

## Gas Chromatography and Mass Spectroscopy of Cuticular and Epicuticular Waxes of *Arabis Serotina*

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### ABSTRACT

*The surface waxes of the endangered plant *Arabis serotina* consist of a plethora of various different hydrocarbons. These hydrocarbons were extracted from the plant's leaves and then analyzed using gas chromatography and mass spectroscopy. The model organism *Arabidopsis thaliana* was used to develop a method for extracting and analyzing these waxes. Using a method refined on *A. thaliana*, it was possible to procure and study the waxes of *A. serotina* without wasting too much of the endangered species. *A. serotina* wax presumptively contains the following hydrocarbons (but may also contain more, as alkanes were not adequately analyzed): hexadecanoic acid, octadecanoic acid, tetracosanoic acid, hexacosanoic acid, octacosanoic acid, eitriacontanoic acid, dotriacontanoic acid, 1-docosanol, 1-tetracosanol, 1-hexacosanol, 1-octacosanol, 1-eitriacontanol, and 1-dotriacontanol. Secondary alcohols of heptacosanol, nonacosanol, hentriacontanol, tritriacontanol may also be present but are difficult to distinguish from organic acids.*

**Keywords:** Wax, Alcohol, Silylation, Alkane, *Arabis Serotina*

### 1. INTRODUCTION

The plants *Arabidopsis thaliana* (mouse-ear cress) and *Arabis serotina* (shale barren rockcress) are both members of the *Brassicaceae* (mustard) family. *A. thaliana* is a widely studied plant specimen and is considered to be a model organism for many plant research projects (including a lot of genomic research). It is the lab rat of the plant world. *A. serotina* is a plant native to the Appalachian regions West Virginia and Virginia and is on the endangered species list. The shale barren rockcress, as its name implies, grows on hot, dry, barren shale cliffs. The microcosm in which this plant lives can only be described as harsh, since the shale cliffs bake in the summer sunlight.



**Figure 1. *Arabidopsis serotina* (Top).  
*Arabidopsis thaliana* (Bottom)**

These plants, like most others, produce a wax-like coating of very long chain hydrocarbons on their surface. These are the cuticular and epicuticular waxes of the plant. The epicuticular wax is the wax coating on the surface of the plant. These waxes form a layer on the surface of the plant consisting of dendrites, filaments, plates, and tubes when viewed through a scanning electron microscope. It is believed that the chemical composition of such waxes may contribute to their geometric structure [3].

The cuticular wax of the plant exists in the plant cuticle and covers all of the plants epidermal cells. The cuticular wax exists to provide a continuous hydrophobic barrier over the surface of plants which limits nonstomatal water loss and gaseous exchanges, controls the absorption of lipophilic compounds, provides mechanical strength, adds viscoelastic properties, prevents organ fusion during plant development, and protects the plant from stressors in the environment [5].

Since these two plants have different habitats, the waxes of the plant would be adapted to suit that plant's particular needs. *A. thaliana* is found in temperate climates almost worldwide. It grows in newly cleared soil (like that around railroad tracks). It can survive in light, medium, or heavy soil; it can survive in acidic, basic, or neutral pHs, as well as moist or dry conditions [7]. Typically, the plant flowers in spring and does not live into the hottest parts of the summer; its growth cycle only takes around 6 weeks to complete [6].

*A. serotina*, on the other hand, has a very limited habitat. It is found only in the Appalachian regions of West Virginia and Virginia. *A. serotina* survives through the summer and into early fall, living on shale cliffs in the mountains. The plant's flowering period is from mid-July to September. Appalachian summers easily reach 60 degrees Celsius on the shale rock during the plants growth cycle. The shale cliffs are not only hot, but also extremely dry [2]. For this reason, it is hypothesized that *A. serotina's* wax will differ from *A. thaliana's* wax and most likely be more heat stable.

Knowing how these molecules are made helps in isolating and identifying them. All of the wax components start out as 16 or 18 carbon fatty acids attached to acyl-coenzyme A. These fatty acids are then elongated by fatty acid elongases. This results in fatty acids containing an even number of carbons that vary in length from 16 carbons to upwards of 36. Primary Alcohols are formed when the acyl-CoA reductase reduces the fatty acid molecule twice to form an alcohol; thus, primary alcohols contain an even number of carbons. Alkanes are formed when the fatty acid is acted upon by acyl-CoA reductase and then by a decarboxylase; the result is an alkane, which is one carbon less than the even chained fatty acid precursor. A hydroxylase

can then create an odd carbon length secondary alcohol from the alkane. The secondary alcohol can then go on to be oxidized into a ketone. Therefore fatty acids, and primary alcohols contain even numbers of carbon, and secondary alcohols, alkanes, and ketones contain odd numbers of carbons [4].

Previous research shows that *A. thaliana* contains the following wax compounds in the stem: hexadecanoic acid, octadecanoic acid, eicosanoic acid, docosanoic acid, tetracosanoic acid, hexacosanoic acid, octacosanoic acid, eitriacontanoic acid, tetracosanal, hexacosanal, octacosanal, eitriacontanal, dotriacontanal, 1-docosanol, 1-tetracosanol, 1-hexacosanol, 1-octacosanol, 1-eitriacontanol, pentacosane, heptacosane, nonacosane, hentriacontane, tritriacontane, as well as C27, C29, and C31 secondary alcohols, various esters, and some unknown compounds [3].

Since the rare *A. serotina* is in the same family with the common *A. thaliana*, *A. thaliana* provides an excellent model organism for studying the extraction and analysis methods for the wax of *A. serotina*. Methods honed on *A. thaliana* were used to isolate and analyze the wax of *A. serotina*. Doing so allowed for as little material as possible to be taken from the endangered species, resulting in less damage to the plant. Since the two plants likely share many of the same wax compounds, the gas chromatograph results for *A. thaliana* could be used to help identify compounds in *A. serotina*. By using these techniques, it is possible to analyze the unique wax composition of *A. serotina*.

## 2. EXPERIMENTAL

To analyze the cuticular and epicuticular wax of the plants *Arabis serotina* and *Arabidopsis thaliana*, it must first be isolated off the surface of the leaves or stem. Since the wax consists mainly of long-chain

hydrocarbons, a non-polar solvent would seem appropriate. As it turns out, during the course of the research, this was not the case. The method from Jenks et al. states that the extraction may be done in hexane (a non-polar alkane, C<sub>6</sub>H<sub>14</sub>) [1], and their extraction method was used a model with which to start developing the extraction method used for this experiment. However research by Suh et al. mentions the use of chloroform (a slightly polar molecule, CHCl<sub>3</sub>) for a wax extraction [3]; this avenue will also be explored in the experiment.

As it turns out, hexane only proved to extract the alkanes and ketones well. The alcohol standard 1-hexacosanol was insoluble in hexane. This is likely due to the fact that 1-hexacosanol is slightly polar. Chloroform, on the other hand, has difficulty dissolving some of the longer non-polar alkanes. Chloroform will, however, readily dissolve long-chain alcohols and organic acids.

Because of this, two extractions were performed on the samples. The first extraction was the chloroform extraction, and the second extraction was performed with hexane. 15ml of solvent is used to the extraction, and the sample is left in the solvent for 30 seconds before decanting the liquid. Each extraction was performed twice on the sample plant.

For the *A. thaliana* wax extraction, the stem of the inflorescence was used. Two stems were used per extraction; the stem was cut into smaller segments to fit in the extraction vessel. For the extraction on *A. serotina* 15 fully developed leaves were used, since the plants had not produced any inflorescence.

The solvent in the wax-solvent mixtures is then evaporated off using a stream of nitrogen gas and low amounts of heat. Once the solvent is entirely evaporated, wax residue is resuspended in a 100 micro liter solution of the appropriate solvent

containing an internal standard. The internal standard used was tetracosane and was made as a 0.5% solution. The internal standard-wax solution can be stored at 4 degrees Celsius until the next steps can be completed.

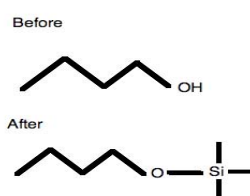
The solution extracted with hexane is complete and is ready to run on the gas chromatograph as is. The chloroform solution containing the alcohols and carboxylic acids will require a little modification before it is ready. A silylating agent will be added to the chloroform mixture to make the alcohols and fatty acids more volatile.

The silylation reaction involves replacing the alcohol group with a trimethylsilyl group. The trimethylsilyl group is a silicone covalently bonded with three methyl groups. Since the silylating reagent and the derivatives created by the silylating reagent are extremely moisture sensitive, special considerations must be made when performing the reaction.

The silylating agent chosen for these reactions was 2,2,2-trifluoro-N-trimethylsilyl-1-trimethylsilyloxy-ethanimine, also known as N, O -Bis(trimethylsilyl) trifluoroacetamide or simply BSTFA. The BSTFA was contained in a sealed container with a wax septum on the top. A syringe flowing nitrogen gas was inserted into container; this allows the BSTFA to be extracted with another syringe. 75 micro liters of BSTFA was added to the 100 micro liter chloroform extract sample. The solution was briefly warmed, then allowed to react for 30 minutes.

In the reaction, the BSTFA reacts with the alcohol group on the hydrocarbon. The BSTFA donates one of its trimethylsilyl groups to the hydrocarbon to form the trimethylsilyl derivative of that hydrocarbon (seen in figure 2). The remaining trimethylsilyl group on the BSTFA does not react. Pyridine may be used to catalyze the

reaction, but it was not used in this experiment. The result of the silylation reaction is a solution containing trimethylsilyl derivatives of primary and secondary alcohols, as well as derivatives of fatty acids. The derivatives in this solution are significantly more volatile than their normal counterparts. Both solutions are now ready to be analyzed using the gas chromatograph and mass spectrometer (Sigma-Aldrich Co. 1997).



**Figure 2. Silylation Reaction: 1-butanol To 1-butanol Trimethylsilyl Ester**

To help determine alkane retention times, a five alkane standard solution was prepared. It used the following alkanes: tetracosane, heptacosane, nonacosane, hentriacontane, and pentatriacontane. Since the parent peaks for alkanes in the wax solution rarely, if ever, show up in the mass spectrum, a plot of the retention times of the five alkane standards could be used to identify unknown alkanes based on retention time.

Settings for the gas chromatograph were borrowed from the previous research conducted by Suh et al [5]. The method was then further altered to suit the particular needs of this experiment. A splitless method was used to maximize sensitivity. A sample of 3 micro liters was injected. The sample was run at an initial temperature of 40 degrees Celsius and is increased at a rate of 40 degrees per minute for four minutes for a

final temperature of 200 degrees. Then the temperature is increased at a rate of three degrees per minute for until 320 degrees is reached. The oven is then held at 320 degrees for 30 minutes. Lastly, the oven is ramped down at 50 degrees per minute until it reaches 50 degrees Celsius. The inlet temperature was set at 250 degrees Celsius. An inlet temperature of 320 degrees was tried, but it turned out to cause problems. The inlet pressure was 6.60 psi, and helium gas was used.

Plant material for the extractions was grown in the Shepherd University green house, and hydrocarbon standards and solvents come from Sigma-Aldrich. No wild *A. serotina* were harmed for this experiment.

### 3. SAFETY

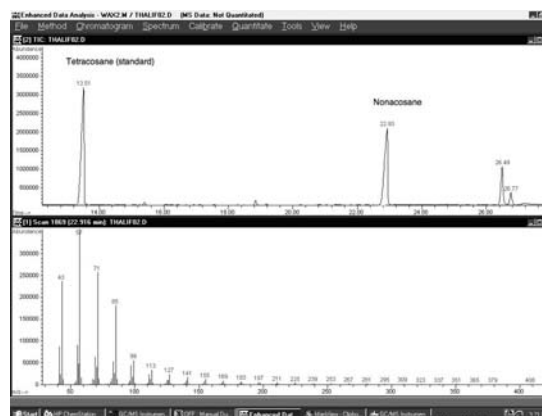
As always, protective eyewear should be worn at all times. Acetone is highly flammable, an eye irritant, may cause dryness or cracking in this skin, and may cause drowsiness or dizziness if vapors are inhaled. Chloroform is harmful if swallowed, irritating to the skin, and has limited evidence of a carcinogenic effect. Hexane is highly flammable, irritating to the skin, harmful if inhaled, dangerous for prolonged contact or inhalation, is dangerous to aquatic organisms, causes a risk of impaired fertility, harmful if swallowed, and may cause drowsiness or dizziness if inhaled. Nitrogen gas may cause rapid asphyxiation if proper ventilation is not used. Long-chain alkanes tetracosane, heptacosane, hentriacontane, and pentatriacontane should not come in contact with eyes or skin. Avoid eye and skin contact with 1-hexacosanol. BSTFA is highly flammable and causes burns, therefore, wear protective clothing and gloves, as well as eye protection.

### 4. RESULTS

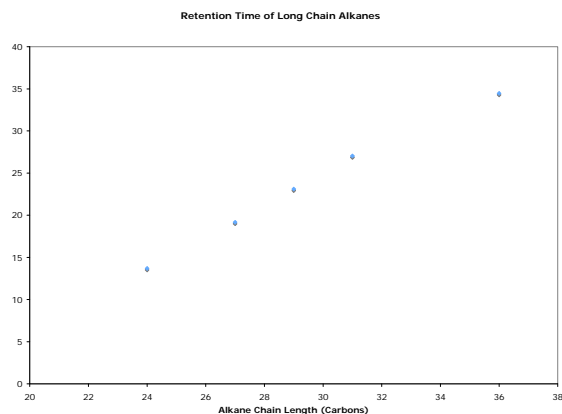
When the extraction and analysis method was refined on *A. thaliana* many of the compounds reported to be present according to Jenks were found. Alkanes proved to be the easiest to find, using the series of long-chain alkane standards. Alkanes fragment in a very specific way; as the alkane is blown through the gas chromatograph, it breaks up into fragments that are one carbon atom and two hydrogen atoms shorter than the last fragment. The result is a bunch of peaks in the mass spectrometer that are separated by a molecular weight of 14. Alkanes that were found in *A. thaliana* are listed in the table below, as well as a mass spectrum an alkane extracted from *A. thaliana* and a plot of the retention times of the five alkane standard solution.

**Table 1. Alkanes Found in *A. Thaliana***

Name	Molecular Formula	Molecular Weight g/mol	Retention Time minutes
heptacosane	C <sub>27</sub> H <sub>56</sub>	380	19.163
nonacosane	C <sub>29</sub> H <sub>60</sub>	404	23.005
hentriacontane	C <sub>31</sub> H <sub>64</sub>	428	26.959

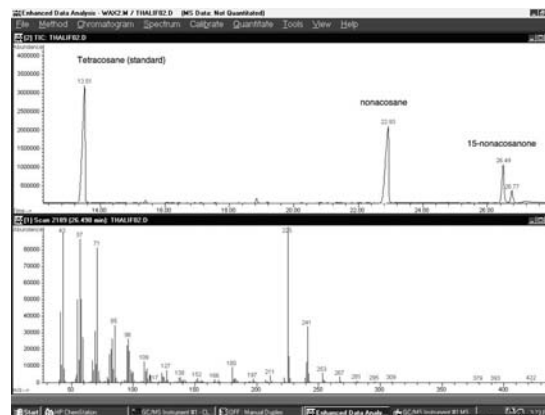


**Figure 3. Hexane extraction of *A. thaliana* wax (top). Mass spectrum of nonacosane (bottom).**



**Figure 5. Graph of Alkane Retention Times Against Carbon Length**

The other easy to identify compound was presumptively 15-nonacosanone. It is the only ketone present in the wax of *A. thaliana*. It had a retention time of 26.490, very close to hentriacontane. It identified by the mass spectrometer by having a hydrocarbon like fragmenting pattern, and a large 225 peak. The 225 peak corresponds to the fragment containing 14 carbons plus the carbonyl group. The 225 peak is so prevalent because a split on either side of the carbonyl group will yield the 225 molecular weight fragment. This is because the carbonyl is in the exact middle of the 29 carbon chain. If the ketone were set up like this, it would be much harder to identify. The mass spectrogram below shows the possible fragmentation pattern of a ketone.



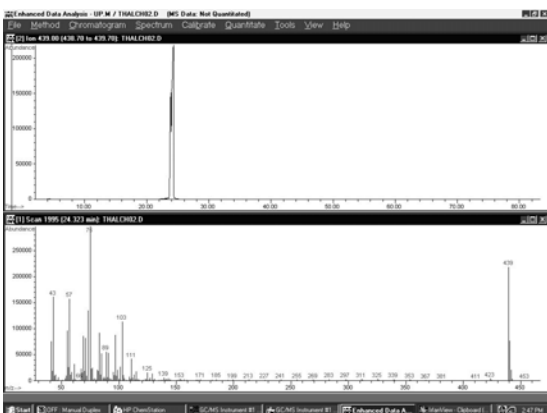
**Figure 6. Hexane Extraction of *A. thaliana* (Top). Mass Spectrum of 15-Nonacosanone (Bottom)**

The primary alcohols (all even carbon numbers), presented a challenge to identify. The parent peak of these compounds was never present, and so they were initially dismissed as something else. The largest fragment in the alcohols was an odd numbered peak. As it turns out, the odd numbered peak is 15 g/mol less than the molecular weight of the alcohol. For example, hexacosanol-trimethylsilyl has a molecular weight of 454 g/mol; the largest molecular weight peak for hexacosanol-trimethylsilyl is 439 g/mol. That is one  $\text{CH}_3$  less than the full molecule. It is hypothesized that this carbon group is lost off of the trimethylsilyl and allows for the formation of a very stable ion with the oxygen forming a double bond with the silicon. Using the model of a large peak 15 g/mol less than the molecular weight, it was possible to identify many of the primary alcohols in the wax of *A. thaliana*. The fragmentation pattern of hexacosanol-trimethylsilyl is seen picture 5. The large 73 peak is most likely the trimethylsilyl group broken off, while most other peaks are various lengths of the molecule. The large 439 peak is presumably the stable ion formed when one methyl group breaks off the trimethylsilyl group. The primary

alcohols found are presented with their retention time below.

**Table 2. Primary Alcohols Found in *A. Thaliana***

Name	Molecular Formula	Molecular Weight g/mol	Retention Time minutes
tetracosanol	C <sub>24</sub> H <sub>50</sub> O	354	19.911
hexacosanol	C <sub>26</sub> H <sub>54</sub> O	382	24.235
octacosanol	C <sub>28</sub> H <sub>58</sub> O	410	27.744
eitriacontanol	C <sub>30</sub> H <sub>62</sub> O	438	36.463



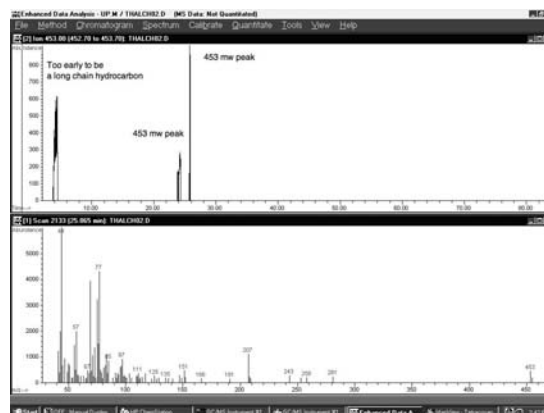
**Figure 6. Extracted ion chromatogram for molecular weight 439 g/mol (hexacosanol-tms) in *A. Thaliana* wax (top). Mass spectrum for hexacosanol-tms (bottom)**

The secondary alcohols and carboxylic acids were the hardest compounds to identify and distinguish. This is because the molecular weight of an alcohol is the same as the molecular weight of a carboxylic acid that is one carbon less. For example, heptacosanol and hexacosanoic acid both have the same molecular weight (396 g/mol). They can also stabilize the same way as the primary alcohols do (15 g/mol less than the molecular weight) when attached to a trimethylsilyl group. When the chromatogram is searched for these molecular weights, 2 distinct peaks appear in the region where long-chain hydrocarbons are found. Presumably, one of these peaks is the acid and the other the alcohol. It is difficult to determine which is which,

because at such a high chain length, it is difficult to distinguish a fragmentation pattern. Also, the carboxylic acid group (COOH) has the same molecular weight as the alcohol group attached to two carbons (CH<sub>2</sub>CH<sub>2</sub>OH), so the groups are not a reliable source of determination either. For these reasons, it was not possible to distinguish between secondary alcohols (odd carbon number) and the carboxylic acid with one carbon less (even chain). Possible secondary alcohols and carboxylic acids found in *A. thaliana* are listed in the table below. The gas chromatogram depicts two peaks near each other with the same molecular weight. The mass spectrum below shows one of the peaks fragmentation pattern.

**Table 3. Secondary Alcohols or Carboxylic Acids of *A. Thaliana***

Name	Molecular Formula	Molecular Weight g/mol	Retention Time minutes
Possible Secondary Alcohols			
n-hencosanol	C <sub>21</sub> H <sub>44</sub> O	312	14.373
n-tricosanol	C <sub>23</sub> H <sub>48</sub> O	340	17.97
n-pentacosanol	C <sub>25</sub> H <sub>52</sub> O	368	21.82
n-heptacosanol	C <sub>27</sub> H <sub>56</sub> O	396	25.8
n-nonacosanol	C <sub>29</sub> H <sub>60</sub> O	424	29.63
Possible Secondary Alcohols			
hexadecanoic	C <sub>16</sub> H <sub>32</sub> COOH	256	9
octadecanoic	C <sub>18</sub> H <sub>36</sub> COOH	284	11.155
eicosanoic	C <sub>20</sub> H <sub>40</sub> COOH	312	14.373
docosanoic	C <sub>22</sub> H <sub>44</sub> COOH	340	17.97
tetracosanoic	C <sub>24</sub> H <sub>48</sub> COOH	368	21.82
hexacosanoic	C <sub>26</sub> H <sub>52</sub> COOH	396	25.8
octacosanoic	C <sub>28</sub> H <sub>56</sub> COOH	424	29.63



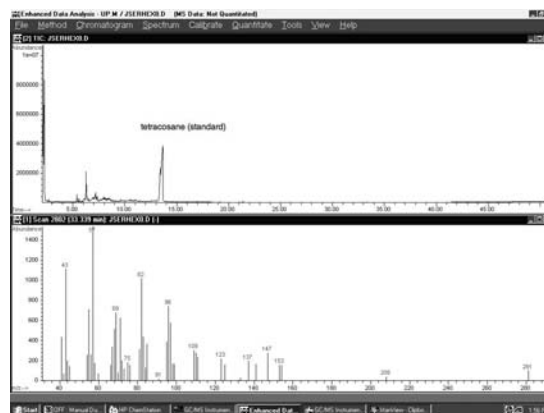
**Figure 7. Extracted Ion Chromatogram for Molecular Weight 453 g/mol (Top). Mass Spectrum of the Larger Peak (Bottom)**

Presumably, one of the peaks in figure 7 is for a carboxylic acid and one is for a secondary alcohol. The large peak coming around 3 minutes is likely not a long-chain hydrocarbon because of its low retention time. The 207 peak in the mass spectrum is a contaminant from the column. More research is needed to determine which peak belongs to which hydrocarbon.

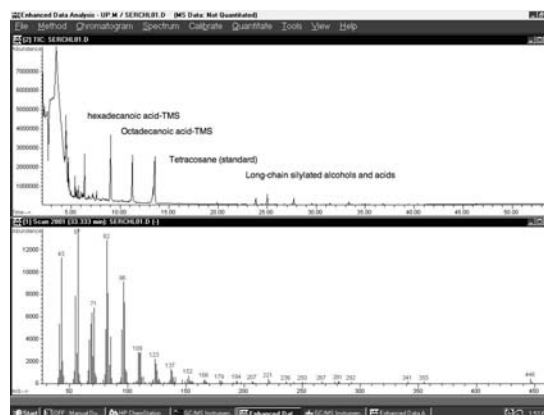
Using the same methods used to identify the hydrocarbons in *A. thaliana*, hydrocarbons of *A. serotina* were presumptively identified. No alkanes were identified. This was probably because they were split up between the two extractions and were so dilute that they were not easily detectable. The primary alcohols and secondary alcohols or carboxylic acids are listed below.

**Chart 4. Hydrocarbons Found in *A. Serotina* Wax**

Name	Molecular Formula	Molecular Weight g/mol	Retention T minut
<b>Primary Alcohols</b>			
1-docosanol	C <sub>22</sub> H <sub>46</sub> O	326	16.12
1-tetracosanol	C <sub>24</sub> H <sub>50</sub> O	354	19.91
1-hexacosanol	C <sub>26</sub> H <sub>54</sub> O	382	23.84
1-octacosanol	C <sub>28</sub> H <sub>58</sub> O	410	27.73
1-eitriacontanol	C <sub>30</sub> H <sub>62</sub> O	438	30.75
1-dotriacontanol	C <sub>32</sub> H <sub>66</sub> O	466	35.02
<b>Possible Secondary Alcohols</b>			
n-pentacosanol	C <sub>25</sub> H <sub>52</sub> O	368	21.86
n-heptacosanol	C <sub>27</sub> H <sub>56</sub> O	396	25.80
n-nonacosanol	C <sub>29</sub> H <sub>60</sub> O	424	29.60
n-hentriacontanol	C <sub>31</sub> H <sub>64</sub> O	452	33.27
n-tritriacontanol	C <sub>33</sub> H <sub>68</sub> O	480	36.74
<b>Possible Carboxylic Acids</b>			
hexadecanoic	C <sub>16</sub> H <sub>32</sub> COOH	256	9.03
octadecanoic	C <sub>18</sub> H <sub>36</sub> COOH	284	11.25
tetracosanoic	C <sub>24</sub> H <sub>48</sub> COOH	368	21.86
hexacosanoic	C <sub>26</sub> H <sub>52</sub> COOH	396	25.80
octacosanoic	C <sub>28</sub> H <sub>56</sub> COOH	424	29.60
eitriacontanoic	C <sub>30</sub> H <sub>60</sub> COOH	452	33.27
dotriacontanoic	C <sub>32</sub> H <sub>64</sub> COOH	480	36.74



**Fig 8. Hexane extraction of *A. thaliana* (top). Mass spectrum of a very small peak corresponding to what may be an alkane (bottom).**



**Fig 9. Silylated *A. serotina* wax in chloroform (top). Silylated hydrocarbon (bottom).**

In the chromatogram of *A. serotina* some of the secondary alcohol/acid molecular weights also showed two peaks. This is most likely due to there being both an acid and a secondary alcohol present. The retention time presented was of the more prevalent peak. More time is needed to analyze both peaks for possible differences. However, the presence of the second peak indicates the likely presence of both the secondary alcohol and the carboxylic acid.

## 5. Conclusion:

While this experiment gleaned a lot of information about plant waxes and their analysis, many problems still remain. The biggest of these problems is finding an easy way to distinguish between the secondary alcohols and the carboxylic acids. One proposed way to do this is by growing the plants in C<sup>13</sup> enriched CO<sub>2</sub> gas. Another way is to obtain standards of the possible compounds present and use the retention times of the standards to identify the compounds.

Another problem was the double extraction using two solvents. This is most likely the culprit behind not seeing any alkanes. A possible solution for this would be to perform a mixed solvent extraction in which all of the wax compounds would dissolve. That mixture could be silylated since (even though it contains alkanes) only acids and alcohols are affected by the reaction.

Also, the quantities of each of the hydrocarbons in the waxes were not analyzed in this experiment. Eventually, once all of the identification and extraction problems are worked out, it will be possible to quantify each of the wax components. This can be done by using internal standards and integrating the gas chromatograph profiles.

In the end, a unique glimpse into the wonder of *A. serotina* was seen. Many wax compounds were preliminarily identified in its mixture. Hopefully, fully understanding its waxes will allow for better understanding of the species and possibly an industrial use for the waxes. Ultimately, having a use may save this plant from extinction.

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