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Optimized analysis and quantification of glucosinolates from *Camelina sativa* seeds by reverse-phase liquid chromatography☆

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ABSTRACT

Gold-of-pleasure or false flax (*Camelina sativa* L. Crantz) is being developed as an alternative oil crop for biodiesel and for food use. The seed meal, which contains three relatively unique glucosinolates, is being evaluated for approval for use as an ingredient in animal feeds and for other uses. The objective of this research was to develop reproducible methods for the isolation of large quantities of pure camelina glucosinolates (glucoarabin, glucocamelinin, and 11-(methylsulfinyl)-undecylglucosinolate) and develop efficient methods for quantifying these compounds. The separation and purification of the camelina glucosinolates were achieved using a combination of reverse phase chromatography, counter-current chromatography and ion exchange chromatography. An efficient reverse phase HPLC separation method was used to quantitate the glucosinolate content in camelina seed and plant extracts. The quantitation methodology was used to measure glucosinolate levels in seeds from 30 cultivars grown in a U.S. field trial and measure glucosinolates levels in sprouted camelina seeds.

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1. Introduction

Camelina sativa L. Crantz, known as gold-of-pleasure, false flax, wild flax, linseed dodder, camelina, German sesame, and Siberian oilseed, is a flowering plant in the Brassicaceae. It is native to Northern Europe and Central Asia, but has been introduced to North America, possibly as a weed in flax. It has been traditionally cultivated in Europe as an oilseed crop to produce vegetable oil and animal feed. There is ample archeological evidence to show it has been grown in Europe for at least 3000 years (Jones and Valamoti, 2005). Camelina was an important oil crop in eastern and central Europe, and has continued to be cultivated for its seed, which was used in oil lamps and as an edible oil. Interest in the use of camelina as a functional food and as a biodiesel feedstock continues to grow (Zeman, 2007; Moser and Vaughn, 2010). Camelina is a good alternative crop because it is much less weather dependent, has more consistent yields, and is cheaper to produce than other new crops (Moloney et al., 1998). Camelina has very low requirements for tillage and weed control (Putnam et al., 1993; Vollmann et al., 2007; Urbaniak et al., 2008). This could potentially allow this unique vegetable oil to be produced more cheaply than those from traditional oil crops, and it would be particularly attractive to biodiesel producers looking for a feedstock cheap enough to allow them to compete with petroleum diesel and gasoline. Significant new crop research is currently being conducted in the northern United States and in a number of Canadian provinces. The oil contains exceptionally high levels of omega-3 fatty acids, which is uncommon in vegetable sources (Budin et al., 1995; Abramovic and Abram, 2005; Abramovic et al., 2007; Schwartz et al., 2008) and over 50% of the fatty acids in cold-pressed Camelina oil are polyunsaturated (Budin et al., 1995; Abramovic et al., 2007).

Finding additional uses for the seed meal press-cake will make the crop more economically competitive. Camelina could be added to the growing list of functional foods. Current research efforts centers on its high levels of omega-3 fatty acids, as well as rich levels of antioxidants such as tocopherols which make the oil naturally

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stable (Budin et al., 1995; Abramovic et al., 2007; Dubois et al., 2007). Defatted camelina seed meal contains significant levels of proteins and carbohydrates as well as a number of phytochemicals including glucosinolates, which could be utilized for additional food, feed, and agricultural uses (Gugel and Falk, 2006; Zubr, 2010). If the meal can be used as quality feed and/or food ingredients, this will significantly increase the market value of the seed meal. Camelina seed meal contains 5–10% residual fat (which contains fairly high levels of omega 3 fatty acids), high-quality protein, and some potentially functional phytochemicals, which can be exploited to develop new feed and food uses.

Glucosinolates occur as secondary metabolites of many plants of the order Brassicales, especially in the family Brassicaceae, as well as in members of the Capparidaceae and Caricaceae families (Fenwick et al., 1983; Daxenbichler et al., 1991; Fahey et al., 2001; Clarke, 2010). Plants use chemicals derived from glucosinolates as natural pesticides and as defense against herbivores; these substances are also responsible for the bitter or sharp taste of many cruciferous vegetables (Fahey et al., 2003; Clarke, 2010). About 120 different glucosinolates are known to occur naturally in plants (Fahey et al., 2003; Clarke, 2010). The plants contain the enzyme myrosinase, which in the presence of water cleaves off the glucose group. The remaining molecule then quickly converts to a thiocyanate, an isothiocyanate or a nitrile; these are the active substances that serve as defensive compounds for the plant (Spencer and Daxenbichler, 1980; Vaughn and Berhow, 2005). To prevent damage to the plant itself, the myrosinase and glucosinolates are stored in separate compartments of the cell and come together only under conditions of stress or injury. Several degradation products of hydroxyl-substituted glucosinolates have been shown to be goitergenic in both man and animals (Hoist and Williamson, 2004; Anilakumar et al., 2006). In contrast, at subtoxic doses, their hydrolytic and metabolic products act as chemoprotective agents against chemically induced carcinogens by blocking the initiation of tumors in a variety of mammalian tissues. They exhibit their effect by inducing Phase I and Phase II enzymes, by inhibiting enzyme activation, modifying steroid hormone metabolism, and protecting against oxidative damages (Shapiro et al., 2001; Talalay and Fahey, 2001; Fahey et al., 2003).

C. sativa accumulates significant levels of three glucosinolates in its seeds (Fig. 1): glucoarabin (9-(methylsulfinyl)nonylglucosinolate – GS9), glucocamelinin (10-(methylsulfinyl)decylglucosinolate – GS10), and 11-(methylsulfinyl)undecylglucosinolate (GS11) (Daxenbichler et al., 1991; Lange et al., 1995; Schuster and Friedt, 1998; Fahey et al., 2001; Vaughn and Berhow, 2005). The levels of glucosinolates accumulated in seeds are affected by genotype and environmental conditions (Farnham et al., 2005). Camelina glucosinolates may potentially be anti-cancer nutriceuticals in both animal and human diets. The structure of the camelina glucosinolates, containing terminal methylsulfinyl groups with varying-length aliphatic chains connecting to the glucosinolate functional group, are similar to that of glucoraphanin (4-(methylsulfinyl)butylglucosinolate), the difference being only the length of the aliphatic connecting chain. In theory, the degradation products of GS9, GS10, and GS11 should behave in a similar fashion to that of sulforaphane, the degradation product of glucoraphanin, which is an anticancer compound produced in broccoli and other crucifer vegetables (Shapiro et al., 2001; Talalay and Fahey, 2001; Fahey et al., 2003).

In order to prove this theory, quantities of purified camelina need to be produced. Preparative chromatography and countercurrent chromatography have been used to purify a variety of glucosinolates from plant extracts on a prep scale including glucoraphanin from broccoli and sinalbin from white mustard (Fahey et al., 2003; Toribio et al., 2009). These methods make it possible to purify larger quantities of glucosinolates for biological studies. The biological effects of the degradation products – the isothiocyanate, thiocyanate and nitrile forms – from the camelina glucosinolates in diets and in agriculture has not been assessed. In this report we have developed methodology to separate and purify the camelina glucosinolates in mg quantities and developed a robust method to analyze glucosinolates in camelina seeds, meal, and sprouts.

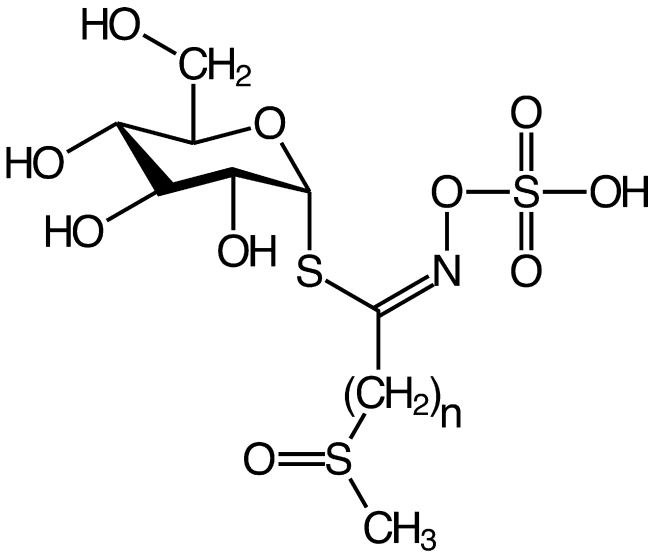

Fig. 1. Basic structure of the glucosinolates found in *Camelina sativa*. GS9 – glucoarabin (9-(methylsulfinyl)nonyl-glucosinolate), $n = 9$; GS10 – glucocamelinin (10-(methylsulfinyl)decyl-glucosinolate), $n = 10$; and GS11 – 11-(methylsulfinyl)undecyl-glucosinolate, $n = 11$.

2. Materials and methods

2.1. Reagents and chemicals

Sinigrin was purchased from Sigma–Aldrich Chemical Company (St. Louis, MO). All other chemicals and solvents were of analytical grade.

2.2. Camelina cultivars and sprouting studies

Camelina accessions were planted in individual cages and grown and harvested for seed bank increase in 2009 at NCRPIS field plots. One to two gram seed samples of each of 30 cultivars were used for comparative glucosinolate analysis.

2 g of camelina seeds were placed in a series of 10 cm diameter glass Petri plates on top of three sheets of filter paper. The plates were wetted with distilled water, covered with a glass top, and placed in a BioChambers plant growth chamber (Winnipeg, Manitoba) set at 24 °C during the day cycle and 20 °C during the night cycle, with a light dark cycle of 16 h of light (300 μE PPF PAR) and 8 h of dark. One set of plates was removed each day for the seven-day sprouting period. The remaining plates were wetted with additional water every 24 h. The sprouts were removed from the filter paper; placed in tared jar; weighed; freeze dried; then weighed again to determine an approximate weight/dry weight ratio.

2.3. Analytical sample preparation and extraction

Seeds were ground to a fine powder with a commercial coffee grinder. Weighed samples were placed in filter paper packets and defatted overnight in a Soxhlet extractor with hexane. After drying in the hood, the percent hexane extractables were determined by the difference in weight. For sprouting studies, sprouts

were weighed, then freeze-dried overnight and reweighed. Dried samples were ground to a fine powder with a mortar and pestle.

For HPLC analysis, typically between 0.25 and 0.5 g of defatted seedmeal and freeze-dried sprout powders were placed in a capped vial with between 2 and 5 mL of methanol. The vials were sonicated for 15 min, and allowed to stand overnight. After another brief sonication, a portion of this extract was filtered through a 0.45 μm filter into an auto sampler vial.

2.4. Apparatus and chromatographic conditions

For glucosinolate quantitation, a modification of a high-performance liquid chromatography (HPLC) method developed by Betz and Fox (1994) was used. The extract was run on a Shimadzu (Columbia, MD) HPLC System (two LC 20AD pumps; SIL 20A autoinjector; DGU 20As degasser; SPD-20A UV–VIS detector; and a CBM-20A communication BUS module) running under the Shimadzu EZStart Version 7.3 software. The column used was an Inertsil reverse phase column (250 mm × 4.6 mm; RP C-18, ODS-3, 5 μm; Varian, Torrance, CA). The initial mobile phase conditions were 40% methanol 60% aqueous 0.005 M tetrabutylammonium bisulfate (TBS) at a flow rate of 1 mL/min. After injection of 20 μl of sample, the initial conditions were held for 10 min, and then up to 100% methanol over an additional 15 min and held at 100% methanol for 5 more minutes. The glucosinolates were detected by monitoring at 237 nm.

To confirm the identity of the glucosinolates found in the seed extracts, aliquots were injected on a Q-TOF LC–MS. Samples were run on an Applied Biosystems/MDS Sciex QStar Elite Q-TOF mass spectrometer with a Turboionspray electrospray source, and an Agilent 1100 series HPLC system (G1379A degasser, G1357A binary capillary pump. G1389A autosampler, G1315B photodiode array detector, and a G1316A column oven) all running under Applied Biosystems Analyst 2.0 (build 1446) LC–MS software. The MS was calibrated at least once daily with a standard calibration mixture recommended by Applied Biosystems and the signal detection was optimized as needed. The data was acquired in the negative TOF MS mode. The MS parameters were as follows: cycle time 1 s., accumulation time – 1 s, mass range 400–1000 Da, source gas 1–45 units, source gas 2–25 units, curtain gas-25 units, ion spray voltage −4200, source heater 400 degrees, declustering potential −60, focusing potential -265, declustering potential 2–15, ion release delay 6, ion release width 5. The column used was an Inertsil ODS-3 reverse phase C-18 column (3 μm, 150 mm × 3 mm, from Varian). The initial HPLC conditions were 40% methanol and 60% 0.25% acetic acid in water, at a flow rate of 0.25 mL/min, then the column was developed to 75% methanol over 20 min, then to 100% methanol in another 5 min. The effluent was also monitored at 237 nm on the PDA. Three glucosinolate peaks were detected which had mass ions of *m/z* 507, 521, and 535, corresponding to the $[M-H]^-$ mass ions for GS9, GS10, and GS11, respectively.

^1H and ^{13}C NMR spectroscopy was performed on a Bruker Avamce 500 spectrometer (Bruker BioSpin, Billerica, MA, USA).

2.5. Glucosinolate standards

A fresh sinigrin standard (Sigma, St. Louis, MO) was prepared in water on a molar concentration basis, and a series of dilutions were prepared to make a standard curve. Standard solutions of semi-purified GS9, GS10, and GS11 were prepared by chromatographic isolation in the laboratory. Concentrations in these standards were determined by sinigrin calibration curve on an nM/mAbs basis. Concentrations of GS9, GS 10, and GS 11 in the samples were calculated from a standard curve of freshly prepared pure sinigrin (Sigma) as nmol injected, the concentration in the samples were determined and the final values calculated as mg/g dry weight. The % hexane extractables for each seed set was used to determine the concentration in the whole fat seeds.

2.5.1. Preparative chromatographic purification of glucosinolates

For glucosinolate purification approximately 500 g of defatted seed meal was used. The meal was extracted with methanol in Soxhlet extractors for 24 h. The resulting methanol extracts were concentrated by rotoevaporation and resuspended in a mixture of methanol and water.

A Büchi (Newcastle, DE) Sepacore flash chromatography system with dual C-605 pump modules, C-615 pump manager, C-660 fraction collector, C-635 UV photometer, with SepacoreRecord chromatography software was used, a 40 mm × 150 mm flash column with approximately 90 g of preparative C18 reverse phase bulk packing material (125 Å, 55–105 μm, Waters Corp, Milford, MA).

The column was equilibrated with 20% methanol and 0.5% acetic acid in water for 5 min at a flow rate of 30 mL/min. After injection of the samples (20 mL), the column was developed with a binary gradient to 100% methanol over 50 min. The effluent was monitored at 237 nm and fractions based on absorbance were collected in the fraction collector by the software program. Three broad overlapping peaks were eluted from the column. The procedure was repeated until all the extract was used. Fractions containing each of the three absorbance peaks were pooled and concentrated by evaporation in the hood at room temperature. As the methanol evaporated greenish-yellow crystals of the flavonoid rutin formed which were removed by filtration during the drying process. Dried fractions were resupended in water for preparative HPLC chromatography.

A Shimadzu (Columbia, MD) preparative HPLC system was used with dual 8 A pumps, SIL 10vp autoinjector, SPD M10Avp photodiode array detector, FRC-10A fraction collector, SCL 10Avp system controller all operating under the Shimadzu Class VP operating system. 10 mL sample aliquots in methanol were injected on a Phenomenex (Torrance, CA) Luna C18(2) semi-preparative reverse-phase column (10 μm, 100 Å, 250 cm × 50 cm). The column was pre-equilibrated with 10% methanol and 90% water with 0.5% ammonium formate (pH 5) at a flow rate of 50 mL/min and the effluent was monitored at 237 nm. The column was developed to 100% methanol over 50 min. Fractions were collected based on time intervals and pooled based on peak separation. The procedure was repeated to obtain sufficient quantity of purified material. Fractions were allowed to evaporate to remove methanol, and then freeze-dried to recover the purified glucosinolates. The resulting yields were 150 mg of GS9, 550 mg of GS10, and approximately 2 mg of GS11.

Alternatively, flash chromatography fractions containing glucosinolates, were pooled and 200–400 mL aliquots loaded on disposable Waters (Milford, MA) 35 cc (10 g) Accell Plus QMA strong anion exchange columns, which were pre-equilibrated with 100 mL of imidazole–formate buffer pH 4.15. The loaded columns were washed with 100 mL of water, then 100 mL of water:isopropanol:formic acid solution (8:2:0.5), and 100 mL water to remove contaminates. The columns were eluted with 80 mL 0.5 M potassium sulfate containing 5% isopropanol in water. The process was repeated until all flash fractions were passed through. The pooled materials was flushed with nitrogen to remove the isopropanol, then lyophilized to remove the water. The residue was dissolved in methanol with sonication, and filtered through Whatman no. 2 filter paper to remove the potassium sulfate, then dried under nitrogen, and resuspended in water for lyophillization.

2.5.2. Preparative counter-current chromatographic purification of glucosinolates

For a second glucosinolate purification approximately 250 g of defatted seedmeal was used. The meal was extracted with

methanol in Soxhlet extractors for 24 h. The resulting methanol extracts were concentrated by rotoevaporation to yield around 25 g of crude extract.

Separation of glucosinolates from camelina was done according to the modified method described by Toribio et al. (2009). The defatted camelina seedcake methanol extract was purified using a Fast Centrifugal Partition Chromatograph (FCPC Kromaton®) apparatus fitted with a preparative 1 L rotor. The two-phase solvent system for CPC consisting of ethyl acetate, *n*-butanol and water (3:2:5, v/v) was prepared by mixing solvents in a separatory funnel, and after thorough equilibration of the solvents, the two resulting phases were separated. Aliquat® 336 was added to the organic phase to the final concentration of 40 mM. This freshly prepared organic phase was pumped into rotor to become a stationary phase, using Waters high-pressure pump (model 590). During this phase the rotor was span at 200 rpm. The sample solution was prepared by dissolving 16 g of camelina extract in 70 mL of a mixture of the upper (organic without Aliquat® 336) and lower phases (60:10, v/v) and filtered immediately before injection. Sample injection was done using a Dynamax SD-200 pump with a 25 mL pump head. After injection the rotation speed was adjusted to 900 rpm and the aqueous phase (mobile phase) was pumped in using "descending mode" valve position at the flow rate 9 mL/min (approx. 2L). Then aqueous phase (mobile phase) containing 40 mM of potassium iodide was pumped through at the same flow rate. During the CPC run 25 mL fractions were collected and analyzed by TLC and HPLC chromatography in order to monitor their composition and purity.

TLC was performed on silica gel 60 F_{254} plates (Merck), developed at room temperature with *n*-butanol:acetic acid:water (60:15:25, v/v/v) and compounds were visualized with universal spray reagent ($Cr^{+3}/Mo^{+6}/H_2SO_4$) after heating. HPLC chromatographic separations were achieved using a Hewlett Packard HP Series 1050 system equipped with an autosampler and a HP 1050 Photodiode Array Detector. The column YMC ODS-AQ (5 μm, 250 mm × 4.6 mm i.d.) was used for the analysis of glucosinolates. A 10 mM Aliquat® 336 in acetonitrile/water 50:50 (v/v) was used as a mobile phase (isocratic mode). Peaks were monitored at 205 and 235 nm, at a flow rate of 0.8 mL/min. HP Chemstation was used to control the operation and to perform data analysis.

2.6. Characterization of purified camelina glucosinolates

9-(Methylsulfinyl)-nonylglucosinolate (Glucoarabin, GS9). Brown amorphous powder. HRESIMS: *m/z* 506.11937 $[M-H]^-$ (calcd. for $C_{17}H_{32}NO_{10}S_3$, 506.11884). ^{1}H NMR (500 MHz, MeOH-d_4) δ: 4.83 (1H, m, H-1′), 3.85 (1H, d, J = 12.1 Hz, H-6′a), 3.66 (1H, dd, J = 12.1, 5.3 Hz, H-6′b), 3.41 (1H, t, J = 8.7 Hz, H-3′), 3.34 (2H, m, H-4′, 5′), 3.27 (1H, dd, J = 9.7, 8.7 Hz, H-2′), 2.84 (1H, m, H-9a), 2.79 (1H, m, H-9b), 2.70 (2H, t, J = 7.4 Hz, H-1), 2.64 (3H, s, H-10), 1.76 (4H, m, H-2, 8), 1.56–1.34 (10H, m, H-3, 4, 5, 6, 7). ^{13}C NMR (500 MHz, MeOH-d_4) δ: 160.8 (C-0), 82.4 (C-1′), 81.0 (C-5′), 78.2 (C-3′), 72.8 (C-2′), 69.7 (C-4′), 61.2 (C-6′), 53.5 (C-9), 36.7 (C-10), 32.2 (C-1), 28.9–28.3 (C-3, 4, 5, 6, 7), 27.2 (C-2), 22.2 (C-8). Acetone standard addition NMR analysis for purity: 7.0 mg of GS9 dissolved in 600 μL MeOH-d_4 with 1.9 μL (12.8 μM) of acetone, resulted in ratios of 2.76 μmol of acetone per μmol of GS9, 9.62 μmol of water per μmol of GS9, and 3.49 μmol of water per μmol of acetone, yielding a solid salt composition of $C_{17}H_{32}NO_{10}S_3$ K × $9H_2O$ of 95% purity.

10-(Methylsulfinyl)-decylglucosinolate (Glucocamelinin, GS10). Brown amorphous powder. HRESIMS: *m/z* 520.13526 $[M-H]^-$ (calcd. for $C_{18}H_{34}NO_{10}S_3$, 520.13449). ^{1}H NMR (500 MHz, MeOH-d_4) δ: 4.82 (1H, d, J = 9.8 Hz, H-1′), 3.85 (1H, dd, J = 12.7, 1.6 Hz, H-6′a), 3.65 (1H, dd, J = 12.1, 5.4, H-6′b), 3.40 (1H, m, H-2′), 3.33 (2H, m, H-4′, 5′), 3.26 (1H, dd, J = 9.8, 8.8, H-2′), 2.84 (1H, m, H-10a), 2.79 (1H, m, H-10b), 2.69 (2H, t, J = 7.6, H-1), 2.64 (3H, s, H-11), 1.76 (4H, m, H-2, 9), 1.56–1.32 (12H, m, H-3, 4, 5, 6, 7, 8). ^{13}C NMR (500 MHz, MeOH-d_4) δ: 160.5 (C-0), 82.4 (C-1′), 81.0 (C-5′), 78.2 (C-3′), 72.8 (C-2′), 69.7 (C-4′), 61.3 (C-6′), 53.5 (C-10), 36.7 (C-11), 32.3 (C-1), 29.0–28.3 (C-3, 4, 5, 6, 7, 8), 27.2 (C-2), 22.2 (C-9). Acetone standard addition NMR analysis for purity: 4.8 mg of GS10 dissolved in 600 μL MeOH-d_4 with 1.3 μL (17 μM) of acetone, resulted in ratios of 2.36 μmol of acetone per μmol of GS10, 4.626 μmol of water per μmol of GS10, and 1.96 μmol of water per μmol of acetone, yielding a solid salt composition of $C_{18}H_{34}NO_{10}S_3$ K × $4H_2O$ of 96% purity.

11-(Methylsulfinyl)-undecylglucosinolate (GS11). Brown amorphous powder. HRESIMS: *m/z* 534.15055 $[M-H]^-$ (calcd. for $C_{19}H_{36}NO_{10}S_3$, 534.15014).

2.7. Method validation

Standard solutions of GS9 and GS10 were prepared and diluted to the appropriate concentration. A range of concentrations of the two glucosinolates and sinigrin were analyzed by four replicate injections. The molar extinction coefficient for GS9, GS10 and sinigrin were determined to be 3.5528×10^{-6}, 3.5457×10^{-6}, and 3.3493×10^{-6}, respectively. The limits of detection under the chromatographic analysis were determined to be 5 ng/μL for the HPLC and 0.1 ng/μL for the LCMS. Recovery of accurate amounts of GS10 added to three 0.25 g samples of defatted canola meal (which does not contain GS10) were 101.8% ± 4.4%.

3. Results and discussion

3.1. Purification and evaluation of camelina glucosinolates

Characterization of the phytochemical composition in plant organs, such as leaves and seeds, provides a good insight to the possible functional uses of these materials in agriculture (as potential pesticides) and in animal and human diets as functional nutriceutical. Camelina has been previously shown to contain flavonoids in the form of glycosylated quercetin derivatives (Onyilagha et al., 2003). We have found two forms of quercetin in seed extracts: rutin (quercetin-3-O-rutinoside) and rutin-2″-O-apioside. Fig. 2 has a typical seed extract chromatogram and the chromatograms of the purified standards. Camelina seeds contain three major glucosinolates (Fig. 1), glucoarabin (GS9), glucocamelinin (GS10) and 11-(methylsulfinyl)undecyl-glucosinolate (GS11). While these compounds have been characterized in previous reports, (Daxenbichler et al., 1991; Lange et al., 1995; Schuster and Friedt, 1998; Fahey et al., 2001; Vaughn and Berhow, 2005) further work on characterizing the role these compounds play in the protection of the plant and what role they play in the nutrition of the animals that consume them have not been conducted due to both the lack of availability of seed meals and purified compounds.

We have developed a method to isolate purified glucosinolates from camelina seed meals by extracting with methanol and using reverse-phase flash chromatography to clean up the glucosinolate fractions, then preparative HPLC to separate the individual glucosinolates and finally by ion exchange chromatography to isolate the potassium salts of the glucosinolates. The resulting products were used as standards to develop a modified analytical HPLC method for evaluating glucosinolate levels in seeds and meal samples. An example chromatogram is shown in Fig. 2. In order to determine the elution times of the three glucosinolates, we partially purified GS9, GS10, and GS11 to the point that each glucosinolate was the major UV absorbing peak in the fraction. As the ion-pairing agent used in our HPLC method could not be used in the LC–MS, we used an alternate LC–MS method to confirm the identity of the three glucosinolates we had partially purified by its characteristic $[M-H]^-$ ion, which was 506 Da for GS9, 520 Da for GS10, and

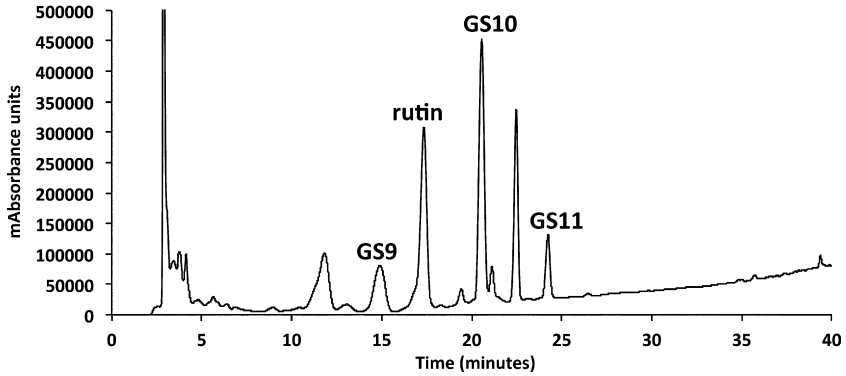

Fig. 2. HPLC reverse phase chromatogram of a seed meal extract from Camelina seed. The glucosinolates were detected by monitoring at 237 nm.

534 Da for GS11. In addition, we converted the glucosinolates in an extract of camelina seed meal to their isothiocyanate forms and ran the dichloromethane extracted products on a GSMS and found the 3 corresponding isothiocyanates, which were confirmed by a MS database match. Then each of the standards was run on the HPLC system to obtain the correct retention times, as shown in Fig. 3. The use of the ion-paring agent TBS gives excellent chromatographic results. When run with acetic acid as the ion-pairing agent the glucosinolates elute as GS9, then GS10, then GS11, but the peaks are not as symmetric (data not shown).

We noted that the yields of purified glucosinolates from the reverse phase chromatography methods were fairly low, so we turned to the larger scale counter current chromatographic method. As a result of CPC purification we have obtained fractions rich in single glucosinolates varying in their purity from 60 to 95% in case of glucocamelinin. All glucosinolates were present in fractions that were eluted with a mobile phase containing potassium iodide. This step allowed us to get purified glucosinolates in quantities suitable for final preparative HPLC separation. Apart from glucosinolates we were also able to obtain enriched fractions containing other phenolic substances present in this plant material, such as the flavonoid rutin.

At the end, we obtained two fractions containing GS9 and GS10 that were chromatographically very pure, and a fraction containing GS11, which contained detectable levels of GS9 and GS11 (Fig. 3). The NMR spectra for GS9 and GS10 were confirmed. The UV spectrum of purified GS9 and GS10 were similar to previously published data. Both samples had an absorbance maximum at 237

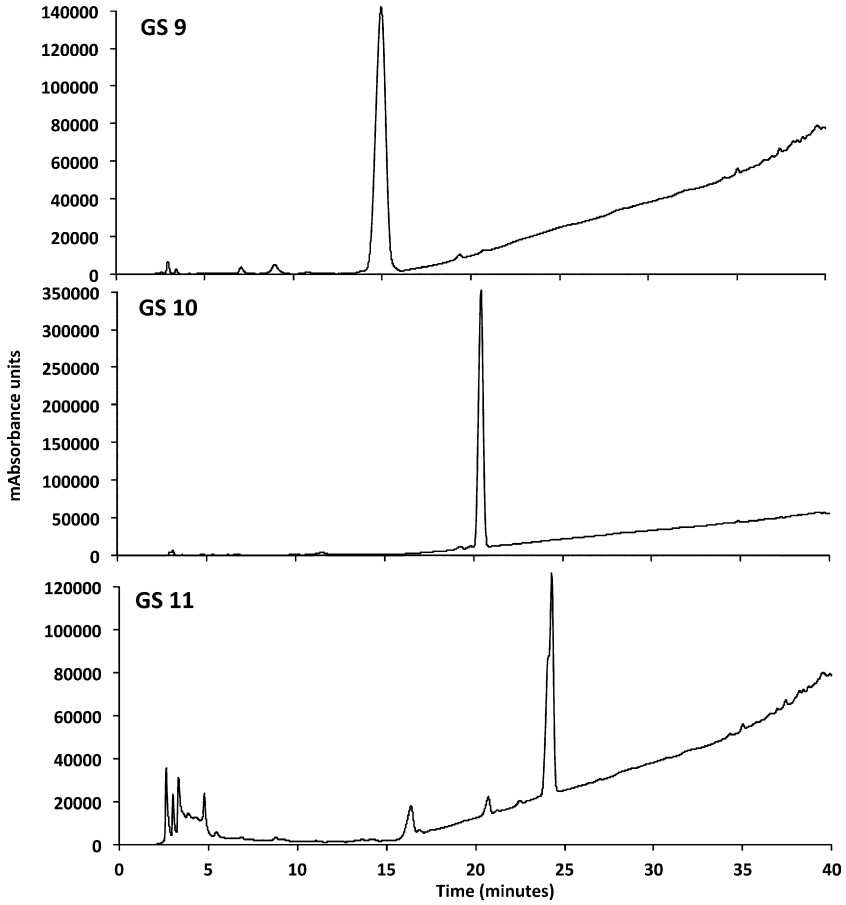

Fig. 3. HPLC reverse phase chromatograms of the semi-purified standards of GS9, GS10, and GS11.

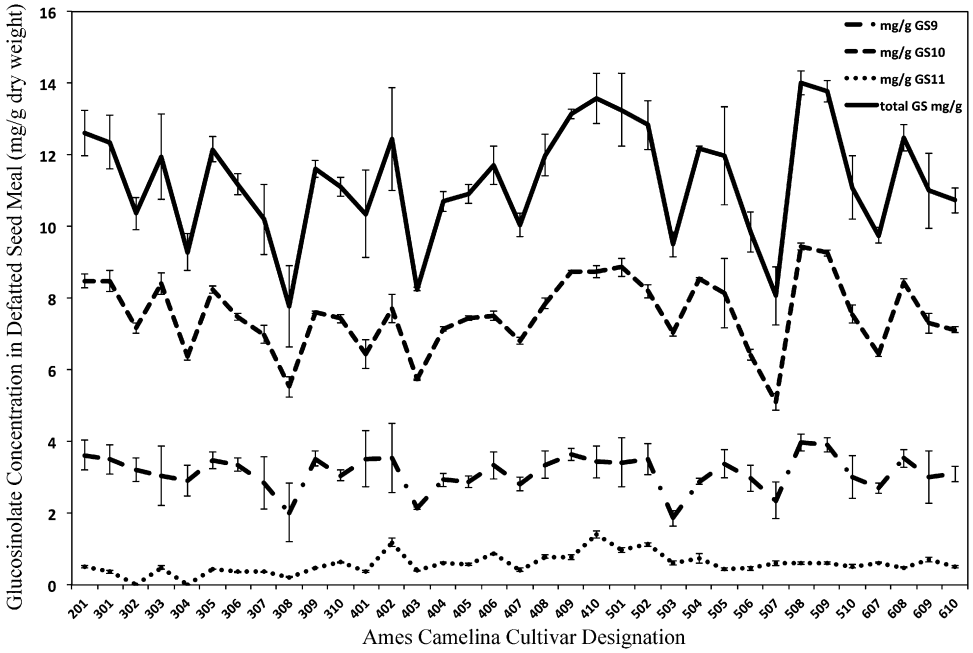

Fig. 4. Glucosinolate levels in seeds of 35 camelina cultivar accessions grown in seed yield trials in Ames, IA in 2009. Error bars indicate standard deviation of the triplicates for each data point.

that matched very closely that of sinigrin in the methanol/water mix. After freeze-drying, both the GS9 and GS10 fractions resulted in dry friable solids which were slightly brown in color. Analysis for potassium and sulfur content by inductively-coupled plasma mass spectrometry gave very ambiguous results, indicating either a mixed salt in the solid or contamination by a sulfate salt.

NMR analysis of a standard addition of acetone to a solution of GS9 and GS10 in deuterated methanol indicated the purity of the solid GS9 potassium salt to be 72% containing 23% water and the solid GS10 potassium salt to be 85% containing 12% water. However, the amount of contaminating inorganic salts and water can vary considerably from lot to lot, and may lend to inconsistent glucosinolate concentrations in the dry samples, especially as many glucosinolates are somewhat hydroscopic. Using the value we determined for our GS9 and GS10 solids, the molar extinction coefficients at 237 nm were determined on a set of four different dilutions in methanol for the GS9 and the GS10 salts by our HPLC method and compared to that determined for the sinigrin standard. The molar extinction coefficient for GS9, GS10 and sinigrin were determined to be 3.5528×10^{-6}, 3.5457×10^{-6}, and 3.3493×10^{-6}, respectively. The largest standard error obtained for the three sets was $\pm 0.6\%$, easily putting these values within the same range as each other. As the sinigrin standard chemical composition is better defined and its molar extinction coefficient at 237 nm is virtually the same as that determined for GS9 and GS10, we feel it can be used as a quantitation standard for the camelina glucosinolates and

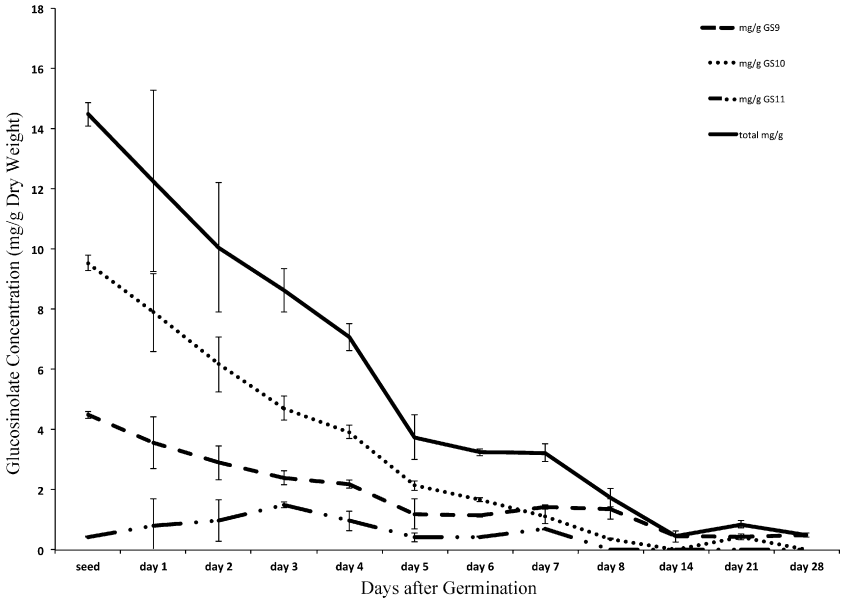

Fig. 5. Glucosinolate levels during the course of seven days sprouting studies shown as mg/g dry weight. The seeds had 38% hexane extractable-lipids, the sprouts were typically 10% or less dry weight. Error bars indicate standard deviation of the triplicates for each data point.

even other aliphatic glucosinolates for which a pure standard is not available.

3.2. Analysis of camelina glucosinolates

This analytical method was used to examine the glucosinolate content in cultivars of camelina being grown in several American states and Canadian provinces. An example of cultivar study grown in Ames, Iowa, during the 2009 growing season is shown in Fig. 4. As usual there are moderate to large variations in accumulation levels of the glucosinolates between the cultivars. These levels are affected by environmental conditions as well. A good robust, reproducible method of analysis is important for the evaluation of this crop. The values shown are for the fully defatted seed meal. The seeds in this study contained between 21% and 36% hexane extractable oil (data not shown). Typically pressed camelina seed meal will retain 5–10% residual oil.

We also examined the levels of glucosinolates in camelina during the course of sprouting as shown in Fig. 5. In general the levels dropped dramatically during the 7-day course of sprouting, but the sprouts do contain measurable levels of GS9 and GS10. Broccoli sprouts are a source of glucosinolates and camelina could be used in similar fashion.

This methodology has been used to quantify glucosinolate levels in meals used for feeds for egg laying chickens (Kakani et al., 2012) and for dairy cattle (manuscript in preparation) in preparation for getting approval to use camelina as a feed ingredient. The next step will be to determine if the camelina glucosinolate isothiocyanates have similar activity to sulforaphane. With the quantities that can be purified with the methods described here it will be possible to produce the quantities required for the biological experiments. This may make camelina seed meal an even more important feed and possibly a food ingredient due to its characterized nutriceutical composition.

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References

Abramovic, H., Abram, V., 2005. Physico-chemical properties, composition and oxidative stability of *Camelina sativa* oil. Food Technol. Biotechnol. 43, 63–70.

Abramovic, H., Butinar, B., Nikolic, V., 2007. Changes occurring in phenolic content, tocopherol composition and oxidative stability of *Camelina sativa* oil during storage. Food Chem. 104, 903–909.

Anilakumar, K.R., Khanum, F., Bawa, A.S., 2006. Dietary role of glucosinolate derivatives: a review. J. Food Sci. Technol. 43, 8–17.

Betz, J.M., Fox, W.D., 1994. High-performance liquid chromatographic determination of glucosinolates in brassica vegetables. In: Huang, M.-T., Osawa, T., Ho, C.-T., Roen, A.T. (Eds.), Food Chemicals for Cancer Prevention I: Fruits and Vegetables, ACS Symposia Series 546. ACS Publications, Washington, DC, pp. 181–195.

Budin, J.T., Breene, W.M., Putnam, D.H., 1995. Some compositional properties of camelina (*Camelina sativa* L. Crantz) seeds and oils. J. Am. Oil Chem. Soc. 72, 309–315.

Clarke, D.B., 2010. Glucosinolates, structures and analysis in food. Anal. Meth. 2, 310–325.

Daxenbichler, M.E., Spencer, G.F., Carlson, D.G., Rose, G.B., Brinker, A.M., Powell, R.G., 1991. Glucosinolate composition of seeds from 297 species of wild plants. Phytochemistry 30, 2623–2638.

Dubois, V., Breton, S., Linder, M., Fanni, J., Parmentier, M., 2007. Fatty acid profiles of 80 vegetable oils with regard to their nutritional potential. Eur. J. Lipid Sci. Technol. 109, 710–732.

Fahey, J.W., Wade, K.L., Stephenson, K.K., Chou, F.E., 2003. Separation and purification of glucosinolates from crude plant homogenates by high-speed counter-current chromatography. J. Chromatogr. A 996, 85–93.

Fahey, J.W., Zalcmann, A.T., Talalay, P., 2001. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry 56, 5–51.

Farnham, M.W., Stephenson, K.K., Fahey, J.W., 2005. Glucoraphanin level in broccoli seed is largely determined by genotype. Hortscience 40, 50–53.

Fenwick, G.R., Heaney, R.K., Mullin, W.J., 1983. Glucosinolates and their breakdown products in food and food plants. Crit. Rev. Food Sci. Nutr. 18, 123–201.

Gugel, R.K., Falk, K.C., 2006. Agronomic and seed quality evaluation of *Camelina sativa* in western Canada. Can. J. Plant Sci. 86, 1047–1058.

Hoist, B., Williamson, G., 2004. A critical review of the bioavailability of glucosinolates and related compounds. Nat. Prod. Rep. 21, 425–447.

Jones, G., Valamoti, S.M., 2005. Lallemantia, an imported or introduced oil plant in bronze age northern Greece. Veg. Hist. Archaeobot. 14, 571–577.

Kakani, R., Fowler, J., Haq, A., Murphy, E.J., Rosenberger, T.A., Berhow, M., Bailey, C.A., 2012. Camelina meal increases egg n-3 fatty acid content without altering egg quality or production in laying hens. Lipids 47, 519–526.

Lange, R., Schumann, W., Petrzika, M., Busch, H., Marquard, R., 1995. Glucosinolates in linseed dodder. Fett 97 (4), 146–152.

Moloney, A.P., Woods, V.B., Crowley, J.G., 1998. A note on the nutritive value of camelina meal for beef cattle. Irish J. Agric. Food Res. 37, 243–247.

Moser, B.R., Vaughn, S.F., 2010. Evaluation of alkyl esters from *Camelina sativa* oil as biodiesel and as blend components in ultra low-sulfur diesel fuel. Bioresour. Technol. 101, 646–653.

Onyilagha, J., Bala, A., Hallett, R., Gruber, M., Soroka, J., Westcott, N., 2003. Leaf flavonoids of the cruciferous species, *Camelina sativa*, *Crambe* spp., *Thlaspi arvense*, and several other genera of the family Brassicaceae. Biochem. Systematics Ecol. 31, 1309–1322.

Putnam, D.H., Budin, J.T., Field, L.A., Breene, W.M., 1993. Camelina: a promising low-input oilseed. In: Janick, J., Simon, J.E. (Eds.), New Crops. Wiley, New York, pp. 314–322.

Schuster, A., Friedt, W., 1998. Glucosinolate content and composition as parameters of quality of Camelina seed. Ind. Crops Prod. 7, 297–302.

Schwartz, H., Ollilainen, V., Piironen, V., Lampi, A.M., 2008. Tocopherol, tocotrienol and plant sterol contents of vegetable oils and industrial fats. J. Food Comp. Anal. 21, 152–161.

Shapiro, T.A., Fahey, J.W., Wade, K.L., Stephenson, K.K., Talalay, P., 2001. Chemoprotective glucosinolates and isothiocyanates of broccoli sprouts: metabolism and excretion in humans. Cancer Epidemiol. Biomarkers Prev. 10, 501–508.

Spencer, G.F., Daxenbichler, M.E., 1980. Gas chromatography–mass spectrometry of nitriles, isothiocyanates and oxazolidinethiones derived from Cruciferous Glucosinolates. J. Agric. Food Chem. 31, 359–367.

Talalay, P., Fahey, J.W., 2001. Phytochemicals from cruciferous plants protect against cancer by modulating carcinogen metabolism. J. Nutr. 131, 3027S–3033S.

Toribio, A., Nuzillard, J.-M., Pinel Bt Boudesocque, L., Lafosse, M., Poype, Fo.D.L., Renault, J.-H., 2009. Pilot-scale ion-exchange centrifugal partition chromatography: purification of sinalbin from white mustard seeds. J. Sep. Sci. 32, 1801–1807.

Urbaniak, S.D., Caldwell, C.D., Zheljazkov, V.D., Lada, R., Luan, L., 2008. The effect of seeding rate, seeding date and seeder type on the performance of *Camelina sativa* L. in the Maritime provinces of Canada. Can. J. Plant Sci. 88, 501–508.

Vaughn, S.F., Berhow, M.A., 2005. Glucosinolate hydrolysis products from various plant sources: pH effects, isolation, and purification. Ind. Crops Prod. 21, 193–204.

Vollmann, J., Moritz, T., Kargl, C., Baumgartner, S., Wagentristl, H., 2007. Agronomic evaluation of camelina genotypes selected for seed quality characteristics. Ind. Crops Prod. 26, 270–277.

Zeman, N., 2007. Crazy for Camelina. Biodiesel Magazine.

Zubr, J., 2010. Cabohydrates, vitamins, and minerals of *Camelina sativa* seed. Nutr. Food Sci. 40, 523–531.