MEASUREMENT AND CONTROL STRATEGIES FOR STEROL GLUCOSIDES TO IMPROVE BIODIESEL QUALITY

Final Report

KLK755

Isolation and Characterization of Acylated Steryl Glucosides in Oilseed Crops of the Pacific Northwest

N10-02

National Institute for Advanced Transportation Technology

University of Idaho

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16. Abstract

Acylated steryl glucosides (ASG), a form of phytosterols, have been identified in a variety of agricultural products, including the oilseeds of soybean and rapeseed. Currently, there are limited data available on the quality and quantity of phytosterols in oilseed crops. During biodiesel production, the esterification of ASG in oil extracts forms steryl glucosides (SG). These steryl glucosides in low concentrations (less than 50ppm) have been implicated in agglomeration of contaminates leading to subsequent filter clogging in biodiesel applications.

Limited methods exist for the direct isolation and characterizations of ASG. Methods rely on multistep extraction, saponification, acidification and derivatization for evaluation with GC-MS. The objective of this study is to characterize the ASG in agronomically significant oilseed crops of the Pacific Northwest. In this study, HPLC-ESI-MS and HPLC-APCI-MS techniques were evaluated as characterization tools. Oilseeds have been solvent extracted; continuing work is the characterization using the validated MS techniques of the glycolipid fraction after isolation with preparatory chromatography. Characterization of these natural products is important to the industry for developing strategies to minimize agglomeration in biodiesel.

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Biodiesel fuels; alternate fuels; fuel processing; fuel composition	Unrestricted; Document is available to the public through the National Technical Information Service; Springfield, VT.

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EXECUTIVE SUMMARY

The biodiesel industry is very concerned about compounds called sterol glucosides (SG). These compounds can be present in neat biodiesel. SG may act as seed crystals or agglomeration centers where contaminates can accumulate and produce particles that will plug fuel filters. Dr. Robert Moreau's team at the USDA has identified sitosteryl-glucoside and campesteryl-glucoside in neat bulk biodiesel tanks and residues from plugged fuel filteres.[1] Acylated steryl glucosides (ASG), related compounds to SG which incorporate a fatty acid chain, have been identified in a variety of agricultural products, including soybean and rapeseed. Currently, there are limited data available on the quality and quantity of phytosterols in oilseed crops. During biodiesel production, the ASG in oil soybeans and other oilseeds are esterified forming SG. These SG in low concentrations (less than 50ppm) have been implicated in the agglomeration of contaminates leading to subsequent filter clogging in biodiesel applications.

Industrial oil extraction from oilseeds is optimized for maximum oil extraction. The extraction of ASG into the oil may vary significantly depending on the industrial process conditions. For example, constant and changing temperature, pressure, solvent systems, seed moisture, seed tempering and history, and multiple solvent extractions may all have an effect. Biodiesel produced from minimally processed vegetable oils is susceptible to these variations. This study proposes to measure the amount of ASG in unprocessed oilseeds that are of commercial significance in the Pacific Northwest (PNW). This will provide a base upper limit for SG in these oilseed crops. Whether these trace contaminates are the only source of biodiesel operational problems is still not established and will not be investigated for this study.

Limited methods exist for the direct isolation and characterization of ASG. The methods rely on multistep extraction, saponification, acidification and derivatization for evaluation with gas chromatography mass spectrometry (GC-MS). The objective of this study is to characterize the ASG in agronomically significant dry land oilseed crops of the PNW (canola, mustard, and camelina). Exhaustive solvent extraction of oilseeds and sample preparation is followed by characterization and quantification of the glycolipids fractions. Our team has validated the use of high performance liquid chromatography – atmospheric pressure chemical ionization (HPLC-APCI) and electrospray ionization mass spectrometry (HPLC-ESI-MS) for characterization of

ASG and SG standards. Quantification may be accomplished with inline UV detection at 210nm in the same instrumentation used for characterization.

In this study, HPLC-ESI-MS and HPLC-APCI-MS techniques were evaluated as characterization tools. Oilseeds have been solvent extracted. The glycolipids fraction will be isolated using preparatory chromatography and characterized using the validated MS techniques. Characterization of these natural products is important to the industry for developing strategies to minimize operational problems with biodiesel.

DESCRIPTION OF PROBLEM

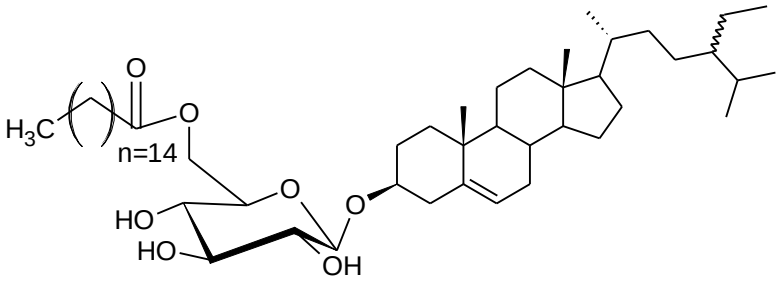
Minnesota and several other states have experienced significant problems with biodiesel at low temperatures. Multiple contaminates have blocked filters in otherwise high quality biodiesel. Currently, the only surefire method to prevent cold flow problems is to winterize the fuel. The fuel is cooled, and sufficient time is allowed for crystallization events to occur. Then a filter aid, like diatomaceous earth, is added and the biodiesel is filtered to remove any non-liquid materials in the fuel. This is expensive, time consuming, requires specialized infrastructure, and is highly energy intensive for fuel production.

Industrial oil extraction of a natural product is a complex process. Industrial extraction has been optimized for maximum oil recovery. The extraction of ASG and SG is not optimized in the process so slight changes in process conditions or seed conditions may significantly affect the total extraction. This may explain some of the variability that has been observed with some researchers seeing significant effects of SG and some seeing no effect. The structure of ASG may also be significant. The structural formulas for some ASG examples are shown in Figure 1.

Most occurrences of filter failure are confounded by an inability to trace the fuel producer, oil processor and the oilseed crop. The problem is further complicated by possibly having multiple reasons for the filter failures. The biodiesel industry is concerned that SG may act as seed crystals or agglomeration centers where contaminates can accumulate. The industry assumes that ASG present in plants and oils are esterified during biodiesel production, forming SG. The hexane solvent extraction system that is most common in industrial practice is optimized to obtain the maximum levels of triglycerides out of oilseeds. The hexane extraction efficiency of SG or ASG may change due to process or agronomic conditions. Understanding the extraction mechanism might be a way to control the level of ASG and SG in the oil and ultimately, in the biodiesel.

The level of ASG and SG present in oilseeds is not readily available. What data are available is obtained by extraction, multistep derivation and GC-MS evaluation. There are limited analytical techniques for the evaluation of SG and ASG. Sterols are commonly evaluated using gas chromatography mass spectrometry/ flame ionization detection (GC-MS/FID). The steps in these analyses include: solvent extraction, acid hydrolyses 6 M HCL (cleaves glycoside bond), solvent

extraction, silylate with trimethylsilyl (TMS) derivatization and GC-MS or GC-FID evaluation. This is time consuming and the multiple steps required allow the introduction of error.



Acylated β-sitosteryl glucoside (Palmitic)

Figure 1: ASG 6th position only verifiable with NMR.

APPROACH AND METHODOLOGY

Dr. Jon Van Gerpen, Dr. Brian He and Keegan Duff are currently developing an analytical method for the evaluation of SG and ASG. The goal of the project is to determine the levels of ASG and SG in agronomically significant oilseeds of the PNW. It's apparent that ASG (also known as esterified sterol glucoside, ESG) is present and is lipophilic. Thus, it is expected that it would be susceptible to solvent extraction. With more information about how these compounds are extracted, the industry can make more informed decisions about how to minimize the level of SG in the fuel and thus minimize low temperature problems with biodiesel in the PNW.

The project objective is to determine the levels of ASG and SG in agronomically significant oilseeds crops of the PNW. Using HPLC-APCI-MS, a soft ionization technique, SG and ASG standards have been detected. With a similar chromatography method, HPLC-ESI has been validated using the same ASG and SG standards. Currently, there is only one supplier of SG and ASG standards.

The University of Idaho seed breeding laboratory has provided seed from research plots of agronomically significant oilseeds for evaluation. The following oilseeds will be investigated:

- **Winter Canola**
  - *Brassica. napus L. cv.* **Athena**
- **Spring Canola**
  - *Brassica. napus cv.* **Gem**
- **Spring Rapeseed**
  - *Brassica. napus cv.* **Sterling**
- **Spring Camelina**
  - Camelina. sativa L. cv. **Columbia**
  - Camelina. sativa L. cv. **Calena**
- **Spring (Yellow mustard, White mustard)**
  - *Sinapis alba L. cv.* **Ida Gold**
- **Spring (Oriental mustard, Indian mustard, Brown mustard)**
  - *Brassica juncea cv.* **Pacific Gold**

Photographs of some are also shown in Figure 2.

Canola, Camelina, Yellow Mustard, and White Mustard

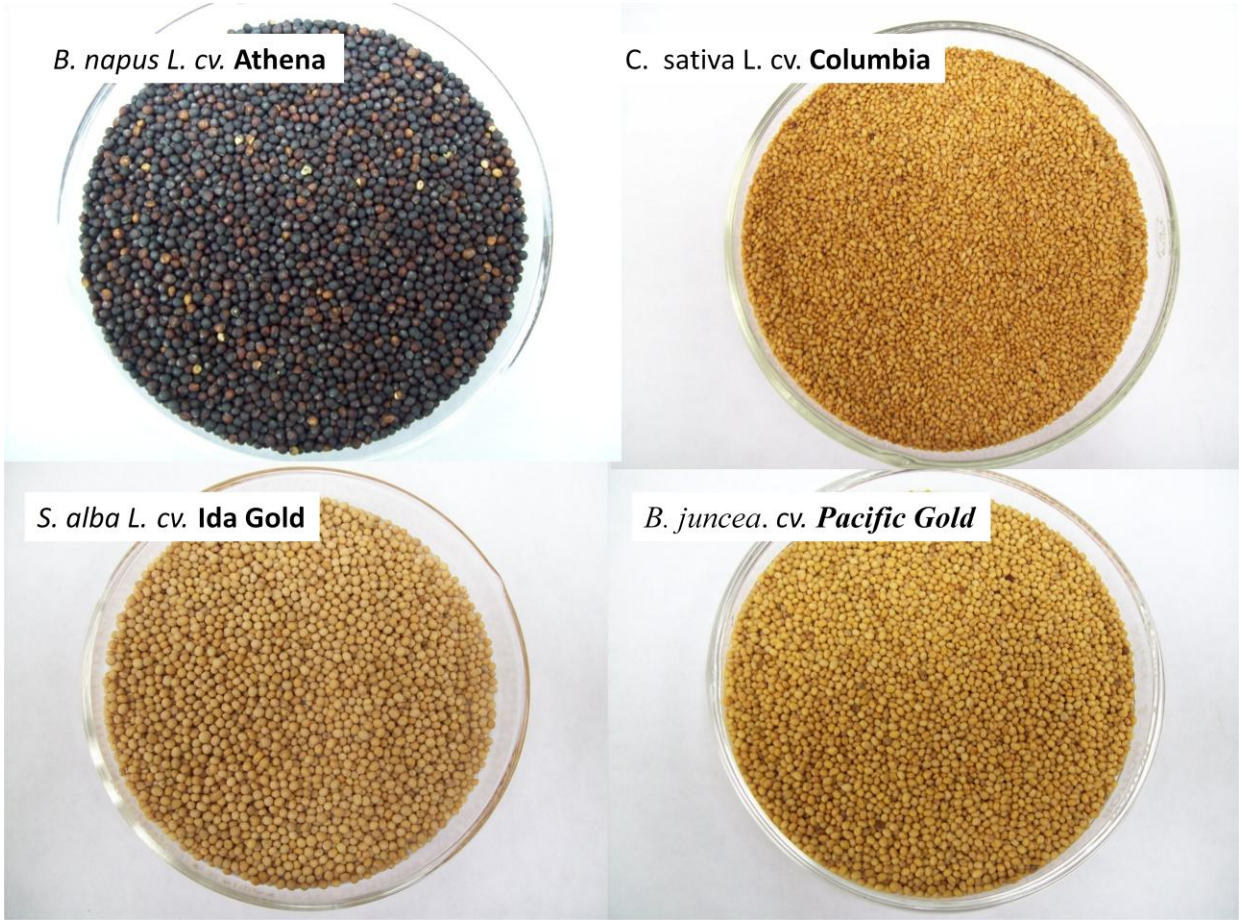


Figure 2: Agronomically Significant Oil Seeds in the PNW.

The team has evaluated several different schemes for extraction of the ASG.

Seeds have been:

a. Manually ground and solvent extracted,
b. Manually ground and exhaustively Soxhlet extracted with chloroform, methanol (2:1),
c. Ball milled (Ultra-Turrax Ball Mill) and exhaustively solvent washed with sequentially more polar solvents (heptane, ethyl acetate, methanol, methanol 40°C).

The ball mill used for option c. is shown in Figure 3.

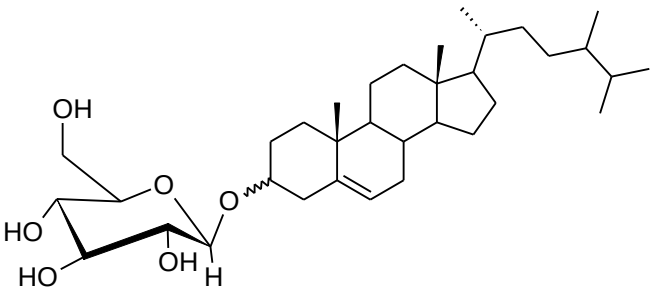
Figure 3: Heptane/Canola slurry and Ultra-Turrax Ball Mill.

From these extractions, several natural products have been identified. The levels of sucrose in canola and camelina were determined after rigorous structure elucidation using H^{1}NMR and C^{13}NMR data. The team is currently optimizing a rapid GC-MS technique for use in detection of steroids as a validation tool for preparatory work. A method for detection of the sterol moieties will allow optimization of the oil extraction techniques.

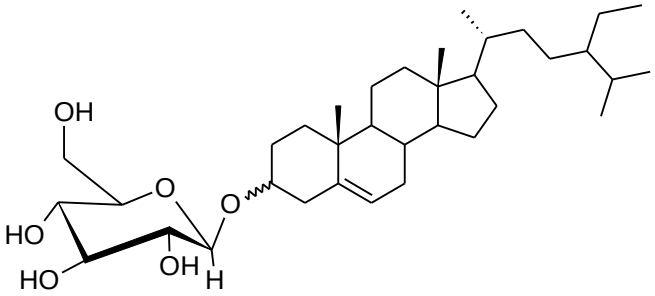
It may prove necessary to perform preparatory work to isolate these analytes from their matrix prior to characterization work. The preparatory methods being investigated include: size exclusion chromatography, silica gel chromatography, chromatography using LH20, Diol SPE (solid phase extraction) cartridge. The ASGs are present in relatively low concentrations (less than 100ppm to several 1000 ppm). Prior to characterization, the triglycerides and phospholipids will have to be isolated from the analytes of interest.

The chromomeric separation on the mass spectrometer was not sufficient with trial extracts that were run in spring 2009. We were able to identify the presence of ASG and SG, but direct chromatography on the instrument was not able to resolve the peaks sufficiently.

Future work will entail method development of HPLC-MS techniques with soft ionization for characterizing SG and ASG. One of the aims of this method is direct analysis of natural products rather than evaluation of derivatives. Once characterization work has occurred, then it is anticipated that quantification can be analyzed using UV at 210nm.



Steryl Glycoside(SG), Capesteryl Glucoside, exact mass 562.4



Steryl Glycoside(SG), Sitosteryl Glucoside, exact mass 576.4

Figure 4: Two common sterol glucosides (SG) in plants with only minor structural difference in the cholesterol moieties.

When the methods have been developed, we will be able to investigate additional questions that have been raised about ASG and SG. One of these questions is where the fatty acid chain that distinguishes the ASG from the SG is located (the acylation point). Acylation is typically assumed to occur at the 6th position of the glucose (see Figure 1); however, South Korean work has demonstrated that in tree extracts, the 4th position was acylated.[2] H^1NMR and C^{13}NMR will likely be required to positively identify the structures. This will require the isolation of purified analytes. This may prove to be beyond the scope of this initial research project.

Another question is what type of fatty acid chain is attached to the ASG. Yamauchi evaluated glycolipids in red bell peppers using HPLC-APCI. This paper showed that a distribution of fatty acids were found in ASG fatty acid moieties including palmitic 16:0, stearic 18:0, linoleic 18:2,

and α-linolenic 18:3. Additionally, the two ASG sterol moieties: campesterol and β-sitosterol, were also identified.[3] In Figure 1 the fatty acid moieties was depicted as stearic acid (18 carbons, no double bonds).

Figure 5: THF run purified though activated alumina column to remove peroxides.

VALIDATION OF HPLC-ESI METHOD

Once a glycolipids fraction has been isolated from oilseeds, a series of analytical techniques are needed to characterize and quantify the natural products. Previous work used HPLC-APCI. A new HPLC-ESI method has since been developed that requires validation with standards.

Steps have been taken to reduce contamination peaks that were present in previous mass spectrometry work. All glass in contact with samples was autoclaved to 450°C in excess of one hour to burn off any phthalates (plastic residues). Additionally, the mobile phase containers were also autoclaved to 450°C. Standards were obtained from Matreya. The following standards were prepared; SG 2.5mg/ml tetrahydrofuran (THF) (Matreya +98%) and ASG 22.5mg/ml DME (1,2-dimethoyethane, glyme) (Matreya +98%). New DME was used.

New THF under nitrogen was run through an activated alumna column and subsequently reactively distilled prior to use for dissolving standards (see Figures 4 & 5). New HPLC grade methanol was used and 17.7mΩ water was used. 23.7_{88} HPLC-ESI ammonium formate was added to both mobile phases. A new Phenomenex, Kinetex 2.6µm, C18, 100A, 150x2.1mm column was used. UV-detection was collected from 190-400nm. The highest level selection was 210nm and looks promising for quantization.

Figure 6: THF reactively distilled over magnesium turnings after peroxide removal using activated alumina.

See appendix A for a list of MS parameters for this and previous runs.

FINDINGS; CONCLUSIONS; RECOMMENDATIONS

Presented

"Evaluation of Acylated Sterols Glucosides (ASG) and Sterol Glycosides (SG) of Oil Seed Crops of the Pacific Northwest" at the 100th AOCS (American Oil Chemists Society) Meeting in Orlando Florida 2009.

Submitted for presentation

"Isolation and characterization of Acylated Steryl Glucosides (ASG) in Oil Seed Crops of the Pacific Northwest (PNW)" at the 101stAOCS (American Oil Chemists Society) Meeting in Phoenix Arizona 2010.

Sucrose

Initial Work with Soxhlet Extraction using Chloroform: Methanol, 2:1
Sucrose Positive Identified with HNMR and correlation spectroscopy (COSY)

Canola	7	ppm
Camelina	18	ppm

Continued Project plan:

1) Work up data currently acquired,
2) Validate GC-MS method for steroid detection
3) Oilseed solvent extraction SG with THF (purified) and ASG with DME
4) Preparatory work and optimization and validation using GC-MS
5) Prepare multiple samples for analysis
6) Evaluating glycolipids fractions with analytical techniques

While other work is occurring, standard samples will be re-run on HPLC-APCI to determine which techniques provide more detailed spectra. In addition, methods may be evaluated with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF). Once an extraction, preparatory and analytical procure is established, it will be repeated for reproducibility and multiple species, cultivars and climatic variations.

REFERENCES

[1] Robert A. Moreau, Karen M. Scott, Michael J. Haas, The Identification and Quantification of Steryl Glucosides in Precipitates from Commercial Biodiesel, 6 June 2008.

[2] WenYi Jin, Byung-Sun Min, JongPill Lee, Phuong Thien Thuong, Hyeong-Kyu Lee, KyungSik Song, Yeon Hee Seong and KiHwan Bae, Isolation of constituents and anti-complement activity from Acer okamotoanum, Archives of Pharmacal Research, 1976-3786, 2007.

[3] Glycolipids in red bell peppers, HPLC-APCI-MS, ASG sterol moieties: campesterol, β-sitosterol, Ryo yamauchi, kichi Aizawa, Takahiro inakuma, Koji kato, 2001.

APPENDIX A HPLC COMPARISON

1/11/2010

Instrumentation:

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 mode subsequently followed by positive mode:
Gas temp 350°C
Vaporizer 350°C
Dry Gas 5.0L/min
Capillary 3000V
Fragmentation voltage four channels (100, 200, 300, 400)
Skimmer 60V
OCT RFV 250
DAD-UV 230-700nm
100-3500mz

UV 190-400nm

Standards:

SG 2.5mg/ml THF (Matreya +98%)
ASG 22.5mg/ml DME
(1,2-dimethoyethane, glyme) (Matreya +98%)

Samples:

1) THF Blank (repeated)
2) 2.5mg/ml THF SG STD
3) DME Blank
4) 2.5mg/ml DME ASG STD
5) Pyridine SG STD (x trace mg/ml)

1/8/2010

Instrumentation:

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,	40°C	.4ml/min,	H_2O:MeOH,	9:1
T=1min,	40°C	.4ml/min,	H_2O:MeOH,	9:1 gradient
T=11min,	40°C	.4ml/min,	100%MeOH	
T=25min,	40°C,	.4ml/min,	100%MeOH	
T=26.50min,	40°C,	.4ml/in,	H_2O:MeOH,	9:1
T=30min,	40°C,	.4ml/in,	H_2O:MeOH,	9:1

Injection:

1 µL

ESI Mass Spec:

Positive mode:
Gas temp 350°C
Vaporizer 350°C
Dry Gas 7.0L/min
Capillary 3000V
Skimmer 60V
OCT RFV 250
DAD-UV 230-700nm

Second ESI run:

Negative mode

Standards:

SG 2.5mg/ml THF (Matreya +98%)
ASG 22.5mg/ml DME
(1,2-dimethoyethane, glyme) (Matreya +98%)

Samples:

1)THF Blank (repeated)
2) 2.5mg/ml THF SG STD
3) DME Blank
4) 2.5mg/ml DME ASG STD
5) Pyridine SG STD (x trace mg/ml)
UV signal strongest at 210, also strong at 205, 215

Spring 2009

Instrumentation:

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

Injection:

0.5 µL

APCI Mass Spec:

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

Standards:

SG 0.1mg/ml chloroform (low solubility) (Matreya +98%)
ASG 2.5mg/ml DME
(1,2-dimethoyethane,(glyme) (Matreya +98%)

Summary of Literature references used for formation of methods:

Moreau, 2008

Method B:

HPLC: Normal phase isocratic HPLC 0.5ml/min Agilent model 1100msd Hexane/isopropanol/acetic acid, 85/14.0/0.1 (v/v/v)

Colum: Varian LiChrosorb five diol Colum 3x100mm with guard column chrompack polar bonded 2.1x10mm

APCI:

Positive mode
200-1,000 m/z
Fragmentor 5V
Drying gas 5 L/min
Nebulizer pressure 50 psi
Drying gas temperature 350°C
Corona current of 4.0 µA
Capillary voltage 4,000 V

Method C:

HPLC: reverse phase isocratic 0.2ml/min

Colum: Prevail C18, 3µm column (2.1x150mm, Alltech Associates, Deerfield**,** IL USA) MeOH/H2O 96/4 (v/v), premixed with 20mM ammonium formate

ESI MS:

200-1,000 m/z,
fragmentor 5V
drying gas 5,
nebulizer pressure 50,
drying gas temperature 300°C,
capillary voltage 5,500

Ryo Yamauchi, 2001

Analysis of Molecular Species of Glycolipis in Fruit Pastes of Red Bell Peper (Capsicum annuum L.) by high-Performance Liquid Chromatoraphy-Mass Spectrometry, 2001, ryo yamauchi, koici Aizawa, Takahiro Inakuma, Koji Kato.

Preparatory work:

Lyophilized red bell pepper past was chloroform/methanol (2:1, v/v) extracted 3x, concentrated and eluted on silica gel column with chloroform. Colum: BW-820MH, 70-200 mesh; Fuji Silysia Chemical Ltd., Kasugi, Japan; 4.5x30cm. Sequential elutions with chloroform (neutral lipids), acetone (glycolipids), methanol (phospholipids).

Instrumentation:
Shimadzu LC-10AV vp pump , Shimadzu SPD-10vp UV-vis detector (Shimadzu Co., Kyoto, Japan.) UV-detection at 205nm and APCI detection. Shimadzu LCMS-QP8000α quadruple mass spectrometer. The MS parameters were optimized by direct infusion of polyethylene glycol standards.

Colum:

Isocratically Luna 3 µm C18 column (2.0x150mm, Phenomenex, Torrance, CA) at 40°C.

HPLC:

Isocratic Flow rate of 0.2ml/min mobile phase;

methanol/water (98:2, v,v) for SG

methanol/ethanol (3:2, v,v) for ASG

APCI Mass Spec:

Positive ion mode
m/z 200-1000 at scan rate 3sec
APCI probe voltage 4.5Kv
Temp of 400°C
Nitrogen for nebulizing gas at 2.5L/min
Curved desolvation line (CDL) voltage of -40V at 250°C
Deflection voltage +70V for ASG and SG