Use of Anion-Exchange Membrane Extraction for the High-Performance Liquid Chromatographic Analysis of Mustard Seed Glucosinolates

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A new one-step extraction using anion-exchange membranes for the HPLC determination of glucosinolates in mustard seeds is reported. The exchange of glucosinolates on the membranes was studied using sinigrin in solutions and sinigrin added as an internal standard to seeds of yellow mustard. By varying time of extraction, membrane size, and sample size, the optimal conditions for maximum glucosinolate recovery were determined and the following procedure was adopted: 0.2 g of ground mustard seeds are heated in 20 mL of boiling water for 5 min. After cooling, samples are transferred to plastic centrifuge tubes, 9-cm^2 membranes are added, and suspensions are shaken on a mechanical shaker for 2.5 h. Glucosinolates are then eluted from the membranes with 25 mL of 1 N KCl by shaking again for 2.5 h. Using this procedure, the sinigrin extraction from solutions and from mustard seeds was linear with 80% recovery. Seeds of yellow, brown, oriental, and Indian mustard were analyzed by this procedure; excellent reproducibility, with coefficients of variation in the range 1.0−4.3% were obtained. This method offers a simple and inexpensive alternative to complicated and tedious procedures for glucosinolate isolation/purification required for chromatographic determinations.

Keywords: *Glucosinolates; mustard seed; extraction; high-performance liquid chromatography*

INTRODUCTION

Development of new mustard varieties for potential use as an oilseed crop (Getinet et al., 1996; Love et al., 1990) as well as nutritional evaluation of mustard meal for use as an animal feed (Bell et al., 1998; Slominski et al. 1999) requires chemical analysis of glucosinolate content in the seeds. Glucosinolates are a group of compounds that occur in plants of the Cruciferae family and especially in *Brassica* genus. When plant tissue is crushed in the presence of moisture, glucosinolates undergo enzymatic hydrolysis, releasing a variety of physiologically active compounds. The effect of these compounds is negligible when consumed by humans in small amounts, however, when consumed by animals in larger quantities as part of their feed, the compounds may reduce palatability or may be toxic (McGregor et al., 1983).

Glucosinolate determination is performed either by measurement of the enzymatic degradation products or analysis of the intact glucosinolates. Volatile "mustard oils" (organic isothiocyanates) are used for the glucosinolate measurements by the mustard industry. For the determination of the intact glucosinolates, gas chromatography (GC) and high-pressure liquid chromatography (HPLC) are most frequently used. Sample preparation before chromatographic determination, first proposed by Thies (1976, 1977, 1979), consists of a multistep isolation/purification procedure. Seeds are homogenized, defatted, and heated in water to extract glucosinolates and inhibit enzyme myrosinase responsible for glucosinolate hydrolysis. The sample is then purified on an anion-exchange column: extract is applied onto the weak anion exchanger, the column is washed with water, and glucosinolates are eluted with pyridinium acetate or imidazole formate. If desulfation is desired, extract after protein precipitation with barium/lead acetate is applied onto the ion-exchange column. Desulfation is carried out overnight on the column with added sulfatase, and desulfoglucosinolates are eluted from the column with water. GC analysis is performed after glucosinolate trimethylsilylation (Getinet et al., 1996; Daun and McGregor, 1981; Heaney and Fenwick, 1980), whereas samples without any further derivatization are injected into the liquid chromatograph. Desulfoglucosinolates are separated by reversed-phase (RP) HPLC with gradient elution (Minchinton et al., 1982; Sang et al., 1983; International Organization for Standardization, 1995; AOCS, 1998) and pyridinium salts of glucosinolates can be analyzed by ion-pair RP HPLC (Helboe et al., 1980). An HPLC separation of intact glucosinolates in sulfate form is also possible by using an aqueous ammonium acetate−acetonitrile mixture as a mobile phase (Björkqvist and Hase, 1988).

Simple and rapid methods for glucosinolate isolation/purification from the seeds are needed. Anion-exchange membranes have been used in our laboratory for extraction of a variety of anionic species (both organic and inorganic) from soils by either membrane burial in the soil or by shaking the membrane with the soil suspensions (Schoenau and Huang, 1991; Qian et al., 1992; Szmigielska and Schoenau, 1995; Szmigielska et al., 1996, 1998). This methodology, like solid-phase microextraction (Supelco, 1995), greatly facilitates extrac-

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tion of an investigated compound or a group of compounds from the sample matrix. The membrane is immersed into a liquid sample, the compounds of interest are adsorbed to the membrane through an ion-exchange process, and then after desorption, the compounds are analyzed in membrane eluate. Thus, the steps of a traditional extraction are omitted. The present study was undertaken to first explore the ability of anion-exchange membranes to exchange glucolsinolates by using sinigrin as a model compound and then develop a simple one-step extraction method for the determination of intact glucosinolates in the seeds by HPLC. Mustard seeds were selected for this study because mustard seeds contain primarily one glucosinolate and therefore provide a simple sample matrix suitable for the investigation of glucosinolate extraction by the membranes from the seed material.

MATERIALS AND METHODS

Anion-exchange membrane strips were cut from a membrane sheet (Hydro Components, Inc., Blue Bell, PA). The total exchange capacity of the membranes, as listed by the manufacturer, was 1.1 mequiv g^{-1} (0.04 mequiv cm^{-2}). The membrane strips were prepared for use by shaking three times on a mechanical shaker (1 h each time) with saturated sodium bicarbonate solution. Membranes were then thoroughly rinsed, and clean membranes were stored in distilled water. Because of the low cost of the membrane material, new membrane strips were used for each analysis.

Pure sinigrin (monohydrate, K salt) was obtained from Sigma. Mustard seeds (*B. juncea, S. alba*) were purchased from the local seed suppliers and local supermarkets.

The HPLC separation of mustard seed glucosinolates was based on the method reported for rapeseed glucosinolates by Björkqvist and Hase (1988). An HPLC system (Waters Associates, Milford, MA) was equipped with a Baseline 810 chromatography Work Station; mobile phase was a mixture of methanol: 0.1 N ammonium acetate (3:97) at a flow rate of 1 mL min^{-1}; ultraviolet (UV) detector was set at 235 nm. Ten microliters of the standards and the samples were injected onto the Supelcosil LC-18 RP column (15 cm $\times$ 4.6 mm i.d., 5-μm particle size) from Supelco (Bellefonte, PA).

Optimization of Ion-Exchange Parameters for Maximum Recovery of Sinalbin from the Seeds of Yellow Mustard Using Sinigrin as an Internal Standard (IS). The following general procedure for sample preparation was used to optimize the conditions for glucosinolate extraction from the seeds. Mustard seeds were ground in a coffee grinder and then preweighed samples were heated for 5 min in 20 mL of boiling water to inhibit myrosinase and to extract glucosinolates from the seeds. After cooling, samples were transferred to plastic centrifuge tubes, membranes (in bicarbonate form) were added, and suspensions were shaken on a mechanical shaker at 160 rpm. Membranes were then removed, rinsed in distilled water, placed in centrifuge tubes containing 25 mL of 1 N KCl solution, and shaken on a mechanical shaker. Glucosinolate concentration in membrane eluates was determined by HPLC.

Membrane size, sample size, and time of shaking were varied to determine the optimal conditions for the extraction of mustard glucosinolates. Separate experiments were carried out to establish these parameters for sinigrin extraction from solutions and for sinalbin extraction from the seeds of yellow mustard with sinigrin added as an IS.

To study the effect of the membrane size on the glucosinolate recovery from solutions and from mustard seeds, membrane pieces in the range of 2 to16 cm^2 were examined. Membrane of each size was placed: (1) in 25 mL of 500 μM sinigrin solution to study the effect of membrane size on sinigrin extraction from solutions, and (2) in 0.2 g of ground and heated mustard seeds with 25 mL of 500 μM sinigrin solution added to study the effect of membrane size on glucosinolate extraction from spiked seed samples. Samples were shaken with the membranes for 3.5 h and membranes were eluted for 3.5 h.

To determine the optimal sample size for the maximum recovery of glucosinolates from mustard seeds, samples in the range of 0.025 to 0.4 g were taken for analysis; 25 mL of 500 μM sinigrin solution was added as an IS to each sample together with 9-cm^2 membranes. Samples were shaken with the membranes for 3.5 h and membranes were eluted for 3.5 h.

The time required for the maximum glucosinolate exchange on the membranes was determined by loading the samples onto the membranes and eluting the glucosinolates from the membranes for a duration of time ranging from 7 min to 3.5 h. Membranes (9 cm^2) were placed in (1) 25 mL of 500 μM sinigrin solution to study the extraction of sinigrin from solutions with time of shaking and (2) 0.2 g of ground and heated mustard seeds with 25 mL of 500 μM sinigrin solution added to study the extraction of sinigrin (IS) and sinalbin from seed samples with the time of shaking.

For the evaluation of sample stability, membrane extracts of mustard seeds were stored at room temperature in the laboratory, and sinigrin and sinalbin concentration in the extracts was measured daily for up to 1 week. Also, membranes with adsorbed glucosinolates from mustard seeds were stored dry and in water, and were eluted daily for up to 1 week to check for glucosinolate stability on the membranes.

Determination of Mustard Seed Glucosinolates with Anion-Exchange Membranes. Based on the parameters just established, the following procedure was adopted for the membrane extraction of the glucosinolates from mustard seeds: 0.2 g of ground seeds were boiled in water; extracts were transferred to centrifuge tubes, 9-cm^2 membranes were added, and glucosinolates were loaded onto the membranes and eluted from the membrane by shaking for 2.5 h on a mechanical shaker.

Using the parameters just described, linearity of the sinigrin extraction from solutions and mustard seed samples was examined. A series of sinigrin solutions in a range of 100 to 500 μM was prepared, and 25 mL of each solution was extracted with the membrane. Also, 25 mL of each of these solutions was added to 0.2 g of ground and heated mustard seeds, and the sinigrin and sinalbin were extracted and measured.

To examine the applicability of the membrane extraction for the determination of glucosinolates in mustard seeds, seeds of yellow, brown, oriental and Indian mustard were analyzed using the procedure just described. Sinigrin solutions were used as quantitative standards for the determination of sinigrin, gluconapin, and sinalbin in mustard seed samples. For sinalbin, the same UV absorption coefficient as that for sinigrin at 235 nm was assumed; the ion-exchange process allowed for the UV detection of sinalbin in yellow mustard using sinigrin solutions as quantitative standards because the sinapin cation in the sinalbin molecule was exchanged to K cation, and the contribution of sinapin to the UV absorption of sinalbin was eliminated.

RESULTS AND DISCUSSION

Optimization of Ion-Exchange Parameters for Maximum Glucosinolate Recovery. *Effect of Membrane Size on Glucosinolate Recovery.* From solutions, sinigrin was recovered most efficiently by the membranes in the size range of ~4 to 11 cm^2. With these membranes, recovery of sinigrin was ~80%, as estimated from the ratio of the HPLC peaks obtained after sinigrin exchange on the membranes and the HPLC peaks of sinigrin standards. Extraction of sinigrin added to mustard seeds and sinalbin from mustard seeds also yielded 80% recovery, but with the membranes in the range of ~7 to 11 cm^2. The difference in the range of membrane size for the maximum of glucosinolate recovery between standard solutions and extracts was

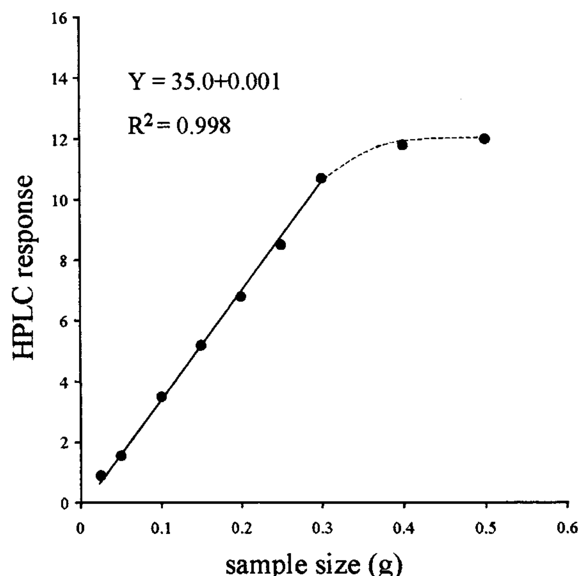

Figure 1. Effect of sample size on the amount of sinalbin extracted from seeds of yellow mustard with the anion-exchange membranes (each data point represents the mean of two replications).

probably because compounds other than glucosinolates from mustard seeds adsorbed to the membranes and made the 4−7-cm^2 membranes less efficient for exchange of glucosinolates. Low recovery from the membranes <4 cm^2 was probably a result of the small membranes not having the capacity to exchange the glucosinolates present in solutions or extracts. Low recovery from membranes >11 cm^2 was attributed to the 1 N KCl not being strong enough for an efficient glucosinolate elution from the large membranes. Membrane size of 9 cm^2 was therefore selected as optimal for extraction of mustard glucosinolates.

Separate measurements showed that no difference in sinigrin recovery was observed when 25 mL volume of 500 μM sinigrin solution was diluted to 50 and 100 mL before the extraction, indicating that the initial volume of a solution (or a mustard seed extract) taken for the extraction with the membranes was not critical for reproducible results and that only the volume of 1 N KCl used for the elution of glucosinolates from the membranes needed to be measured accurately. For the latter measurement, a dispenser mounted on top of a bottle containing 1 N KCl solution was used.

Sample Size Requirement for Maximum Glucosinolate Recovery. A clear relationship between the sample size taken for the extraction and the amount of glucosinolates recovered from the samples with the 9-cm^2 membranes was obtained (Figure 1). Sinalbin peak increased linearly ($R^2 = 0.998$) up to an ∼0.3-g sample size and started to level off for larger samples, whereas the peak of sinigrin (IS) remained constant and also decreased for seed samples >0.3 g. These results indicated that 9-cm^2 membranes are suitable for glucosinolate extraction from mustard seed samples <0.3 g, and that within this range of sample size, a linear response was observed for sinalbin.

Effect of Extraction Time on Glucosinolate Recovery. The measurements of the glucosinolate recovery with time of shaking revealed that sinigrin in solutions exchanged very quickly on the membranes; after only 7 min of sample loading and elution, the sinigrin peak represented ∼50% recovery, whereas after 1 h, the recovery was 80%, which is the maximum of sinigrin exchange rate as already determined (vide supra). However, glucosinolate extraction from the seed samples was slower than from solutions: sinigrin added to the seeds and sinalbin yielded the highest HPLC peaks after ∼2.5 h of shaking. Shaking the samples with the membranes and eluting glucosinolates from the membranes longer than 2.5 h did not yield larger HPLC peaks, indicating that 2.5 h was sufficient for maximum recovery.

Sample Stability. Excellent sample stability was observed for glucosinolates extracted with the membranes. Membrane extracts of mustard seeds that were stored at room temperature in the laboratory for up to 1 week showed no decrease in glucosinolate content; thus, samples can be kept easily in the laboratory until analysis. Membranes with adsorbed glucosinolates from mustard seeds were stored dry and in water. When stored dry, glucosinolate concentration decreased only by ∼10% within 5 days; after that, the glucosinolate concentration eluted from the membranes decreased more rapidly. However, the fact that membranes with mustard glucosinolates can be kept dry until analysis time for up to 5 days with only a minimal loss of glucosinolates offers a convenient method for storing the samples. The membranes with adsorbed glucosinolates should not be kept in water because a significant decrease in glucosinolates retained on the membranes was observed.

Determination of Mustard Seed Glucosinolates. Several kinds of mustard seeds were analyzed for glucosinolate content using the procedure just described. Figure 2 illustrates the HPLC chromatograms of the membrane-extracted glucosinolates from yellow, oriental, and Indian mustard seeds. Under the conditions used for the HPLC separation, sinigrin, sinapin, and gluconapin eluted at R_T of 2.7, 4.7, and 5.8 min, respectively. Sinapin was the only glucosinolate identified in yellow mustard seeds. Oriental and brown mustard seeds contained mainly sinigrin, with traces of 4-OH glucobrassicin, and seeds of Indian mustard contained primarily gluconapin, with small amounts of sinigrin and traces of 4-OH glucobrassicin. The peaks were identified based on the injections of seeds of known glucosinolate composition obtained from Agriculture & Agri-Food Canada (Saskatoon, Saskatchewan, Canada).

The results of the analysis of the selected mustard seeds are listed in Table 1. These results were corrected for 80% recovery. Excellent reproducibility of the glucosinolates determination by this method was obtained. The coefficient of variation ranged from 1.0 to 4.3% based on the analysis of five replications, demonstrating that anion-exchange membranes are a precise tool for a simple, one-step extraction of glucosinolates from mustard seeds.

As can be seen from Figure 3, a linear relationship between sinigrin extracted from solutions and sinigrin standards was obtained within the concentration range tested with the 80% rate of sinigrin exchange on the membranes. When varying amounts of sinigrin IS were added to 0.2-g mustard seed samples, sinigrin exchange was also linear with the same rate of exchange, whereas the amount of sinalbin extracted from the samples remained constant. This result demonstrated that the membrane extraction combined with the HPLC detection is a reliable method for the determination of glucosinolates in mustard seeds.

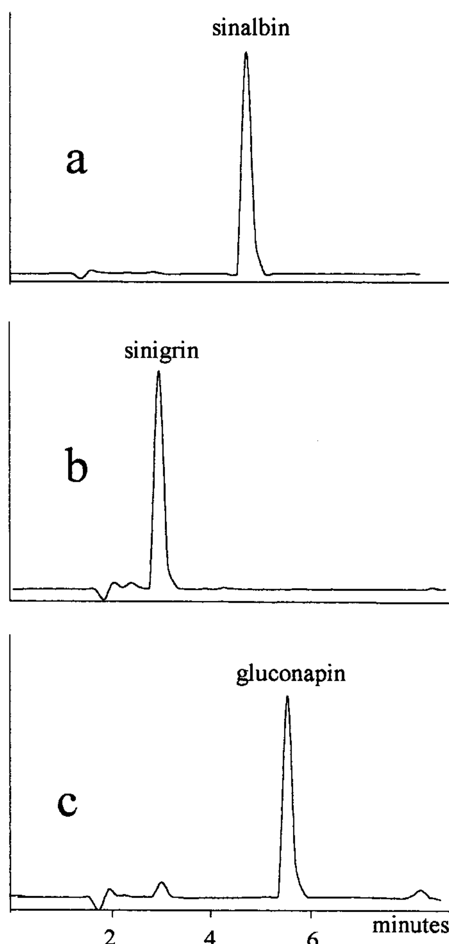

Figure 2. Liquid chromatograms of glucosinolates extracted with the anion-exchange membranes from the seeds of (a) yellow mustard, (b) oriental mustard, and (c) Indian mustard.

Table 1. Glucosinolate Content in Mustard Seeds[a]

seed	sinalbin	sinigrin	gluconapin
yellow mustard 1	154.9 ± 6.7	nd[b]	nd
yellow mustard 2	177.1 ± 4.4	nd	nd
brown mustard 1	nd	95.9 ± 2.6	nd
brown mustard 2	nd	92.9 ± 1.1	nd
brown mustard 3	nd	83.4 ± 1.8	nd
Oriental mustard 1	nd	105.1 ± 1.6	nd
Oriental mustard 2	nd	105.9 ± 1.1	nd
Oriental mustard 3	nd	112.3 ± 1.1	nd
Indian mustard	nd	5.7 ± 0.4	106.9 ± 4.3

[a] Values expressed as μmol/g seed; mean ± standard deviation, n = 5. [b] nd, Not detectable.

Conclusions. The developed method for glucosinolate extraction from mustard seeds is fast, simple, and inexpensive. No special reagents, organic solvents, or complicated procedures are required for this extraction method. The extraction is carried out in aqueous solutions making it easy and safe to perform. The cost of this procedure is very low as the price of each membrane is less than one cent, and only 1 N KCl solution is needed for the glucosinolate elution from the membranes. The RP HPLC separation with the aqueous ammonium acetate−MeOH as mobile phase allows for the injections of intact glucosinolates in sulfate form and, therefore, sample modification or derivatization before the injection is not required. The membrane extraction has the potential to simplify the currently used methods for the determination of glucosinolates in other economically important crops, such as canola.

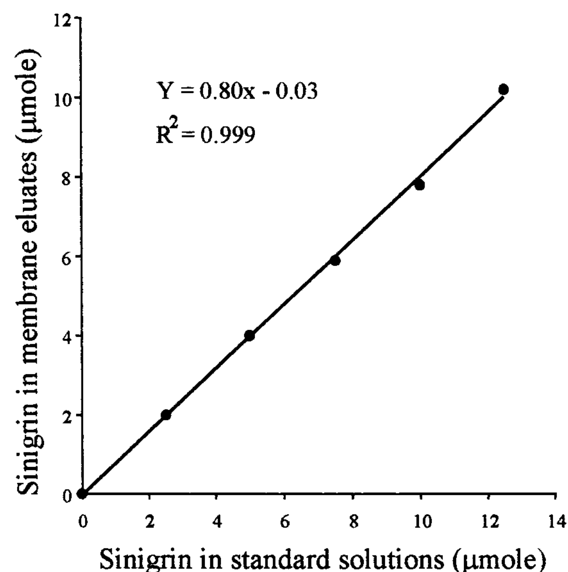

Figure 3. Relationship between the amount of sinigrin in standard solutions and in membrane extracts (each data point represents the mean of two replications).

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