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Direct and Simultaneous Analysis of Sinigrin and Allyl Isothiocyanate in Mustard Samples by High-Performance Liquid Chromatography

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A reversed-phase HPLC method for the simultaneous determination of the glucosinolate sinigrin and its major degradation product allyl isothiocyanate (AITC) was developed and used for direct analysis of aqueous extracts from Oriental mustard (*Brassica juncea* L.) related materials (ground and cracked seeds, powders, and bran) and from soil samples. The lowest detection limit was 0.1 μg/mL for both sinigrin and AITC). The developed method was used to trace the degradation of sinigrin to AITC in aqueous extracts. One of the major advantages of this method is the complete estimation of sinigrin content. The simultaneous analysis of both sinigrin and AITC in a single run avoided the underestimation caused by separate analyses.

KEYWORDS: Glucosinolates; sinigrin; allyl isothiocyanate; mustard; simultaneous determination; HPLC

INTRODUCTION

Glucosinolates are natural products that are characteristic of plants of the mustard family (Brassicaceae) (*1*). Although in most cases glucosinolates themselves are not directly responsible, they can frequently be correlated with flavor changes (*2*, *3*) and with protection against plant pathogens, insects, and other herbivores (*4*). Glucosinolates are broken down enzymatically by myrosinase mainly to isothiocyanates, cyanides, and thiocyanates, many of which are the actual active principles responsible for biological activity (*5*). The release of glucosinolates from plant material and their subsequent enzymatic conversion to breakdown products such as allyl isothiocyanate (AITC) therefore become important as the two processes are directly related to biological activity (*4*, *6*, *7*).

The enzymatic conversion of glucosinolates has been well studied, and there are thorough reviews of analytical methods for the separation and analysis of individual glucosinolates and their breakdown products. Paper chromatography, high-voltage electrophoresis and isotacophoresis, TLC, GC, HPLC (*6*, *8*, *9*), and CE (*10*, *11*) have been used.

Reversed-phase (RP) HPLC has generally been the method of choice in recent years because it has the versatility of analyzing glucosinolates in both intact and desulfated forms. Although the latter is more sensitive, the ion-exchange and desulfation steps are time-consuming and sometimes can lead to losses of glucosinolates. Because the separated compounds are not suitable for physiological or biological studies, the use of such a method as a preparative technique is limited to specific purposes (*12*). RP-HPLC methods for direct analysis of intact and nonderivatized glucosinolates have, therefore, been developed to overcome the above disadvantages (*12*−*15*).

The glucosinolate composition of Oriental mustard seed (*Brassica juncea* L.) is predominantly sinigrin, which primarily degrades to AITC (*16*). AITC has been frequently analyzed by GC (*17*), although RP-HPLC methods have also been explored for the analysis of isothiocyanates (*18*−*20*).

Glucosinolates are usually extracted from plant material with hot or aqueous methanol (*6*). Heating prior to extraction may not completely inactivate the enzyme when the moisture level in the seed is <8% (*9*). We also found that heating at 100 °C for 1 h did not completely destroy the indigenous enzymes in Oriental mustard bran (Tsao et al., unpublished data). In aqueous solutions, depending on the pH, sinigrin was shown to degrade to AITC even without the presence of enzyme (*7*). Incomplete deactivation of the enzyme and nonenzymatic conversion could result in underestimation of the total sinigrin content if only sinigrin is analyzed. On the other hand, because degradation of the indigenous sinigrin by the coexisting myrosinase in Oriental mustard bran takes ∼2 h to complete at room temperature (at 1% bran/water, w/v), any shorter time could result in incomplete conversion of sinigrin to AITC (*7*). This again can result in underestimation of the sinigrin content, regardless of the targeted analyte, sinigrin or AITC. Moreover, the effect of the remaining enzyme on the further degradation of AITC is unknown, and

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extended enzymatic reaction could potentially result in further degradation of AITC, thus leading to false evaluation of the analytes (the half-life of AITC in aqueous solution at room temperature in the absence of enzyme presence was 31 days) (*7*). Therefore, a method in which glucosinolates can be quickly extracted and immediately analyzed, together with their degradation products, would be very valuable. However, no methods have been published to date for the direct and simultaneous analysis of both glucosinolates and their breakdown products in a single analytical run.

In our study on the nematicidal effect of Oriental mustard bran, it was critically important to trace the dynamics of enzymatic conversion of sinigrin to AITC. The method reported here was, therefore, developed to simultaneously monitor concentrations of both sinigrin and AITC. In this paper, we report for the first time an RP-HPLC method for the direct and simultaneous determination of sinigrin and AITC in aqueous extracts from mustard seed and related materials without tedious cleanup and preparation procedures.

MATERIALS AND METHODS

Materials and Chemicals. Mustard products (bran, ground and cracked seed) were provided by G. S. Dunn and Co. Ltd., Hamilton, ON, Canada. HPLC grade water and acetonitrile (ACN) were from Caledon Laboratories Ltd., Georgetown, ON, Canada. Sinigrin (a monohydrate potassium salt) was purchased from Sigma Chemical Co. (St. Louis, MO) and AITC from Aldrich Chemical Co., Inc. (Milwaukee, WI). Ammonium acetate (NH_4OAc) was purchased from BDH Inc., Toronto, ON, Canada.

HPLC Apparatus and Operating Conditions. A Hewlett-Packard series 1100 HPLC system was used throughout the method development and subsequent sample analysis. This HPLC instrument was equipped with a photodiode array detector and ChemStation software, which was used in data acquisition and analysis. A Sphereclone ODS-2 column (5 μm, 15 cm × 4.6 mm, Phenomenex, Inc., Torrance, CA) was employed, with a precolumn packed with Spherisorb (5 μm, 3 cm × 4.6 mm, Phenomenex, Inc.). The detector was set at 228 nm (λ_{max}) for sinigrin and at 242 nm (λ_{max}) for AITC. The binary mobile phase was composed of 0.025 M NH_4OAc (pH 6.75) (A) and ACN (B). The flow rate was kept constant at 1.0 mL/min for a total run time of 12 min. The system was run with the following gradient program: 99% A/1% B (v/v) isocratic for 2 min, then linearly increased to 50% A/50% B in 0.5 min, held for 7.5 min, and then brought back to 99% A/1% B at 12 min. There was a 2-min postrun period between each sample injection. Injections (20 μL) were made using an HP1100 autosampler.

Extractions of Sinigrin from Oriental Mustard Bran and Seed. Five different extraction methods using 50% ACN in water (v/v) (A−D) and pure water (E) were studied to compare the extraction efficiency for total sinigrin content in mustard bran and seed samples.

(A) Soxhlet Extraction. One gram of bran or ground seed was weighed into a coffee filter and put in a Soxhlet extractor. The extractor was mounted on a 150-mL round-bottom flask with 100 mL of 50% acetonitrile. The flask was kept boiling with a heating mantel for 24 h at ∼1 cycle per hour. The extract was then cooled to room temperature, filtered through filter paper (no. 601-25, Ahlstrom, Mt. Holly Spring, PA), rinsed with 50% acetonitrile, and brought to volume (100 mL).

(B) Boiling Extraction. One gram of bran or ground seed was added to 100 mL of boiling 50% acetonitrile in a 200-mL round-bottom flask connected to a reflux condenser and refluxed for 30 min.

(C, D) Extractions by Soaking in Hot or Cold Solvent, Respectively. One gram of ground seed or bran was soaked in 100 mL of boiling (hot) or room temperature (cold) 50% acetonitrile for 30 min. No further heat was provided to the former (hot) flask during the extraction.

(E) Extraction with Room Temperature Water. One gram of bran or ground seed was soaked in 100 mL of water with constant mechanical shaking for 2 min. Extracts of methods B, C, and E were cooled to room temperature with a water bath, filtered, and brought to volume (100 mL) (when necessary). All sample solutions were filtered through a 0.45 μm syringe filter (Gelman Sciences Inc., Ann Arbor, MI) and analyzed by HPLC.

Analysis of Sinigrin and Allyl Isothiocyante in Mustard Products. Commercial mustard products (1 g) from G. S. Dunn and Co. Ltd. were extracted with water (10 mL) by mechanical shaking for 2 min before they were syringe-filtered for HPLC analysis.

Soil Samples. An air-dried loamy sand soil (Delhi, ON) was passed through a 2-mm sieve and mixed with a mustard sample at 1.0% w/w. The moisture content was then adjusted to 10% w/w with distilled water. The above treated soil was extracted with an equal weight of water or an ACN/water mixture (50/50, v/v), and the sample was subjected to constant mechanical shaking for 2 min at room temperature. The supernatant was filtered through a 0.45-μm syringe filter and analyzed by HPLC.

Preparation of Stock Standard and Buffer Solutions. Stock solutions (1000 μg/mL, w/v) of sinigrin (monohydrate) and AITC were prepared in HPLC grade water individually or as a mixture and stored at 4 °C. These were diluted to various concentrations (0.1−100 μg/mL) to prepare calibration curves. Each point of the calibration curve was measured three times, and means and standard deviations were calculated. Standard solutions at the same concentrations were also prepared in 50/50 and 80/20 ACN/water (v/v). AITC standard solutions were made fresh each time because of its lack of stability, whereas the same sinigrin solutions were used for a month because they were stable at 4 °C.

Data Analysis. All data were analyzed using SAS/STAT 6.12 (SAS Institute Inc., Cary, NC). The General Linear Model procedure was used for the analysis of variance and mean separations. Differences between treatments were determined by Fisher's Protected LSD test.

RESULTS AND DISCUSSION

The UV spectra of sinigrin and AITC (λ_{max} at 228 and 242 nm, respectively), and HPLC chromatograms are shown in **Figure 1**. The relationship between the concentration and the peak area was linear between 0.1 and 100 μg/mL for both sinigrin and AITC in a mixed standard solution in water, with a linear correlation coefficient r^2 of 1.0000 for both compounds. Standard curves of sinigrin and AITC in separate solutions were similarly linear between 0.1 and 100 μg/mL, with r^2 values of 1.0000 and 0.9999, respectively. Linearity ($r^2 > 0.9941$) was also found in standards prepared in both 50/50 and 80/20 ACN/water (v/v) solutions. The lower end of the standard curve (0.1 μg/mL) represents the minimum detectable concentration of sinigrin or AITC standard (signal/noise, or S/N, ≥ 3 at 228 and 242 nm for sinigrin and AITC, respectively). Concentrations of sinigrin or AITC in the samples were calculated using standard curves generated from the mixed standards.

One of the major advantages of this method is the complete estimation of sinigrin content. By simultaneously analyzing both sinigrin and AITC in a single run, underestimation (incomplete detection) caused by separate analysis was avoided. As shown in **Tables 1** and **2**, increased total sinigrin content was found in most samples using this simultaneous monitoring method. Underestimation could be up to 68% otherwise, depending on sample types and method of extraction (**Tables 1** and **2**). The percent underestimation for all five extraction methods (**Table 1**) was <3% except for the extractions of seed (seed, D) and bran (bran, E) at room temperature. The higher percentage of underestimation rates in seed, D, and bran, E (20 and 17%, respectively), might be caused by incomplete deactivation of myrosinase (seed, D) (**Table 1**). Although different extraction methods were used in this study, a specific method should be used for different types of samples. Extraction of seed meal with water at room temperature for 2 min was not as efficient as the Soxhlet (seed, A) and boiling (seed, B) extractions but was not significantly different from soaking in hot 50/50 ACN/water (seed, C) (**Table 1**). For the bran, the efficiencies of

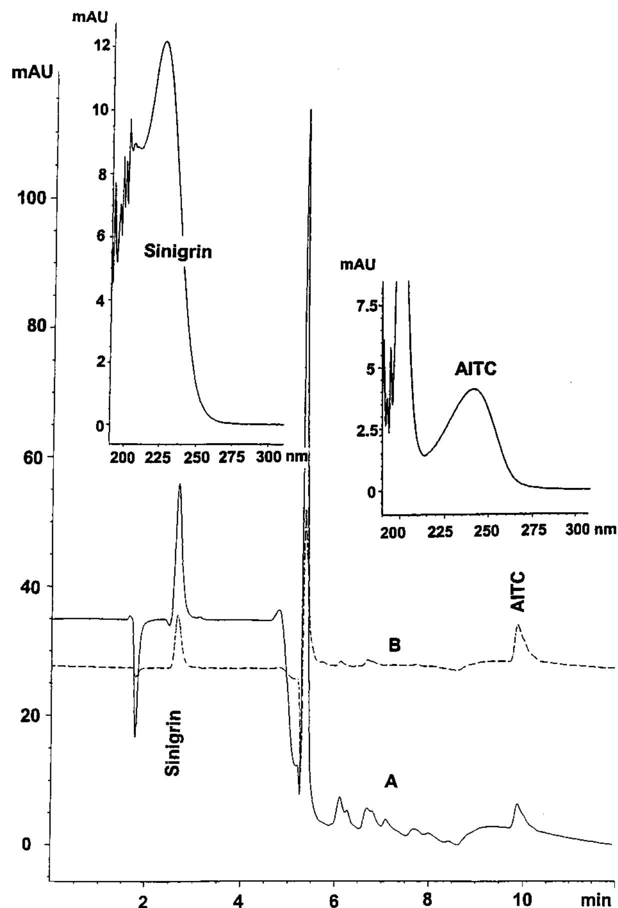

Figure 1. HPLC chromatograms and UV spectra of a standard mixture (10 ppm) of sinigrin (t_R = 2.80 min, λ_{max} = 228 nm) and AITC (t_R = 9.80, λ_{max}= 242 nm): (A) recorded at 228 nm; (B) recorded at 242 nm.

extraction were similar except for soaking in hot 50/50 ACN/water (seed, C) (**Table 1**). ACN was chosen over methanol for the extractions because the latter was reported to react with isothiocyanates (*18*).

The improved estimation of sinigrin using the current analytical method was apparent when water was used to extract the different mustard products (**Table 2**). The extraction was conducted for only 2 min in order to minimize the enzymatic conversion of sinigrin. As shown in **Table 2**, the estimation of total sinigrin may be significantly lower if only sinigrin is analyzed. The degree of underestimation varied from 0 to 68%, depending on sinigrin content, which differed between species and among different products of the same species (**Table 2**).

Table 2 also shows that whole ground Oriental mustard seed (202) had the highest sinigrin content, followed by Oriental mustard bran (403), pure Oriental mustard flour (130), a 50/50 (w/w) mixture of yellow and Oriental mustard brans (401), coarse ground Oriental seed (302), whole ground yellow mustard (140), and the bran of yellow mustard (402). The significant difference in "hotness" between the yellow and Oriental mustards is considered to be the direct result of sinigrin content, although yellow mustard contains other glucosinolates besides sinigrin. The lower sinigrin content of coarse ground Oriental seed (302) may be due to bigger particles in the cracked seed (which are hard for water to penetrate in such a short time period, 2 min).

Acetate buffer has been used in the HPLC mobile phase for glucosinolate analysis; however, the purpose of using it was not clear (*13*, *20*). We found that ammonium acetate buffer could retain sinigrin longer than pure water on the C_{18} column; therefore, the buffer was used in this method to intentionally delay the elution of sinigrin and AITC to avoid interference with other compounds. Without the presence of ammonium acetate, sinigrin eluted before 2 min under either isocratic or gradient ACN/water conditions, where several interfering peaks are also present. In our present method, sinigrin and AITC eluted at 2.8 and 9.9 min, respectively, and were free of interference (**Figure 2**).

Figure 2 also shows the process of enzymatic conversion of mustard bran-derived sinigrin to AITC; sinigrin, being water soluble, dissolved in the extracting water nearly instantly (D). The amount of sinigrin decreased over time, whereas that of AITC increased (C) until all sinigrin was converted (B). This conversion is considered to be important for biological activity.

This method was developed for the simultaneous monitoring of sinigrin and its conversion to AITC in soil. Soils mixed with Oriental mustard bran were simply extracted with water or a mixture of ACN and water. The latter extracts both sinigrin and AITC more efficiently from soil. AITC concentration in water extract was <20% than that in ACN/water extract. Syringe-filtered soil extracts were directly analyzed by HPLC immediately after extraction. Sinigrin and AITC were free of interference from coextractables in the soil (**Figure 3**). Our method also avoided sample concentration by vacuum evapora-

Table 1. Total Sinigrin Content of Oriental Mustard Seed and Bran Using Different Extraction Methods

sample	extraction method[a]	sinigrin[b] (%)	AITC[b] (%)	total sinigrin[c] (%)	underestimation[d] (%)
seed	A	5.17 ± 0.03	0.07 ± 0.00	5.24 A	1.36
seed	B	5.12 ± 0.05	0.01 ± 0.00	5.12 B	0.13
seed	C	4.55 ± 0.02	0.00 ± 0.00	4.55 C	0.05
seed	D	3.36 ± 0.01	0.85 ± 0.01	4.21 D	20.16
seed	E	4.43 ± 0.03	0.07 ± 0.05	4.50 C	1.56
bran	A	1.86 ± 0.02	0.06 ± 0.01	1.92 F	3.00
bran	B	2.16 ± 0.09	0.00 ± 0.00	2.16 E	0.00
bran	C	1.74 ± 0.05	0.00 ± 0.01	1.75 G	0.17
bran	D	2.13 ± 0.03	0.00 ± 0.00	2.13 E	0.12
bran	E	1.75 ± 0.07	0.36 ± 0.04	2.10 E	16.95

[a] A, Soxhlet extraction; B, boiling; C, hot soaking (added at boil, but no further heat was supplied); D, cold soaking [room temperature (22 °C]; E, water soaking (with mechanical shaking for 2 min, room temperature). [b] Values are the means of three replications. [c] Means separated within column by Fisher's Protected LSD test ($P \leq 0.05$). [d] Percent underestimation was calculated as follows: sinigrin from AITC/total sinigrin × 100 (for further details, see Materials and Methods). Refer to the text for extraction solvent.

Table 2. Sinigrin and AITC Contents of Mustard Products Extracted with Water

mustard[a]	sinigrin[b] (%)	AITC[b] (%)	total sinigrin (%)	underestimation[c] (%)
130	2.24 ± 0.04	0.98 ± 0.20	3.22	31
140	0.04 ± 0.00	0.08 ± 0.03	0.12	68
202	4.43 ± 0.03	0.07 ± 0.05	4.50	2
302	1.35 ± 0.07	0.39 ± 0.02	1.74	23
401	1.75 ± 0.07	0.36 ± 0.04	2.10	17
402	0.10 ± 0.00	0.00 ± 0.00	0.10	0
403	3.26 ± 0.07	0.08 ± 0.03	3.35	2

[a] 130, Oriental mustard flour; 140, whole ground yellow mustard; 202, whole ground Oriental mustard seed; 302, coarse ground Oriental seed; 401, 50/50 (w/w) mixture of yellow and Oriental mustard brans; 402, bran of yellow mustard; 403, Oriental mustard bran. [b] Values are the means of three replications. [c] Percent underestimation was calculated as follows: sinigrin from AITC/total sinigrin × 100 (for further details, see Materials and Methods).

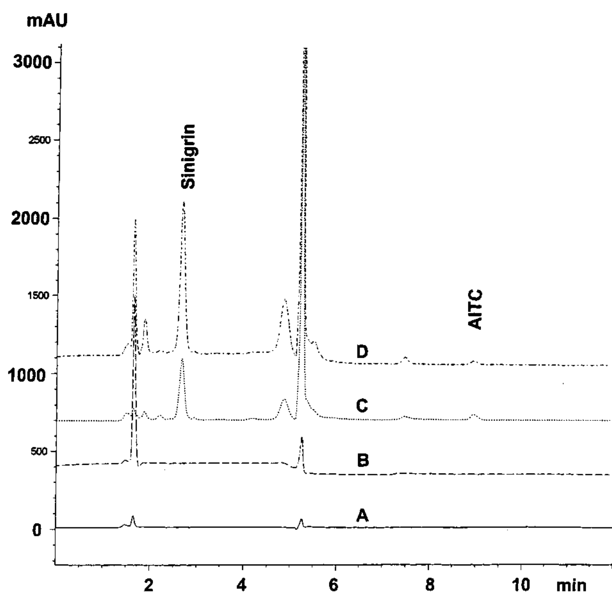

Figure 2. Chromatograms (recorded at 242 nm) showing the change in sinigrin and AITC concentrations due to the enzymatic conversion of Oriental mustard bran-derived sinigrin to AITC in water: (A) blank (water); (B) 240 min; (C) 60 min; (D) 2 min. Mustard bran was suspended in water at 0.1 g/mL, and the supernatant was analyzed after 2, 60, and 240 min.

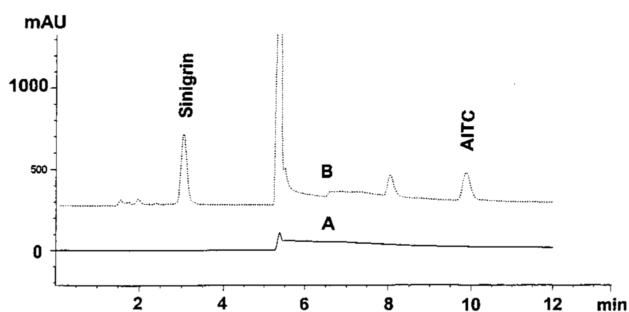

Figure 3. Chromatograms of ACN/water extracts from soil samples. The Oriental mustard bran was mixed into air-dried soil (Jordan Station, ON) at 10% (w/w) and adjusted to 5% moisture: (A) blank (soil only, 242 nm); (B) treated soil (242 nm). The soil was extracted with 50/50 ACN/water (1/1, w/v) 24 h post-treatment.

tion, which due to the volatile nature of AITC, may lead to low recovery. Mathäus and Fiebig (*19*) found that recovery of propyl isothiocyanate was dependent on pressure and time during vacuum evaporation; lower pressure (<190 mbar) and higher temperature (shorter evaporation period) gave lower recoveries.

In conclusion, this method provided a simple sample cleanup and preparation procedure for extracts from plant materials and soil samples and increased the accuracy of total sinigrin estimation in mustard by the simultaneous determination of both sinigrin and AITC. The method can be successfully applied to follow the degradation of sinigrin to AITC in detail.

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