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Determination of Glucosinolates in Canola Seeds Using Anion Exchange Membrane Extraction Combined with the High-Pressure Liquid Chromatography Detection

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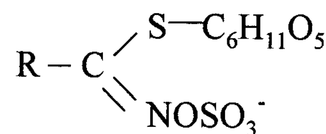
A rapid, simple, and reliable method for the determination of individual glucosinolates in canola seeds was developed using a semiquantitative extraction of glucosinolates with anion exchange membranes and HPLC detection. In this one-step extraction procedure, a membrane (7 cm^2) is placed in the seed suspension prepared by grinding and boiling 0.8 g of seeds in 20 mL of water. After 10 min of shaking on the mechanical shaker, the membrane is removed from the suspension, washed, and transferred to a vial containing 5 mL of 1 N tetramethylammonium chloride. The glucosinolates are eluted from the membrane by shaking the membrane for 10 min with the eluting solvent. The glucosinolate content in membrane eluates is determined by HPLC using sinigrin standards. A coefficient of variation ranging from 1.9 to 7.6% for aliphatic glucosinolates indicated very good reproducibility of the method. Because of the instability of 4-hydroxyglucobrassicin, the coefficient of variation for the determination of this indolyl glucosinolate was 13.9%. To verify the results of the membrane extraction/HPLC detection, this new method was compared with the existing colorimetric and GC procedures. Very good correlation ($R^2 = 0.98$) was obtained between the total glucosinolates determined by the membrane extraction/HPLC method and the palladate colorimetric procedure for 17 canola varieties. Concentrations of individual glucosinolates in five canola varieties were compared with the GC data. Very good agreement between these two methods was obtained for aliphatic glucosinolates. However, the membrane extraction/HPLC method yielded slightly higher values for 4-hydroxyglucobrassicin than the GC method, possibly indicating that the decomposition of this glucosinolate was reduced during the sample extraction with the membranes. The simplicity and low cost of the membrane extraction/HPLC method make it an attractive alternative to the existing procedures for glucosinolate analysis in canola seeds.

Keywords: *Canola; glucosinolates; anion exchange membranes; extraction; HPLC*

INTRODUCTION

With increasing canola production in western Canada and ongoing plant breeding programs aimed at developing low-glucosinolate canola varieties, there is a need for simple and rapid methods for glucosinolate determination in the seeds. Canola oil is used for nutritional (cooking oil) and industrial (lubricants) purposes, and the meal is used for animal feed. Glucosinolates are found in varying amounts in rapeseed (Figure 1). After the crushing process, glucosinolates remain in the meal and, in the presence of moisture, undergo enzymatic hydrolysis, releasing a variety of compounds. These compounds have antinutritional properties and may be toxic to the animals (Fenwick et al., 1983). Rapeseed varieties with low levels of glucosinolates (<18 μmol g^{-1} of seed) are desired for feed; therefore, routine testing of the seeds for glucosinolate content is necessary.

Glucosinolate analysis in seed or plant tissue is performed either through indirect measurements of the glucosinolate breakdown products or through direct determination of intact glucosinolates. In indirect methods, enzymatically or chemically released products such as isothiocyanates, oxazolidinethiones, nitriles, thiocyanate ion, sulfate, and glucose are measured (McGregor, 1978); these procedures usually require controlled conditions and frequently are time-consuming. Direct measurements of intact glucosinolates are accomplished by gas chromatography (GC) (Underhill and Kirkland, 1971; Thies, 1977; Heaney and Fenwick, 1980; Daun and McGregor, 1981), high-pressure liquid chromatography (HPLC) (Minchinton et al., 1982; Helboe et al., 1980; Björkvist and Hase, 1988), or colorimetry (Thies,



progoitrin:
R= 2-hydroxy-3-butenyl,
napoleoferin:
R= 2-hydroxy-4-pentenyl,
gluconapin:
R= 3-butenyl,
4-OH glucobrassicin:
R= 4-hydroxy-3-indolylmethyl,
glucobrassicanapin:
R= 3-pentenyl,
sinigrin:
R= 2-propenyl

Figure 1. Structures and names of glucosinolates.

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1982). The colorimetric method allows for the determination of the total glucosinolate content in the seeds. The chromatographic methods provide greater precision and sensitivity and are suitable for the identification and quantitation of individual glucosinolates. However, the sample preparation required before chromatographic analysis is complex and tedious. In a typical procedure, oil is first removed from the seeds, glucosinolates are extracted with hot water or methanol, and the sample is purified on a weak anion exchange column. Desulfation is carried out overnight on the column with added sulfatase, and desulfoglucosinolates are eluted with water. Sample trimethylsilylation (TMS) is required before the GC analysis, whereas purified extracts can be injected directly into the HPLC.

We have recently studied a simple one-step procedure for the extraction of glucosinolates from mustard seeds using anion exchange membranes (Szmigielska and Schoenau, 2000). In this method, membranes are shaken with the seed suspensions to allow for glucosinolates to be adsorbed onto the membranes through the ion exchange process. Glucosinolates are then released from the membranes by shaking the membranes with an eluting solvent, and the glucosinolate concentration in membrane eluates is determined by HPLC. In this procedure, the steps of a traditional extraction are omitted, similar to solid phase microextraction procedures (Supelco, 1995). The objective of this study, therefore, was to extend the application of the membrane extraction method to canola glucosinolates and to compare the newly developed method with existing procedures for the determination of glucosinolates in canola seeds.

MATERIALS AND METHODS

Extraction of Glucosinolates from Canola Seeds with Anion Exchange Membranes followed by HPLC Detection. Seeds were ground in a coffee grinder, and 0.8 g samples were heated in ~20 mL of boiling water in small beakers for 5 min. After cooling, samples were transferred to plastic centrifuge tubes, 7 cm^2 anion exchange membranes (Hydro Components, Inc., Blue Bell, PA) in bicarbonate form were added, and the suspensions were shaken on a mechanical shaker at 160 rpm for 10 min. Membranes were then removed, washed in running water, and rinsed in methanol and isooctane by shaking the membranes by hand in ~20 mL of each solvent to remove any oil that adhered to the membrane surface and possibly interfered with the ion exchange process. Membranes were then transferred to vials containing 5 mL of 1 N tetramethylammonium chloride (Aldrich Chemical Co., Milwaukee, WI) and were shaken for 10 min on a mechanical shaker to elute the glucosinolates from the membranes.

The glucosinolate concentration in membrane eluates was determined by HPLC (Waters Associates, Milford, MA) with UV detection at 235 nm. The mobile phase was a mixture of methanol and 0.1 N ammonium acetate (3:97) pumped at a flow rate of 1 mL min^{-1}. A Supelcosil LC-18 column (15 cm × 4.6 mm i.d., 5 μm particle size) from Supelco (Bellefonte, PA) was used for the separation of glucosinolates, and the injection volume was 10 μL. Sinigrin (Sigma Chemical Co.) solutions in the range of 100−500 μM were used as quantitative standards for the determination of glucosinolates in canola seeds. Peak areas of the sinigrin standards and of individual glucosinolates in membrane eluates were determined with the Baseline 810 Chromatography Work Station. The same coefficient of absorption at 235 nm was assumed for sinigrin and the naturally occurring glucosinolates in canola seeds because the contribution to the UV absorption at this wavelength comes from the glucosinolate group and not from the R groups (Helboe et al., 1980; Björkqvist and Hase, 1988).

Anion exchange membrane pieces (7 cm^2) were cut from a membrane sheet and were prepared for use by shaking the pieces three times with saturated sodium bicarbonate solution (1 h each time) on a mechanical shaker. Membranes were then thoroughly washed and stored in distilled water for further use. New membranes were used for each analysis. The total exchange capacity of the membranes, as listed by the manufacturer, was 1.1 mequiv g^{-1} (0.04 mequiv cm^{-2}).

Canola seeds (*Brassica napus* and *Brassica rapa*) were obtained from various seed suppliers and from Agriculture and Agri-Food Canada (Saskatoon, SK). The seeds supplied by Agriculture and Agri-Food Canada had been analyzed according to an official method of the Canadian Grain Commission for the determination of glucosinolate content in rapeseed/canola (Daun and McGregor, 1981). In this method, TMS derivatives of desulfoglucosinolates are determined by GC. Seeds are crushed and extracted with methanol; lead−barium acetate and benzyl glucosinolate are added, and samples are shaken. Samples are then centrifuged, and the supernatants are transferred to DEAE-Sephadex columns. Desulfation of glucosinolates is carried out overnight on the columns after the addition of purified sulfatase. Desulfoglucosinolates are then eluted from the columns with methanol or water, and the solvent is evaporated. Next, samples are derivatized for GC analysis, and samples are injected into the GC equipped with a DB-1 capillary column.

Colorimetric Determination of Glucosinolates in Canola Seeds. The method described by Thies (1982) was scaled up to allow the measurements using a spectrophotometer (Beckman DU^R-64). Seeds were ground in a coffee grinder, and 0.6 g of low-glucosinolate or 0.2 g of high-glucosinolate varieties was transferred to 4.5 mL of boiling water. Samples were boiled for another 20 min and then cooled and filtered through Whatman paper under vacuum. The volume of each extract was adjusted to 4.5 mL, and samples were filtered again through Millipore filters (0.45 μm) under vacuum. The filtration steps were added to the original procedure and resulted in improved reproducibility of the measurements. As recommended, sodium tetrachloropalladate (Aldrich Chemical Co.) solution (0.588 mg L^{-1} water) was freshly prepared before mixing with the extracts; 200 μL of the extract was transferred to a vial, and 4 mL of the palladate solution was added. After 30 min at room temperature, absorption was measured at 450 nm. Timing was critical in this method as the intensity of the brown color of the sodium tetrachloropalladate solution and of the samples changed with time.

A standard curve was constructed using rapeseed meal 226-4 (Canadian Normalization Standard) containing a known concentration of glucosinolates, that is, 35.9 μmol g^{-1} of meal; 0.18 g of the rapeseed meal was extracted with 4.5 mL of water as described above for the samples, and the volumes of the extract in the range of 50−250 μL were taken for the color reaction. Calculating the glucosinolate content in seeds against a standard curve rather than against one known seed sample greatly improved the results, especially for the high-glucosinolate varieties for which falsely low glucosinolate content was obtained if it was calculated against one reference sample.

RESULTS AND DISCUSSION

HPLC Separation of Canola Glucosinolates. The HPLC separation of canola glucosinolates is shown in Figure 2. The glucosinolate peaks were identified on the basis of the elution order as compared to the HPLC separation reported by Björqvist and Hase (1988). Also, the HPLC analysis of the samples of known glucosinolate composition obtained from Agriculture and Agri-Food Canada helped in peak identification based on the relative concentration of individual glucosinolates in canola seeds. Under the HPLC conditions used in this study, aliphatic glucosinolates, that is, progoitrin, napoleoferin, gluconapin, and glucobrassicanapin, eluted at t_R of 2.7, 5.0, 5.9, and 15.5 min, respectively, while

Table 1. Comparison of the Glucosinolate Contents in Canola Seeds (Micromoles per Gram of Seed) Determined by the Membrane Extraction/HPLC and GC Methods

	canola variety									
	Legend		Parkland		GF689		GF549		GF833	
	HPLC	GC	HPLC	GC	HPLC	GC	HPLC	GC	HPLC	GC
progoitrin	3.85	3.82	5.61	5.10	2.78	2.85	10.16	10.88	2.06	2.12
napoleoferin	0.04	0.11	0.97	0.89	0.35	0.43	1.32	1.55	0.41	0.04
gluconapin	1.81	1.71	2.22	2.58	1.72	1.63	7.23	7.53	1.15	1.03
4-hydroxyglucobrassicin	4.96	4.19	4.17	3.40	3.95	3.48	5.20	3.71	4.18	3.49
glucobrassicanapin	0.15	0.40	1.78	2.12	0.82	0.95	4.90	4.82	0.08	0.22
total	**10.81**	**10.23**	**14.75**	**14.09**	**9.62**	**9.34**	**28.82**	**28.49**	**7.88**	**6.90**

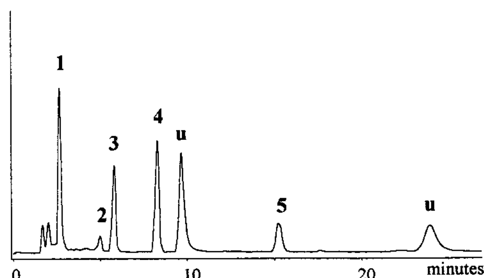

Figure 2. Liquid chromatogram of glucosinolates extracted with the anion exchange membrane from canola seeds. Peaks: 1 = progoitrin; 2 = napoleoferin; 3 = gluconapin; 4 = 4-hydroxyglucobrassicin; 5 = glucobrassicanapin; u = unknown.

4-hydroxyglucobrassicin, an indolyl glucosinolate, eluted at 8.5 min. Similarly to Björqvist and Hase (1988), unknown peaks were detected in the chromatograms of canola extracts (t_R of 9.9 and 24.5 min). The identity of these peaks was not investigated in this study. Sinigrin eluted at t_R of 2.9 min and did not overlap with any of the naturally occurring glucosinolates in rapeseed; this allowed us to use sinigrin as an internal standard (IS) in the development of the extraction method.

Development of the Extraction Method for Canola Glucosinolates Using Anion Exchange Membranes. Sinigrin IS was added to the seed suspensions of a selected loww-glucosinolate variety to monitor the rate of glucosinolate exchange on the membranes. Peaks of sinigrin (IS) and progoitrin (one of the naturally occurring glucosinolates in canola seeds) were measured. The optimal sample size was determined by taking 0.2−1.4 g of ground seeds for analysis with 25 mL of 100 μM sinigrin solution added. Samples were shaken for 3.5 h with the membranes, and the membranes were eluted with 25 mL of 1 N tetramethylammonium chloride for 3.5 h. The progoitrin peak increased linearly while the peak of sinigrin (IS) remained constant up to ~1.0 g sample size. For samples >1.0 g, the size of the progoitrin peak leveled off while the peak of sinigrin decreased in size. The recovery of sinigrin was 80%, as calculated from the ratio of the peak area of the extracted sinigrin and the sinigrin standard, demonstrating that the percent recovery of glucosinolates from canola seeds was satisfactory for the sample size up to ~1.0 g.

To determine the time required for maximum recovery, 0.8 g of ground and heated seeds together with 25 mL of 100 μM sinigrin solution was shaken with the membranes and eluted from the membranes for duration of times ranging from 10 min to 3.5 h. As can be seen in Figure 3, the peak response for sinigrin and progoitrin reached its highest value after 2.5 h of shaking and did not change with longer shaking time.

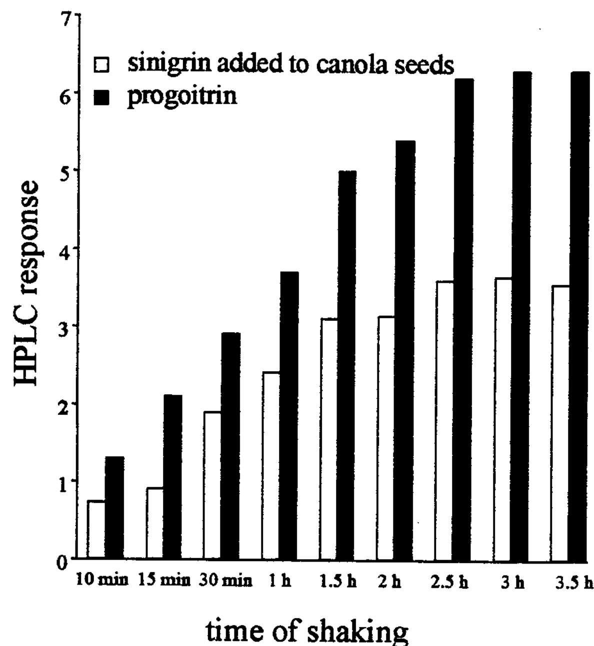

Figure 3. Effect of shaking time on the amount of sinigrin (internal standard) and progoitrin extracted from canola seeds with anion exchange membranes (each data point represents the mean of two replications).

Peaks of other glucosinolates in canola seeds, except 4-hydroxyglucobrassicin, also increased to their maximum values at 2.5 h of extraction time. 4-Hydroxyglucobrassicin is an indolyl glucosinolate known to decompose during sample preparation (DeClercq and Daun, 1989; Spinks et al., 1984). Use of 1 N tetramethylammonium chloride (Hanley et al., 1983) instead of potassium chloride (Szmigielska and Schoenau, 2000) for the elution of glucosinolates from the membranes did not prevent 4-hydroxyglucobrassicin from partial decomposition.

As can also be seen from Figure 3, after 10 min of sample loading onto the membrane and 10 min of glucosinolate elution from the membrane, 20% of sinigrin (IS) and progoitrin was recovered as determined from the ratio of peak areas obtained after 10 min and 2.5 h of extraction. When the volume of 1 N tetramethylammonium chloride used for the elution of glucosinolates from the membranes was reduced from 25 to 5 mL and samples were shaken for 10 min, the resulting chromatograms had glucosinolate peaks equivalent in size to the 2.5 h shaking with 25 mL of 1 N tetramethylammonium chloride. Thus, by reducing the volume of the eluting solvent by a factor of 5, the time needed for extraction was only 10 min. In this semiquantitative extraction, only a portion of total glucosinolates present in 0.8 g of seed sample was removed.

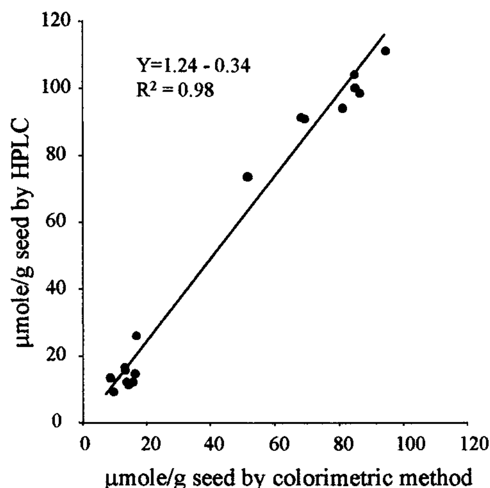

Figure 4. Correlation between the total glucosinolate contents in canola seeds determined by the membrane extraction/HPLC method and by the palladate colorimetric method.

However, the long extraction time of 2.5 h needed for sample loading onto the membrane and the 2.5 h needed for glucosinolate elution from the membrane were significantly shortened to 10 min. Another advantage of the semiquantitative extraction was that less 4-hydroxyglucobrassicin decomposed during the short extraction. The peak of 4-hydroxyglucobrassicin in the chromatograms obtained by the semiquantitative extraction was larger than the peak obtained by the long extraction.

All glucosinolates in membrane extracts were stable except 4-hydroxyglucobrassicin. Thus, samples should be injected immediately after extraction because prolonged storage at room temperature may result in decreased peak of 4-hydroxyglucobrassicin. Refrigeration of samples for up to 1 day was helpful for storing the samples as only a small decrease in 4-hydroxyglucobrassicin content was observed.

Very good reproducibility was obtained for the determination of aliphatic glucosinolates by the developed method. The coefficient of variation ranged from 1.9 to 7.6% based on four determinations. Because of the instability of 4-hydroxyglucobrassicin in the membrane extracts, the coefficient of variation for the determination of 4-hydroxyglucobrassicin was 13.9%. The glucosinolate content in the seeds obtained by the membrane extraction/HPLC method was corrected for 80% recovery.

Comparison of the Membrane Extraction/HPLC Method with the Colorimetric and GC Procedures. Figure 4 shows the relationship between the results obtained by the membrane extraction/HPLC method and the palladate colorimetric method for 17 different varieties of canola. The palladate colorimetric method allows for the determination of total glucosinolates in seed samples. Therefore, the concentrations of individual glucosinolates in the seeds obtained from the membrane extraction/HPLC method were summed and compared with the results of the colorimetric measurements. Very good correlation ($R^2 = 0.98$) was obtained for the two methods, demonstrating that the newly developed membrane extraction/HPLC procedure is a reliable tool for the determination of glucosinolates in the seeds.

The concentrations of individual glucosinolates in five varieties of canola seeds evaluated by the membrane extraction/HPLC method were also compared to the GC data (Table 1). Very good agreement between the two methods was obtained for aliphatic glucosinolates. However, the membrane extraction/HPLC method consistently yielded slightly higher values for 4-hydroxyglucobrassicin than the GC method, possibly indicating that the decomposition of this glucosinolate during sample preparation was reduced in the membrane extraction/HPLC method. The differences between the total glucosinolate contents obtained by the two methods were approximately equal to the differences in the levels of 4-hydroxyglucobrassicin.

Conclusions. The membrane extraction/HPLC method for the determination of glucosinolates in rapeseed is simple and inexpensive. Only the membrane strips and the 1 N tetramethylammonium chloride solution are required for the sample preparation. The time needed for the extraction is short; also, no specialized techniques or instruments are needed to perform the extraction. The RP-HPLC separation with ammonium acetate/MeOH mobile phase allows for the glucosinolates to be injected as tetramethylammonium salts directly after elution from the membranes without any further sample modification or derivatization. The HPLC quantitation of glucosinolates is accomplished using sinigrin, a commercially available glucosinolate, instead of a reference seed or meal sample, which may vary from laboratory to laboratory.

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