MEASUREMENT AND CONTROL STRATEGIES FOR STEROL GLUCOSIDES TO IMPROVE BIODIESEL QUALITY – YEAR 2

Final Report

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University of Idaho

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16. Abstract

This project had the objective of measuring trace compounds in biodiesel called sterol glucosides (SG) so strategies to reduce their concentration could be investigated. A MALDI-TOF-MS (matrix assisted laser desorption ionization time of flight mass spectrometry) analytical method for rapid evaluation of sterol glucosides has been developed and validated. Sitosteryl-glucoside, campesteryl-glucoside, and stigmasteryl-glucoside were identified in residues that temporarily shut down the Inland Empire biodiesel plant in Odessa, Washington. Standards were created by purification and recrystallization of these problematic residues. The standard's purity was validated with mass spectrometry (MS) and nuclear magnetic resonance (NMR) measurements. Preparative methods are necessary to isolate the trace concentrations of acylated sterol glucosides (ASG) and SG from triglycerides and phospholipids in oilseed extracts. We have evaluated several preparative procedures, a modified silica gel chromatography procedure, and developed a size exclusion chromatography (SEC) preparative method for evaluation of glycolipids with an emphasis on SG. These preparative methods were evaluated using this MS analytical technique. SEC was selected as the best preparative procedure for evaluating tetrahydrofuran (THF) oilseed extracts. A winter canola seed sample (*Brassica. napus L. cv.* Amanda) was analyzed.

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INTRODUCTION

In September 2005, the state of Minnesota instituted a 2% biodiesel mandate. Shortly after starting, widespread cold flow problems required two moratoriums. The failures were ultimately attributed to one producer with quality control problems. However, some of the problems could not be explained by poor fuel quality. In these cases, the vehicle fuel filters were plugged even though the fuel used met all specifications. Plant based sterol glucosides (SG) were found in these residues. These discussions were conducted in closed sessions, so documentation is limited.

In the fall of 2009, eastern and central Oregon also experienced problems with cold flow properties with fuel that met all specifications. This occurred shortly after instituting a 2% biodiesel mandate. Prior to the biodiesel mandate, problems were reported with the incremental transition to an ultra low sulfur (15ppm) diesel fuel standard requiring full implementation in 2010. The time of the failures coincided with an abnormally rapid temperature drop.

Multiple contaminants have been identified in blocked filters in otherwise high quality fuel. Currently, the only reliable method to prevent cold flow problems is to winterize the fuel. In this process, the fuel is cooled and sufficient time is allowed for crystallization events to occur. Then, the fuel is filtered to remove any non-liquid materials in the fuel. This is expensive, time consuming, requires specialized infrastructure, and high energy input for fuel production. During the course of this research project, a new American Society for Testing and Materials (ASTM) procedure similar to the winterization process was added to detect if any cold-precipitated residues will block a filter.

The biodiesel industry assumes that SG present in neat biodiesel is the source of the problem. Sterol glucosides may act as seed crystals or agglomeration centers where contaminants can accumulate. Dr. Robert Moreau's team at the United States Department of Agriculture (USDA) has identified sitosteryl-glucoside and campesteryl-glucoside in neat bulk biodiesel tanks and filter residues.[1]

Most occurrences of filter failure are difficult to trace back to the fuel source, oil processor, or oilseed crop. Acylated steryl glucosides (ASG) as shown in Figure 1 are present in plants and vegetable oils. The industry assumes that ASG are cleaved and esterified under anhydrous conditions during biodiesel production, forming SG like those shown in Figure 2. The hexane solvent extraction system is optimized to obtain maximum levels of triglycerides from oil feedstocks. The extraction efficiency of SG or ASG may change due to process or agronomic conditions, and this might be a way to control the level of SG in the oil and ultimately in the biodiesel.

The actual level of ASG and SG present in oilseeds is not known for most oilseeds. The majority of studies report the total phytosterol content of oilseeds. There are limited analytical techniques for the evaluation of SG and ASG.[2,3] In literature, the data available for phytosterol compounds has been obtained by extraction, multistep workup and derivatization, and evaluation with gas chromatography mass spectrometry (GC-MS).

The University of Idaho (U of I) research team has developed an analytical method for the evaluation of SG. The goal of the project was to determine the levels of ASG and SG in agronomically significant oilseeds of the Pacific Northwest (PNW). With this information the industry can make more informed decisions about how to minimize low temperature problems when using biodiesel in the PNW.

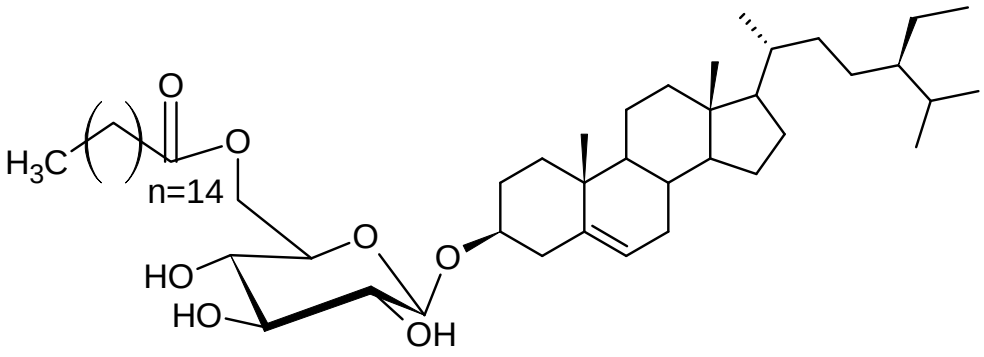


Figure 1: Acylated β-sitosteryl glucoside (palmitic) $C_{51}H_{90}O_7$.

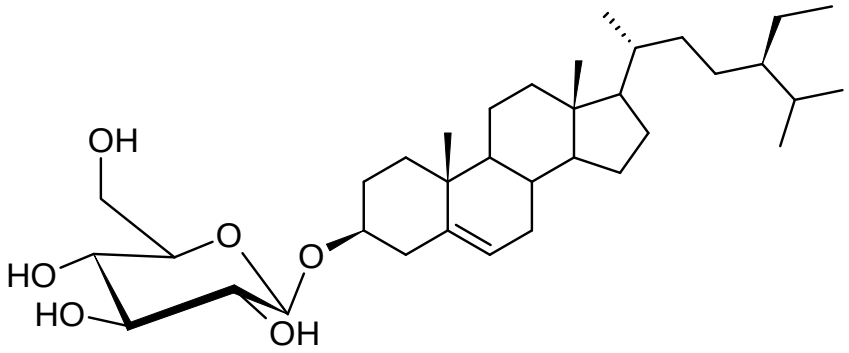


Figure 2: β-sitosteryl glucoside $C_{35}H_{60}O_6$.

Acylated sterol glucosides naturally occur in plant tissues. Acylation is typically assumed to occur at the 6th position of the glucose (see Figure 1). However, South Korean researchers have demonstrated that in tree extracts, the 4th position was acylated.[4] ^{1}H NMR and ^{13}C NMR will likely be required to positively identify the structures. For a pure analyte, a sweet of nuclear magnetic resonance (NMR) spectroscopy techniques can show the intermolecular relationship between protons and carbons, providing structural elucidation. This requires the isolation of purified analytes. This was beyond the scope of this initial research project. It's important to note that the MS data presented in this paper does not distinguish stereo centers. There are multiple stereo centers present in the three SG identified.

Glycolipids are natural products with carbohydrate and lipid moieties. SG and ASG fall into the class of glycolipids. Yamauchi evaluated glycolipids in red bell peppers using a technique called high performance liquid chromatography atmospheric pressure chemical ionization mass spectrometry (HPLC-APCI-MS).[5] The glycolipids fraction of the extract underwent further separation with the HPLC prior to analysis with MS. Prior to MS the analytes of interest must be ionized. HPLC-MS instruments are commonly fitted with an Electrospray ionization (ESI) source. APCI is an alternative ionization method that for SG achieves higher ionization efficiency. Yamauchi showed that a distribution of fatty acids were found in ASG fatty acid moieties including palmitic 16:0, stearic 18:0, linoleic 18:2, α-linolenic 18:3. Additionally, the two ASG sterol moieties: campesterol and β-sitosterol were also identified.[6] In Figure 1, the fatty acid moiety depicted is palmitic acid.

VEGETABLE OIL PRODUCTION IN NORTH AMERICA

The following oils are produced in significant quantities in North America: Soya, Argentine canola, Polish canola, sunflower, safflower, flax, almond, walnut, peanut, cotton seed, mustard, camelina, grape seed, meadowfoam, rapeseed, palm (Mexico), and Jatropha (preproduction Mexico). However, vegetable oil production in North America is dominated by soya oil and canola oil.

In the Midwest, soya is typically grown in rotation with corn. Soybeans are a legume (nitrogen fixer). This organic nitrogen is bioavailable for the following crop, reducing the requirement for inorganic nitrogen-based fertilizers. Soybean meal and its protein concentrates are used for animal feed. The meal is heated during oil extraction and toasted after being defatted. This is done to denature and inactivate a group of mainly proteins that act as trypsin inhibitors. In particular these inhibitors reduce weight gains in swine and poultry.[7] To make high protein concentrates, residual oligosaccharides are removed from defatted soya meal flours by an aqueous ethanol wash or acidified water.[8] These trypsin inhibitors are only present in soya and this is a major advantage of canola meal.

The majority of vegetable oil produced in the United States and Canada is processed via seed extraction, chemical refining, bleaching, deodorization, and filtration. This process starts when cleaned seed is run through a two-step mechanical extrusion press, flaked, and then the macerated seed tissue is subsequently solvent-extracted with food grade hexane. The hexane is recovered from this extract and reused.

The raw triglyceride oil is then refined. This may consist of two steps. The oil can be degummed by reacting with concentrated phosphoric acid and centrifuged to remove the precipitated gums. This degumming step strips the oil of phospholipids, solid particulates, and heavy metals that may be present in the oil. A lecithin product may be produced out of these phospholipids. The second refining step is neutralization. The oil is washed with caustic soda (NaOH); this base reacts with any free fatty acids in the oil producing soap stock. The soap stock is of low value and typically ends up in animal feed for disposal.

After refining, the heated oil is run through bleaching clays. The loading, exact composition, activity, temperature, and exposure time of this step affects the final color of the oil; although these effects are not visible until after deodorization. This step removes any other components that are not soluble in the oil.

The oil is then run though deodorization columns. These large vacuum columns fractionate the oil and remove volatile compounds from the oil. Superheated steam is injected into the column to facilitate stripping of free fatty acids and pesticides during deodorizing. The hot oil finally runs through fine filtration and is distributed to the subsequent user.

Depending on government incentives, significant quantities of canola/rapeseed and soya are converted into biodiesel at plants in North America. These plants may be situated at existing oil crushing/refining infrastructure or at destination locations. Some biodiesel producers crush some of their own vegetable oil and purchase the remainder. These two are typically run as independent businesses. This industry has grown to satisfy consumer and governmental incentives based on demand for green alternative biofuels.

Biodiesel (fatty acid methyl ester-FAME) production is a relatively simple chemical process. Triglycerides (vegetable oils/animal fats) are reacted with strong alkoxide base in a methanol solvent, forming methyl esters and glycerin. Water has to be eliminated from the system to prevent solvent leveling. If water is present in the system, the reaction is limited by solvent leveling to the aqueous pH system where the strongest basic ion formed is the OH^- species. This causes the reaction to proceed poorly and excessive soap formation occurs. In nonaqueous solvent systems lacking water significantly stronger acid and basic species are generated. When methanol is used as a solvent stronger basic species are generated CH_3O^- due to the deprotonation of methanol in anhydrous sodium alkoxide. The reaction normally proceeds via a S_N2 (backside attack) reaction mechanism. Commonly, in commercial systems, the reagents are heated to just below the boiling point of methanol with the goal of reducing the reaction time.

IDENTIFICATION OF SG IN INDUSTRIAL BIODIESEL FACILITY

Figure 3: Crude residue that shut down Inland Empire Oilseeds, LLC.

Our research team has worked with a local biodiesel producer, Inland Empire Oilseeds, LLC, and purified a residue that built up throughout their biodiesel plant. This crude residue is shown in Figure 3. This problematic residue resulted in the shutdown of the plant for over two weeks, and caused significant difficulty in restarting the plant. A railcar of refined and bleached (RB) canola oil was being processed. This oil was not deodorized. At the time of offloading the oil, a slight increase over normal turbidity was observed. Shortly after reacting the oil through the standard base catalyzed process, problems occurred. The residue built up systemically, depositing in piping, tanks, and the ion exchange resin towers. Our team was contacted shortly after this problem occurred. The crude and purified residues have been evaluated using a variety of analytical techniques. The target natural products have been identified in these residues.

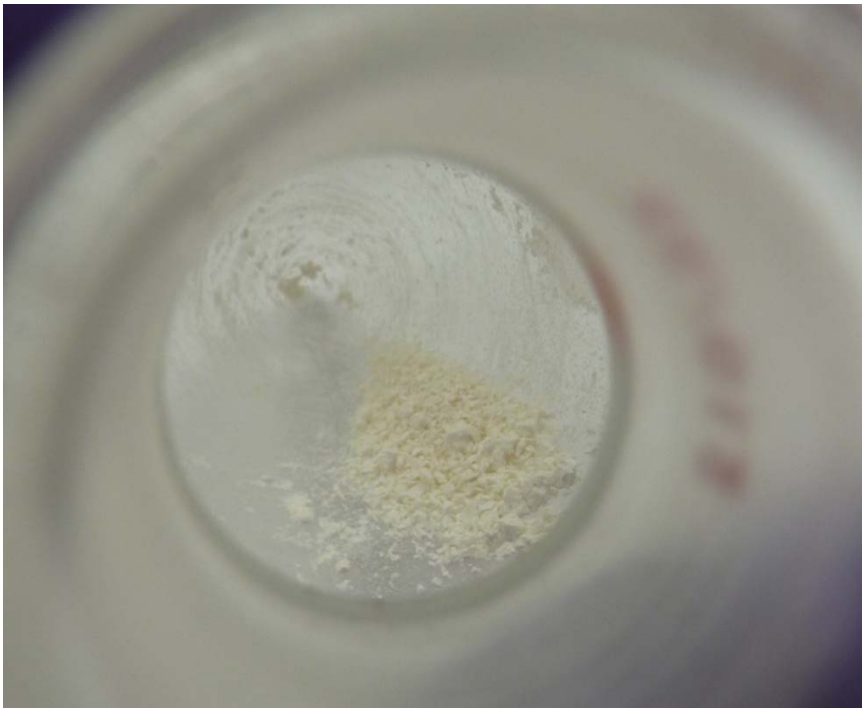


Figure 4: Purified white powder from the problematic residue at the Inland Empire biodiesel plant.

The Purified White Powered Residue

The crude SG based residue from the Inland Empire biodiesel plant was purified for use as a standard. The following process was used. The residue was slurried with freshly distilled hot THF. Magnetic particles were removed with a Teflon stir bar, as this slurry was vacuum filtered through a glass frit. The crude product was rapidly precipitated out of solution with temperature reduction as the solvent was rapidly vaporized through a glass frit and the vapors removed by vacuum. The collected precipitate was subsequently washed twice with aliquots of fresh THF, centrifuged and the supernatant was pipetted off with yellow color bodies. Then, it was washed twice with aliquots of anhydrous ethanol, centrifuged and the supernatant was pipetted off. Finally, it was vacuum dried at 50mbar and 40ºC for over 12 hours. After determining the high purity of product, the process was repeated with a large quantity of residue for use as standards for the remainder of the analytical work. The purity of these standards were validated with MS and NMR techniques. The NMR techniques used are beyond the scope of this report but will be included in the author's thesis.

ANALYTICAL WORK UP OF RESIDUE

Soft ionization techniques allow the identification of large molecular weight compounds like the natural products of interest ASG and SG. Soft ionization methods, like APCI and MALDI, produce high populations of unfragmented parent ions or adducts as opposed to hard ionization techniques which produce spectra dominated by fragments. With a sufficient population of a parent ion or adduct, the species can be further evaluated with Mass Spec/Mass Spec (MS/MS). Using a collision cell where parent ions are subjected to a high voltage producing fragments, those fragments can be evaluated with MS/MS. In MS/MS, the controlled generation of multiple daughter ions from these parent species allows for fingerprinting and determination of chemical substituents. Because it breaks molecules into fragments, mass spectrometry with hard ionization techniques is limited to spectra dominated by fragmentation patterns. For example, with phytosterols, a characteristic fragment can be related to a specific steroid moiety. However, with only the MS from only one characteristic fragment, it is difficult to discriminate sterol ester (SE), sterol glucoside (SG), acylated sterol glucoside (ASG), steryl ferulate (SF), and free sterols (FS) from the MS alone. Gas chromatography mass-spectrometry (GC-MS) methods for analyzing these compounds rely on complicated workup methods to isolate individual phytosterol compounds so each class of compounds requires its own sequential analysis.

In any sample workup, there are losses and efficiencies. If an analyte of interest can be ionized, it can be directly analyzed with a soft ionization technique. If analytes are ionized, they can be discriminated by their parent ions/sodium adducts and further evaluated using MS/MS to provide a high certainty of identification. An additional technique the author has used is comparison of predicted isotopic abundance patterns to the observed patterns. For the characteristic SG sodium adducts observed with MALDI-TOF-MS isotopic abundance peaks were observed. A noise peak or contamination peak does not show the same isotopic abundance pattern and this allows for rapid screening.

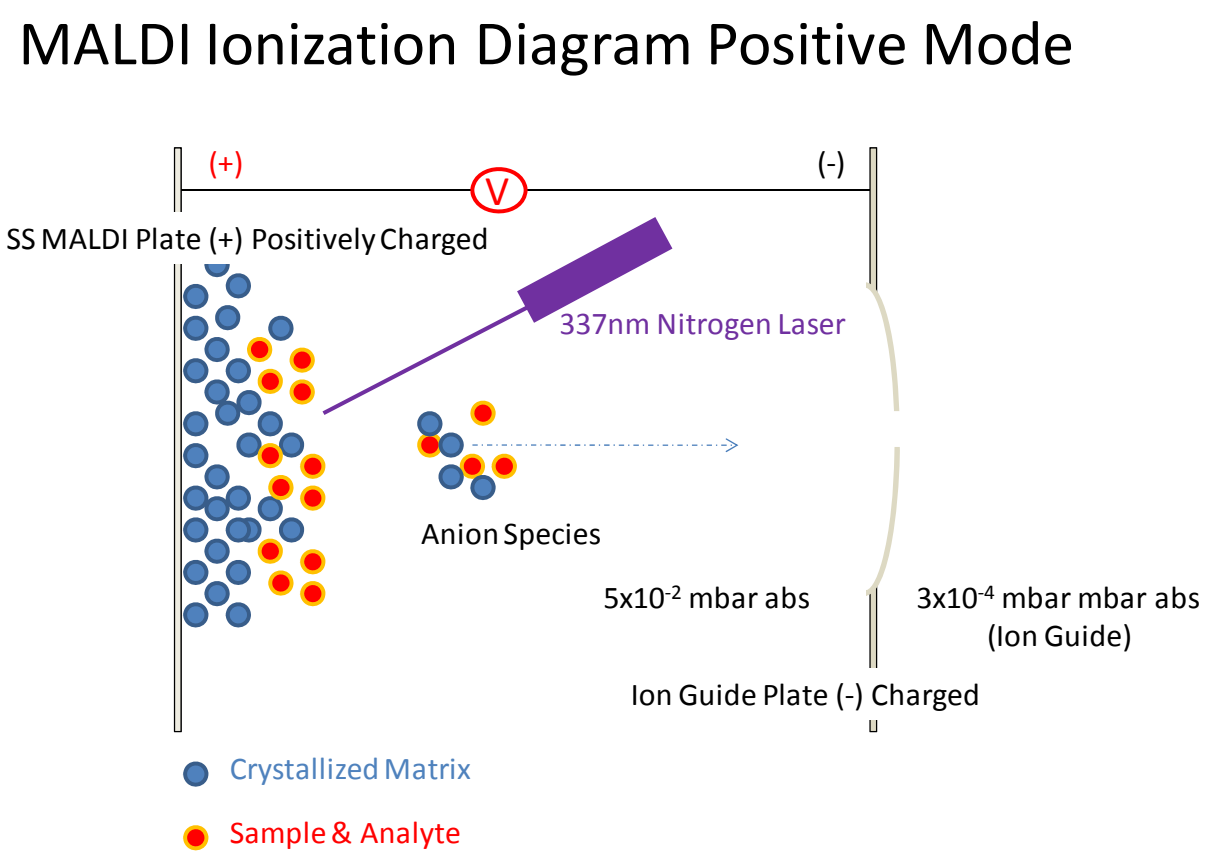


Figure 5: Visual depiction of MALDI ionization source in positive mode analyzing for anions, using the thin film technique.

The soft ionization used for this research was achieved with a technique called matrix assisted laser desorption ionization time MALDI. Figure 5 depicts a visualization of this ionization process. Deposited on a stainless steel target plate, crystallized matrix and sample is positively charged with respect to the stainless steel ion guide plate. Under $5x10^{-2}$ mbar absolute vacuum conditions, a laser is fired on the crystallized matrix material in direct contact with the sample. As the matrix is ionized, some of the analytes and sample are also ionized. Then, the ionized species are pulled by pressure variations and a driving voltage into the ion optics of the instrument. This pressure drop is on the order of $5x10^{-2}$ mbar in the ionization chamber eventually dropping to $6x10^{-7}$ mbar in the time of flight (TOF) detector. The mass separation and detection is conducted in the MS region of the instrument. Only the ionized species can be directed by the magnetic optics of the instrument. A TOF detector has very high mass resolution; however, it is prone to mass saturation. Resolution and resolving

power is a technical term that relates to the ability of a spectroscopy technique to distinguish discrete signal peaks. If the TOF reaches mass saturation, the detector signal plateaus. Figure 5 depicts positive ionization mode for the detection of anions. Changing the polarity to negative ionization mode allows the detection of cations. The instrument's detector reports species on a mass to charge ratio (M/Z) scale. For species with only one charge unit these correspond to Daltons (Da). Theoretical isotopic masses are also calculated in Daltons. The spectrum for multiply charged species is significantly more complicated to interpret.

The mass spectroscopy work was conducted with a Waters MALDI-Q-T Premier instrument. A thin film technique was used, where the matrix and then the samples were spotted on the stainless steel MALDI plates. First, one microliter of matrix consisting of 3.1mg/ml 2-(4-Hydroxyphenylazo) benzoic acid (HABA) dissolved in methanol is spotted. Second, after the matrix evaporates to dry crystals one microliter of sample is spotted. The matrix solvent was changed to THF after initial work. For all but Figure 7, the solvent used was THF. This change reduced the evaporation time and improved consistency in signal which is attributed to more uniform crystal formation. The ease of spotting also increased due to more desirable surface tension properties. A polyethylene glycol oligomers PEG 600 lock mass standard with 3,5-dihydroxybenzoic acid (DHB) matrix was spotted to center wells. PEG 600 is a group of oligomers that monomer units are joined in discreet units. They are commonly used as a standard in high-resolution mass spectroscopy. See Appendix A for the lock mass procedure. This procedure allows for accurate mass correction for the instrument without contaminating the samples. A complete description of the spotting procedure and instrument conditions is included in the appendix.

An example MALDI plate is displayed in Figure 6. Samples have already been spotted on the matrix and lock mass standards spotted in center wells. Several of the wells have been evaluated, and a spiral pattern caused by laser ablation during firing can be observed on D2, D3, and D4 wells. The bottom most row H has not been spotted and is blank.

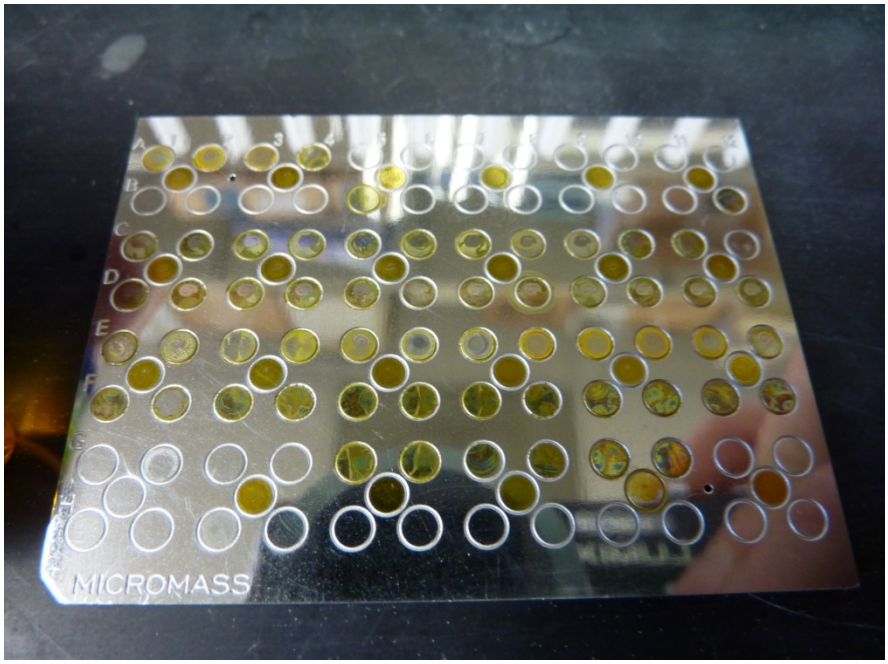


Figure 6: Example of a stainless steel MALDI sample plate.

The stainless steel sample plate is inserted into a vacuum chamber in the instrument. Under an absolute $5x10^{-2}$ mbar vacuum, a pulsing nitrogen laser with an emission max of 337nm excites the matrix and analyte ions for subsequent MS or MS/MS. The collision cell voltage was set to 5KEV, and the mass to charge ratio measured in the range of 200-2000m/z. The preliminary work has been presented orally at the American Oil Chemistry Society (AOCS).[9]

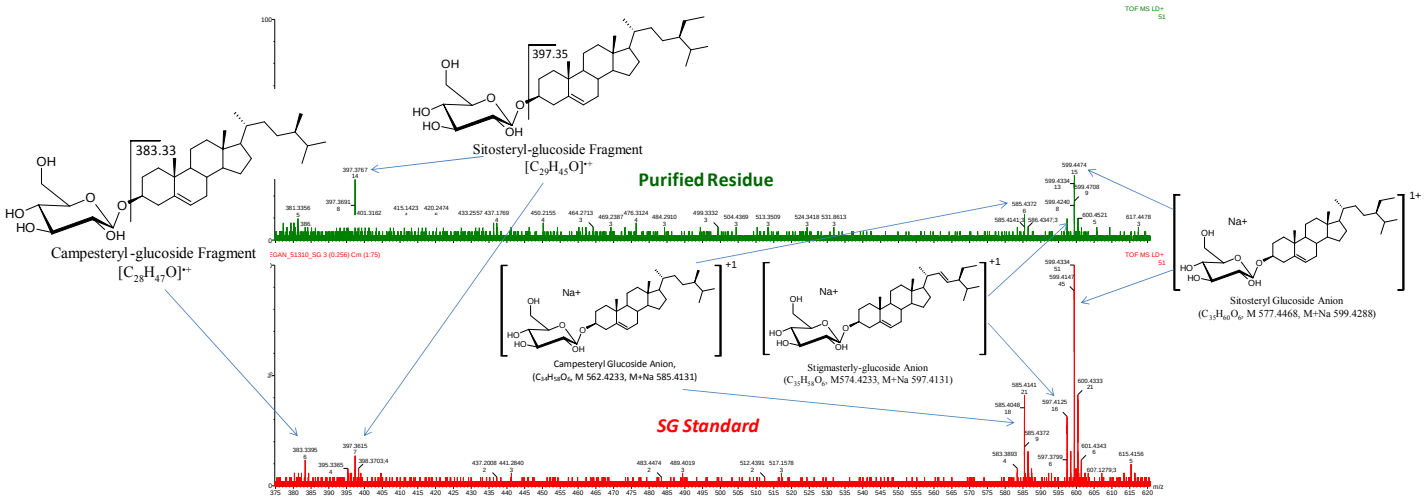


Figure 7: MALDI-TOF-MS of (Matreya >98%) SG standards and purified residue, sodium adducts of sterol glucosides - sitosteryl-glucoside and campesteryl-glucoside, and characteristic fragments in the range of 370-630m/z.

The target natural products have been identified in these residues. Sitosteryl-glucoside, campesteryl-glucoside, and stigmasteryl-glucoside have been positively identified using a novel MALDI-TOF-MS technique. This soft ionization technique has produced sodium adducts and characteristic fragments as seen in Figure 7. These spectra match well to SG standards (Matreya >98%) and reported fragmentation patterns in the literature where APCI-MS atmospheric pressure chemical ionization mass spectrometry has been used to form ammonium adducts and fragments.[1] The three sterol glucosides (SG) detected are common in plants with only minor structural differences in the steroid moieties. See Appendix B for the full MS spectrum.

BULK PROPERTIES OF PURIFIED STEROL GLUCOSIDES

To the best of our knowledge, due to the difficulty in purification, these may be the first reported bulk properties of sterol glucosides. Morris reports infrared absorption spectra for β-sitosterol-D-glucoside purified from defatted peanut flour.[10]

Fourier Transform Infrared Spectroscopy (FTIR)

A Fourier transform infrared spectroscopy (FTIR) study of the purified SG powder was run. A Fourier transform (FT) is an algorithm that converts the sinusoidal data into discrete units. Infrared spectroscopy (IR) is a nondestructive technique that works by measuring the reflectance or absorbance of a sample of a broadband light source. These absorption bands correspond to specific molecular vibrational modes that can be correlated to discreet functional groups. These absorption bands can be used to identify the presence of specific chemical moieties, for example hydroxyl groups.

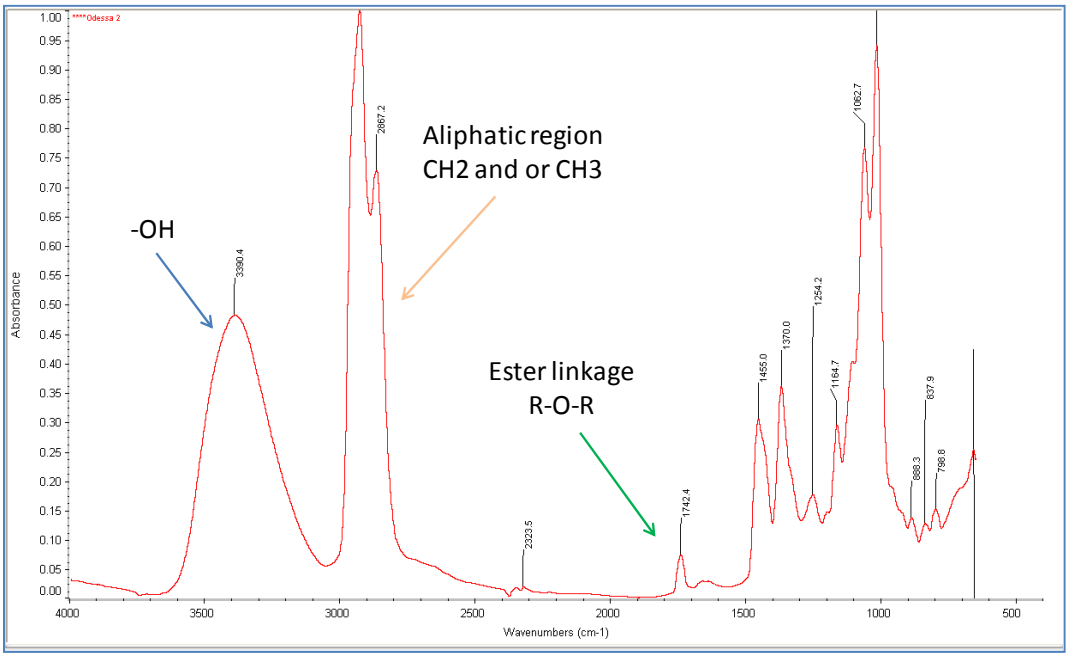


Figure 8: FTIR spectrum of purified residue confirms SG functional groups.

The powdered and purified sterol glucoside sample was put into a Nicolet Avatar 370 with an attenuated total reflectance cell. The FTIR trace and corresponding functional groups are

shown in Figure 8; evident in the spectrum are the hydroxyl, ester linkage, and an aliphatic region. The peaks presented mach well with Morris's reported IR spectra.[11] These data confirm the functional groups previously assigned from the mass spectra for a sterol glucoside.

DIFFERENTIAL SCANNING CALORIMETRY (DSC)

Differential scanning calorimetry (DSC) is an analytical technique used to measure thermodynamic events of samples when heated or cooled. The difference in the applied heat flux required to maintain a reference and sample at a predetermined heating and cooling profile is observed. As a sample undergoes a phase transition, the variation in the heating flux is observed. For example, detection of a solid to liquid transition is observed as an endothermic event. DSC is commonly used to measure molecular order in polymeric systems as a function of temperature. These transitions are commonly thermal history dependent.

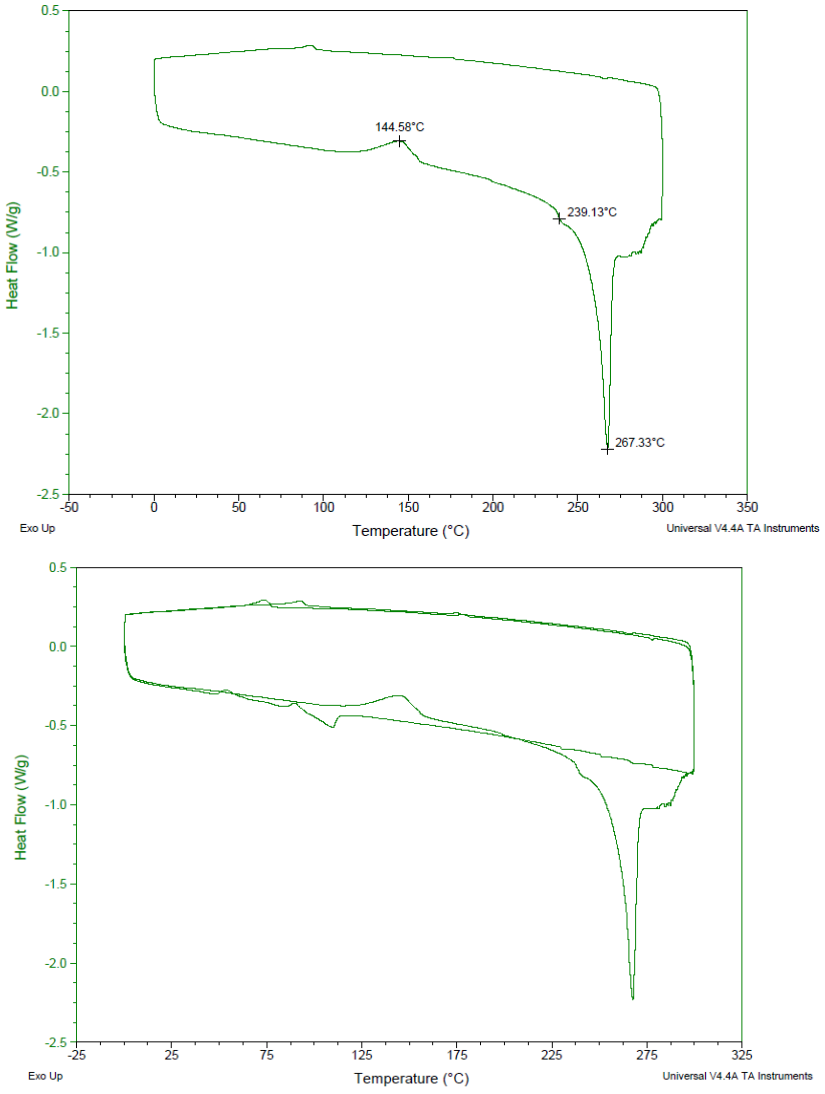


Figure 9: (A) The first DSC trace of purified residue with deep endothermic event at 267ºC, and (B) the second DSC imposed on first trace lacking deep endothermic event.

A TA instruments Q200 was used, and scanned at 10ºC/min from 0 to 300ºC. The first and second DSC traces of the purified product are shown in Figure 9. For the first trace presented, there was a deep endothermic event at 267.33ºC. Figure 9B shows the second trace imposed on top of the first trace. The second trace lacks the deep endothermic event. The lack of the event in the second trace shows that a nonreversible phase transition occurs above 200ºC. Because this phase transition is nonreversible, a simple solid to liquid transition did not occur. It is possible that a phase change occurred and was directly followed by a degradation reaction or only a degradation reaction occurred. It was observed that the temperature of the phase transition is dependent on the heating rate. As the heating rate increased, the temperature of the endothermic event was depressed. For a pure compound heated at thermodynamic equilibrium, a discreet melting point is expected, making this rate dependent finding unusual. The melting point is commonly used as an assessment of purity. Impurities typically depress the melting point of pure compounds. Morris reports a melting point of 285-289ºC for β-sitosterol-D-glucoside extracted from defatted peanut flour.[12] Matrya LLC, as supplier of specialized lipid standards reports 283-287ºC for their (>98%) SG standards.[13] These commercial SG standards were compared to in-house SG standards and found to have the same purity by NMR and MS. However, slight variations in the ratio of steroid composition were observed. These commercial standards were also used for the initial APCI-MS work presented later in this report.

THERMO GRAVIMETRIC ANALYSIS (TGA)

Thermogravimetric Analysis (TGA) works by measuring the change in the mass of a sample as it is subjected to ramped heating. This can show how individual volatile components evolve off the sample. Oxidation is typically associated with an increase in mass. Comparing the TGA traces using an ambient atmosphere and a nitrogen atmosphere can show variations due to oxidation. A TGA Q50 TA instruments 2009 was used with aluminum pans (rated for use on samples up to 600ºC).

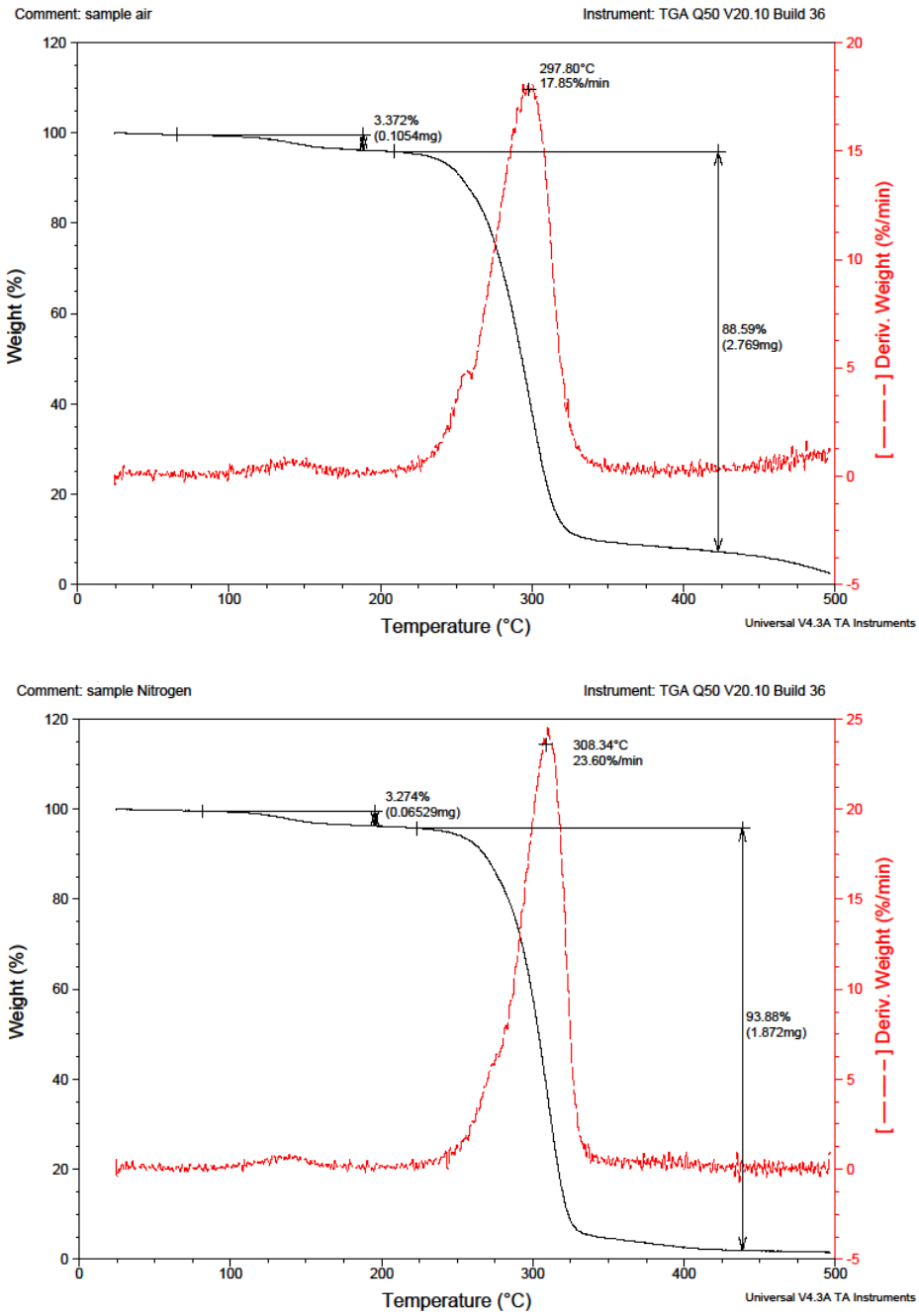


Figure 10: Thermo Gravimetric Analysis TGA of purified extract; top under ambient, bottom under nitrogen Atmosphere.

In Figure 10, TGA traces under nitrogen and air atmospheres show minimal differences. This indicates that the presence of oxidation is not significant to their decomposition kinetics. The decomposition is not occurring due to oxidation. This is complementary to DSC traces in Figure 9. These TGA and DSC traces indicate that SG compounds do not undergo melting or oxidation on heating, but may undergo polymerization or some other decomposition

reactions. Heating these compounds with the intention of causing decomposition will not remove these problematic compounds but will change them into some other product.

X-RAY DIFFRACTION

X-ray diffraction is an analytical technique used to measure lattice spacing in crystalline structures. Amorphous materials produce only a noise signal. To conduct the measurement, a sample is placed into a holder and exposed to an X-ray source. The sample or the source is moved, changing the angle of incidence. The signal response is measured at discreet angles of incidence and these can be related to the spacing of the crystal lattice. Crystallography of inorganic complexes is commonly done to elucidate structures by knowing these lattice spacings. DNA's helically structure was first elucidated with a single crystal X-ray diffraction technique.

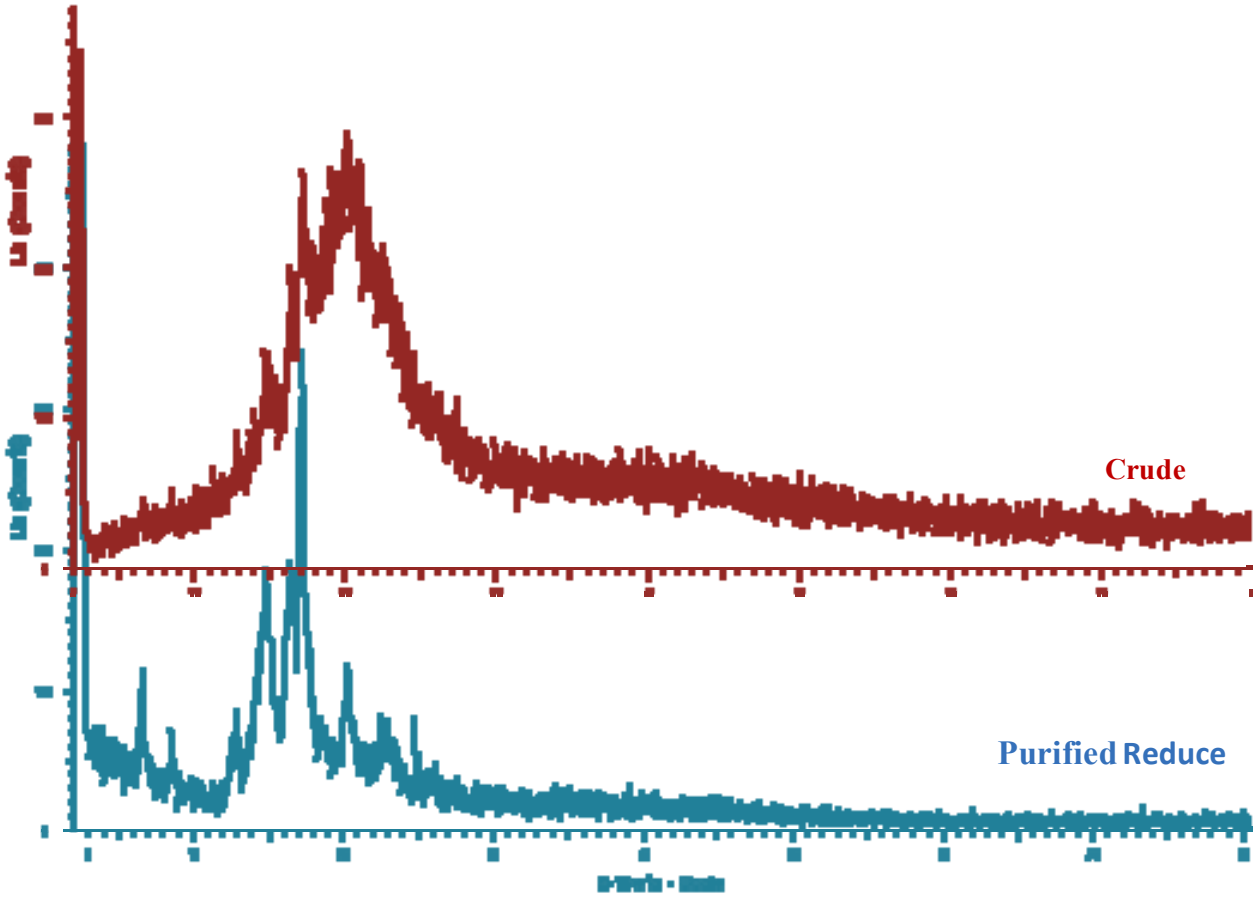


Figure 11: Powder x-ray diffraction of crude and purified reduced crystallinity is present.

A Siemens D5000 powder X-ray diffractometer was used for this experiment. The X-ray diffraction patterns for the crude residue from bag filters at the Inland Empire biodiesel plant and the purified residue are shown in Figure 11. Discreet peaks are present indicating crystallinity. However, with this experiment the level of crystallinity is confounding; it could be a minor or major portion of the residue. On purification, the signal clarity improved as shown in the bottom trace. However, the thermal history of the crude residue is different than that of its initial conditions in the biodiesel plant. These peaks are at low angle of incident which indicates an organic compound.

Second Phase Seed Extract Evaluation for Sterol Glucosides

Seed Selection

Amanda, a winter canola cultivar agronomically significant to the Palouse and the Colombia Basin of the Pacific Northwest, was selected for investigation of its sterol glucoside content. This new release, *Brassica. napus L. cv.* Amanda, from the University of Idaho Brassica breading program is expected to replace *Brassica. napus L. cv.* Athena. Canola is a biennial that is typically grown as an annual. Winter canola planted in fall germinates with two cotyledon leaves, then establishes five or more leaves in the rosette growth stage for sufficient winter survival before going dormant during the winter. It then produces the bulk of its vegetation in spring, flowers prior to the onset of high temperatures and the oilseed is harvested during the summer. Canola can be grown under irrigation or dryland conditions. Dryland canola typically has lower yield and a higher risk of crop failure. Under irrigation or in areas of high humidity like North Dakota, canola can have problems associated with blackleg and other pathogens.

It should be noted that the seed evaluated looked visually mature, but the extract had the characteristic green color associated with chlorophyll usually found in immature seed. The seed lab acknowledged that the seed was swathed prematurely; however, the fatty acid profile was typical of other Amanda samples. The fatty acid profile of Amanda is dominated by oleic acid with three primary cis-isomers. Immature canola seed should have minimal difference in the fatty acid profile. However, for some immature seed from rapeseed cultivars

with high erucic acid (22:1), variations could be expected in profiles of longer chain fatty acids as they are incorporated in the seed just prior to harvest.

OVER VIEW OF SIZE EXCLUSION CHROMATOGRAPHY (SEC)

An oil extract from the seed samples was fractionated with size exclusion chromatography (SEC) as a preparative procedure to resolve the low concentration of sterol glucosides from the triglycerides matrix. The collected fractions were evaluated using the MALDI-TOF-MS method developed for evaluation of sterol glucosides found in biodiesel production. SEC has the advantage that it allows for direct analysis of samples without derivation.

In a simplistic view, SEC or gel permeation chromatography (GPC) takes advantage of the greater mobility of lower molecular weight compounds to resolve compounds based on their molecular weight rather than their chemical properties. A visual representation of SEC is shown in Figure 12. This is typically done under isocratic (constant) flow and isothermal conditions. In polymer science, this physical separation process is commonly calibrated for a linear region where the bulk polymers can be resolved according to their chain length, and analyzed with light scattering detection. For this experiment, the larger triglyceride molecule is eluted first, and then the slightly lower molecular weight sterol glucoside is eluted off the column. A fraction collector was employed to collect the fractions in one ml increments from the constant flow rate column. These one ml samples were then evaluated with MS for SG and triglycerides.

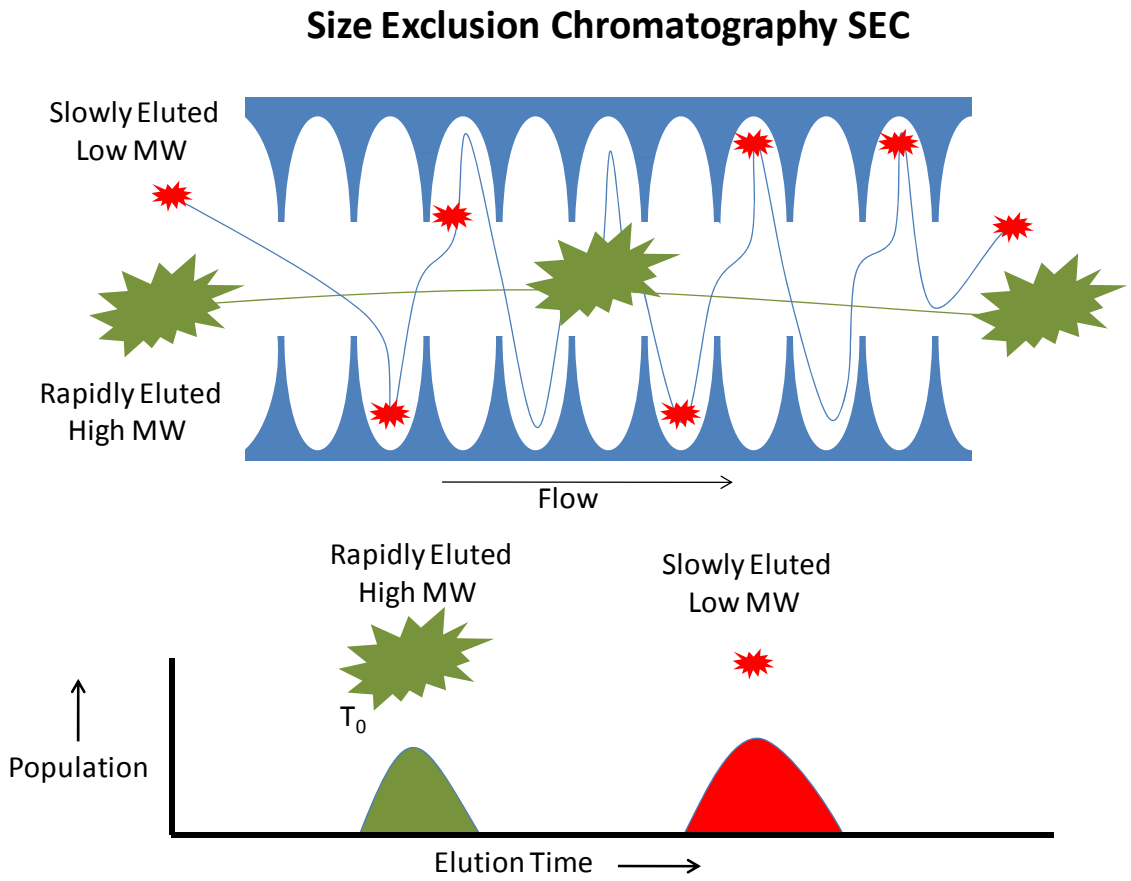


Figure 12: Visual representation of SEC showing species resolved by molecular weight.

IN DEPTH EXPLANATION

All glassware was heated to 450ºC for in excess of one hour to eliminate phthalate contamination. An Amanda seed cultivar from the University of Idaho Brassica breeding program was ground with a glass mortar and pestle and then mixed with acid treated sand to improve extraction efficacy in a Soxhlet crucible. Then, the sample was Soxhlet extracted to exhaustion with freshly distilled tetrahydrofuran (THF). This solvent was removed from the extract with a rotary evaporator at 50mbar and 35ºC.

The SEC column was operated on a Hewlett Packard Series 1090 II HPLC, (1988). The THF mobile phase was sparged with helium. The mobile phase was held at 40ºC under isothermal conditions and at a 1ml/min isocratic flow rate. The chromatography column used was a Jordi Gel DVB 100Å, 250mm long, 10mm ID. See Appendix C for the manufacturers sample chromatogram. THF is commonly inhibited with antioxidants like butylated hydroxytoluene (BHT) to prevent the formation of peroxides. Uninhibited high purity HPLC grade tetrahydrofuran was used to lower the UV cutoff (background UV absorption of solvent) and prevent the inhibitory compound from contaminating later MS analyses. The limited solubility characteristics of SG restricted possible mobile phase solvents. SG has the highest solubility in pyridine and THF of any solvents used experimentally during the course of this work. Pyridine was not chosen because of its high toxicity. THF is also toxic and incompatible with the PEEK™ tubing commonly used in chromatography systems.

To determine the T_0 time for the SEC column, a 24,150 Dalton polystyrene standard was dissolved in THF and injected under the same conditions (1ml/min THF, 40Cº, 10-20µl injection volume). This T_0 time represents the fastest rate that compounds will elute through the column. The T_0 time was determined as 18.54-18.77 min. The analytes of interest eluted between 25 min and 29 min. The extracts were found to elute prior to 30 min, so a minimum of 150 min (5 x 30 min) was used between runs to insure that there was no cross contamination between SEC runs.

After SEC, one ml fractions were collected, and the THF was evaporated under nitrogen at 35ºC with a heating block. Then, the samples were dissolved into 20µL of THF and 1µL was

spotted onto the MALDI plates. Finally, analyses were performed with MALDI-TOF-MS. MS conditions were as previously described in this report.

METHOD VALIDATION

The SEC method was validated to be sure that the SG and the triglyceride in the extract were sufficiently resolved by the difference in their molecular weight. A 3.15±0.03mg/ml triolein standard and 3.15±0.04mg/ml SG standard dissolved in THF was injected on the SEC column under the standard conditions (20μL). The triolein standard was obtained from Nu-Chek Prep, Inc (>99% purity).

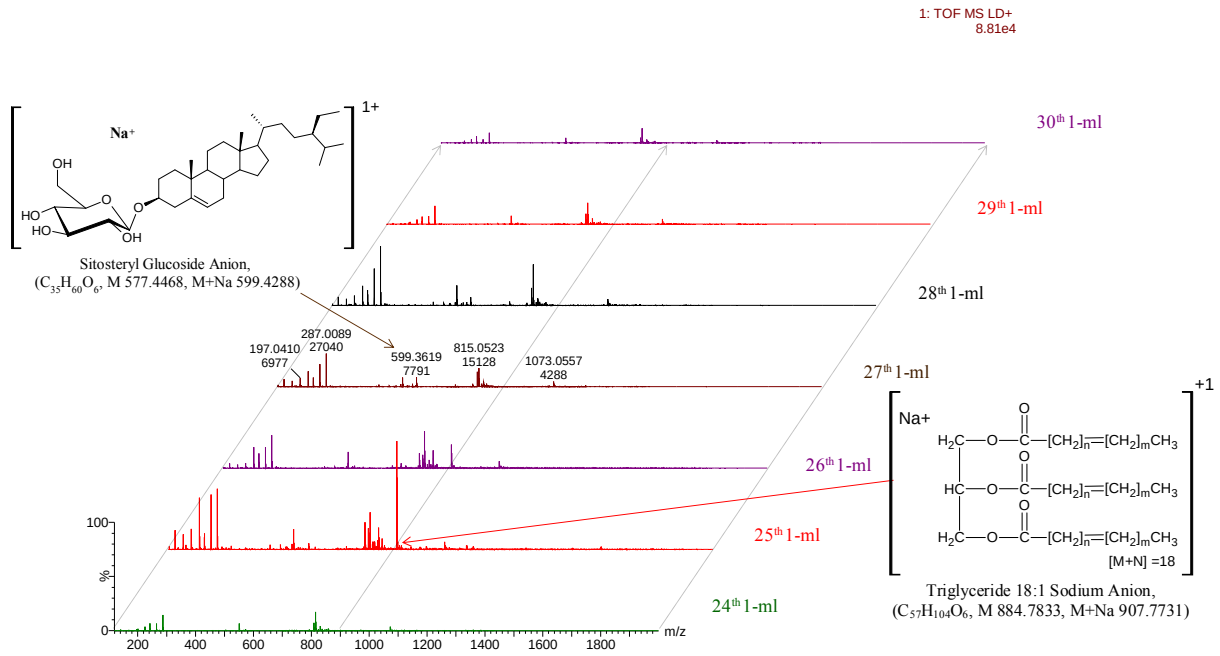


Figure 13: Method validation for the 24th 1-ml to 30th 1-ml fractions showing characteristic sodium adducts for triglyceride standard and SG standards.

MS traces for the 24th 1-ml fraction through the 30th 1-ml fractions are shown on a cascading scale in Figure 13 for the triolein standard and SG standard. The MS traces show that the two natural products of interest were sufficiently resolved which validates this method. As can be seen in the UV traces shown in Appendix D, only the peak for the triolein standard is observed on the 210nm trace. Both the triglyceride and SG are designated on the spectra. The highest intensity signal corresponds to the sodium adduct mass of the triglyceride occurred in the 25th 1-ml fraction and showed some peak tailing into the 26th 1-ml fraction. The signal corresponding to the SG sodium adduct reached its highest intensity at the 27th 1-ml fraction

with some tailing into the 30th 1-ml fractions. From the MS data, the location of the double bond cannot be differentiated.

Matrix Effects

It is possible for matrix effects in a complex mixture like a crude solvent seed extract to interfere with the chromatographic separation or ionization processes. Matrix effects describe all the effects of the unknown compounds present in a sample in addition to the analyte. These matrix compounds can significantly alter chromatographic separation and suppress or complicate analytical signals. In natural products, the matrix may bind to a target analyte making it inaccessible to detection. A relevant example is the use of acid hydrolysis with 6 molar hydrochloric acid (HCl) in the workup of cereal grains to make phytosterols accessible to extraction prior to further workup, derivation, and GC-MS analyses. However, it is known that Δ7-sterols, a subclass of phytosterols with double bonds at the seventh carbon, are susceptible to decomposition or isomerism under acid hydrolysis conditions.[14,15]

Spiked Extract

A SG spiked extract was run to validate that the SG analyte of interest could be chromatographically resolved and detected with the MS analytical technique. A SG spiked canola extract in tetrahydrofuran was prepared. The sample was composed of 35.80±.03mg/ml extract and 3.60±.03mg/ml SG standard. Ten microliters of this standard mixture were injected onto the SEC column. This sample was then run with the same chromatography and sample preparative procedures.

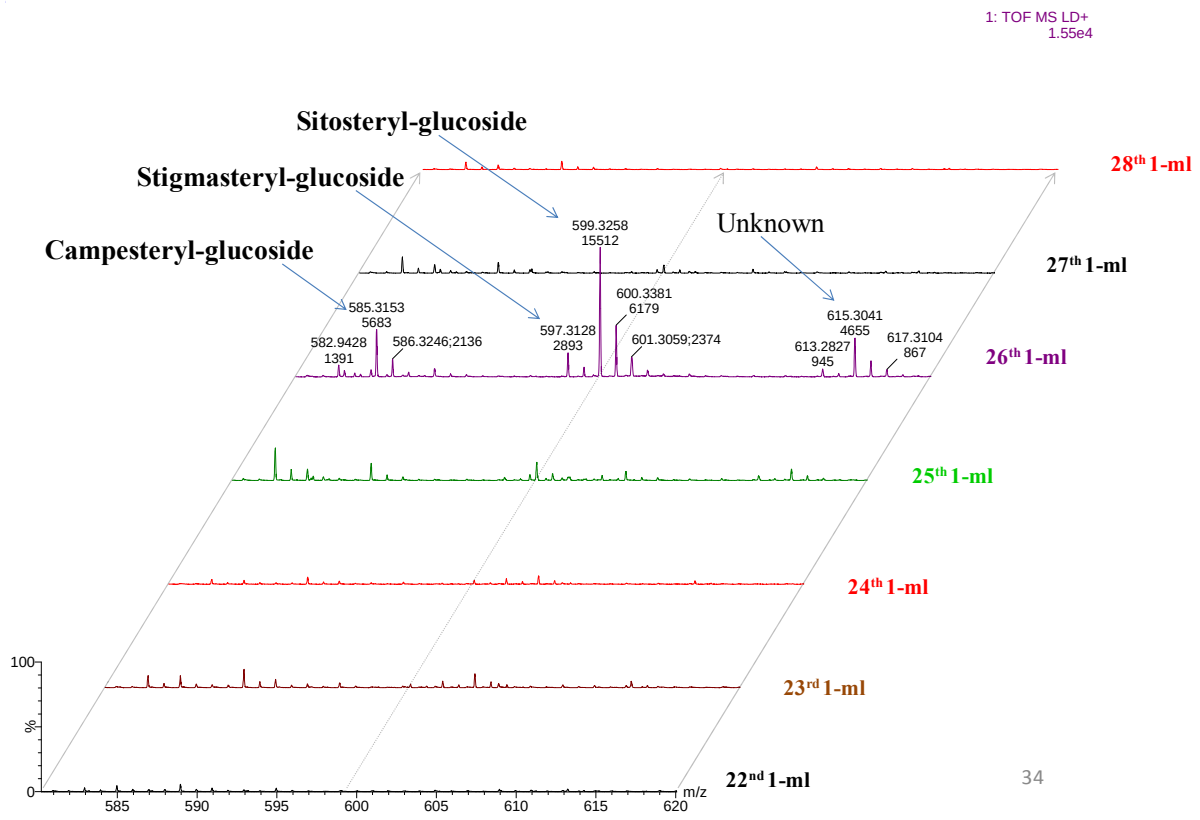


Figure 14: The SG adducts are fully resolved in the MS traces of the 22nd 1-ml to 28th 1-ml fractions of the SG spiked THF canola extract.

MS traces for the 22nd 1-ml fraction through the 28th 1-ml fractions are shown on a cascading scale in Figure 14 for the SG spiked canola extract. See Appendix E for the 210 UV trace showing two resolved peaks. The MS traces in the region of interest, 580-620m/z, of the SG spiked canola extract show prominent peaks attributed to sitosteryl-glucoside, campesteryl-glucoside, and stigmasteryl-glucoside. Sterol glucosides provide good resolution of the analyte species discreetly eluted in the 26th 1-ml fraction.

MS TRACES: SG STANDARD AND SPIKED CANOLA EXTRACT 26TH 1-ML

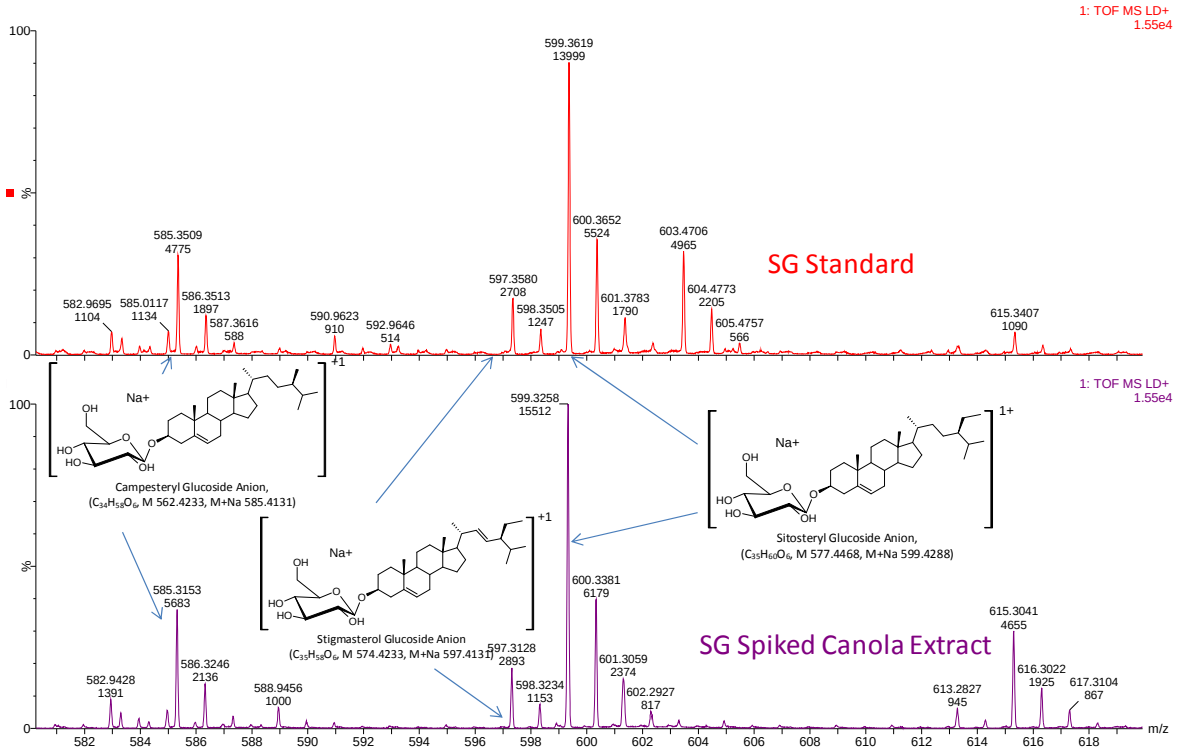


Figure 15: MALDI-TOF-MS traces of a SG standard and a SG spiked canola extract sitosteryl-glucoside, campesteryl-glucoside, and stigmasteryl-glucoside are identified.

In Figure 15, two MS traces are shown. First, a standard was directly spotted on the MALDI plate consisting of 1.05±0.03mg/ml triolein standard and 1.31±0.04mg/ml SG standard dissolved in THF. Second, the highest intensity signal for the SG sodium adduct (26^{th} -1ml) trace of the 3.60±.03mg/ml SG with 35.80±.03mg/ml extract. Recall this second trace is the 26^{th} -1ml trace shown in Figure 14. Figure 15 is a close-up of the spectra in the region of interest for sterol glucosides [580 to 620m/z]. In both MS traces in Figure 15, sitosteryl-glucoside, campesteryl-glucoside, and stigmasteryl-glucoside sodium adducts have been identified and their assigned peaks are designated.

ISOTOPIC ABUNDANCE OF LOCK MASS CORRECTED SPECTRUM

The sterol glucoside sodium adducts observed in this study had characteristic isotopic abundance patterns. An 8.4 mg/ml sterol glucoside standard in THF was spotted onto a

HABA matrix for MALDI-TOF-MS. The raw data traces and the corrected masses are displayed in Figure 16 magnified in the region of interest 580-610 m/z for the three sterol glucosides. A lock mass correction was performed on the samples. The variation from this discreet PEG fragment [*569.3149Da*] and the observed m/z signal of the same fragment is used to generate a correction algorithm; and the corrected output from this is displayed as the lock mass corrected spectrum. The lock mass standard is in the center wells adjacent to the sample wells on the sample plate, as was displaced in Figure 6. This accurate mass correction is small, but corrects for slight measurement variations caused by factors like the effect of temperature variations on the instrument.

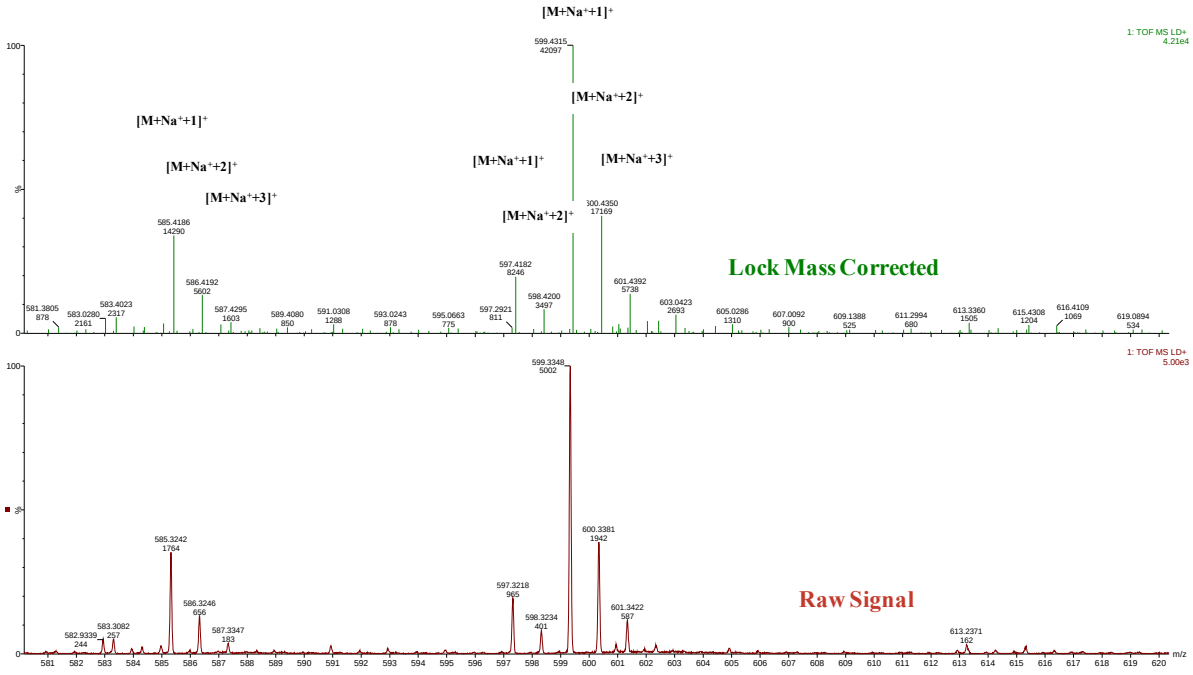


Figure 16: Isotopic distribution of sterol glucosides MALDI-TOF-MS raw signal and lock mass corrected.

The ISOFORM 1.02 NIST program was then used to calculate the isotopic abundance for all values reported in this paper. These calculated values are compared to the observed values in Table: 1.

Table 1: The Observed Isotopic Abundance Values Normalized to the Parent Monoisotopic Sodium Adduct

	Monisotopic Na Adduct	2nd Isotopic Abundance	3nd Isotopic Abundance
	[M+Na]	[M+Na+1]	[M+Na+2]
		Theoretical (observed), [% Difference]	Theoretical (observed), [% Difference]
Sitosteryl-glucoside $[C_{35}H_{60}O_6+Na]^+$	599.4888	39.98, (40.7), [1.8]	8.98, (13.6)[51]
Stigmasteryl-glucoside $[C_{35}H_{58}O_6+Na^+]^+$	597.4131	39.95, (42.4), [6.1]	8.97 (Masked)
Campesteryl-glucoside $[C_{34}H_{58}O_6+Na^+]^+$	585.41319	38.84, (39.2), [0.92]	8.54, (11.2), [31]

In nature there is a distribution of isotopes for all elements. The monoisotopic peak corresponds to the exact mass of a particular formula. Larger organic compounds statistically will have cascading peak populations. For example in the simplest case, the masses will increase by M+1, M+2, and so on where additional neutrons are incorporated somewhere into the formula. These isotopic distributions are very predictable in natural systems without enrichment. The observed isotopic distribution patterns match closely with the theoretical isotopic distribution patterns.

The use of isotopic patterns was found to be extremely useful for quickly assessing other unknown peaks and ascertaining if there were large organic compounds present in the sample rather than noise/contamination peaks. Several other compounds have been positively identified in the spectrum and these will be included in the thesis.

Table 2: Accurate Mass of Identified Sterol Glucosides

Sterol Glucoside	Theoretical Monoisotopic Na Adduct (Da)	Observed (M/Z)	Mass Accuracy (PPM)
Sitosteryl-glucoside $[C_{35}H_{60}O_6+Na^+]^+$	599.4288	599.4315	4.50
Stigmasteryl-glucoside $[C_{35}H_{58}O_6+Na^+]^+$	597.4131	597.4182	8.54
Campesteryl-glucoside $[C_{34}H_{58}O_6+Na^+]^+$	585.41319	585.4186	9.24

The accurate masses of the theoretical monoisotopic sodium adducts and lock mass corrected observed spectra are shown in Table 2. Both uses of accurate mass identification and isotopic distribution patterns had good correlation for sitosteryl-glucoside, campesteryl-glucoside, and stigmasteryl-glucoside.

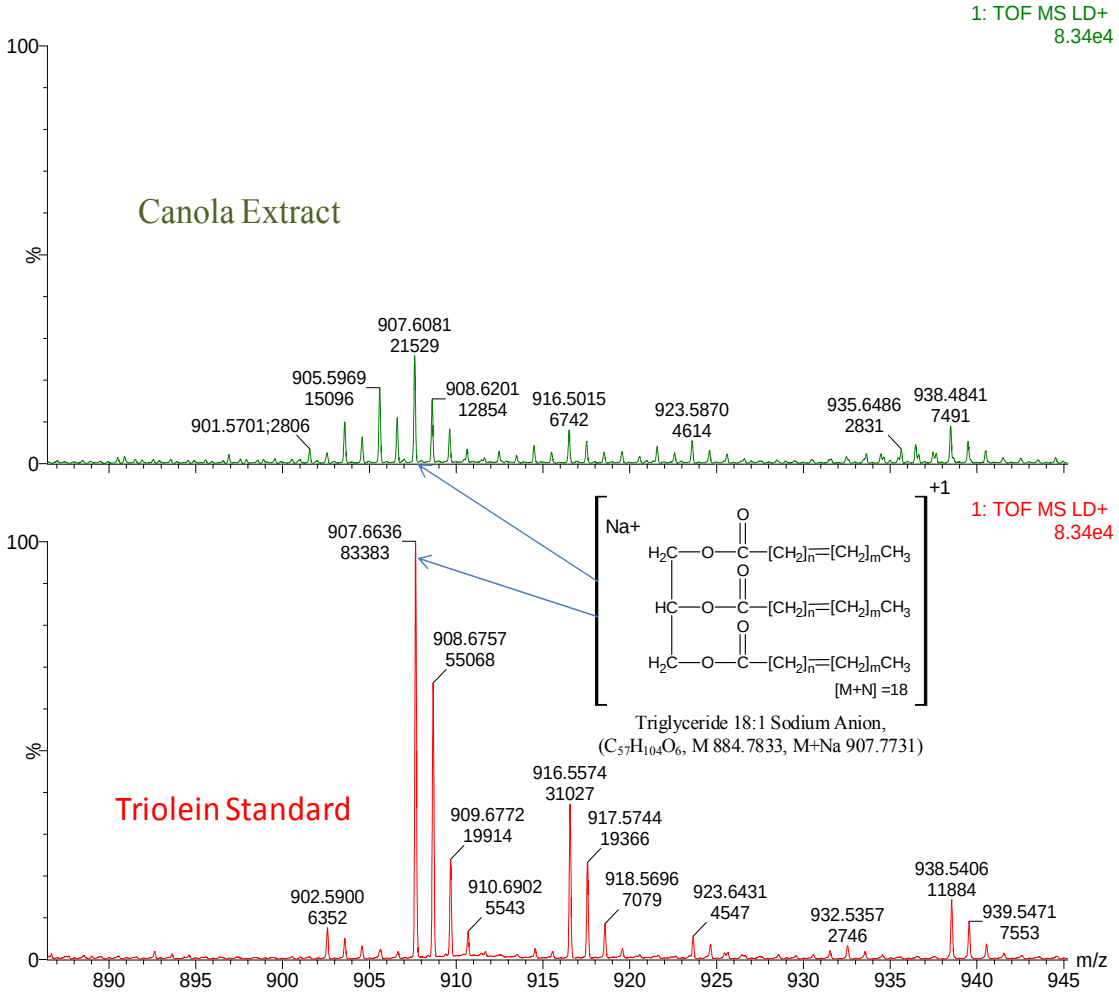


Figure 17: MADI-TOF-MS of triolein standard and highest intensity signal 23rd -1ml fraction of SG spiked *Brassica. napus L. cv.* Amanda THF seed extract.

Triglyceride: *Brassica. napus L. cv.* Amanda THF Seed Extract

In the interest of being thorough, the highest intensity signal trace (23rd 1-ml) for the triglyceride in the SG spiked extract is compared to the same SG and triolein standard with the region of 885-945m/z enlarged. The MS traces in Figure 17 have the characteristic peak pattern corresponding to a triglyceride 18:1 Sodium Anion [M +Na] 907.7731Da as validated with standard and matching literature.[16] Additional peaks are also present. In the triolein standard, an isotopic distribution for triolein 18:1 can be observed. In a seed extract, a distribution of triglycerides is expected in addition to the isotopic abundance patterns. These

masses correspond to the fatty acid profile of this seed lot observed by the plant breeding program. If peak 907m/z is assigned for a triglycerides composed of three fatty acids 18:1, then the mass change associated with additional double bonds can explain the observed peaks at 905, 903, and 901m/z.

CANOLA EXTRACT

An aliquot of the same canola seed extract as used for the spiked sample was analyzed. A 115.91±.05 mg/ml of extract in THF was run under the same SEC and MS conditions. See the SEC inline UV trace shown in Appendix F. In this UV trace, only a peak corresponding to the triglyceride is observed.

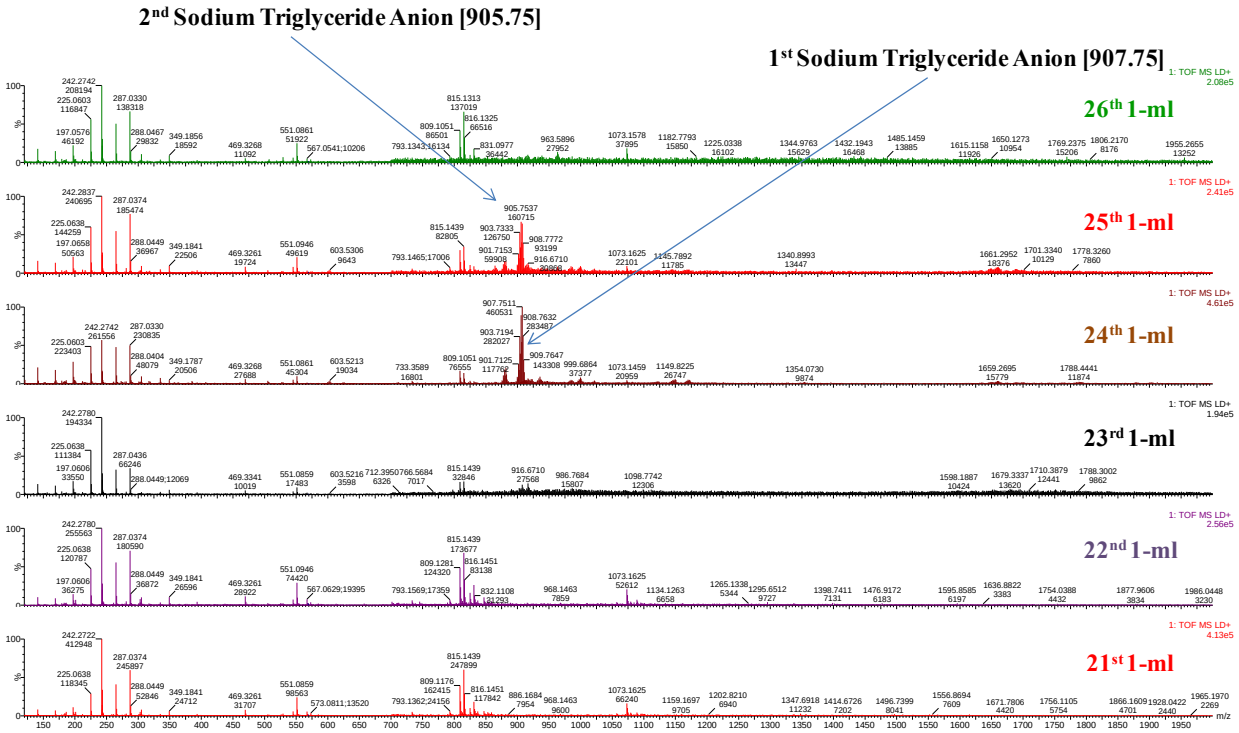


Figure 18: MS traces for the 21st 1-ml fraction to 26th 1-ml fractions of the 115.91±.05 mg/ml of THF canola extract with two sodium triglyceride anions resolved.

Shown in Figure 18 are the MS traces for the 21st 1-ml fraction to the 26th 1-ml fractions of the canola extract. As observed on previous MS traces, a sodium triglyceride anion is designated as the 907m/z ion. Resolved in the next trace is a second sodium triglyceride anion; its mass 905m/z indicates that it is lacking one additional unit of saturation. It was not anticipated that the SEC would be able to resolve triglycerides differing by only one unit of saturation.

It should be noted that significant counts were obtained for the peaks of interest to obtain an accurate mass, however their signal intensities were not sufficiently resolved above the background. From the accurate mass data in the 26th 1-ml ms trace, sitosteryl-glucoside sodium adduct (42.5ppm) and campesteryl-glucoside sodium adduct (6.99ppm) were observed with the corresponding mass accuracy with respect to their calculated theoretical masses. To obtain qualitative data relating the concentration of SG in the trace, and ultimately back to the seed, will require additional method development. A more precise calibration curve and accompanying validation experiments will be required.

GLYCOLIPIDS ISOLATED WITH SILICA GEL FROM CAMELINA SEED:

Cleaned camelina seed was manually macerated with a mortar and pestle, and subsequently Soxhlet extracted to exhaustion with 1,2-dimethoxyethane (DME or glyme). The preparative procedure was modeled after the procedures of Moreau and Yamauchi.[17,18] An aliquot of this concentrated extract was run on a silica gel column. The silica gel used was Fisher Chem silica gel, 230-400 mesh (S825-1). All solvents used were HPLC grade or higher in purity. An excess of one and a half bed volumes of each elution solvent was run to condition the silica. The column was equilibrated with chloroform and an aliquot of seed extract was added. Then, sequential elutions with chloroform, acetone, and methanol were performed. Each solvent was expected to resolve triglycerides, glycolipids, and phospholipids respectively, as reported. [19]

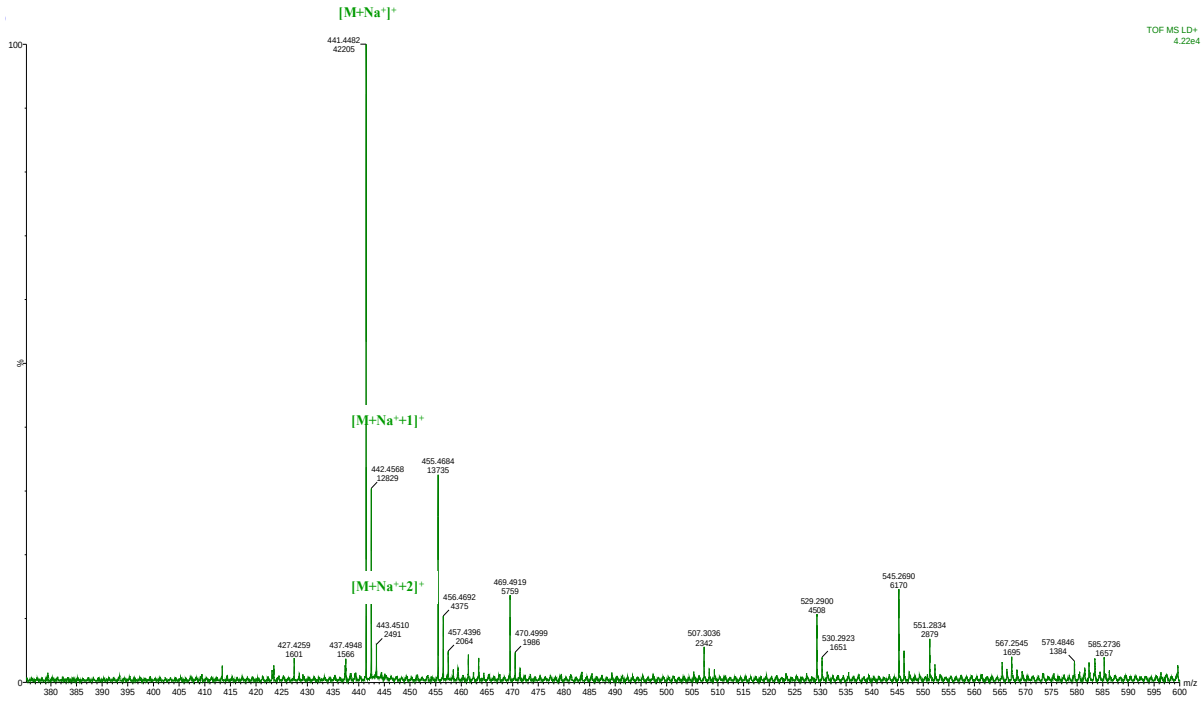
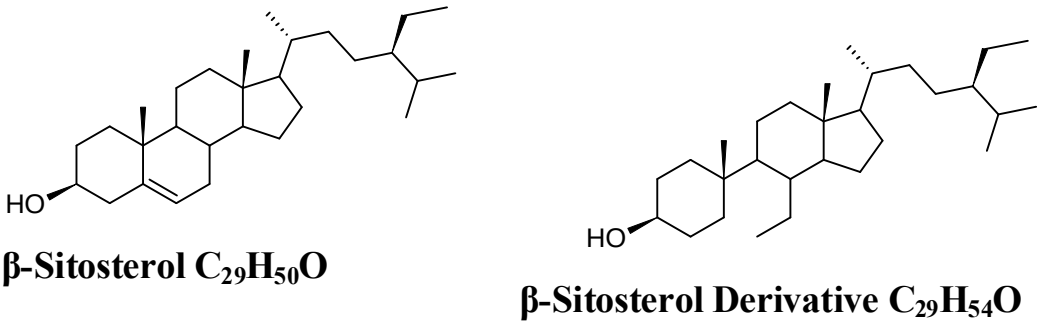


Figure 19: Proposed β-sitosterol derivative and isotopic distribution ($C_{29}H_{54}O$, $[M+Na^+]^+$ 441.4072).

The glycolipid fraction was analyzed using MALDI-TOF-MS and its spectrum is shown in Figure 19 in the range of 375-600M/Z. The isotopic distribution is labeled in this MS spectra. This spectrum is not lock mass corrected. See Appendix G for the full MS spectrum of the proposed β-sitosterol derivative.

The exact mass from the mass spectrum was used with "formula calculator" a USDA software program from which the formula $C_{29}H_{54}O$ was calculated.[20] The variation between the observed mass and the theoretical mass for $C_{29}H_{54}O$ is 92.9 PPM. The ISOFORM 1.02 NIST program was then used to calculate the isotopic abundance that is expected for this formula. This formula matches the theoretical value for this formula to within a percentage point for the first abundance. [21] From the literature, the most common phytosterol in refined camelina oil is β-sitosterol $C_{29}H_{50}O$.[22] If the double bond was cleaved and saturated, an additional four hydrogen atoms could be present forming a β-sitosterol derivative $C_{29}H_{54}O$. No theoretical explanation for the formation of this product is proposed. An attempt to acquire a HNMR spectrum was made, but it was unsuccessful, possibly due to a breakdown of the analyte.



β-Sitosterol $C_{29}H_{50}O$

β-Sitosterol Derivative $C_{29}H_{54}O$

Figure 20: Structure for β-sitosterol $C_{29}H_{50}O$ a free sterol common in plants and a proposed β-sitosterol derivative $C_{29}H_{54}O$.

In Figure 20, the structure of β-sitosterol and the proposed β-sitosterol derivative are shown. From the isotope calculator, the theoretical isotopic distribution for the sodium adduct of the proposed β-sitosterol derivative is shown in Table 3.

Table 3: Theoretical and Observed Isotopic Distribution for Proposed β-Sitosterol Derivative Na Adduct $[C_{29}H_{54}ONa]^+$

β-Sitosterol Derivative Na Adduct $[C_{29}H_{54}ONa]^+$	Monisotopic Na Adduct [M+Na]	2nd Isotopic Abundance [M+Na+1]	3rd Isotopic Abundance [M+Na+2]
Theoretical	441.4072	33.04	5.480
Observed	441.4482	30.4	5.90

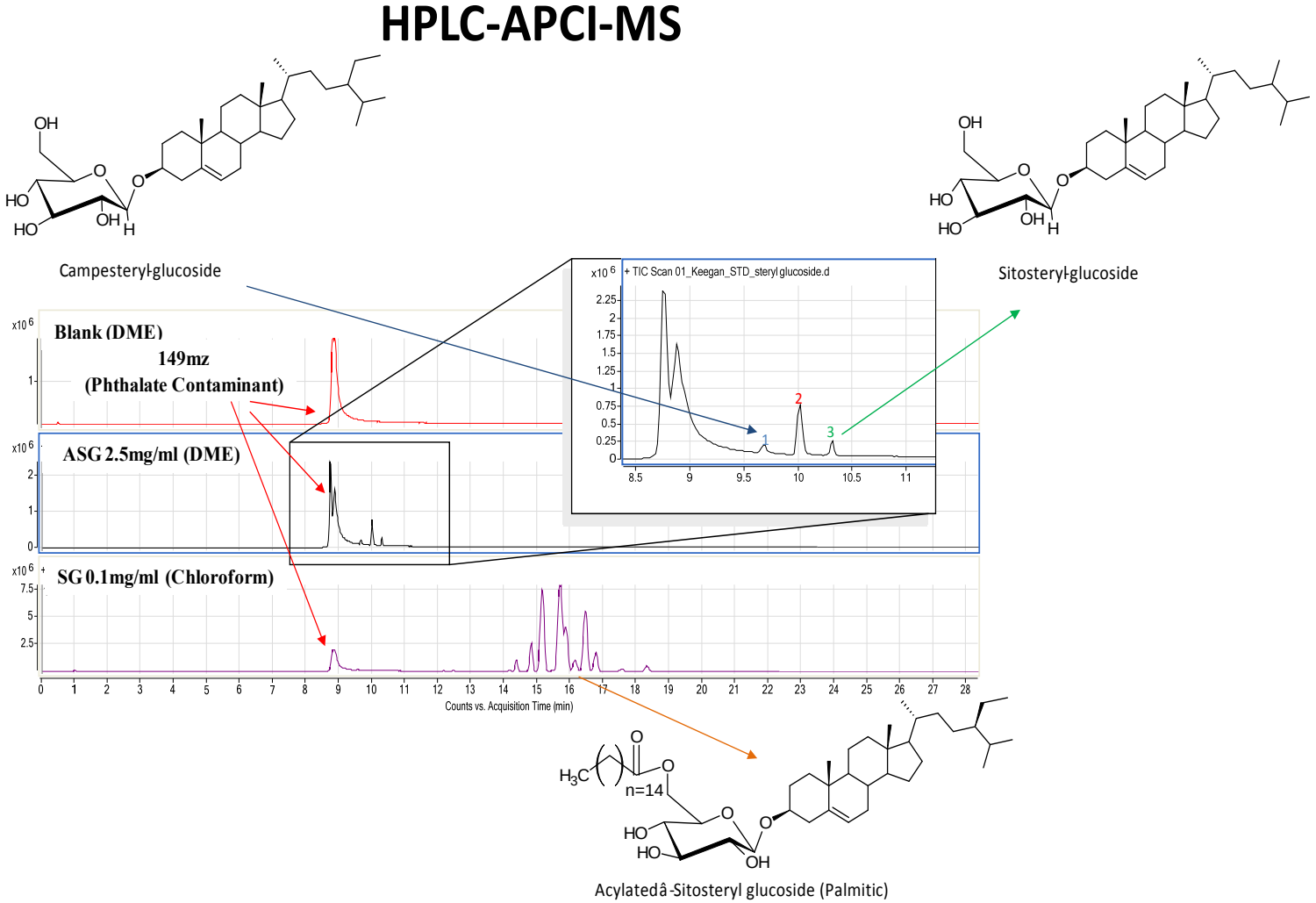


Figure 21: HPLC-APCI-MS of solvent blank, SG standard and ASG standard.

During the initial phases of this project, a method modeled after Moreau's HPLC-APCI-MS method for SG was evaluated. Additionally, ASG (also esterified sterol glucoside - ESG) were evaluated with the same conditions on the same instrument. Three total ion chromatogram traces are shown in Figure 21. First, a solvent blank (1,2-dimethoxyethane) is shown. Then, a SG standard 0.1mg/ml (chloroform) was analyzed and shown below the solvent blank. Third, an ASG standard 2.5mg/ml (1,2-dimethoyethane) is also shown. These standards were obtained from Matreya, LLC both were represented as greater than 98% pure. In the blank, a significant phthalate peak was observed with a characteristic 149 m/z fragment. The 149 Da mass is one of the characteristic fragments of phthalates which have been extensively examined.[23] The presence of the phthalate contamination is problematic if the contaminate elutes at the same time as an analyte of interest. It can act as anion suppressant and prevent the analyte from sufficiently ionizing. In ion suppression, the deleterious compound either preferentially ionizes or in some other way inhibits the ability of the analyte species to ionize. Ion suppression leads to poor and inconstant spectra. This contaminant also eluted in the two subsequent SG and ASG standards runs. See Appendix H for the procedure used.

Ammonium adducts of sterol glucosides and characteristic fragments for campesterol and sitosterol moieties are identified in Figure 19. There are several peaks resolved in the ASG spectrum. The total ion chromatogram (TIC) of the ASG standard was scanned for the mass fragments 398.39, and 384.37 corresponding to the sitosterol and campesterol moieties. From these mass scans, multiple peaks were resolved. For a given phytosterol multiple, ASG compounds were resolved by retention time.

As reported by Moreau, a strong signal at 205nm was detected for SG above the baseline.[24] Additionally the ASG was also detected at 205nm. Due to the solvent gradient as the proportion of methanol increases, the baseline changes to the UV cutoff of methanol. This baseline shift is reproducible, but significant change is observed at 205nm.

This experiment was repeated with the same instrument with the comparable column with the same chromatographic conditions, and standards with a change of the mass spectrometry source to electro spray ionization (ESI). However, the results were poor, which was

attributed to poor ionization efficiency of these analytes under ESI conditions. See Appendix I for the procedure used. Moreau conveyed that they also had poor ionization efficiency with ESI as compared to APCI for SG analytes. (R. A. Moreau, personal communication, AOCS 2010)

CONCLUSION

Sterol glucosides (SG) were identified in residues that shut down a commercial biodiesel plant. Biodiesel plant operators, biodiesel distributors and vegetable oil crushing facilities in the biodiesel supply chain should be alert for tank residues that may contain these compounds. Methods for evaluation of SG have been reviewed, and a new preparatory procedure and a MALDI-TOF-MS technique has been developed and validated for these analytes.

Using the new analytical techniques developed, the occurrence and accumulation of sterol glucoside compounds can be determined. Significant variations in the broader category of phytosterol compounds are expected due to soil and other agronomic factors. In addition, a more thorough understanding of phytosterol extraction and accumulation in vegetable oil production is needed to determine if these compounds can be left in the seed meals, or collected as a high-value co-product. Understanding the mass balance of these compounds from seed crop to biodiesel production is necessary to optimize phytosterol removal for winter operation.

The following conclusions can be drawn from this study.

- A MALDI-TOF-MS method was developed for the detection of sterol glucosides (SG).
- In a residue that shut down a commercial biodiesel plant, sitosteryl-glucoside, campesteryl-glucoside, and stigmasteryl-glucoside were identified.
- Material properties of purified sterol glucosides are reported including DSC traces, TGA traces, FTIR, and X-ray diffraction.
- A preparative method to separate triglycerides in seed extract from sterol glucosides with size exclusion chromatography (SEC) was developed.
- The SEC preparative method developed was able to identify sitosteryl-glucoside and campesteryl-glucoside with exact mass in a winter canola seed sample (*Brassica. napus L. cv.* Amanda). Further method refinement is required to obtain quantitative data.

APPENDIX

Appendix A: Lock Mass Procedure

This lock mass procedure is a modified version of a Waters procedure for measuring the monoisotopic masses of sodium adducts of polyethylene glycol oligomers for lock mass correction.

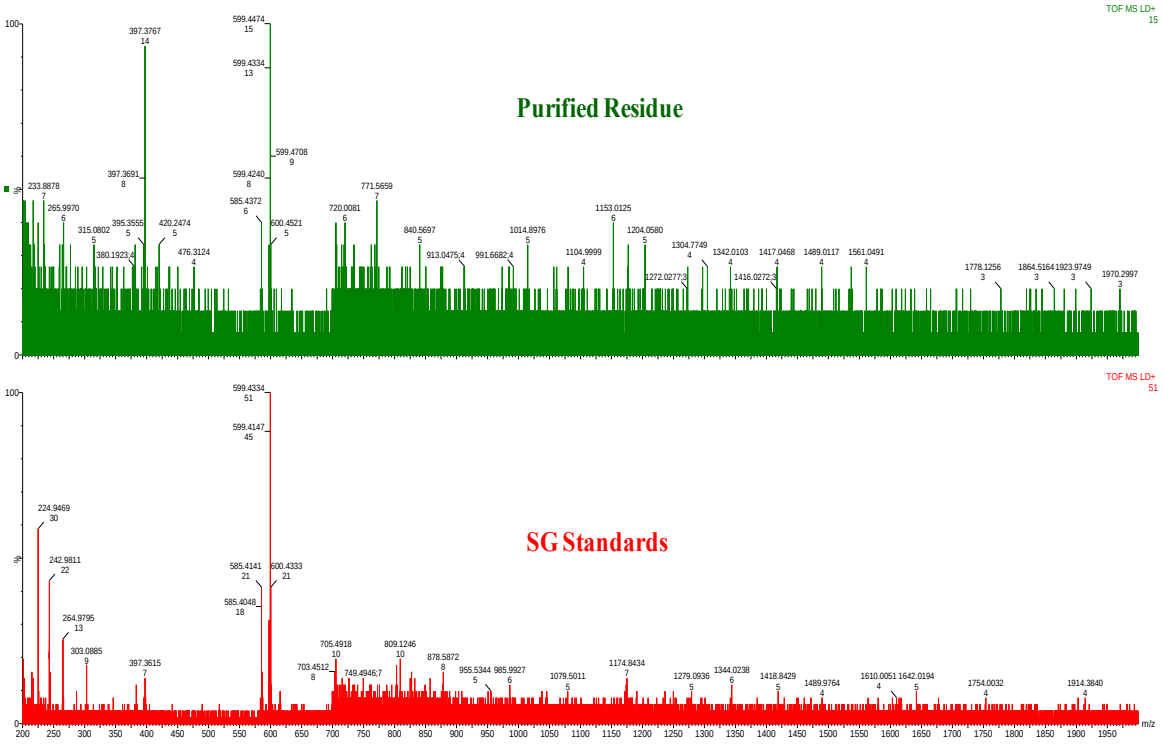
10mg/ml PEG 600 in 1:1 water:acetonitrile
2mg/ml NaI in 1:1 water:acetonitrile
Mix PEG and NaI30:6 v/v

Matrix 3mg/ml DHB

Mix 1:1 with mix/matrix

Spot 1µl on lock mass wells (targets) allow to air dry.

Appendix B: SG Standards and Purified Residue



MALDI-TOF-MS of (Matreya >98%) SG standards and purified residue, sodium adducts of sterol glucosides: sitosteryl-glucoside and campesteryl-glucoside, stigmasteryl-glucoside, and characteristic fragments 200-2000 M/Z.

Appendix C: Sample SEC Chromatogram

JORDI

QUALITY ASSURANCE CHROMATOGRAM

DESCRIPTION Serial Number: 1116071A
Packing Material: Jordi Gel DVB 100A
Length: 250 mm ID: 10 mm

Catalog Number: 15020

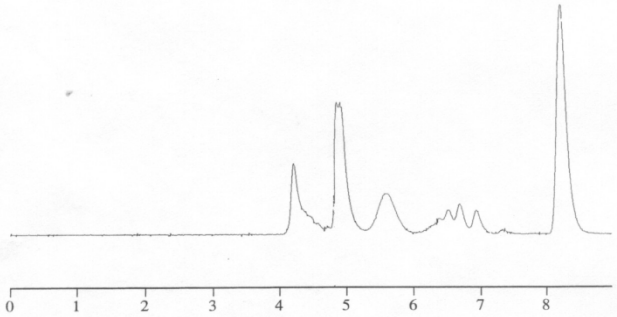
Fitting Code: B

TEST CONDITIONS
Mobile Phase: Chloroform
Flow Rate: 1.5 mL/min
Detector: UV 254 nm

Temperature: Ambient
Pressure: 500 PSIG
Sensitivity: 0.2 AUFS

Sample: P-Sty 1 Inj. vol.: 7 µL

Component	Conc (mg/ml)
1. 2180K	1.0
2. 34.3K	1.0
3. 12.1K	1.0
4. 2.38K	1.0
5. 0.374K	1.0
6. 0.092K	2.0



Peak	Retention Time (min)	Capacity Factor	Symmetry	Efficiency (Plates/m)
1	8.23	0.00	1.0	65278

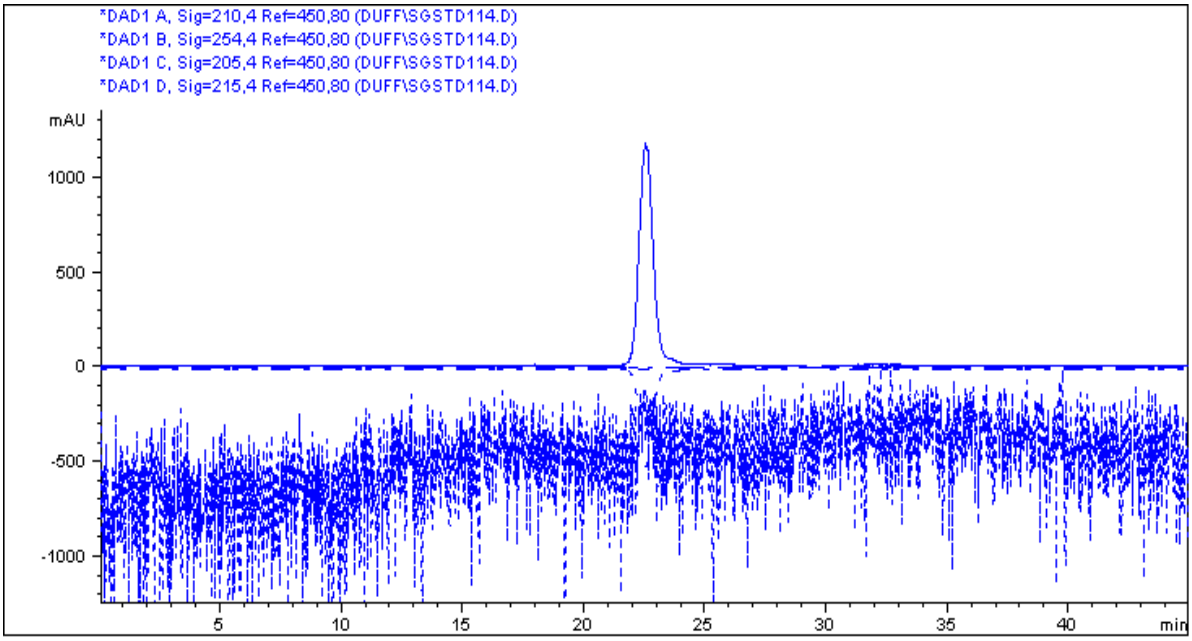
Void Time: 8.23 min.

Remarks:

Jordi Associates, F. L. P. • 4 Mill Street • Bellingham, Massachusetts 02019 • Phone: (508) 966-1301 • Fax: (508) 966-4063

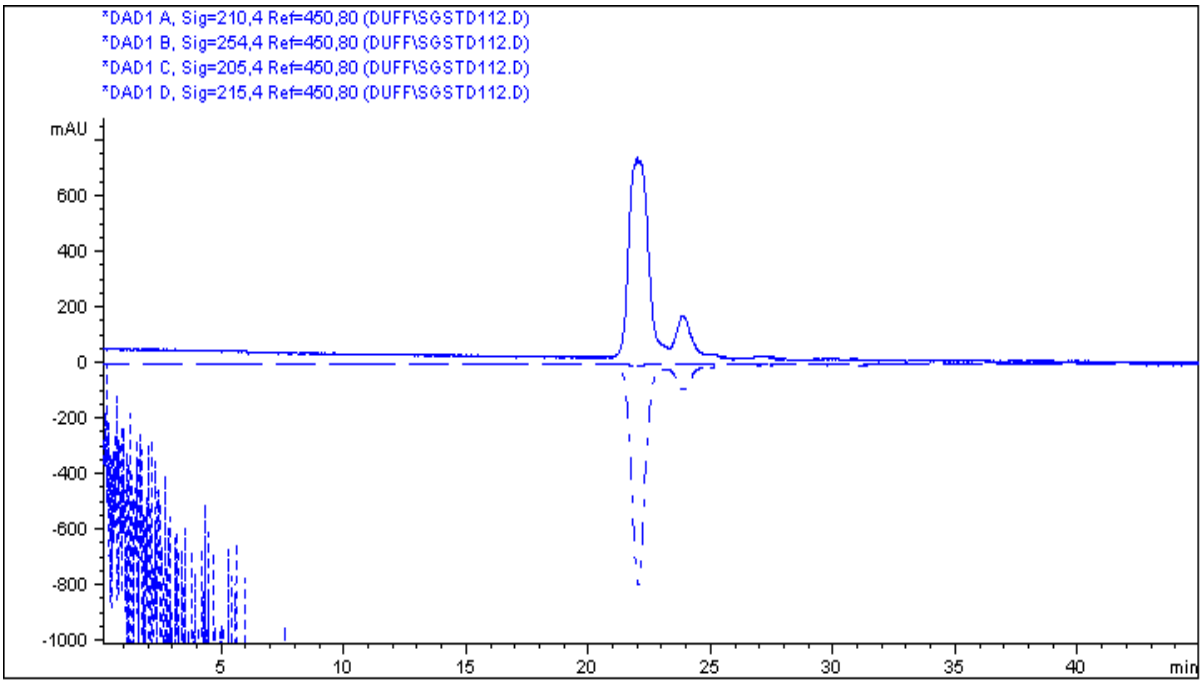
9/19/02 F155D

Appendix D: 210nm UV Trace Triolein & SG Standards



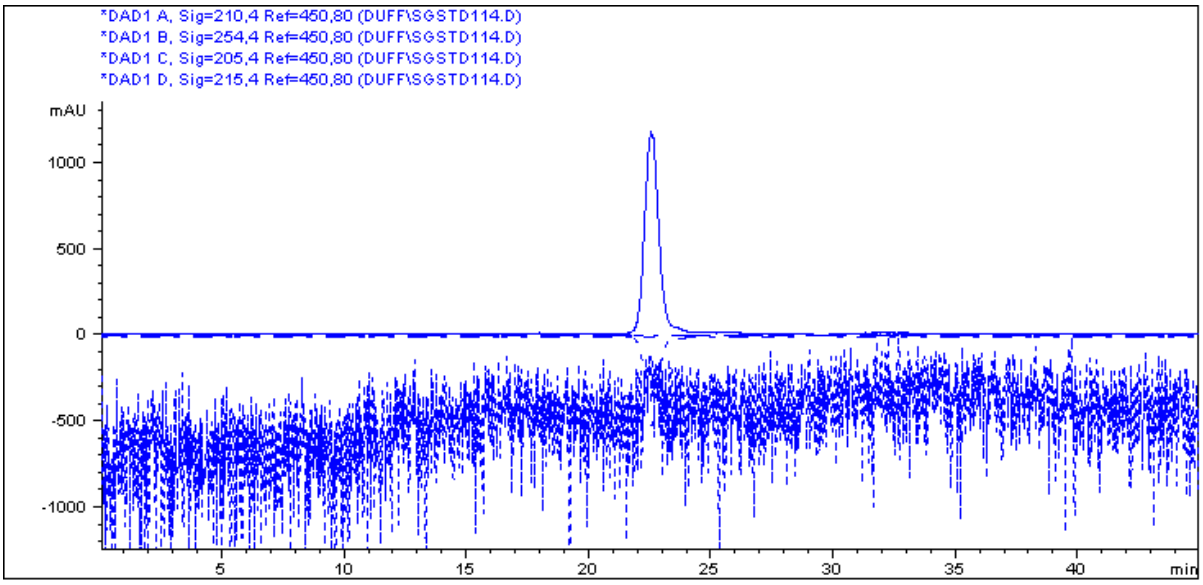
A 210nm UV trace on top for a 3.15±0.03mg/ml triolein standard and 3.15±0.04mg/ml SG standard dissolved in THF.

Appendix E: 210nm UV Trace SG Spiked Canola Extract



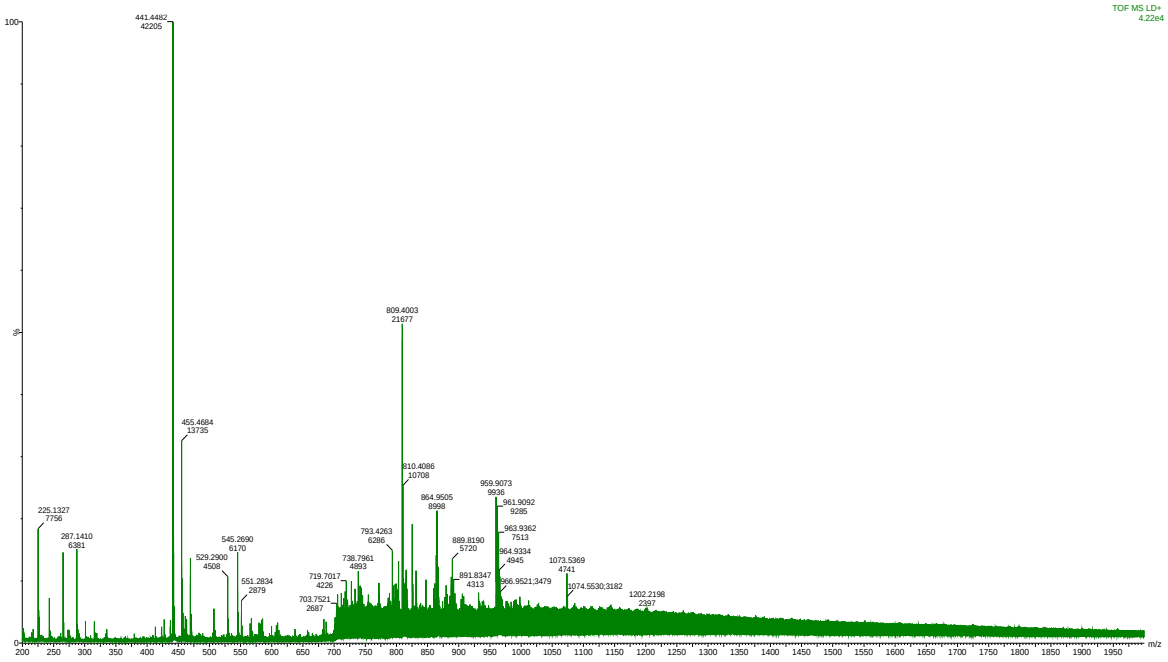
Top a 210nm UV trace and bottom 215nm inverted UV trace for the 35.80±.03mg/ml extract and 3.60±.03mg/ml SG standard.

Appendix F: 210nm UV Trace Canola Extract



Top a 210nm and bottom 215nm inverted UV traces for a 22.542mg/ml canola extract.

Appendix G: SG Standards and Purified Residue



MS spectrum of the proposed β-sitosterol derivative ($C_{29}H_{54}O$, $[M+Na^+]^+$441.4072) in the range of [200-2000M/Z].

Appendix H: HPLC-APCI-MS Procedure

Colum: Agilent XDB-C18 Ultra High Pressure 1.8µm, 4.6x50mm, 927975-902, USWDYD4362

HPLC Gradient:

T=0 min,	60°C	1ml/min,	H_2O:MeOH, 3:7
T=0min,	80°C	1ml/min,	100%MeOH
T=30min,	80°C,	1.3ml/min,	100%MeOH
T=60min,	60°C,	1.3ml/in,	100%MeOH

Both mobile phase solvents have 0.1% ammonium formate to promote ammonium adduct formation.

Injection: 0.5 µL

APCI Mass Spec Positive mode:

Gas Temperature	350°C
Vaporizer	350°C
Dry Gas	5.0L/min
Capillary	200V
Skimmer	60V
Corona	4.0µA
OCT RFV	250
DAD-UV	230-700nm

Appendix I: HPLC-ESI-MS Procedure

Colum: Phenomenex, Kinetex 2.6µm, C18, 100A, 150x2.1mm

HPLC Mobile Phase:
A: H_2O 17.7mΩ premixed 1.5% (23.7_{88}mM) ammonium formate
B: MeOH premixed 1.5% (23.7_{88}mM) ammonium formate

Gradient:

T=0 min,	50°C	.4ml/min,	H_2O:MeOH,	9:1
T=1min,	50°C	.4ml/min,	H_2O:MeOH,	9:1 gradient
T=11min,	50°C	.4ml/min,	100%MeOH	gradient flow rate
T=25min,	50°C,	.75ml/min,	100%MeOH	gradient flow rate
T=26.50min,	50°C,	.4ml/in,	H_2O:MeOH,	9:1
T=30min,	50°C,	.4ml/in,	H_2O:MeOH,	9:1

Injection: 5 µL

ESI Mass Spec Negative & Positive mode:

Gas temperature	350°C
Vaporizer	350°C
Dry Gas	5.0L/min
Capillary	3000V
Fragmentation voltage four channels	(100, 200, 300, 400V)
Skimmer	60V
OCT RFV	250
DAD-UV 2	30-700nm
Mass Range	100-3500mz

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