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Extraction and Characterization of Lipids From *Salicornia Virginica* and *Salicornia Europaea*

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Abstract

The lipid content from *Salicornia virginica* and *Salicornia europaea* is investigated. The plants are leafless halophytes with seeds contained in terminal nodes. The lipids, in the form of cell membranes and oil bodies that come directly from the node cells, are observed using fluorescence microscopy. Two extraction methods as well as the results of extracting from the seeds and from the entire nodes are described. Characterization of the fatty acid components of the lipids using Gas Chromatography in tandem with Mass Spectroscopy is also described. Comparisons are made between the two methods and between the two plant materials as lipid sources.

Background

There are two major challenges to humankind's energy-intensive lifestyle that make research in alternative fuel sources a necessity. First, petroleum is a non-renewable resource; global oil production will undoubtedly peak. The production of oil reaches this peak when half of the original supply has been pumped from the ground. This occurrence has an indefinite timeline; estimates for peak oil production range from the present to near mid-century (Ref. 1). Considering even the most optimistic estimate, research in alternative fuels is a critical undertaking. Secondly, it is evident that the Earth's global average temperature is increasing with time. Evidence suggests that our use of fossil fuels as an energy source is most likely a contributor to the recent warming trend (Ref. 2). This is because the burning of fossil fuels results in carbon dioxide formation, and it's formation from carbon outside of the carbon cycle creates a

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net-positive increase of greenhouse gases in the Earth's atmosphere. Thus, a fuel source with a net-zero greenhouse gas emission seems likely to reduce humankind's contribution to climate change.

Two promising constituents of plant material for use as a fuel feedstock are lipids and cellulose. Lipids can be used to produce fuels with relatively long-chained hydrocarbons resembling petroleum-derived diesel fuel. Cellulose is more suited for the production of aromatic hydrocarbons found in petroleum-derived gasoline. We describe the extraction and characterization of lipids from halophyte plants with the ultimate goal of determining the feasibility of using lipids as a feedstock for synthetic aviation fuel.

Lipids are a large class of molecules that are naturally occurring, insoluble in water, and comprised of long-chain hydrocarbon groups that may also contain oxygen, nitrogen, phosphorus and sulfur. The most common types of plant lipids are waxes, triglycerides, and phospholipids (Ref. 3). Waxes provide a layer on the plant's surface that acts as interface between the plant and its surroundings. This layer serves many vital purposes, including a barrier to limit the diffusion of water and solutes while controlling the release of volatiles. Waxes are complicated mixtures of components, primarily wax esters. The nature of the other lipid constituents can vary greatly with the source of waxy material, but they include hydrocarbons, aldehydes, alcohols, ketones, and several others. The molecular structure of triglycerides consists of three fatty acid molecules bonded to one glycerol molecule. The distribution of fatty acids is dependent on the plant species, but typically consists of fatty acids with sixteen and eighteen carbons and some percentage of fatty acids with one or two double bonds (unsaturated). At a given temperature, the overall amount of saturation determines if the triglyceride is called a "fat" or an "oil". Triglycerides are used as an energy source stored mostly in seeds and to a lesser extent within the plant itself. The molecular structure of phospholipids consists of two fatty acids bonded to a glycerol with a phosphate functional group. Thus, the molecule has a polar head and a non-polar tail. They form bilayers in water by arranging the heads to point outward towards the aqueous media with the tails inward. The bilayers are the structural basis of cellular membranes.

The lipids investigated in the work come from halophytes grown in the GreenLab Research Facility located at the NASA Glenn Research Center. Halophytes by definition are salt-tolerant plants and thus grow in habitats that are rich in salts. The benefits of obtaining fuel from halophytes are that it does not compete with freshwater human needs, and it does not exploit arable land that can otherwise be used for agriculture with traditional food crops (Ref. 4). The specific halophytes investigated in this work belong to the Salicornia genus including the perennial species, *S. virginica* and the annual species, *S. europaea*. They are leafless plants with jointed and succulent stems that form terminal nodes. The seeds are produced and carried within the nodes. The quantity of lipids from the seeds is reported to be near 30 percent (Ref. 5) The lipids contained within the nodes include lipids from the developing seed as well as the lipids from the cells and the waxy surface of the nodes. The overall amount of node lipids increases as the plant matures due to the lipid accumulation in the growing seeds.

Experimental

Microscopy

To qualitatively study the lipid content of the node cells, fluorescence microscopy was utilized. Nile red was used as the fluorescent probe since it fluoresces strongly in hydrophobic lipids and is fully quenched in water. Preparation of the Nile red dye was based on a procedure by Fowler et al. (Ref. 6). The staining of the sample with the dye was based on a procedure by Lersten et al. (Ref. 7). A stock solution of Nile red of 500 μg/mL in acetone was prepared and stored in the dark. The dye was made by addition of 100 μL of stock solution to 10 mL of 75 percent glycerol in water, followed by brisk stirring. The dye was then degassed by vacuum to remove bubbles. For staining, thin layers of epidermal and mesophyll cells were sectioned freehand from the node of a fresh *S. virginica* sample using a razor blade. To avoid solvation of the lipids, staining was carried out on an unfixed layer of the node by simply wetting the sample with the solution.

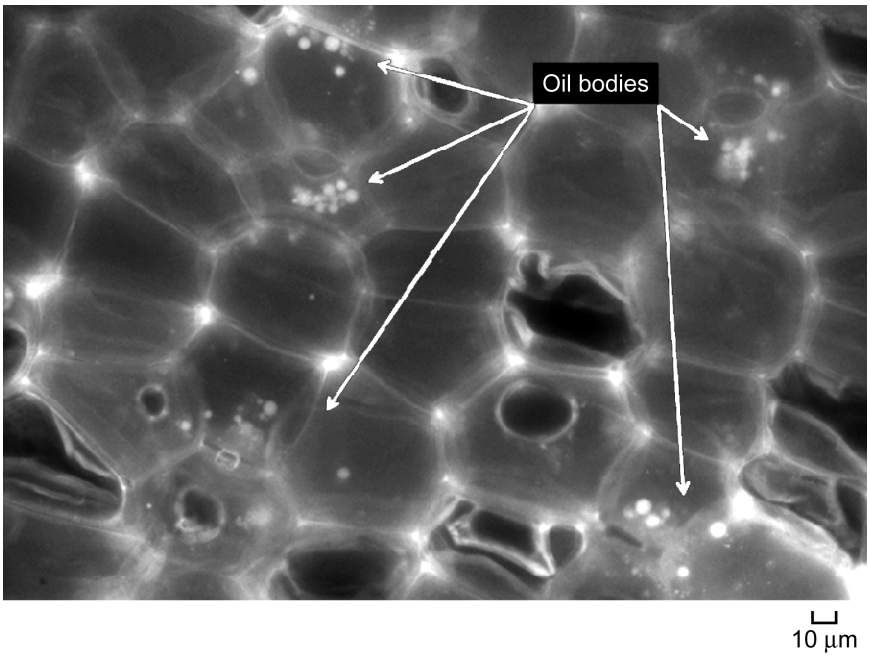

Figure 1.—Spherical oil bodies in the cytoplasm of plant cells.

The microscopy studies were carried out using a fluorescence microscope equipped with 100 W halogen lamp and a 20 times plane objective lens. The Nile red fluorescence was viewed using green fluorescence 560/40 nm band pass exciter filter, a 595 nm chromatic mirror, and a 675/75 nm suppression filter.

Oil bodies within the cytoplasm as well as the lipids that make up the cell membranes of *S. virginica* are clearly seen in Figure 1 with Nile red fluorescence. Interestingly, the oil bodies were mostly clustered in random cells with only a few individual oil-containing spheres, typically ranging in size from 0.25 to 3 μm.

Extraction

The general method of extracting plant constituents consists of dehydration, comminution, and solid-liquid extraction (Ref. 8). Drying the plant material is a necessary step as the percent yield of extracted material is based on a standard dry weight of the raw material. Also, water interferes with the catalysts during transesterification, the reaction used to prepare the extract for characterization (Ref. 9). Comminution refers to the process of grinding the sample material into a fine powder. Solid-liquid extraction involves dissolving components with a particular solvent to remove them from a solid matrix.

Freezing is a necessary step for storing and drying the plant material. Samples are kept frozen and in the dark to reduce enzymatic hydrolysis of the lipids which produces free fatty acids (Ref. 8). For long-term storage, the temperature of liquid nitrogen, near –210 °C, is best for minimal enzymatic activity. However, a temperature near –30 °C is typically adequate for short-term storage of less than a month. Water is removed from the frozen sample by sublimation in a vacuum, as opposed to evaporation at an elevated temperature, in order to reduce loss of constituents.

Comminution of plant material is one of the most important steps in the overall method. The sample is broken down into relatively small particles to efficiently extract plant constituents of interest. Seeds were ground using a high speed micro-mill with the capability of grinding samples from 20 to 50 mL using a blade rotating at speeds up to 12,000 rpm. The sample chamber temperature is maintained by heat exchangers around the housing of the micro-mill and can be lowered by circulating cool water, methanol-dry-ice liquid or liquid nitrogen. The nodes, which are relatively elastic, are ground using a blender with a suspension solvent. Isopropyl alcohol is typically used as it evaporates at a relatively low temperature and

also acts as a preservative by inhibiting lipolytic enzymes that hydrolyze triglycerides (Ref. 10). The ground plant material is dried under mild heat or vacuum in order to remove the suspension solvent.

There are two methods used in this work to extract the lipids from the seeds or nodes. One method of extraction involves the use of an automated Soxlet extractor. The pre-dried and weighed samples are sealed in pre-weighed filter bags. Up to 15 bags can be loaded into the extractor at once. The instrument automatically extracts and rinses the sample by performing 40 extraction cycles in 1 hr at a temperature of 90 °C and a pressure of 12 psig. After extraction, both the remaining cellulosic material and the extracted lipids are collected and weighed. The difference between the encapsulated sample before and after extraction is divided by the dry weight of the sample and multiplied by 100 to determine the percent lipid yield of a particular sample. The overall yield is the average across all samples in a particular extraction, expressed as a percentage of weight. The error is taken as the 95 percent confidence interval of the mean.

A second method of extraction used in this work is ultrasonication. During ultrasonication, the formation and collapse of micro-bubbles interacts with the liquid/solid boundary surfaces of the suspension, causing the solid material to break apart. The goal is to break down the plant cell in order to expose the phospholipids that make up the cell membranes and the oil bodies stored within the node cell. Thus, comminution and extraction occur in one step. A typical extraction employing this method occurs as follows: A sample weighing 10 g in a 50 mL suspension of isopropyl alcohol is sonicated for 10 min at 20 kHz for a total energy output of 5.4 kJ. After, extraction is complete the non-extracted cellulose is filtered, dried and weighed. The percentage of extracted material is simply the difference between the weight of the original material and the non-extracted material divided by the original material and multiplied by 100. The yield in each aliquot of a sample can be used to determine an average yield with a 95 percent confidence interval.

The solvent determines the types of lipids extracted from the sample. Neutral lipids such as triglycerides and waxes are extracted with a non-polar solvent. Phospholipids require polar solvents, such as alcohols, to disrupt hydrogen bonds and electrostatic forces. In this study, a mixture of hexane isomers was used as the non-polar solvent. This solvent was used to extract the triglycerides from the seeds and initially as the solvent to extract triglycerides and waxes from the entire nodes. To extract the phospholipids along with the triglycerides in the node cells, a 2:3 mixture of isopropyl alcohol and hexanes was used as the polar solvent. Chlorophyll contained in both the seeds and the nodes was also consequently extracted using these solvents. Particularly for the extraction involving the nodes, chlorophyll was exploited as a simple indicator of the progress of the extraction by gradual pigment loss from the cellulosic material.

Results

The results of five extractions are shown in Table 1. The feedstocks were the seeds and nodes from *S. virginica* and the nodes from *S. europaea*. Method 1 involved the use of the automated Soxlet extractor, while Method 2 used ultrasonication. The extracting solvents were a mixture of hexanes and a mixture of isopropyl alcohol and hexanes as described above. The percent content of extracted material consists of both lipids and chlorophyll.

TABLE 1.—EXTRACTION RESULTS

Sample number	Plant	Source	Method of extraction	Extraction solvent	Yield (% by weight)
1	*Salicornia virginica*	Seeds	[a]Method 1	Hexanes	15.4 ±0.3
2	*Salicornia virginica*	Nodes	[b]Method 2	Hexanes	2.4 ±0.6
3	*Salicornia virginica*	Nodes	Method 1	Hexanes	3.2 ±0.5
4	Salicornia *virginica*	Nodes	Method 1	Isopropyl alcohol/hexanes	3.6 ±0.5
5	*Salicornia europaea*	Nodes	Method 1	Isopropyl alcohol/hexanes	3.5 ±0.5
6	*Salicornia europaea*	Nodes	Method 1	Isopropyl alcohol/hexanes	7.1 ±0.6

[a]Method 1 involves an automated Soxlet extractor for extraction

[b]Method 2. involves the Ultrasonicator for both comminution and extraction.

From the results of Table 1, several observations are made. Firstly, Method 1 provided a higher yield from the same plant material. One possible reason for this was that it was difficult to determine when the extraction was near completion using Method 2, possibly resulting in a premature end to the process. Secondly, the more polar solvent mixture of isopropyl alcohol and hexanes was expected to give larger yields of extracted lipids from plant nodes because of the additional extraction of the phospholipids that make up the cell membranes. However, this is not apparent here since the slight increase in percent yield of Sample 4 was within the margin of error of the yield of Sample 3 which used only hexanes as the extracting solvent. Lastly, an interesting result was the significant difference in percent yield of extract between Samples 5 and 6. Both samples contained the same plant type and same extracting solvent, however, Sample 6 provided a 50 percent increase in extracted material. The only noted difference in the two samples was that the nodes of Sample 5 had green pigmentation and nodes of Sample 6 were red in color indicating that the source plant was near the end of its life cycle.

Characterization

To determine the fatty acid distribution of the extract, fatty acids are removed from the glycerol structure and derivatized into fatty acid methyl esters (FAMEs) using the transesterification reaction. FAMEs are commonly used as fatty acid derivatives, and as a result, there is a large quantity of available information on their chromatographic and spectroscopic properties. There are two types of transesterification reactions which are distinguished by the type of catalyst used: base-catalyzed and acid catalyzed transesterifications.

Base-catalyzed transesterification of triglycerides is the most common process for FAME production. It is a relatively rapid reaction, typically requiring 30 min to completion with a 99 percent yield (Ref. 9). The stoichiometry of the reaction requires a 3:1 methanol/lipid molar ratio. However, the reaction is carried out with an extra amount of alcohol in order to shift the equilibrium to the product side of the reaction, typically near 7:1 for the base-catalyzed reaction (Ref. 11). Unfortunately, the reaction is sensitive to the quality of the feedstock, requiring it to have less than 0.5 percent free fatty acid (FFA) content (Ref. 12). If FFAs are present, saponification will occur as a competing reaction when using the typical alkaline metal alkoxide as the base. The presence of water will cause the formation of some of FFAs by hydrolysis of the produced methyl esters. Saponification results in a reduced yield of methyl esters as the resultant soap is formed instead of the desired methyl esters. In addition, the presence of soap leads to the formation of an emulsion making the purification process much less efficient (Ref. 9).

A base-catalyzed transesterification was used for the FAME conversion of triglycerides extracted from seeds. The extract was dissolved in 100 mL of toluene and combined with 40 mL of 0.5 M of sodium methoxide in methanol. The mixture was heated to 60 °C and stirred for 1 hr. The resultant FAMEs were separated from the mixture with 100 mL of hexanes and washed with warm distilled water. Excess methanol and the hexanes were removed using rotoevaporation. The FAMEs were then dissolved in 100 mL of toluene and diluted further for analysis.

Acid-catalyzed transesterification is more suitable for unrefined lipid extracts. FFAs are converted to methyl esters and there is no soap formation. However, the reaction is much slower than the base-catalyzed reaction, taking approximately 48 hr to obtain a 99 percent yield (Ref. 13). Also, a very high methanol to sample molar ratio is required, typically 250:1 (Ref. 14).

An acid-catalyzed transesterification was used for the FAME conversion of lipids extracted from the nodes. The extract was dissolved in 100 mL of toluene and combined with 200 mL of 5 percent sulfuric acid in methanol. The mixture was heated to 60 °C and stirred for approximately two days. The resultant FAMEs were separated from the mixture with 100 mL of hexanes and washed with warm distilled water. Excess methanol and the hexanes were removed using rotoevaporation. The FAMEs were dissolved in 100 mL of toluene and diluted further for analysis.

Gas chromatography in tandem with mass spectroscopy was used to qualitatively determine the individual components of the extract. Separation was achieved with a cross linked 5 percent Phenyl-, 95 percent methyl-siloxane capillary column (HP-5MS, 30- by 0.22-mm i.d., film thickness 0.25 um).

The FAMEs were dissolved in toluene 1:40 by volume and 0.1 μL of the sample was injected into a capillary injector at 120 °C in splitless mode. The helium carrier gas flow rate was kept at 2.6 mL/min. The oven temperature was initially held at 90 °C. For the first ramp, the temperature was increased to 200 °C at a rate of 5 °C/min and then held at 200 °C for 30 min. For the second ramp, the temperature was increased to 300 °C at a rate of 10 °C/min and again held for 30 min. A mass spectrometer with an electron impact ionization of 70 eV was used for detection and identification of the separated components.

FAMEs from extracts of both seeds and nodes were characterized in order to determine the fatty acid distribution of the extracted lipids. The chromatographic peaks were identified based on their retention times using FAME standards made from pure vegetable oils with known fatty acid distribution. The chromatogram of the separated FAMEs from the fatty acids of triglycerides extracted from *S. virginica* seeds is shown in Figure 2. The following FAME distribution was determined: 14 percent methyl palmitate which has sixteen carbon and no double bonds (C16:0), 73 percent methyl oleate (C18:1), and 13 percent methyl stearate (C18:0). The chromatogram of the FAMEs from the lipids extracted from *S. europaea* nodes is shown in Figure 3. The following FAME distribution was determined: 24 percent methyl palmitate (C16:0), 45 percent methyl oleate (C18:1), 47 percent methyl linoleate, and 8 percent methyl stearate (C18:0).

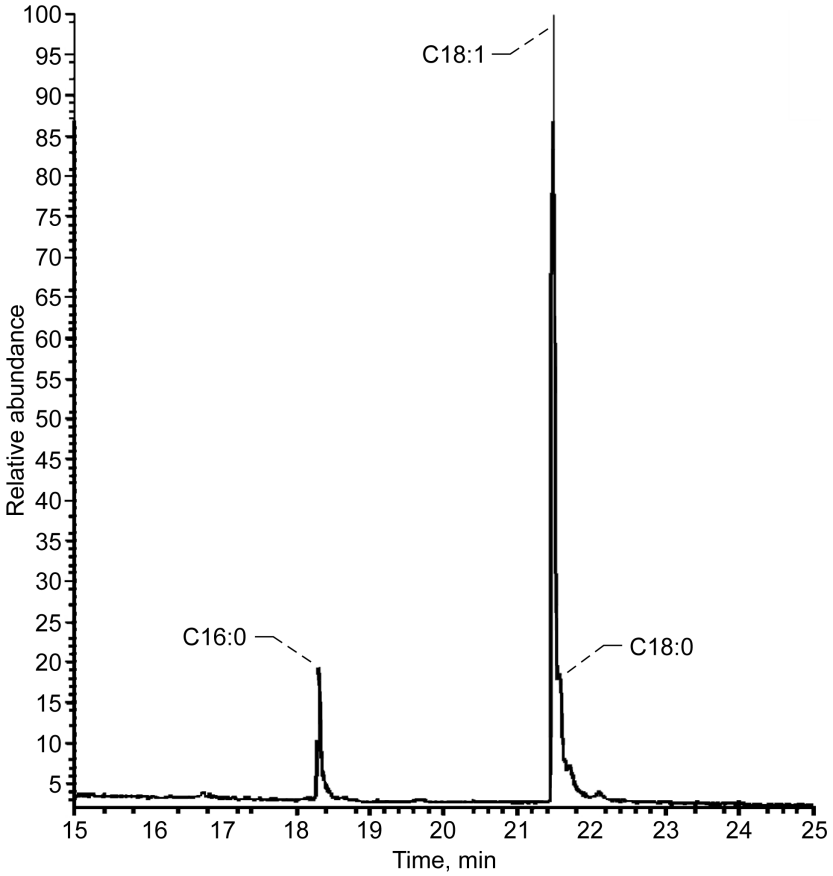

Figure 2.—Chromatogram of methyl esters from the fatty acids of triglycerides extracted from seeds of *S. virginica*. Methyl palmitate (C16:0), methyl oleate (18:1), and methyl stearate (18:0) were identified from their retention times and mass spectra.

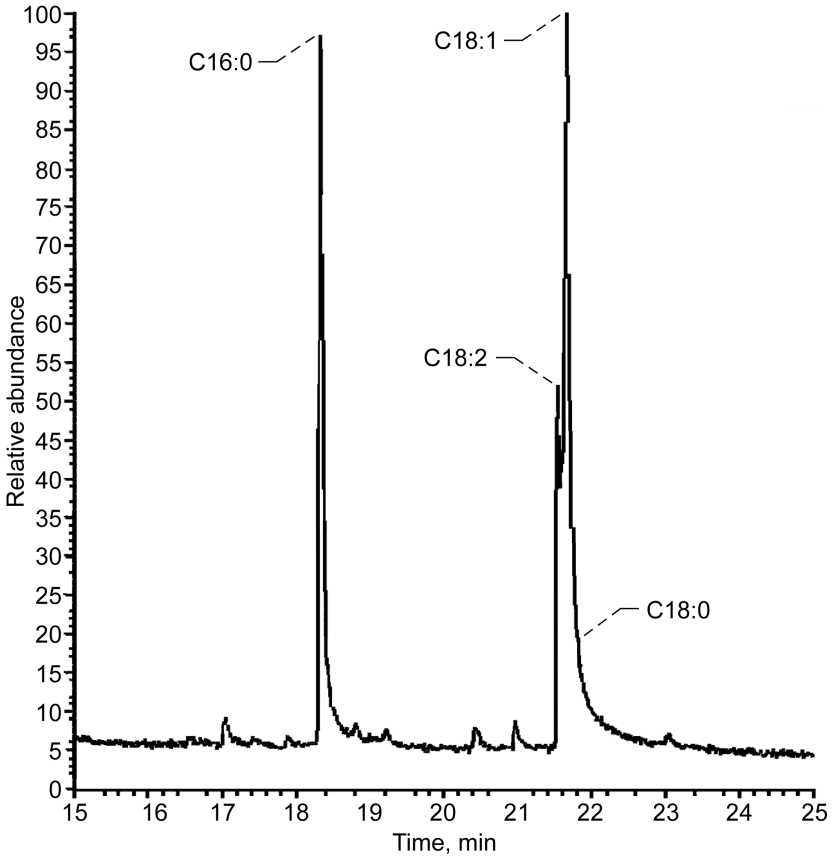

Figure 3.—Chromatogram of methyl esters from the fatty acids of triglycerides extracted from the nodes of *Salicornia europaea*. Methyl palmitate (C16:0), methyl linoleate (18:2), methyl oleate (18:1), and methyl stearate (18:0) were identified from their retention times and mass spectra.

The identity of each chromatographic peak was confirmed from their mass spectrum. For example, the mass spectrum of peak identified as methyl palmitate (C16:0) is shown in Figure 4. The molecular ion is apparent at 270 m/z suggesting that molecule has a molecular weight near 270 g/mol. The loss of the methoxy group can be inferred from the line at 239 m/z. The line at 227 m/z represents the loss of a C3 unit via a complex rearrangement. The long homologous series of related lines at m/z = 199, 157, 143, 129, 115, 101, and 87 are considered to be ions 14 amu apart that have the general formula $[CH_3OCO(CH_2)n]^+$. The homologous series is evidence of a long hydrocarbon chain with no other functional groups. Lastly, the McLafferty rearrangement is seen at 74 m/z and confirms that the chromatographic peak represents a methyl ester (Ref. 15).

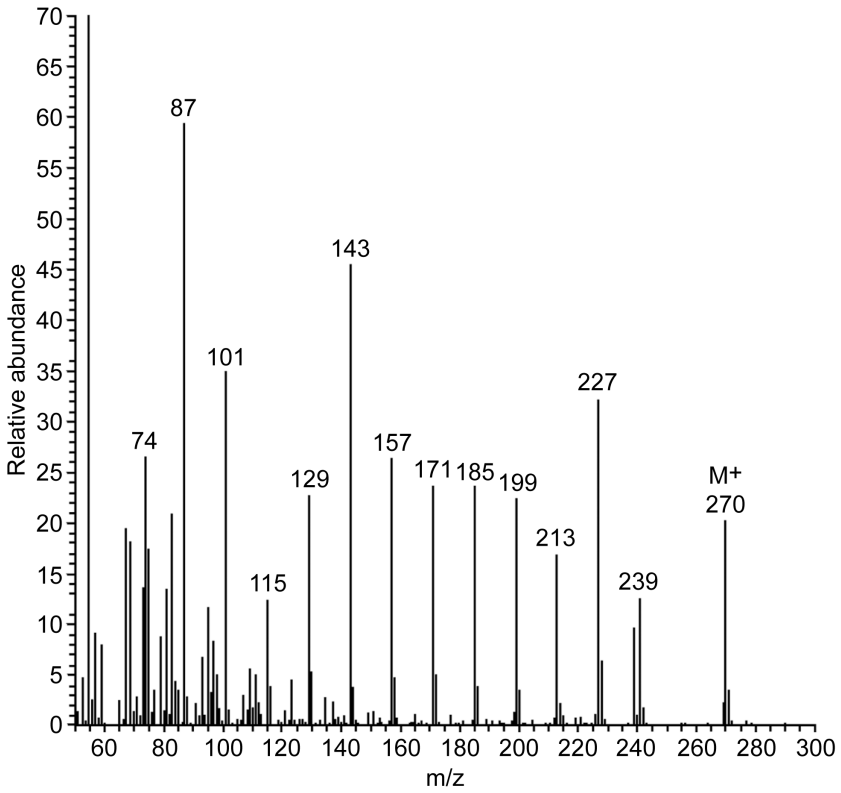

Figure 4.—Mass spectrum of methyl palmitate, (C16:0).

Conclusion

The lipid content of the nodes of *S. virginica* was qualitatively studied using fluorescence microscopy. Both the phospholipid containing cell membranes and the oil bodies were clearly visible under fluorescence. Fluorescence microscopy has potential use as a screening method. Using plant material with known lipid content as standards, it may be possible to quickly estimate the lipid yield of different plant samples without the need to extract the lipids.

Comparing the two extraction methods, Method 1 is mostly superior as it provides higher yields and is less work-intensive and time-consuming. Method 2 has fewer steps since comminution and extraction are combined. However, it involves more work and time because vacuum filtration is required to separate the cellulosic material from the extract. For Method 1 extractions are performed under pressure, while for Method 2 extractions take place in atmospheric pressure. More solvent is required to extract an equivalent amount of lipids using Method 2 since the extraction is limited by atmospheric boiling points of the solvent. However, the ultrasonicator used in Method 2 is able to process much larger quantities than the automated Soxlet extractor used in Method 1. Thus, Method 2 is more appropriate for large-scale processing.

Overall, the extraction efficiency was lower than expected. For example, seeds have been reported to provide lipid yields near 30 percent (Ref. 5). However, the maximum yield in this work was only 15 percent. Several measures can be used to improve the yields. The samples were typically mixed with debris such as soil and remnant cellulosic material. Additional preprocessing to remove debris would improve yields by reducing the initial weight of the sample. Also, improvement in the communition process will most likely improve extraction efficiency as the finer the material is ground the more efficient the extraction. More studies are needed to determine the optimal conditions for extraction. For instance, a thorough investigation of solvent mixtures used to extract the lipids is needed. An acidic solvent that is more polar than the solvent used in this study, may provide higher yields by improving the

disintegration of cells as well as the solvation of polar lipids such as phospholipids. Higher temperatures and pressures in Method 1 and longer ultrasonication times in Method 2 can be investigated as well.

There are advantages to using the seeds of the plant instead of the nodes as a source of lipids. The extract mostly consists of triglycerides with only a negligible amount of FFAs and other lipids. Thus, the base-catalyzed transesterification reaction can be used instead of the acid-catalyzed transesterification which is more time-consuming and expensive in terms of chemicals. The disadvantage is that the seeds are only available after seed dispersal, typically 6 to 8 months after germination for annuals. The nodes are available at any time. However, as evidenced by the yields from Sample 5 and Sample 6 in Table 1, there is significantly larger quantity of storage lipids in the nodes just before seed dispersal mostly from the seed themselves. According to the results in this work, there is a 2 to 5 times greater yield of extract from the seeds than from the nodes depending on when the extraction took place in the plant's life cycle. There is an even a greater percentage of lipids extracted from the seeds since there is a larger quantity of chlorophyll in the extract of the nodes.

The GC/MS characterization showed that both the seeds and the nodes have a significant percentage of unsaturated lipids. Interestingly, the chromatogram of the lipids extracted from the nodes contained methyl linoleate which was not present in the chromatogram of lipids extracted from seeds. A large quantity of methyl oleate and methyl linoleate can be beneficial in a source that is used as precursor in fuel production. Their double bonds provide reaction sites on the molecular structure which could be used for the addition of functional groups or for cracking the molecule in order to produce fuel with different properties.

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
The lipid content from *Salicornia virginica* and *Salicornia europaea* is investigated. The plants are leafless halophytes with seeds contained in terminal nodes. The lipids, in the form of cell membranes and oil bodies that come directly from the node cells, are observed using fluorescence microscopy. Two extraction methods as well as the results of extracting from the seeds and from the entire nodes are described. Characterization of the fatty acid components of the lipids using Gas Chromatography in tandem with Mass Spectroscopy is also described. Comparisons are made between the two methods and between the two plant materials as lipid sources.

15. SUBJECT TERMS
Extraction; Lipids; Characterization; Gas chromatography; Mass spectroscopy

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