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The Development of a GC/MS Protocol for the Analysis of Polysaccharides in Echinacea purpurea

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Abstract:

Echinacea purpurea is an herbal supplement used to reduce symptoms of common colds and flu-like illnesses. As of right now, it is not well understood what chemical components in the plant are immunologically active. The four main potentially immunologically active chemical components in *E. purpurea* are caffeic acid derivatives, alkamides, polyacetylenes, and polysaccharides. In this investigation, a protocol was developed in an attempt to identify polysaccharides present in Puritan's Pride *E. purpurea* (aerial) "non-irradiated" herbal supplement. First, an estimate of the crude total polysaccharide content in Puritan's Pride and in a ChromaDex certified botanical standard (*E. purpurea* -powdered root) was performed and yielded 7.1% of the aerial plant material and 0.12% of the root material; respectively. To identify the individual polysaccharides present in *E. purpurea,* conversion into a form amenable for analysis by GC/MS (gas chromatography/mass spectrometry) required a sequential process of methylation, hydrolysis, reduction and acetylation to produce partially methylated alditol acetates (PMAAs). Although the proposed protocol was not completely successful, much has been learned to allow this project to continue with protocol modifications.

INTRODUCTION

Ethnobotany:

Throughout history, many plants and herbs have been used for their medicinal properties. Many drugs originated out of the simple plant form, and are now derived and altered into a pill or capsule for use by the pharmaceutical world. One of the most common herbal supplements throughout the world is *Echinacea*, the American coneflower. The name *Echinacea* comes from the Greek word echinos, meaning sea urchin, due to the prickly seed head of this flower. There are nine known species of *Echinacea*, but only three of these are used as medicinal herbal supplements (*E. purpurea, E. angustifolia, and E. pallida).*[1] Most of the supplements are derived from the aerial or underground parts of *E. purpurea*, and from the roots of *E. angustifolia* or *E. pallida*. The roots have high concentrations of volatile oils while the above-ground parts of the plant tend to contain more polysaccharides.[10] The various parts of the plants are approved for different treatments. In Europe the German Commission E. regulates approved uses based upon primary literature. They medicate or determine minimum standards of product. Germans have approved the above ground parts of *E. purpurea* to treat colds, upper respiratory tract infections, urinary tract infections, and slow healing wounds, while the root of *E. pallida* is also approved for flu-like infections. [10]

Echinacea has not always been a popular supplement. Natural herbal supplements, including commercially obtained *Echinacea* (usually one or more of the following *E. purpurea, E. angustifolia, or E. pallida)* declined in popularity in the United State with the advances of antibiotics. However, *Echinacea* is now one of the most popular natural supplements in the U.S. and Europe. Native Americans first used

Echinacea over 400 years ago to treat all infections and wounds. [10] Other sources believe Native Americans used *Echinacea* to create teas, mouthwashes, and medicinal bandages for treatments of coughs, dyspepsia, toothaches, stomach cramps, and a variety of other ailments. It has also been reported that *Echinacea* can be used to treat snakebites, headache, and the common cold.[1] *Echinacea* species are endemic to North America and different species occur in various grassland habitats between the Appalachian and Rocky Mountains. [1]

Many herbal supplements use a combination of these two *Echinacea* species. There are four main potentially immunologically active components in *Echinacea*. The main chemical components within *Echinacea* are caffeic acid derivatives (cichoric acid, echinacoside), alkamides, polyacetylenes, and glycoproteins/ polysaccharides.[1] *Echinacea* has many chemical components that when combined with one another produce the medicinal benefits observed originally by Native Americans and that continue to be observed by many cultures today.

Medicinal/Chemistry:

The mechanism for the immunostimulating effects of *Echinacea* is not well understood, and it is still not known which constituents of *Echinacea* are the bioactive compounds.[18] Some research states that the combination of *Echinacea* polysaccharides and the cell matrix surrounding human tissue cells can create a resistance to local inflammations, which limits bacterial access, increases phagocytosis and other immune functions to help fight the infection and decrease toxic by-products, which in turn supports the growth of new tissue.[16] There have been many studies attempting to analyze each of these components to determine their immunological effects. The active

substances in *E. purpurea* may enhance the immune system, relieve pain, reduce inflammation, and have hormonal, antiviral, and antioxidant effects. For this reason *Echinacea* can also be used to treat urinary tract infections, yeast infections, ear infections, athlete's foot, sinusitis, hay fever and slow-healing wounds. [10]

Many liquid *Echinacea* herbal supplements are made using water or alcoholic extracts. Each of these extracts yields different chemical components within the herbal plant supplement. The immunostimulatory activity of the alcoholic extracts is largely due to the lipophilic amides, as well as the polar caffeic acid derivatives, whereas the water soluble polysaccharides are implicated in the expressed juice or aqueous preparations.[4] Another form of *Echinacea* herbal supplement is in a capsule form. However, with most herbal supplements each brand may use multiple species of *Echinacea*, various parts of the plant (aerial or root), different growing conditions, and preparation methods which may affect the amount and or activity of the active ingredients.

Chemical Analysis:

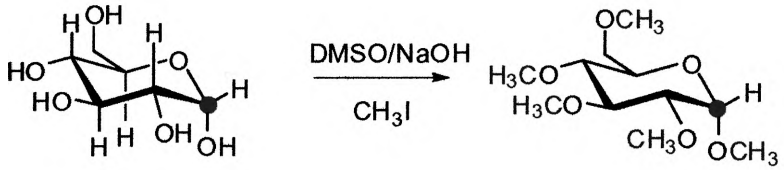
In this investigation, the polysaccharide chemical composition was determined using Puritan's Pride (aerial) herbal supplement and a ChromaDex Botanical Standard (root). By identifying the polysaccharides present in *E. purpurea,* each polysaccharide can later be analyzed for its immunological activity. Polysaccharides are complex carbohydrates. A single sugar unit is called a monosaccharide, two sugars units linked together is called a disaccharide, three sugar units linked together is called a trisaccharide, three to 100 sugar units is called an oligosaccharide, and 100+ sugars linked together is called a polysaccharide (a polymer of monosaccharides).

Some studies indicate that *E. purpurea* is rich in polysaccharides and phytosterols, which causes immune cells to search out bacteria. Previously, scientists have used nuclear magnetic resonance spectroscopy (NMR), a non-destructive technique to determine the structure of various organic compounds. However, NMR uses a much larger sample than Gas Chromatography-Mass Spectroscopy (GC/MS) and is unable to conclusively identify polysaccharides.

To analyze polysaccharides by GC/MS, they must be chemically converted into volatiles by a sequential process of methylation, hydrolysis, reduction and acetylation. This conversion process allows the polysaccharides to be broken down into partially methylated alditol acetates (PMAAs) which are volatile enough to be analyzed in a GC/MS. This process also permits the structural elucidation of how the monosaccharides are linked together to form the original polysaccharide. The general chemical modification scheme is illustrated below for a simple monosaccharide (glucose- Scheme 1) and a disaccharide (sucrose-Scheme 2):

Scheme 1: Structural procedure for glucose:

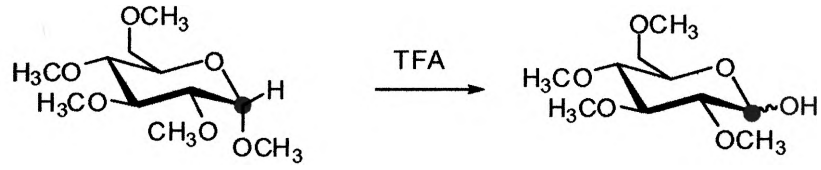
1) Methylation



α-D-glucose

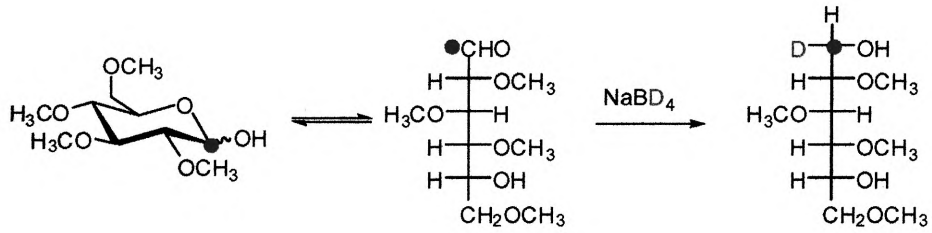
A DMSO/NaOH slurry is used to deprotonate the OH groups and then CH_3I is used for methylation. The red dot represents the anomeric carbon (the alcohol group can occupy two different orientations- up or down).

2) Hydrolysis (only one component since monosaccharide)



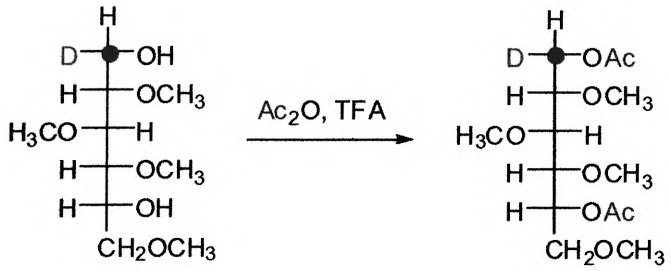
Hydrolysis of the anomeric carbon occurs as a result of trifluoroacetic acid.

3) Reduction(necessary to prevent identical, symmetrical derivatives in GC/MS)



Reduction results in opening of the ring, creation of an aldehyde, and the conversion of the aldehyde to a primary alcohol.

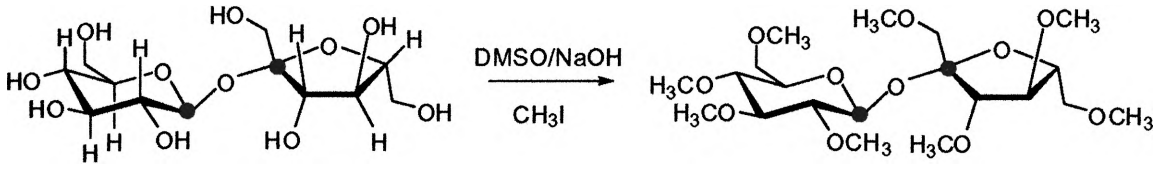
4) Acetylation



The alcohol groups are acetylated by acetic anhydride and TFA.

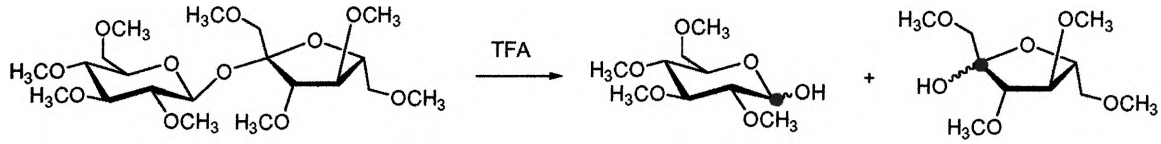
Scheme 2: Structural procedure for sucrose:

1) Methylation



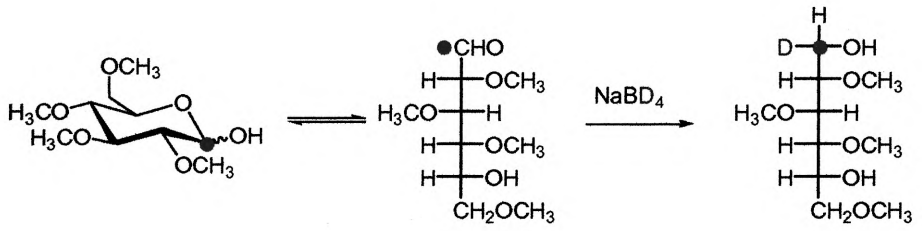
A DMSO/NaOH slurry is used to deprotonate the OH groups and then CH_3I is used for methylation. There are two anomeric carbons associated with a disaccharide.

2) Hydrolysis

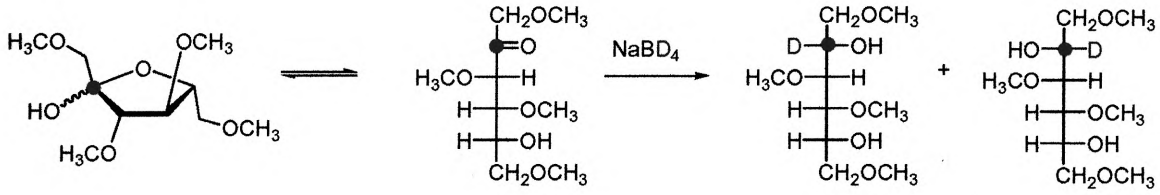


The glycosidic bond is broken using TFA and hydrolysis occurs at the anomeric carbons.

3) Reduction



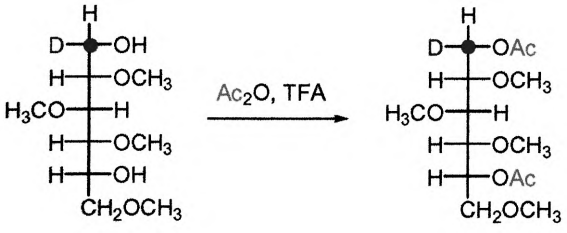
Equilibrium Aldehyde **Reduces to primary alcohol**



Equilibrium Ketone **Reduces to secondary alcohols**

The resulting deuterated products can be easily distinguished with GC/MS.

4) Acetylation



The alcohol groups are acetylated by acetic anhydride and TFA. Each PMAA should be distinguishable with GC/MS due to the differences in positions of the anomeric carbons.

GC/MS is an instrument that chemists use to analyze a complex mixture of volatile compounds. A gas chromatograph first separates the mixture into its individual components and then a mass spectrometer detector is used to conclusively identify these components. The combination of these two techniques allows a quantitative and qualitative analysis of a volatile mixture. In order for a compound to be analyzed by GC/MS it must be sufficiently volatile since it will be introduced into a gas phase and thermally stable since the components will need to remain structurally intact prior to entry into the mass spectrometer.

Gas Chromatography:

The separation of a sample occurs when the sample is injected into the inlet at a high temperature to ensure volatilization. This is followed by introduction into the mobile phase (an inert gas, typically helium). The mobile phase then carries the sample through a capillary column containing a stationary phase to which components are selectively attracted to varying extents (either polar or non-polar). The column temperature can also be altered to aid in the separation of the mixture. The compounds that have lower boiling points will elute or exit the column first while those that have higher boiling points will elute last. As the compounds elute from the column, they enter a detector which indicates the presence of a compound: the greater the concentration of

the compound, the greater the signal. This signal can be measured by determining the peak area of each elutant's gas chromatogram. The time interval between the injection and individual compound detection is called the retention time (t_R). Retention time is useful in determining the identity of a compound; however similar molecular structures may have similar retention times.

Mass Spectroscopy:

As the individual components elute from the GC column, they enter an ionization source in a mass spectrometer. Here components are ionized into charged fragments. The resulting fragments are separated in a mass analyzer and ultimately detected. A total ion chromatogram (total ion current vs. t_R) and a mass spectrum (relative abundance vs. m/z or mass/charge) are produced.

Guiding Research Questions:

1. In what portion of the plant are polysaccharides most abundant?
2. Can PMAAs be successfully produced from *E. purpurea*?
3. Can polysaccharides in *E. purpurea* be identified using GC/MS through linkage analysis?

EXPERIMENTAL METHODOLOGY

The first procedure, the extraction of polysaccharides from *E. purpurea* plant material was adapted from Brovelli (2005).[5] This procedure was used to compare the polysaccharide content in Puritan's Pride herbal supplement (*E. purpurea*-aerial) and a ChromaDex certified botanical standard (*E. purpurea* -powdered root).

I. Determination of Crude Polysaccharide Content in E. Purpurea

A crude extract was prepared by adding 35mL of deionized water to 5g of plant material (Puritan's Pride *E. purpurea* (aerial) "non-irradiated" herbal supplement) then this suspension was incubated in a 90°C water bath for 1 hour. Two samples were run to ascertain reproducibility. Each sample was cooled to room temperature and then centrifuged at 4000rpm for 15 min. The supernatant was filtered using Whitman # 1 filter paper and gravity filtration, then the volume was adjusted to 20 mL with deionized water. Polysaccharide precipitation was induced by the addition of 20mL of 99% ethanol, the samples were vortexed and then refrigerated for 15 min. The sample was then centrifuged for 25 min at 4000 rpm, and the supernatant was discarded. The pellet was resuspended in 8 mL of water. After the addition of 32 mL of 99% ethanol, the sample was vortexed and refrigerated for 15 min. The sample was then centrifuged for 5 min at 4000rpm. The supernatant was discarded, and the pellet was dried in an oven at 65°C for about 15 hrs. The dry mass was then recorded as the crude polysaccharide fraction and expressed as a percentage. [5]

The procedure detailed above was also completed with a ChromaDex certified botanical standard (*E. purpurea* -powdered root). The percent by mass content of polysaccharides was determined and compared to that of the Puritan Pride *E. Purpurea.*

A subsequent procedure as outlined below used the extracted Puritan Pride polysaccharides for conversion into volatile alditol acetates using a combined procedure adapted from Brovelli (2005) [5] and Blakeney (1983) [3].

II. Preparation of Alditol Acetates

A. Conversion of Polysaccharide Pellet into Component Monosaccharides

This step was inadvertently left out of the protocol, but it is necessary to add acid (e.g., HCl) to cleave the glycosidic bond(s) between monosaccharide units.

B. Reduction

The internal standard, *myo-Inositol* was added (0.05mL of a 20mg/mL solution) to the polysaccharide pellet. Next, 2.0g of deuterated sodium borohydride ($NaBD_4$) was dissolved in 100 mL of anhydrous dimethyl sulphoxide (Me_2SO or DMSO) at 100°C. Monosaccharides were reduced for 90 min in a 40°C water bath by adding 3.0 mL of the $NaBD_4$ solution to 0.1 mL of the monosaccharide mixture. After reduction, the excess $NaBD_4$ was decomposed by the addition of 0.1 mL of 18M glacial acetic acid ($C_2H_4O_2$). [3]

C. Conversion of Monosaccharides into Alditol Acetates

0.2 mL of 1-methylimidazole followed by 2.0 mL of acetic anhydride was added to the reduced monosaccharides and mixed. After 10 min at room temperature (~21°C), 5.0 mL of deionized water was added to decompose the excess acetic anhydride. When cool, 1.0 mL of dichloromethane was added and the mixture was vortexed. After the phases had separated, the lower, organic phase was removed with a Pasteur pipette and stored in a 1.0mL septum-cap vial at -20°C. [3]

D. Analysis of Alditol Acetates using GC/MS

The gas flow rate of high purity helium (carrier gas) was set at 1mL/min. The oven temperature was kept for at 190°C for 4 min following injection and then raised at 4°C/min to 230°C, where it was held for 8 min. The injection port and detector were heated to 250°C and 300°C respectively. A 1.0 μL sample was injected for analysis. [3]

III. Preparation of Partially Methylated Alditol Acetates

Further examination of the literature revealed that a useful way to elucidate carbohydrate structure is linkage analysis, accomplished by the production of permethylated and peracetylated derivatives prior to GC/MS. A third protocol was attempted since it provides more information about the structure of the original polysaccharides, which was not available with the previous protocol. This procedure was adapted from Kim et. al (2006).[15] Prior to working with *E. purpurea*, it was decided that establishing the experimental protocol with a less complex carbohydrate was prudent. A monosaccharide (glucose), a disaccharide (sucrose), and an internal standard (*myo*-inositol) were all subjected to the experimental protocol below to ensure that PMAAs could be produced successfully. Once this protocol worked, the procedure would presumably be useful for *E. purpurea*. The internal standard, myo-inositol, was used as a check to insure that the methylation process worked since once it is methylated, it will not be affected by any other steps during the production of PMAAs. Its presence should be easily revealed in the mass spectrum ultimately obtained.

A. Polysaccharide Sample Preparation

300μL of 1.0mg/mL (1000ppm) aqueous polysaccharide solutions (sucrose and glucose) and an internal standard *myo*-inositol were oven-dried at 100°C until a crystal or powder was formed.

B. Preparation of PMAAs

1.3.1 Preparation of NaOH-Me_2SO slurry

400μL methanol and 150μL of 50% NaOH (w/v) were added to a Teflon screw-cap test tube and vortexed until a clear mixture was obtained. 2.0 mL of DMSO was

added, followed by vortexing and centrifugation at 6000rpm for 10 min to pellet the NaOH (an opaque white pellet was formed). The supernant was decanted and the pellet was washed two times with 2.0 mL of DMSO. The pellet was then resuspended in 2mL DMSO.

1.3.2 Methylation

The next steps were conducted quickly in a fume hood to minimize the introduction of moisture into the samples. 100μL of DMSO was added to each of the samples. The samples were then vortexed until a clear mixture was obtained. 500μL of the NaOH slurry was added, then vortexed until clear. 100μL of CH_3I was added and the samples were vortexed intermittently for 10 minutes until a clear solution remained. Samples were then dried using nitrogen gas to remove excess CH_3I.

1.3.3 Sample clean-up

The samples were partitioned with 2.0mL deionized H_2O and 2.0 mL CH_2Cl_2 using a separatory funnel to collect the bottom organic layer. The samples were then washed 3 times with deionized water, and the final organic phase was dried with nitrogen gas.

1.3.4 Hydrolysis

250 μL of 2M (mol/L) CF_3CO_2H (TFA) was added followed by incubation in a 121°C oven for 2 hrs. The samples were brown in color after the 2 hour incubation at 121°C. The samples were dried, followed by two additional dryings with a few drops of methanol.

1.3.5 Reduction

Samples were reduced with 0.1mL of 10mg/mL $NaBD_4$ for 1 hr. at room temperature. The reduction was quenched with one drop of glacial acetic acid and then dried. Since glacial acetic acid is not very volatile, it took about 4 hrs. to dry each sample. The samples were dried three more times with the addition of a few drops of methanol, followed by evaporation under a stream of nitrogen.

1.3.6 Acetylation

100μL of acetic anhydride (AC_2O) and 80μL of 2M TFA were added to each sample, followed by a 10-min incubation at 50°C. 0.5 mL of acetone was added, and the samples were dried. The samples were then dried an additional two times with a few drops of acetone, followed by evaporation under nitrogen. The samples were then cleaned up by partition as previously described in section 1.3.3, and the final organic phase was dried. The final samples were solubilized in 2.0 mL of CH_2Cl_2 and 1.0 μL of the sample was injected into the GC-MS.

1.4 Gas chromatography-mass spectrometry (GC-MS) instrumental parameters

A Thermo-Electron capillary GC column (15m x 0.25 id x 0.25μm, 5% diphenyl) was used in this experiment. Helium was the carrier gas at a constant flow rate of 1mL/min. The oven conditions were an initial temperate of 50°C held for 2 min, 30°C/min to 150°C, 3°C/min to 220°C and finally 30°C/min to 300°C for a 10 min bakeout. The inlet temperature was kept constant at 250°C, and the MS transfer line was set at 300^0C. MS acquisition parameters were set up to scan from m/z 50-550 in the electron impact (EI) mode for routine analysis. Peak assignments were made based on retention times and mass spectra with the aid of a NIST mass spectral library.

RESULTS AND DISCUSSION

Determination of Crude Polysaccharide Content in E. Purpurea

The aerial material of *E. purpurea* contains 7.1% polysaccharides, while the root only contains 0.12%. Since polysaccharides are believed to be immunologically active, the aerial part of the plant is generally used for herbal supplements, although many herbal supplements also use a combination of the aerial and root material. These values agree with literature, stating that polysaccharide content found within the aerial part of the plant is greater than the roots.[10]

Analysis of Alditol Acetates using GC/MS

A TIC (total ion chromatogram) and MS (mass spectrum) were obtained from the analysis of alditol acetates from Puritan's Pride *E. purpurea.* It was expected that mass fragments characteristic of alditol acetates would be seen. A NIST library search that is part of the XCalibur software package for our Thermo-Electron Trace GC/Focus DSQ instrument was performed for each peak of interest obtained in the TIC. The solvent peaks from the procedure II GC/MS spectrum (figure 1) were identified as methylene chloride (t_R = 1.64 min) and DMSO (t_R =3.60 min).

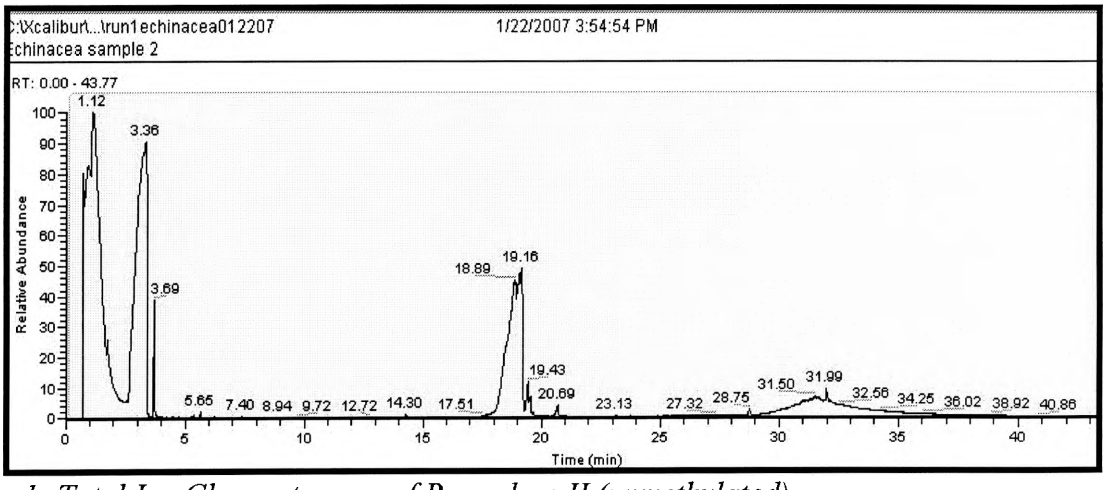


Figure 1: Total Ion Chromatogram of Procedure II (unmethylated)

The peak with a t_R of 19.01 min was identified as myo-inositol hexaacetate, the internal standard after acetylation, as a result of a NIST library search. This peak indicates that the acetylation step was somewhat successful, but may not have been complete after the 10 min incubation period. The structure of myo-inositol hexaacetate is shown in figure 2.

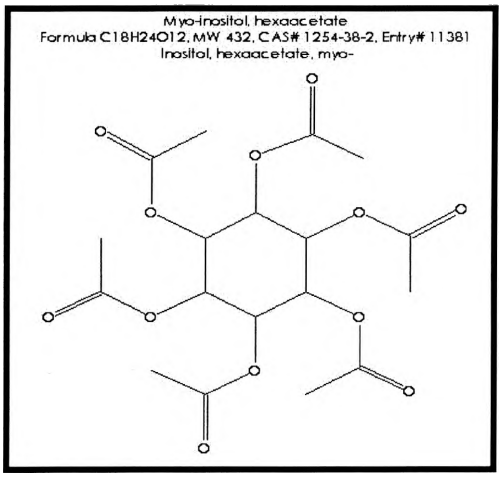


Figure 2: Acetylated myo-Inositol (myo-inositol hexaacetate)

Figure 3 demonstrates the similarities between the MS of the myo-inositol hexaacetate peak from the procedure II sample and the NIST standard, similarities are circled in red.

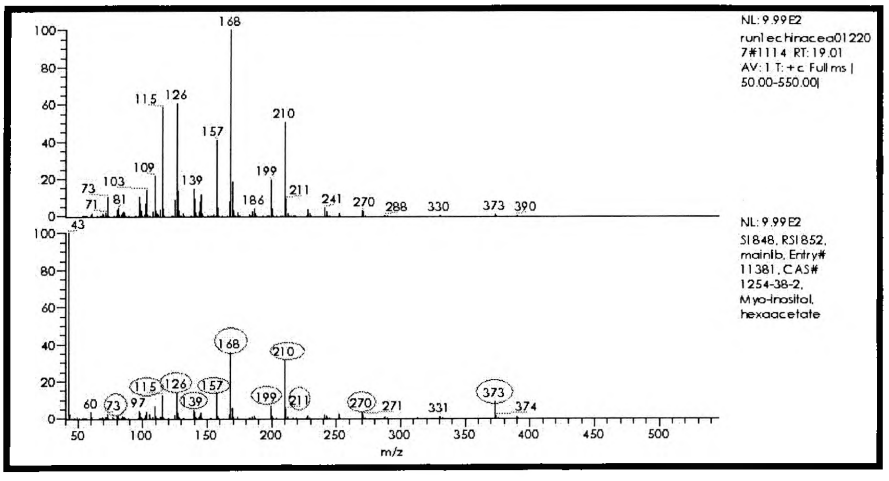


Figure 3: Procedure II (Unmethylated) sample vs. NIST library match MS of myo-inositiol hexaacetate

None of the expected alditol acetates were produced from Puritan's Pride E. Purpurea as evidenced from the total ion chromatogram (Figure 1).

GC/MS Analysis of Partially Methylated Alditol Acetates

Inspection of the TIC and MS of glucose, sucrose, and myo-inositol revealed only the presence of the solvent peak (CH_2Cl_2). This clearly indicates that the expected PMAAs were not produced in any case. It was noticed that during the production of PMAAs, dark brown products resulted from the hydrolysis step. This is usually indicative of degradation and loss of the desired product. All steps prior to this one revealed a clear solution or white crystals. Figure 4 demonstrates the solvent peak for methylene chloride.

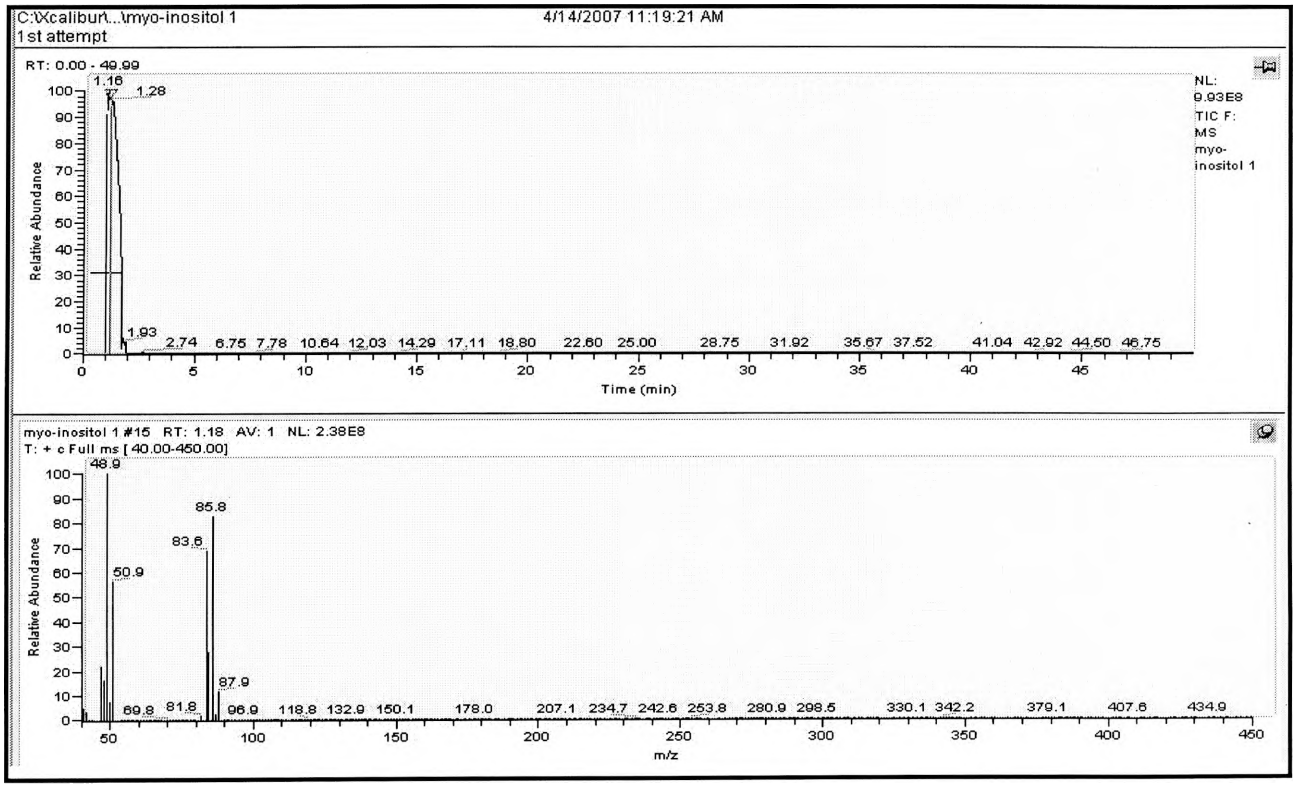


Figure 4: Total Ion Chromatogram and Mass Spectrum: Procedure III (methylated)

The identity of the solvent peak at t_R=1.16 was verified using the NIST library, with a 92% probability of being methylene chloride. The main fragments for methylene

chloride should be 46, 48, 49, 51, 84, 86, and 88. The resulting mass spectrum revealed fragments due to the solvent at 49, 51, 84, 86, and 88.

Future Research:

If more time were allotted for this project, the second procedure could be tried again, adding in a methylation and hydrolysis step with just sucrose first prior to working with *E. purpurea*, to ensure the successful production of PMAAs. The sample size could also be scaled up to ensure that enough of the intended product from each step is produced. Once PMAAs are successfully produced, the reaction could be scaled back down to a smaller sample size as described in the literature and followed in procedure three to ensure the successful recovery of PMAAs. Once PMAAs are successfully produced, then the procedure should be attempted again with polysaccharides isolated from the E. purpurea aerial herbal supplement (Puritan's Pride). After each step, TLC (thin-layer chromatography) or NMR (nuclear magnetic resonance spectrometry) could be used to ensure the completion of each of the desired reactions. The third procedure could also be attempted again with a decrease in the oven temperature to hopefully prevent the decomposition (brown coloration) from occurring during the hydrolysis reaction. Once the PMAAs are successfully produced, GC/MS instrumental conditions should be optimized for detection of said PMAAs. After successful identification of the polysaccharides in *E. Purpurea*, the polysaccharides can be tested individually to determine their immunological response in cell cultures. If one polysaccharide is, then it can be isolated and used for possibly treating upper respiratory infections, colds, and the flu virus.

Acknowledgements:

I would like to thank Dr. Gannicott, Dr. Freier and Dr. Cowden for all their support and encouragement throughout my research; Dr. Reeve for explaining the organic chemistry behind this procedure; the Harold H. Garretson Undergraduate Research in Chemistry Fund for supplies and the purchase of the new GC/MS; the School of Sciences for their continual support, funding, and patience with me during my long evenings and weekend research time.

Literature Sources:

1. Bauer, R. (1998) *Echinacea*: biological effects and active principles. In *Phytomedicines of Europe: Chemistry and Biological Activity*. Lawson, L.D., Bauer, R., Eds. ACS Symposium series 691, American Chemical Society: Washington, DC, 140-157.

2. Bergeron, C., Gafner, S., Batcha, L. L., Angerhofer, C.K. (2002) Stabilization of caffeic acid derivatives in *Echinacea purpurea* L. glycerin extract. *Journal of Agriculture and Food Chemistry*, 50, 3967-3970.

3. Blakeney, A.B., Harris, P.J., Henery, R.J., Stone, B.A. (1983) A simple and rapid preparation of Alditol acetates for monosaccharide analysis. *Carbohydrate Research*, 113, 291-299.

4. Blumenthal, M., Golberge, A., Brinckmann, J. (2000) Herbal Medicine: Expanded Commission E Monographs. Intergrative Medicine Communications, Newton, MA. 88-102.

5. Brovelli, E.A., Rua, D., Roh-Schmidt, H., Chandra, A., Lamont, E., Noratto, G.D. (2005) Human gene expression as a tool to determine horticultural maturity in a bioactive plant (*Echinacea purpurea* L. Monench). *Journal of Agriculture and Food Chemistry,* 53, 8156-8161.

6. Calis, I., Saracoglu, B.I., Sticher, O., Ruedi, P. (1990) Phlinosides A, B, and C, three phenylpropanoid glycosides from *Phlomis linearis*. *Phytochemistry*, 29, 1253-1257.

7. Classen, B., Mau, S.L., Bacic, A. (2005) The arabinoglaactan-proteins from pressed juice of Echinacea purpurea belong to the "hybrid" class of hydroxyproline-rich glycoproteins. *Planta Med*, 71, 59-66.

8. Classen, B. Witthohn, K., Blaschek, W. (2000) Characterization of an arabinoglactan-protein isolated from pressed juice of *Echinacea purpurea* by precipitation with the β-glucosyl Yariv reagent. *Carbohydrate Research*, 327, 497-504.

9. Dlaby-Brown, L., Barsett, H., Landbo, A.K.R., Meyer, A.S., Molgaard, P. (2005) Synergistic antioxidative effects of alkamides, caffeic acid derivatives, and polysaccharide fractions from *Echinacea purpurea* on in vitro oxidation of human low-density lipoproteins. *Journal of Agriculture and Food Chemistry*, 53, 9413-9423.

10. Echinacea-Complementary Medicine. University of Maryland Medical Center. (2006). http://www.umm.edu/altmed/articles/echinacea-000239.htm

11. Egert, D., Beuscher, N. (1991) Studies on Antigen Specifity of immunoreactive arabinoglactan proteins extracted from *Baptisia tinctoria* and *Echinacea purpurea*. *Planta Med*, 58,163-165.

12. Hakomori, S.I. (1964) A rapid permethylation of glycolipid, and polysaccharide catalyzed by methylsulfinyl carbonion in dimethyl sulfoxide. *The Journal of Biochemistry*, 55, 205-208.

13. Harris, P.J., Henery, R.J., Blakeney, A.B., Stone, B.A. (1984) An improved procedure for the methylation analysis of oligosaccharides and polysaccharides. *Carbohydrate Research*, 127, 59-73.

14. Hobbs, Christopher. Echinacea: From Native American Panacea to Modern Phytopharmaceutical. 1998. www.christopherhobbs.com/website/library/articles/article_files/echinacea_01.html

15. Kim, J et. Al. (2006) Addition of glycerol for improved Methylation linkage analysis of polysaccharides. *Carbohydrate Research*, 341, 1061-1064.

16. Luettig, B., Steinmuller, C., Gifford, G.E., Wagner, H., Lohmann-Matthes, M.L. (1989) Macrophage activation by the polysaccharide arabinogalactan isolated from plant cell cultures of Echinacea purpurea. *Journal of the Nation Cancer Institute*, 81, 669-675.

17. Mulsliarakathbacker, J. et al. (2004) Glycosyl composition of polysaccharide from *Tinosporoa cordifolia*. II. Glycodyl linkages. *Acta Pharm*, 54, 73-78.

18. Mazza, G., Cottrell, T. (1999) Volatile components of roots, stems, leaves, and flowers of *Echinacea* species. *Journal of Agriculture and Food Chemistry*, 47, 3081-3085.

19. Rossi, S.A., ElSohly, M.A. (1996) The Volatile Oil Composition of Fresh and Air-Dried Buds of *Cannabis sativa*. *Journal of Natural Products*, 59, 49-51.

20. Sharma, M., Arnason, J.T., Burt, A., Hudson, J.B. (2006) Echinacea extracts modulate the pattern of chemokine and cytokine secretion in rhinovirus-infected and uninfected epithelial cells. *Phytother. Research*, 20, 147-152.

21. Wagner, H., Stuppner, H., Schafer, W., Zenk, M. (1988) Immunologically active polysaccharides of *Echinacea purpurea* cell cultures. *Phytochemistry*, 27, 119-126.