EPL BIO ANALYTICAL SERVICES

Determination of Total Desulfglucosinolates in Canola Seed by Ultra High Performance Liquid Chromatography with Ultraviolet Detection.

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Abstract

Glucosinolates exist as a subset of water soluble anionic organic compounds that contain sulfur and nitrogen. They are secondary metabolites that are divided into three categories: aliphatic, aromatic aryl and aromatic indole glucosinolates. These groups are differentiated by the chemical structure of their side chains. There are multiple ways to analyze this particular class of compounds. Most use high performance liquid chromatography with ultraviolet detection (HPLC-UV) or high performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS). The methodology being explored in this paper is the transfer of this method from conventional instrumentation to ultra high performance liquid chromatography with ultraviolet detection (UHPLC-UV). Excellent resolution and separation of 14 desulfonated glucosinolates, using glucotropaeolin as the internal standard, was obtained using reverse-phase chromatography on a Waters BEH Shield RP-18 (2.1 x 100mm 3µm particle size) column. Analysis time was decreased from 30 minutes to 13 minutes between sample injections, thus greatly increasing sample throughput and efficiency.

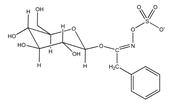
Objective

The objective was to validate an analytical method for the determination of total glucosinolates using UHPLC-UV detection.

This method is referenced from the publications "Determination of Glucosinolates in Rapeseed Improvement of the Official HPLC ISO Method (Precision and Speed)" *(1)* and "Rapeseed – Determination of Glucosinolate content – part 1: Method using high performance liquid chromatography." *(2)*

Chemicals and Reagents

- Glucotropaeolin analytical standard was obtained from POS Pilot Plant Corp, Saskatoon, Saskatchewan, Canada. 0.2300 g (0.2250 – 0.2350 g) of benzyl glucosinolate tetra-methyl ammonium salt reference substance was weighed into a 25mL volumetric flask and filled to volume with deionized water.
- The resultant solution was stored in a freezer at -4° C with an expiration of 365 days.



Chemicals and Reagents

- Acetonitrile (ACN), HPLC Grade, EMD, Billerica, MA
- Formic Acid (FA), Fisher, Fair Lawn, NJ
- Methanol (MeOH), HPLC Grade, EMD
- Ethanol, HPLC Grade, EMD
- Imidazole, Sigma Aldrich, St. Louis, MO
- Sodium Acetate, Fisher
- Deionized (DI) Water, as delivered by the Barnstead NANOpure system or equivalent

Solution Preparation

Purified Sulfatase *Helix pomatia*

- Weigh 0.075 g (0.065 – 0.085 g) of sulfatase *Helix pomatia* enzyme into a 15 mL centrifuge tube. Pipet 7.0 mL of 40% ethanol solution, cap and mix.
- Centrifuge at approximately 2000 rpm for 10 minutes, as controlled by the centrifuge.
- Decant the upper supernatant into a clean 15 mL centrifuge tube and discard the precipitate.
- Pipet 7.0 mL of 70% ethanol solution to the tube, cap and mix well.
- Centrifuge at approximately 2000 rpm for 10 minutes.
- Decant the upper liquid phase off and discard it.
- Add 2.5mL of deionized water to dissolve the sulfatase pellet.
- Transfer the solution of purified sulfatase into a glass vial or other suitable container.
- Store frozen.

Equipment

- Balances, 0.1 and 1 mg accuracy, Mettler-Toledo, Models XP6035 and XP204,
- Columbus, OH
- Class A volumetric glassware
- Acquity UHPLC with UV Detector, Waters Corporation, Milford, MA
- Clear autosampler vials and storage bottles, VWR, Batavia, IL
- 15 mL disposable glass culture tubes, VWR
- Pasteur pipettes, VWR
- Glass beakers, VWR

Equipment

- Bottle top dispenser
- Adjustable pipetter
- Centrifuge, Eppendorf 5810, Hauppauge, NY
- Water bath capable of 75° C
- Weigh boat or weigh paper
- Glass Wool
- Column: Waters Acquity UPLC BEH Shield RP18, 2.1 x 100 mm, 1.7 µm

Test Matrix

The method was validated using a commercially available canola seed sample. Canola samples were ground with dry ice using a Retsch Centrifugal Grinder (Haan, Germany) equipped with a 0.75 mm screen.

Extractions and Procedures

- Approximately 0.200 g of ground canola seed was weighed into disposable glass 15mL culture tubes. Two mL of methanol:DI water solution (70:30 v/v) was transferred to each tube.
- Adding 0.100mL of the glucotropaeolin internal standard.
- The tubes were placed in a 75° C water bath for 10 minutes, shaking the tubes every few minutes.
- After removing the tubes from water bath they were centrifuged for 3 minutes at 2500 RPM. The supernatant was decanted into individual 5mL volumetric flasks. This step was repeated again for a total of two extractions.
- The 5mL volumetric flask was brought to volume using DI water.

Preparation of desulfatation column:

- A glass wool plug was placed in the neck of a disposable Pasteur pipet (5 ¾" x 7.0 mm O.D.) and suspended vertically on a stand.
- There was one pipet for each sample. To each pipet, 0.5mL of a well-mixed DEAE Sepharose ion exchange resin was added and allowed to settle.
- Each pipet was rinsed with 2mL of 6 M imidazole formate and twice with 1mL portions of DI water allowing each wash to drain to waste.
- A 1mL aliquot of the sample extract from the 5mL volumetric flask transferred to a prepared pipet without disturbing the resin surface and allowed to drain to waste.
- Two 1mL portions of sodium acetate buffer was added and allowed to drain to waste after each addition
- Purified sulfatase solution (75µL) was added to each pipet and allowed to react overnight approximately 18 hours.
- A scintillation vial was placed under each pipet and each pipet was treated with two 1mL additions of DI water allowing the water to drain after each addition.
- The vials were mixed and transferred sample to UHPLC vials for analysis. Samples can be stored refrigerated overnight prior to analysis.

HPLC Mobile Phase Gradient Program

Table 1.

Time (min.)	Flow Rate (mL/min)	%A	%B	Curve
0.00	1.50	100	0	6
1.00	1.50	100	0	6
21.0	1.50	0	100	6
26.0	1.50	100	0	6
30.0	1.50	100	0	6

A: DI Water **B:** 20:80 ACN:DI Water

HPLC Parameters

- Column Temp: 30°C
- Sample Temp : 20°C
- Injection Volume: 40 µL
- Wavelength: 229 nm
- Column: Lichrosorb RP18-5, 150 x 4.6 mm, 5µm

UHPLC Mobile Phase Gradient Program

Table 2.

Time (min.)	Flow Rate (mL/min)	%A	%B	Curve
0.00	0.200	99	1	6
2.00	0.200	99	1	6
10.0	0.200	70	30	6
12.0	0.200	99	1	6
13.0	0.200	99	1	6

A: DI Water **B:** ACN

UHPLC Parameters

- Column Temp: 30°C
- Sample Temp : 20°C
- Injection Volume: 3µL
- Wavelength: 229 nm

HPLC and UHPLC Mobile Phase Gradient Program

Both the HPLC-UV and UHPLC-UV systems were calibrated by analysis of internal standard (Glucotropaeolin) in a control sample for retention time verification. One quality control sample was injected and analyzed with the samples. Atlas version 8.3 was used to collect the chromatographic data and calculate sample solution concentrations

Results/Discussion

Eight replicate sub-samples of canola seed were analyzed to determine the precision of the developed method via calculation of the relative standard deviation (%RSD). Sample glucosinolate concentrations were expressed on a percent dry weight basis (%DB). The mean (n=8) glucosinolate content was 0.180 %DB for progoitrin, 0.133 %DB for gluconapin and 0.0300 %DB for glucobrassicin. The RSD was 5.26% for progoitrin, 5.12% for gluconapin and 13.9% for glucobrassicin. Method bridging data fell within control chart tracking ranges (+/- 3σ) from the previous validated method for the three quality control analyte peaks, progoitrin, gluconapin and glucobrassicin. (see Table 3)

The HPLC chromatogram of a canola seed sample and corresponding UPHLC chromatogram of the same sample appear in Figures 1a and 1b, respectively. UHPLC provides a faster run time (13 minutes vs 30 minutes), superior resolution and greater sensitivity than conventional HPLC.

Figure 1a. Chromatogram comparison

1a. HPLC Chromatogram of Desulfoglucosinolates

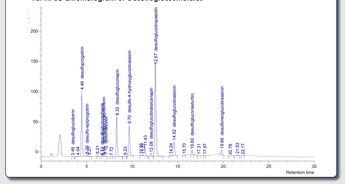
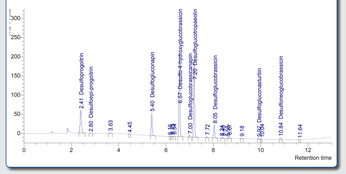


Figure 1b. Chromatogram comparison

1b. UHPLC Chromatogram of Desulfoglucosinolates



Method Bridging and Precision Data

Table 3. Summary of Sample Analysis Results

Analyte	Mean (n=8) (% DB)	Std. Dev.	RSD (%)	Method Bridging Ranges (%DB) +/- 3σ
Progoitrin	0.180	0.00945	5.26	0.0148-0.249
Gluconapin	0.133	0.00680	5.12	0.0369-0.140
Glucobrassicin	0.0300	0.00418	13.9	0.00542-0.0306

Conclusions

An analytical method for the determination of desulfoglucosinolates in canola seed was successfully validated. This method demonstrated acceptable precision, sensitivity and improved chromatography. The validated method will be used routinely to provide nutritional and compositional data to support the registration of GM crops.

References

(1) Wathelet, Jean-Paul, Mabon, Nicolas and Marlier, Michael. "Determination of Glucosinolates in Rapeseed Improvement of the Official HPLC ISO Method (Precision and Speed)".

(2) Internal Organization for Standardization 9167-1:1192(E) "Rapeseed - Determination of Glucosinolate content – part 1: Method using high performance liquid chromatography."