Isolation and Characterization of Sphingolipids in *Arabidopsis thaliana*

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by

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF SPHINGOLIPIDS IN *ARABIDOPSIS THALIANA*

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Total lipids were extracted from *A. thaliana* plant tissue and differential hydrolysis was utilized to determine the presence of sphingolipids. Silicic acid chromatography was utilized to separate lipids based upon their polarity, and fatty acid methyl esters were prepared from isolated *A. thaliana* sphingolipids. Gas chromatography and mass spectrometry were used to analyze the fatty acid content of the isolated sphingolipids. It was determined that the *A. thaliana* sphingolipids contained 16:0 and 18:0 fatty acids. The isolation and characterization scheme employed in this study will be used to investigate the sphingolipid fatty acid content of *A. thaliana* in the future.

Isolation and Characterization of Sphingolipids in *Arabidopsis thaliana*

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INTRODUCTION

Sphingolipids are a class of lipids found in all eukaryotes and some bacteria and are important components of plant, animal and microorganism signal transduction systems and plasma membranes. In eukaryotic cells, sphingolipids are thought to increase the stability and decrease the permeability of membranes and have been implicated in regulating ion permeability (1). There is also growing evidence that sphingolipids are important in yeast cellular signaling (2). Clearly, the biosynthesis of sphingolipids is an integral part of a cell's life-sustaining function.

The general sphingolipid structure contains three main components: a very long chain fatty acid (VLCFA), a long chain base and a head group (Figure 1). A characteristic amide linkage attaches the VLCFA to the long chain base. A ceramide, the simplest sphingolipid, consists of a long chain base, a very long chain fatty acid and hydroxy head group (Figure 1, R = OH). Cerebrosides such as galactocerebroside and glucocerebroside have a monosaccharide residue as the head group (Figure 1, R =

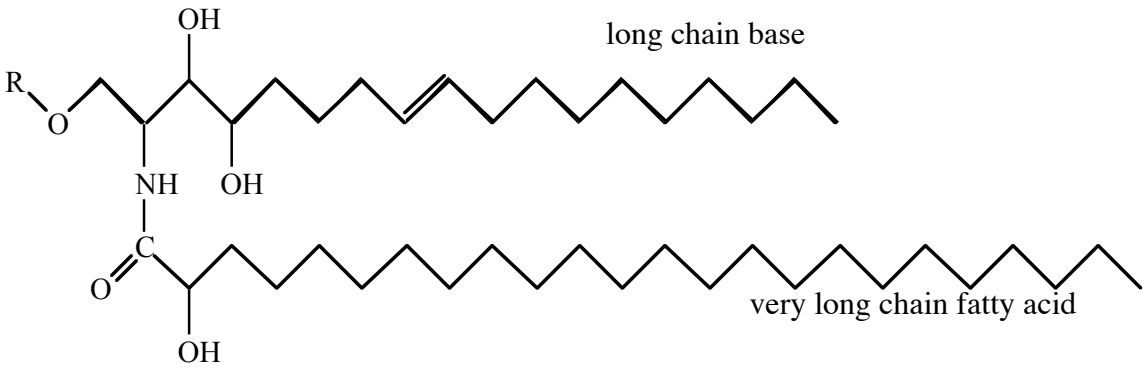

Figure 1. Representative structure of a sphingolipid having 4-hydroxy-8-sphingenine and 2-hydroxylignoceric (cerebronic) acid as the long chain base and fatty acid component, respectively. R represents a head group.

monosaccharide) (3).

Our research laboratory is especially interested in the mechanisms by which the VLCFA moiety of sphingolipids is produced in plants. VLCFAs (fatty acids of 20 carbons and greater) are found in membrane sphingolipids, waxes, seed oil, and the extracellular pollen coat (1,4). The variety of chain lengths of VLCFAs and the diversity of lipids they are found in suggests that production of VLCFAs in plants is very complex. A better understanding of the metabolism of VLCFAs may provide us with genes for pharmaceutical and nutritional products as well as engineering plant products used for lubricants and fuel resources.

VLCFAs are produced by the elongation of saturated and mono-unsaturated C_{18} fatty acids. Elongases (ELOs) are involved in the biosynthesis of VLCFAs in yeast. Studies have shown that disrupting expression of ELO genes in yeast reduces cellular levels of sphingolipid VLCFAs (5). However, little is known of the elongation of fatty acids to yield sphingolipid VLCFAs in plant systems. ELO homologues have been found by homology searches of the recently sequenced *Arabidopsis thaliana* genome; however, their role in sphingolipid biosynthesis is unclear. It is suspected that ELO homologues are involved in sphingolipid biosynthesis in the plant, particularly in the production of VLCFAs incorporated into sphingolipids. In our laboratory, the expression of several *A. thaliana* ELOs have been disrupted using techniques in molecular biology. However, no methods exist for isolating and characterizing the sphingolipid VLCFAs of resulting transgenic plants to determine the role of *A. thaliana* ELOs in sphingolipid biosynthesis. If the disrupted ELO is involved in sphingolipid VLCFA biosynthesis, then it is expected

that its disruption will significantly alter the biosynthesis of sphingolipids in the plant system.

The purpose of this study was to develop methods for the isolation and characterization of sphingolipids in *A. thaliana* that will be used in future studies involving sphingolipid metabolism. Methods based on those developed for yeast and other plant systems were utilized to establish a scheme for the isolation of sphingolipids from *A. thaliana* (6-9). Total lipids were extracted from whole plant tissue and column chromatography was used to isolate polar lipids. Subjecting the polar lipids to mild alkaline conditions isolated sphingolipids by hydrolyzing the ester bond found in glycerolipids. After purification of the sphingolipid fraction, fatty acid methyl esters (FAMEs) were prepared and analyzed by GC/MS. Both 16:0 and 18:0 fatty acids were found in the isolated sphingolipid fraction.

MATERIALS AND METHODS

Growth, collection and storage of yeast and A. thaliana

S. cerevisiae were grown in YPD media containing 1% yeast extract, 2% peptone and 2% dextrose. Cultures were grown for 17 h at 28 °C in a rotary shaker (300 cycles/min). Of this culture, 200 □l was transferred to 200 ml of complete minimal dropout media lacking uracil (cm-ura) supplemented with 0.34 g of yeast nitrogenous base (DIFCO), 1.0 g of $(NH_4)_2SO_4$ and 2% dextrose per liter (10). This culture was grown for 48 h at 28 °C in a rotary shaker (300 cycles/min). Cells were harvested by centrifugation at 500 *g* (Centra-8, International Equipment Company) and immediately

treated with 5% trichloroacetic acid at 0 °C for 1.5 h to stop any phospholipase activity. The cell material was washed twice with distilled water, twice with 0.5% KH_2PO_4, resuspended in 0.5% KH_2PO_4, and then heated for 7 min at 100 °C to remove any residual trichloroacetic acid. The cell material was stored at -20 °C (11,12).

A. thaliana plants were grown at 24 °C for 25 days with 16 h light / 8 h dark cycle. Freshly harvested *A. thaliana* stems, leaves and flowers were frozen on dry ice and homogenized with a liquid nitrogen-cooled mortar and pestle. The resulting powder was stored at -20 °C.

Extraction and separation of lipids

Lipids were extracted from yeast or 5 g of *A. thaliana* material with 2 ml of ethanol/water/ether/pyridine/NH_4OH (15:15:5:1:0.018) at 60 °C (11). The solvent was evaporated under vacuum and the dried extract was dissolved in $CHCl_3$ before storage at -20 °C.

Silicic acid (100-mesh, Sigma) or Biosil (100-mesh, Biorad) column chromatography was used to separate the extracted material. The column system consisted of a 5.75-inch glass Pasteur pipette filled with approximately 2 ml of packed column material. The assembled column was washed with 5 ml of $CHCl_3$ and 5 ml of acetone prior to loading a sample. Solvent was pushed through the column with a steady stream of N_2. Lipid material was eluted with $CHCl_3$ followed by acetone to separate neutral lipids and glycolipids, respectively. Each fraction was dried under N_2, resuspended in $CHCl_3$ and stored at -20 °C.

Extracted lipid material was applied to a TLC plate (Silica Gel, glass-backed, hard layer, fluorescent (GHFL), Analtech) and developed in $CHCl_3/CH_3OH/NH_4OH$ (66:17:3) (9). The plate was subjected to one of four staining systems (H_2SO_4/H_2O (1:1), $H_2O/H_2SO_4/KCrO_4$ (27:20:3), 1% ceric ammonium nitrate in 20% H_2SO_4, or acetic acid/H_2SO_4/anisaldehyde (500:10:2)) (13,14). For the first three systems, the TLC plate was immersed in the appropriate staining solution for approximately 15 min, removed from the stain and then heated at 80 °C for 15 min. For the anisaldehyde stain system, the TLC plate was lightly sprayed with the staining solution and heated at 80 °C for 10 min (14).

Separation of sphingolipid standards via silicic acid chromatography

Approximately 5 μg of bovine brain galactocerebrosides and glucocerebrosides (Sigma) were loaded onto a silicic acid column and sequentially eluted with 2 ml of each of the following solvents: $CHCl_3$, $CHCl_3$:acetone (8:2), $CHCl_3$:acetone (1:1), and acetone. Each solvent fraction was collected separately and dried under N_2. The contents of the fractions were applied to a TLC plate (Silica Gel GHFL, Analtech) and developed in $CHCl_3/CH_3OH/NH_4OH$ (66:17:3). Spots were visualized with the anisaldehyde stain.

Differential hydrolysis of lipid standards

Lipid standards (galactocerebrosides, glucocerebrosides, ceramides, and phosphatidyl choline, Sigma) were subjected to either mild alkaline hydrolysis (0.4 M methanolic KOH, 39 °C, 2 h) or strongly acidic hydrolysis (2% methanolic H_2SO_4, 80 °C, 2 h). Hydrolyzed material was extracted twice with 2 ml $CHCl_3$. The products were dried under N_2 and stored at -20 °C.

Differential hydrolysis of A. thaliana extract

Figure 2 gives an overall scheme for the separation and hydrolysis procedures. *A. thaliana* extracted material was separated into neutral and glycolipid fractions via silicic acid column chromatography as described above. The glycolipid fraction was subjected to mild alkaline hydrolysis (0.4 M methanolic KOH, 39 °C, 2 h), extracted with $CHCl_3$ and then applied to another silicic acid column (8). The column was sequentially washed with 2 ml of each of the following solvent systems: $CHCl_3$, $CHCl_3$:acetone (8:2), $CHCl_3$:acetone (1:1), and acetone (15). Each solvent fraction was collected and dried under N_2. Half of each fraction was dissolved in $CHCl_3$ and loaded directly onto a TLC plate (Silica Gel, GHFL, Analtech). The remaining half of each fraction was subjected to strongly acidic conditions (2% methanolic H_2SO_4, 80 °C, 2 h) to hydrolyze the lipid

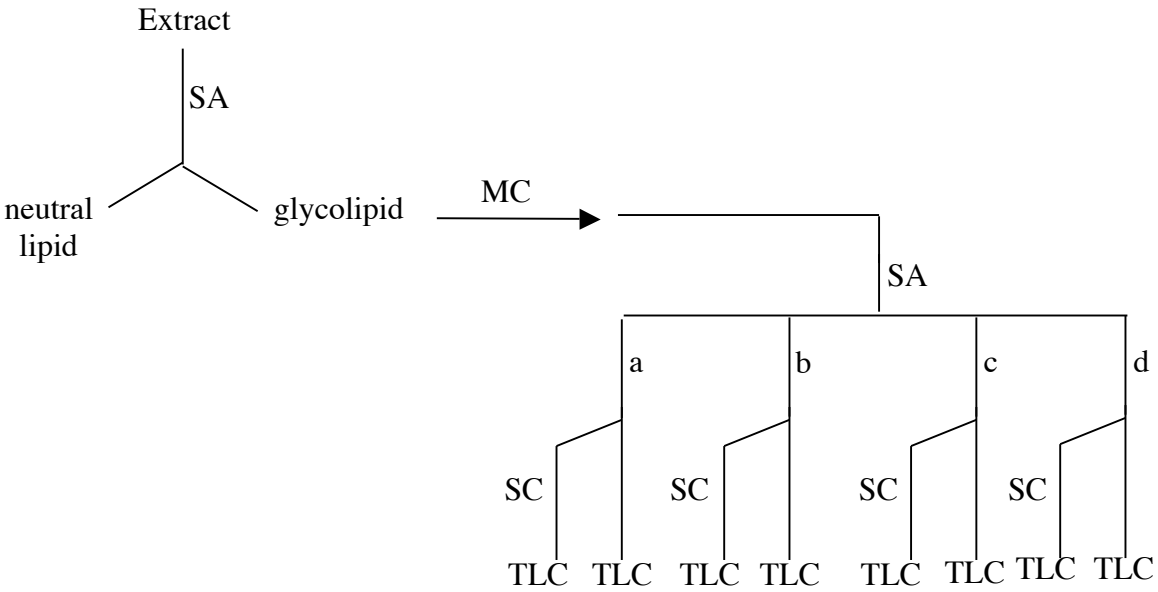

Figure 2. Diagram representing the sequential treatment of the extract during the differential hydrolysis of *A. thaliana*. Abbreviations: SA - silicic acid chromatography, MC - mild alkaline conditions, SC - strong acid conditions, a - $CHCl_3$, b - $CHCl_3$:acetone (8:2), c - $CHCl_3$:acetone (1:1), d - acetone.

material (Figure 2) (8). Hydrolyzed lipid was extracted with 2 ml of $CHCl_3$ and then loaded on the TLC plate. All of the TLC plates were developed using $CHCl_3/CH_3OH/NH_4OH$ (66:17:3) as the mobile phase. Lipid material was visualized with the anisaldehyde stain.

Preparation of fatty acid methyl esters (FAMEs)

Fatty acid methyl esters were prepared by reacting the lipid material with 1 ml of 2% H_2SO_4 in MeOH at 80 °C for 2 h. FAMEs were extracted twice with 2 ml $CHCl_3$. The products were dried under N_2 and stored at -20 °C.

A. thaliana FAME analysis via GC/MS

The fatty acid composition of *A. thaliana* extracted lipid material was analyzed by gas chromatography and mass spectroscopy (GC/MS) following acid hydrolysis. The FAMEs were dissolved in hexane and analyzed via a Hewlett-Packard model 5890 GC coupled to a Hewlett-Packard model 5971 mass selective detector (MSD). The FAMEs were separated using a 30 m x 0.25 mm DB23 capillary column. Column temperature was programmed from 100 °C (3 min hold) to 250 °C at 20 °C/min. The mass range of the MSD was 50 to 550 mass units.

RESULTS AND DISCUSSION

The purpose of this study was to develop methods for the isolation and characterization of sphingolipids in *A. thaliana*. Several techniques including column chromatography, thin layer chromatography, differential hydrolysis of lipid material, and GC/MS were utilized to isolate and characterize plant lipids. Total lipids were extracted from whole *A. thaliana* plant tissue and column chromatography was used to isolate polar

lipids. Subjecting the polar lipids to mild alkaline conditions distinguished sphingolipids from acyl lipids by hydrolyzing the ester bond found in glycerolipids. After purification of the sphingolipid fraction, fatty acid methyl esters (FAMEs) were prepared and analyzed by GC/MS.

The study was exploratory and often required the application of techniques from diverse areas of lipid research. Several experiments were designed as reactions to previous results.

TLC plate stain selection

The ability to detect lipid material was essential to utilizing and understanding the various techniques employed to separate material extracted from *A. thaliana*. Thin layer chromatography was used to visualize the separation of lipid classes after column chromatography. Originally, the TLC plates were soaked in a H_2SO_4:H_2O (1:1) solution to stain organic material (16). Because of the high concentration of H_2SO_4 and large volumes of staining material required for successful staining, a less hazardous and more efficient stain was desired. Several staining methods (H_2O/H_2SO_4/$KCrO_4$, ceric ammonium nitrate in H_2SO_4, and acetic acid/H_2SO_4/anisaldehyde) were tested against the standard H_2SO_4:H_2O staining method. It was found that all four solutions equally stained lipid standards (bovine brain galactocerebrosides and ceramides) run on TLC. However, the anisaldehyde staining method proved to be the cleanest and most time efficient. Less stain was required and less hazardous staining conditions were achieved because the TLC plate was sprayed with the stain. The anisaldehyde staining procedure was therefore used for all subsequent studies involving TLC.

Biosil versus silicic acid column chromatography

In this study, column chromatography was used to separate lipid material on the basis of polarity. As columns have unique separation properties, it was necessary to test the ability of different columns (Biosil and silicic acid) to separate extracted lipid material.

Lipid material extracted from yeast was loaded onto both Biosil and silicic acid columns. A series of increasingly polar solvents ($CHCl_3$, acetone, acetone:H_2O (1:1), and MeOH) were washed through each column and the resulting fractions were applied to a TLC plate.

The rates at which material flowed through the Biosil and silicic acid columns were 0.05 and 0.2 mL/min, respectively. Figure 3 shows the thin layer chromatograms of acetone:H_2O and MeOH eluates. Lipid-□ and lipid-□ are unknown lipids extracted from yeast. The Biosil column retained lipid-□ in both the acetone:H_2O and the MeOH washes and lipid-□ in only the MeOH wash. The silicic acid column retained lipid-□ exclusively in the acetone:H_2O wash and lipid-□ exclusively in the MeOH wash. Silicic acid column chromatography proved to be the best and most efficient method for separating lipid material. Because lipid material flowed through the silicic acid column at a much faster rate than the Biosil column, it allowed for four times the amount of material to flow through the column in a given time. Also, the silicic acid column separated yeast lipid material better than the Biosil column. The Biosil column failed to completely isolate lipid-□ in the acetone:H_2O wash, indicating a separation failure (Figure 3). Complete separation of the lipid material was found only in the silicic acid column. Silicic acid

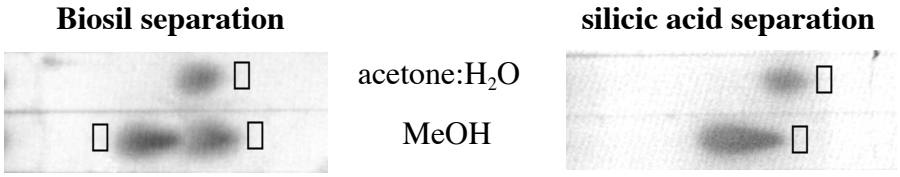

Figure 3. TLC plates of yeast lipid extract separated by Biosil and silicic acid column chromatography. Lipid material was loaded onto both Biosil and silicic acid columns and was sequentially eluted with $CHCl_3$, acetone, acetone:H_2O (1:1), and MeOH solvent washes. Fractions were loaded onto the plates and developed with $CHCl_3$/methanol/NH_4OH (66:17:3). Spots were visualized with the anisaldehyde stain.

column chromatography was used throughout the remainder of the experiments due to its superiority.

Separation of sphingolipid standards via silicic acid chromatography

Once the silicic acid column was identified as the best separation tool, it was necessary to locate the fraction in which sphingolipids would elute. This was done by loading two classes of sphingolipids (bovine brain galactocerebrosides and

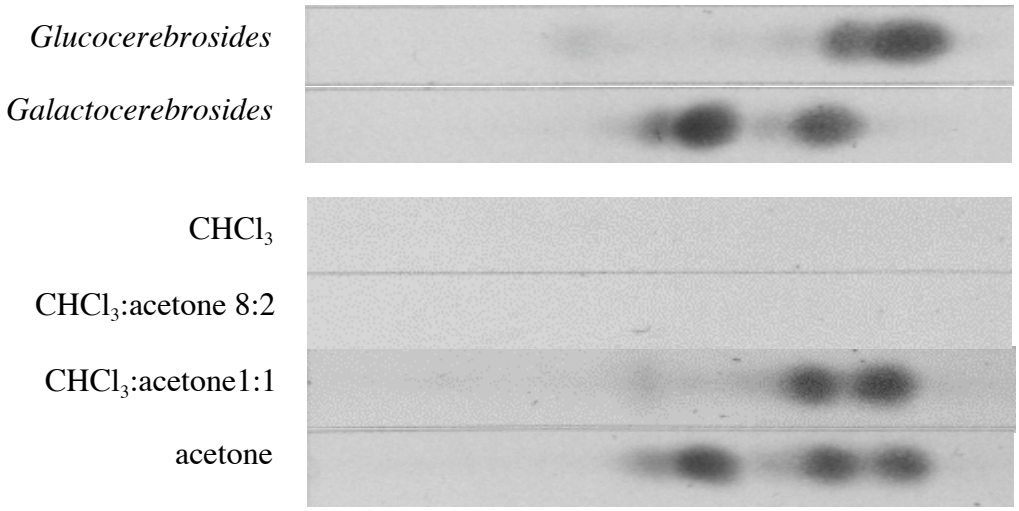

Figure 4. TLC plate of sphingolipid standard controls (top) and separated sphingolipid standards via silicic acid column chromatography (bottom). Standard sphingolipid material was loaded onto a silicic acid column and sequentially eluted with $CHCl_3$, $CHCl_3$:acetone (8:2), $CHCl_3$:acetone (1:1), and acetone solvent washes. Fractions and standards were loaded onto the TLC plate and developed with $CHCl_3$/CH_3OH/NH_4OH (66:17:3). Spots were visualized with the anisaldehyde stain.

glucocerebrosides) onto a silicic acid column and sequentially eluting them with $CHCl_3$, $CHCl_3$:acetone (8:2), $CHCl_3$:acetone (1:1), and acetone. Figure 4 shows that the galactocerebroside and glucocerebroside standards came off the column in the $CHCl_3$:acetone (1:1) and acetone washes. No lipid material was present in the $CHCl_3$ or $CHCl_3$:acetone (8:2) washes. This experiment showed that cerebrosides elute in the final two, most polar solvent washes. This information was later utilized for identifying sphingolipids extracted from *A. thaliana* plant material.

Differential hydrolysis of lipid standards

The concept of differential hydrolysis is explained by the structural difference between sphingolipids and acyl lipids. Sphingolipids contain an amide bond that is not present in other classes of lipids, while the ester linkage found in most lipid classes is absent from sphingolipids (Figure 5). Mild alkaline conditions (0.4 M methanolic KOH) cleave lipids in the ester bond lipid class but do not affect the amide bond found in sphingolipids. However, strongly acidic conditions (2% H_2SO_4 in MeOH) cleave both

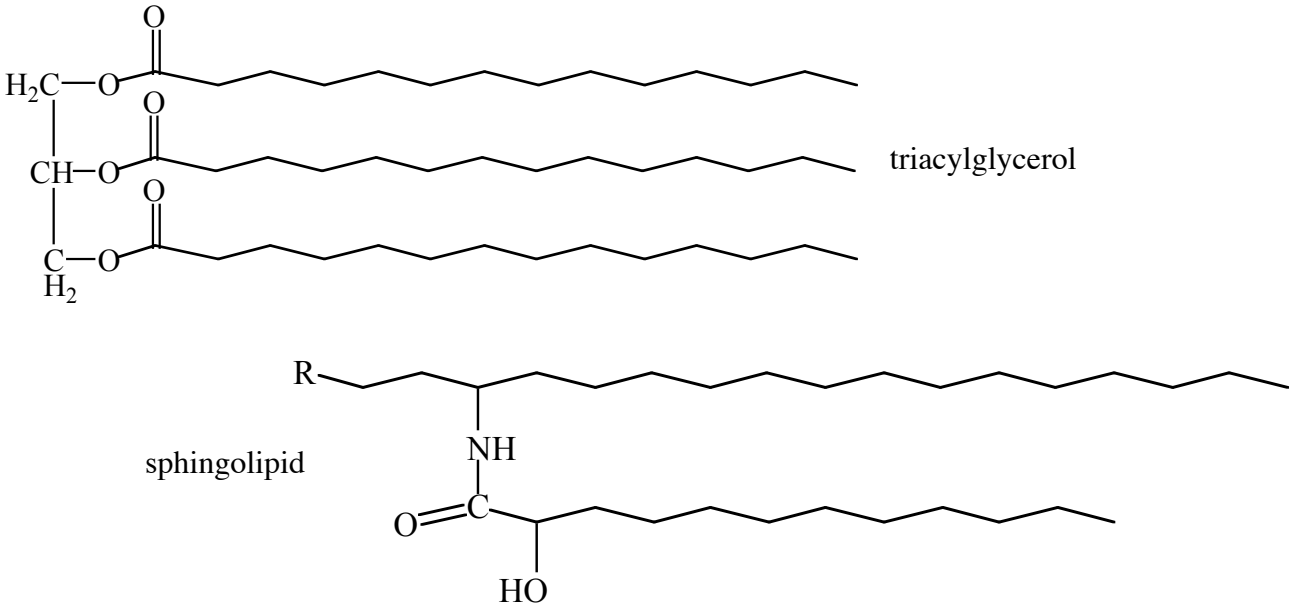

Figure 5. Representative diagrams of a triacylglycerol and a sphingolipid. The triacylglycerol contains three ester bonds and the sphingolipid contains the characteristic amide bond.

ester and amide bonds (8). It is possible to differentiate sphingolipids from other lipid classes by subjecting the total lipid sample to mild alkaline conditions. The unaffected sphingolipids can then be isolated from the resulting methyl esters via column chromatography and analyzed via GC/MS.

Several lipid standards were used to demonstrate the effectiveness of the differential hydrolysis method. Each standard was subjected to strongly acidic conditions and mild alkaline conditions. TLC was used to determine whether or not the lipid standard was hydrolyzed by the conditions. Due to the change in polarity from the hydrolysis, the hydrolyzed lipids were expected to migrate differently than the unreacted lipids. Figure 6 shows that all of the sphingolipid standards that were subjected to mild alkaline conditions (galactocerebrosides, hydroxy ceramides and non-hydroxy ceramides) produced spots that lined up well with the unreacted standards. However, the acyl lipid standard that was subjected to mild alkaline conditions (phosphatidylcholine) did not have spots that lined up with the unreacted standard. Also, Figure 6 shows that none of samples hydrolyzed in strong acid have spots that lined up well with the unreacted lipid lanes. Products of acid hydrolysis of both hydroxy fatty acid ceramides and non-hydroxy ceramides had similar R_f values and are likely to be the long chain base and long chain fatty acid of the original sphingolipid standards.

No lipid material can be seen in the strong acid reaction lane of the galactocerebrosides and phosphatidyl choline standards. However, close examination of the actual TLC plate reveals that lipid material is present in both of these lanes. Regardless, none of the spots lined up with the unreacted lipid standards.

These results suggest that none of the sphingolipids reacted under mild conditions, whereas the acyl lipid did react. They also suggest that all lipids were

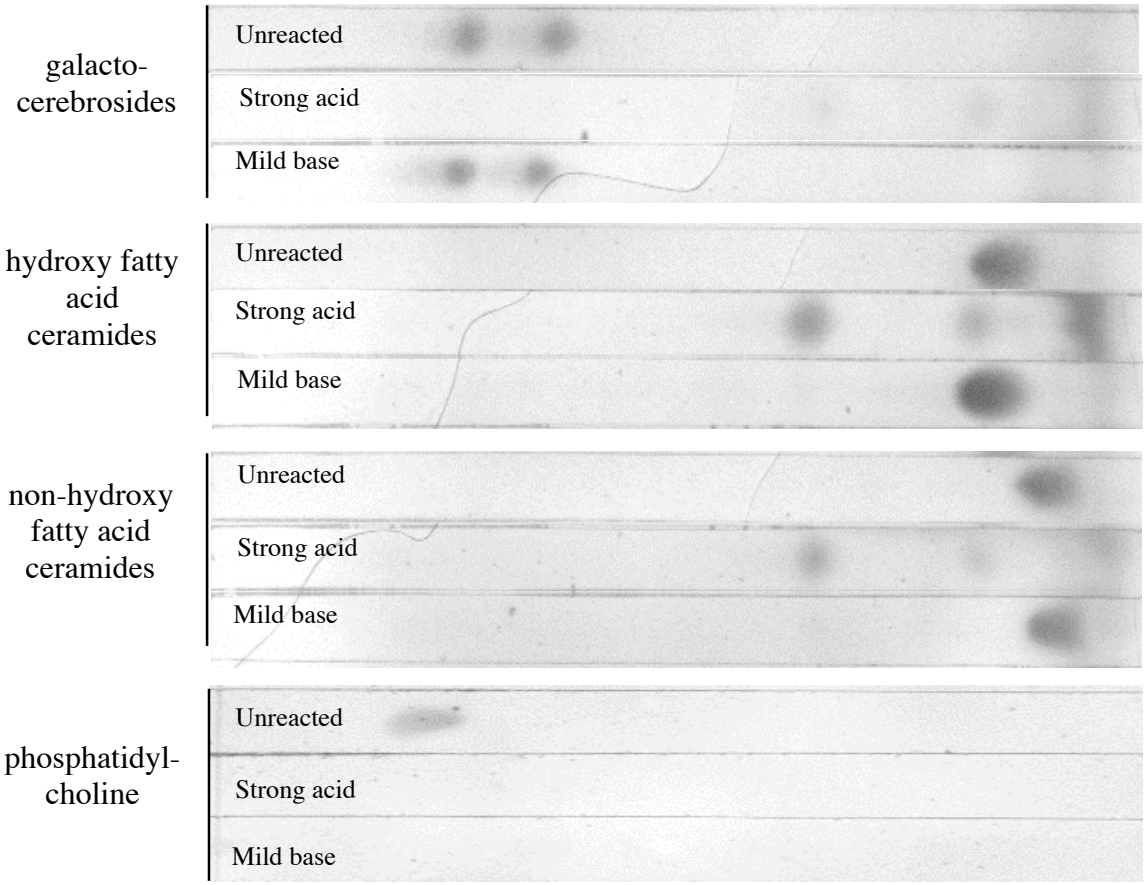

Figure 6. TLC plate showing differential hydrolysis of lipid standards. Lipid standards were reacted under strong acidic conditions (2% H_2SO_4 in MeOH) or mild alkaline conditions (0.4 M methanolic KOH). Reacted lipids were run next to unreacted standards to determine effectiveness of the hydrolysis conditions. Lipid material was loaded onto the plate and developed with $CHCl_3/CH_3OH/NH_4OH$ (66:17:3). Spots were visualized with the anisaldehyde stain.

hydrolyzed under the strong conditions. The results were expected and demonstrate that differential hydrolysis is an effective method for distinguishing sphingolipids from acyl lipids (15).

Isolation of yeast and A. thaliana lipids

Yeast was used to test the effectiveness of the lipid extraction procedure because *A. thaliana* takes significantly more time to grow and collect. Once the extraction technique was demonstrated on yeast, material was extracted from *A. thaliana*.

Figure 7 shows the material extracted from both yeast and *A. thaliana* compared with galactocerebroside standard. The galactocerebroside standard contains a mixture of cerebrosides (with hydroxy fatty acids or non-hydroxy fatty acids) giving rise to two spots on the thin layer chromatogram. Although there is no proof that the spots are lipids,

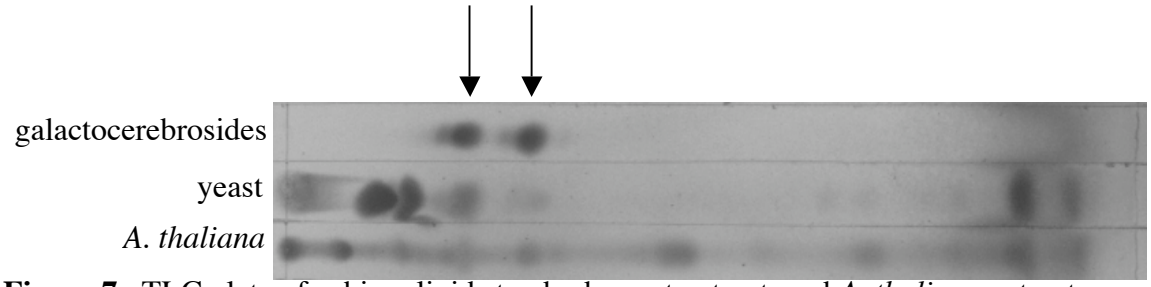

Figure 7. TLC plate of sphingolipid standard, yeast extract, and *A. thaliana* extract. Material was extracted from yeast and *A. thaliana* twice with 2 ml of ethanol/water/ether/pyridine/NH_4OH (15:15:5:1:0.018) at 60 °C as described (7). Extract was loaded onto the plate and developed with $CHCl_3$/CH_3OH/NH_4OH (66:17:3). Spots were visualized with the anisaldehyde stain. The arrows indicate the spots that may be sphingolipids.

both the yeast and *A. thaliana* extracts had spots that lined up with the sphingolipid standard, galactocerebroside (Table 1). This is a good indication that sphingolipids were present in the lipid extracts. Also, more spots were located in the *A. thaliana* lane suggesting that plants have more lipid variety than yeast. This suggested that sphingolipids could be isolated from *A. thaliana*.

Table 1. R_f values of galactocerebroside standard and suspected sphingolipids from yeast and *A. thaliana*.

	$\mathbf{R_f}$	$\mathbf{R_f}$
galactocerebrosides	0.21	0.29
yeast	0.21	0.29
A. thaliana	0.21	0.28

Differential hydrolysis of A. thaliana extract

Once it was suspected that sphingolipids were present in the *A. thaliana* extract, it was necessary to isolate them and verify that they were indeed sphingolipids. This was done by differentially hydrolyzing the *A. thaliana* extract as described above and then isolating the sphingolipids via silicic acid chromatography (Figure 2).

Figure 8 shows two spots present in the $CHCl_3$:acetone (1:1) eluate (Fraction c, Figure 2) treated under mild alkaline conditions. One spot was present in the acetone fraction (Fraction d, Figure 2) treated under mild alkaline conditions. No other spots were present in either fraction treated under harsh acidic conditions. These results indicate that neither compounds found in the $CHCl_3$:acetone (1:1) nor the acetone fractions were affected by mild alkaline conditions. However, when this same material was subjected to harsh acidic conditions, it was hydrolyzed. This suggests that these compounds have a bond that is unaffected by mild conditions and hydrolyzed by harsh conditions. Also, spots found in both mild lanes line up well with a sphingolipid standard, galactocerebroside.

These results indicate that the spots present in the $CHCl_3$:acetone (1:1) and the acetone fractions could be sphingolipids. Further evidence supporting this claim is that, in previous experiments, standard sphingolipids (galactocerebrosides and glucocerebrosides) were exclusively found in the $CHCl_3$:acetone (1:1) and acetone fractions (Figure 4). It is likely that the material in both the $CHCl_3$:acetone (1:1) and acetone fractions may be from the sphingolipid class.

It is possible that the material found in these two lanes is not sphingolipid or that

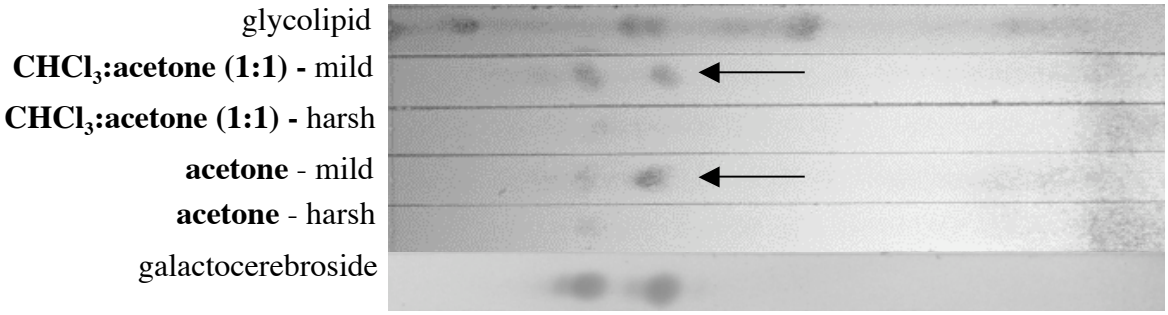

Figure 8. TLC plate showing the differential hydrolysis of the glycolipid fraction of *A. thaliana* extract. The glycolipid fraction was subjected to mild alkaline conditions, separated via silicic acid chromatography and loaded onto the plate (Figure2). The plate was developed with $CHCl_3/CH_3OH/NH_4OH$ (66:17:3). Spots were visualized with the anisaldehyde stain. The arrows indicate suspected sphingolipids.

the sphingolipid is contaminated with other organic material. For instance, steryl glycosides (a sugar residue covalently bound to a sterol moiety through an ether linkage) have been shown to elute in the $CHCl_3$:acetone (1:1) and the acetone washes (15). As steryl glycosides do not have an ester linkage or an amide linkage, it was suspected that they would not be affected by the hydrolysis conditions in this experiment. Since the material in both the $CHCl_3$:acetone (1:1) and acetone lanes was affected, it is suspected that no steryl glycosides were present. However, future experiments need to be

conducted to show that steryl glycosides are not affected by the hydrolysis conditions. Also, future experiments need to be conducted to show that steryl glycoside standards do not line up with the spots suspected of being sphingolipids (Figure 8). Noting that the spots observed in this experiment lined up well with the sphingolipid standard, it is likely that these are sphingolipids and not other lipids.

***A. thaliana* FAME analysis via GC/MS**

Once sphingolipids had been tentatively identified in the *A. thaliana* extract, fatty acid methyl esters were prepared for GC/MS analysis as described above. The reaction of this derivatization is shown in Figure 9. A similar reaction applies for the derivatization of sphingolipids.

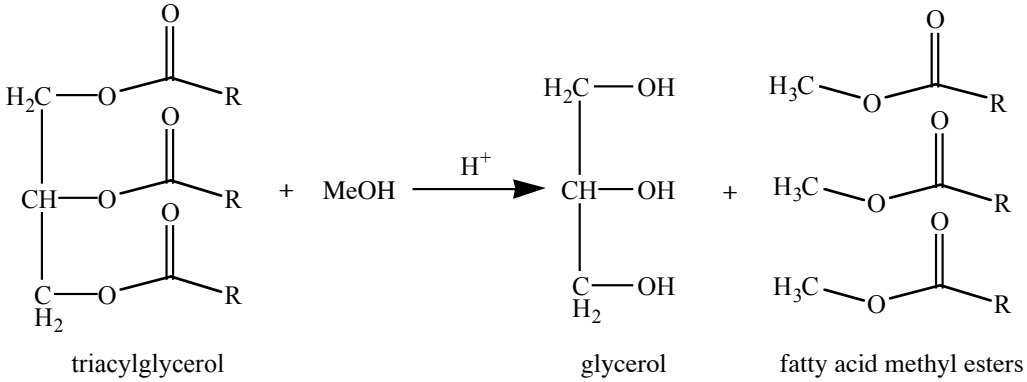

Figure 9. Reaction of an acyl lipid with MeOH which produces glycerol and fatty acid methyl esters.

Several saturated fatty acid methyl ester standards (C_{16}-C_{26}) were run on GC/MS to determine their approximate elution times. The mass spectrum of each standard was analyzed to verify the parent ion value for each FAME standard. Table 2 gives elution times and parent ion values for each fatty acid standard. In addition, FAMEs were made

from galactocerebroside and glucocerebroside standards and analyzed by GC/MS. In the galactocerebroside sample, 18:0, 22:0, and 24:0 fatty acids were identified, and in the glucocerebroside sample, 16:0, 18:0, 20:0, 22:0, and 24:0 fatty acids were identified (Table 2). The fatty acid composition of purified *A. thaliana* sphingolipid was analyzed via GC/MS following acid hydrolysis. Comparing the chromatograms of the sphingolipid fractions to those of the FAME standards showed that 16:0 and 18:0 fatty acids were present in the putative sphingolipid fractions from *A. thaliana* (Table 2).

Table 2. Mass spectral identification of methyl ester derivatives of standard saturated fatty acids, standard glucocerebrosides, standard galactocerebrosides and *A. thaliana* sphingolipid fatty acids.

Unsaturated fatty acids

Fatty acid	Elution time (min)	M^+ (M/Z)
14:0	9.26	242
16:0	11.43	270
18:0	13.39	298
20:0	15.18	326
22:0	16.83	354
24:0	18.36	382

Glucocerebrosides

Fatty acid	Elution time (min)	M^+ (M/Z)
16:0	11.47	270
18:0	13.42	298
20:0	15.20	326
22:0	16.82	354
24:0	18.36	382

Galactocerebrosides

Fatty acid	Elution time (min)	M^+ (M/Z)
18:0	13.41	298
22:0	16.83	354
24:0	18.36	382

***A. thaliana* sphingolipid ($CHCl_3$:acetone 1:1 fraction)**

Fatty acid	Elution time (min)	M^+ (M/Z)
16:0	11.47	270
18:0	13.46	298

***A. thaliana* sphingolipid (acetone fraction)**

Fatty acid	Elution time (min)	M^+ (M/Z)
16:0	11.47	270
18:0	13.40	298

It was expected that a wider range of fatty acid chain lengths would be identified in the *A. thaliana* sphingolipids. The fact that many fatty acid moieties of sphingolipids are hydroxylated may explain why only two fatty acids were identified. The hydroxyl group of a fatty acid makes it significantly more polar. This change in polarity may prevent the hydroxy fatty acid from travelling through the column and being detected by the mass spectrometer. One way to detect hydroxy fatty acids by GC/MS is by first protecting the hydroxy group with trimethylsilyl (TMS) (15,17). This protecting group significantly decreases the polarity of the fatty acid and allows it to enter and travel through the column.

Another possible reason why only two fatty acids were identified is that the sphingolipid fraction may contain contaminant pigments. These pigments could make it difficult to detect less prevalent very long chain fatty acids. Methods for separating the pigments from the sphingolipid are currently being explored (18).

Through developing a scheme for isolating and characterizing sphingolipids from *A. thaliana*, several techniques including differential hydrolysis, silicic acid chromatography, thin layer chromatography, and GC/MS analysis were learned and utilized.

CONCLUSION

Total lipids were extracted from whole plant tissue and column chromatography was used to isolate polar lipids. Sphingolipids were further purified by differentially hydrolyzing the ester bond found in unwanted glycerolipids. After purification of the sphingolipid fraction, fatty acid methyl esters were prepared and analyzed by GC/MS. The results suggested that 16:0 and 18:0 fatty acids were present in the sphingolipids extracted from *A. thaliana.*

Future studies involving TMS derivatization of the sphingolipid fractions will be developed to further characterize the fatty acid content of *A. thaliana* sphingolipids. Techniques will be utilized to further purify the sphingolipids from contaminants such as pigments. This may improve detection of the longer chain fatty acids of interest. Also, experiments will be conducted to show that sphingolipid fractions are not contaminated with steryl glycosides.

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