauba wax and adding an ester-rich fraction to sorghum DDGS wax. This resulted in an increase of melting temperature to above 80°C for the 1:2 DDGS to fraction 1 mixture. Since increasing the ester presence in sorghum kernel wax seemed to increase the melting point to competitive threshold, we wanted to explore a report that sorghum can produce esters.

By finding the kernels that were reported to contain esters, I was able to detect alkyl esters in the wax extracts. This supports the hypothesis that sorghum can produce esters naturally and that the composition of the sorghum wax can differ across varieties.

Future directions for this project could include investigating the processing of the sorghum kernels and wax. Various extraction methods and solvents are used which could impact the composition of the wax. This could be tested by using various extraction and processing methods for sorghum kernel wax to see how that affects its melting point. Other directions could include using previously published methods to create esters in sorghum kernel wax (Domergue and Miklaszewska, 2022) as well as improving the quantitative analysis using the APCI-MS. The main experiment would be to try to create esters in sorghum kernel wax by combining the acids and alcohols using a chemical catalyst or biological method, following literature (Joseph et al., 2005). Another future endeavor would be to extract waxes from the multiple varieties we have and run on the APCI-MS to get full chemical characterization. This was not done in this project due to time constraints, but could be done in the future.

In conclusion, the main differences between carnauba and sorghum kernel waxes is that carnauba has high molecular weight esters and sorghum has fatty acids and aldehydes. It is hypothesized that one, or both, of these are responsible for carnaubas high melting point. In addition, sorghum plants have the ability to create esters, but currently are not producing a high enough amount to create a high melting point. Increasing the ester presence in sorghum, either by making the plant product it or introducing esters post-production, would result in a significant increase in melting point of sorghum kernel

wax. These processes must be done in a way that does not hydrolyze esters, as that would result in a lower melting point.

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