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Determination of Sinigrin in Vegetable Seeds by Online Microdialysis Sampling Coupled to Reverse-Phase Ion-Pair Liquid Chromatography

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A hollow fiber microdialysis sampling coupled online to ion-pair liquid chromatography was investigated as an alternative to sample pretreatment for the direct determination of sinigrin in cruciferous vegetables without desulfation. After microdialysis, the dialysate was online injected into the chromatographic system to analyze the sinigrin with UV detection at 227 nm. Parameters affecting the microdialysis efficiency, such as flow rate, polarity modifier, pH in perfusion stream, pH, and salt added in sample solution, were studied. Through ion suppression in the donor phase and ion-pair formation in the acceptor phase, the microdialysis efficiency of sinigrin was enhanced. Experimental results revealed that the microdialysis of a sample solution (pH 2.0) using 0.1 M tetrabutylammonium (pH 12) as a perfusate at 10 μL/min flow rate maximized the extraction efficiency. Detection was linear in the concentration of 1.0−100 mg/L with a detection limit of 0.3 mg/L. Three seed samples were analyzed, with sinigrin at 49.8 (3.15% RSD), 20.0 (4.43% RSD), and 19.8 mg/g (4.22% RSD) for brussels, cauliflower, and cabbage, respectively. When 40 mg/g sinigrin was spiked in brussels seed powder, the recovery was 102.4% with 3.15% RSD ($n = 3$). The proposed method was proven to provide a very simple, rapid, and eco-friendly procedure to determine sinigrin in the seeds of cruciferous vegetables.

KEYWORDS: Sinigrin; glucosinolates; microdialysis; ion-pair HPLC; cruciferous vegetables

INTRODUCTION

Glucosinolates are anionic and hydrophilic plant secondary metabolites found in cruciferous plants (e.g., *Brassica* spp. vegetables: broccoli, Brussels sprouts, cabbage, cauliflower, and kale) but also occur in 15 other plant families. Aside from being considered to have potential anticancer action, glucosinolates have also been suspected to have a role in the resistance mechanism of pests and diseases. Their physiological effects have been of interest and have been studied consistently (*1*−*4*). Among glucosinolates, sinigrin and its breakdown products are considered powerful antifungal compounds (*5*,*6*). Thus, a simple, fast, and reliable method to determine the sinigrin content in a plant matrix is required in plant pathology studies.

In conventional analytical methods, glucosinolates in Cruciferae samples are usually extracted and purified prior to high-performance liquid chromatography (HPLC) determination (*7*−*10*). Although the procedure yields good results, the desulfation of glucosinolate and the complicated cleanup process are relatively laborious, time-consuming, and solvent-consuming procedures. An anion-exchange membrane used for one-step extraction has been reported in the HPLC determination of glucosinolates in mustard seeds to simplify the sample pretreatment procedure. However, it is still time-consuming (*11*). With the application of HPLC−tandem mass spectrometry techniques, the detection of glucosinolates has become more sensitive because it simplifies the cleanup process (*8*,*10*,*12*,*13*). However, the cost of such noble instrument is too steep for most laboratories. To decrease the analysis time, capillary electrophoresis methods have been developed to determine glucosinolates directly without desulfation (*14*,*15*). In our previous study, a reverse-phase ion-pair liquid chromatography was developed for the direct analysis of sinigrin in mustard seed without desulfation (*16*). Although the duration of separation was shortened to 6 min, it took more than 1 h for the tedious pretreatment process. Later, Rangkadilok et al. also used an ion-pair HPLC method to determine sinigrin and glucoraphanin in *Brassica* species after an extraction procedure (*17*). Microdialysis, which is easy to operate, rapid, and free or uses less organic solvents, has been applied to isolate components from a sample matrix. Online HPLC with microdialysis perfusion provides simplified sample preparation and has been widely applied to biosamples (*18*−*23*), cosmetic samples (*24*), wastewater (*25*), milk (*26*), and fermented drinks (*27*). It has the potential to be an alternative to conventional pretreatment processes in the determination of sinigrin in plant samples. However, there has not been any report related to the application of microdialysis sampling as the cleanup process in the determination of sinigrin so far. In this paper, a microdialysis technique coupled online to HPLC

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is systematically investigated and examined to develop an eco-friendly analytical process to determine the sinigrin content in cruciferous vegetables.

MATERIALS AND METHODS

Apparatus. The HPLC used in this work was equipped with a Dynamax SD-200 solvent delivery system (Varian, Walnut Creek, CA) and a Dynamax UV-1 detector with a 20 μL flow cell. The detection wavelength was set to 227 nm. A reverse-phase Nucleosil C-18 column (25 cm ×4.6 mm i.d., 5 μm particle size) (Supelco, Bellefonte, PA) was used for separation. A Rheodyne 7010 injector/switching valve (Rohnert Park, CA) with a 20 μL external loop was used as the interface between the hollow fiber microdialysis system and the HPLC system for sample introduction. The Varian Star chromatography workstation (system control version 5.3) was used to control the operation of HPLC to obtain the chromatogram and to perform data calculations. The hollow fiber microdialysis system was comprised of a baby bee syringe pump, a worker bee controller, and a 1 mL syringe (Bioanalytical System, Inc., Indiana, IN). A home-assembled linear cellular membrane probe (cellulose acetate; 15 cm length; 5000 Da; i.d., 220 μm; and o.d., 310 μm) microdialysis sampling system was prepared and set up. The syringe pump containing perfusate was connected to the inlet of the probe with polyethylene (PE) tubing (i.d., 380 μm; o.d., 1090 μm). The outlet of the microdialysis probe was connected to the sample loop with PE tubing for chromatographic determination. The assembly of the hollow fiber microdialysis/HPLC system was illustrated in our previous studies (*25*, *27*).

Chemicals and Reagents. Deionized water was produced using a Barnstead Nanopure water system (Barnstead, New York, NY) for all aqueous solutions. All chemicals and solvents were of ACS reagent grade. A standard stock solution of 1000 mg/L sinigrin (Sigma, St. Louis, MO) was prepared by dissolving 0.100 g of sinigrin in 90 mL of water and diluted to 100 mL with water. The solution was stored at 4 °C in a silanized brown glass bottle with a Teflon-lined cap. Fresh working solutions were prepared daily by appropriately diluting the stock solution with water. The HPLC eluent was prepared as a 20% (v/v) acetonitrile (Baker, Phillipsburg, NJ) and 80% aqueous solution containing 0.02 M tetrabutylammonium (TBA) bisulfate (Acros Organics, Belgium) and 0.01 M phosphate buffer at pH 7.0. Sulfuric acid, sodium hydroxide, and sodium dihydrogen phosphate (Riedel-deHaën, Hanover, Germany) were used to adjust the pH. All eluents were filtered through a 0.45 μm poly(vinylidene difluoride) (PVDF) membrane filter and were degassed ultrasonically.

Sample Preparation and HPLC Analysis. Vegetable seeds were heated in an autoclave at 121 °C for 10 min to inactivate the seed myrosinases and were ground in a food processor (HF-366, Supa Fine, Taiwan) for 2 min to form seed meal powder. The seed meal powder (0.05−0.2 g) was added into a 50 mL measured flask containing 40 mL of water. The mixture was stirred for extraction. Sulfuric acid (3.6 M) was used to adjust the pH to 2.0 under stirring. After it was diluted to 50 mL with water (pH 2.0), the solution was transferred into the dialysis cell (50 mL) for microdialysis sampling. The diffusate was collected online in the sample loop for HPLC analysis. The elution of sinigrin in HPLC was achieved isocratically at a flow rate of 1.0 mL/min by using the eluent prepared as 20% (v/v) acetonitrile and 80% aqueous solution containing 0.02 M TBA bisulfate and 0.01 M phosphate buffer at pH 7.0.

Conventional Sample Pretreatment Method. Inactivation of the seed myrosinase and grinding to form seed meal powder were as described in the sample preparation. Ten grams of seed meal powder was collected and weighed in a 100 mL beaker and then heated in a boiling water bath for 1 min. Fifty milliliters of boiling phosphate buffer (20 mM, pH 7.0) was added to the preheated sample, mixed, and shaken well in the boiling water bath at 100 °C for 10 min. After it was cooled with ice, the suspension was centrifuged at 1000*g* and 4 °C for 10 min. A 2 mL aliquot of the clear supernatant was transferred to another centrifuge tube, and 0.5 mL of a 1:1 solution of barium and lead acetate (0.5 M each) was added. The centrifugation procedure was repeated, and the supernatant was suspended in 15 mL of distilled water and was filtered through a 0.45 μm PVDF membrane filter. The filtrate was analyzed by HPLC.

RESULTS AND DISCUSSION

In the microdialysis sampling process, the low molecular weight analytes were allowed to diffuse across the hollow fiber semipermeable dialysis membrane into the perfusate due to the concentration gradient between the sample solution and the fluid in the hollow fiber, while the matrix species with higher molecular weight were prevented from diffusing through the membrane. The microdialysis sampling efficiency can be enhanced through the optimization of conditions in both sample solution and perfusate. Therefore, the factors influencing microdialysis sampling efficiency, including flow rate, polarity modifier, pH in perfusion stream, and the pH, and the addition of salt in sample solution were studied thoroughly to optimize the hollow fiber microdialysis pretreatment technique for the HPLC determination of sinigrin in vegetable seeds.

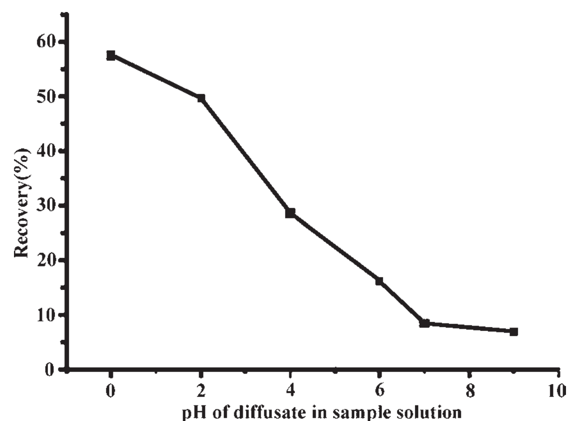

Figure 1. Influence of sample pH on the recovery of sinigrin in the microdialysis. Concentration of sinigrin, 50 μg/mL. Microdialysis was achieved with 0.1 M TBA aqueous solution (at pH 12) at a 10 μL/min flow rate.

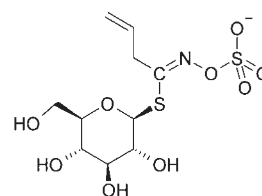

Figure 2. Chemical structure of deprotonated sinigrin.

Effect of Sample Solution pH on the Microdialysis Sampling. Similar to other extraction methods, the pH of the sample solution affects the microdialysis efficiency. **Figure 1** shows the influence of the sample pH on the recovery of sinigrin in the microdialysis process applied in the proposed method. It can be seen from the online analysis that the microdialysis efficiency of sinigrin decreases with an increase in the pH. Furthermore, during microdialysis, sinigrin diffuses unfavorably through the cellular fiber in its anionic deprotonated form (as in **Figure 2**) at a high pH of the sample solution. In low pH, the neutral molecular form of sinigrin increases, and the repulsion between sinigrin and cellulose acetate fiber decreases, thus increasing the microdialysis efficiency of sinigrin. Although the recovery of sinigrin at the sample pH of less than 2.0 will be higher than those in the tests, it is less for the cellulose acetate fiber in such an acidic condition from the results of our studies. The pH of sample solution was thus adjusted to 2.0. At this pH, the recovery of microdialysis was around 50%.

Effect of Salt Addition in the Sample Solution on the Microdialysis Sampling. A salting-out effect is often applied to improve the recovery in extraction processes. However, the increase or decrease of recovery (efficiency) due to the salting-out effect depends on the analyte and the perfusate system in microdialysis (*18*). In the study, various amounts of NaCl (0−1.0 M) were

added into the sample solution to investigate the salting-out effect on the microdialysis efficiency. Results reveal that the recovery of sinigrin decreases quickly with the NaCl addition in the microdialysis process. This shows that the neutral form of sinigrin is salted out onto some other species in the sample matrix during the addition of NaCl. The recovery of microdialysis thus decreased. Therefore, no NaCl was added.

Effect of Polarity Modifier in Perfusion Stream on the Microdialysis Sampling. As described previously, the sinigrin that diffused through the cellular membrane is in its neutral form. On the basis of the popular aphorism "like dissolves like" used for predicting solubility, the diffusion efficiency of species may then be influenced by the polarity of the perfusate. Considering the eco-impact and the cost between methanol and acetonitrile, methanol has been used to adjust the polarity of perfusate. A series of studies have indicated that the microdialysis recovery of sinigrin decreases with an increasing content of methanol from 0 to 50%. This shows that the solubility of the highly hydrophilic sinigrin in perfusate decreases when the polarity of the perfusate decreases due to the addition of methanol. Therefore, no polarity modifier is required to simplify the process.

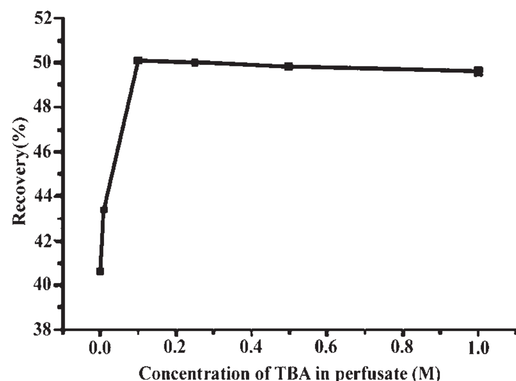

Figure 3. Effect of TBA in perfusate on the recovery of sinigrin. Concentration of sinigrin, 50 μg/mL. Microdialysis sampling was achieved in a pH 12 aqueous solution at a 10 μL/min flow rate, and the sample pH was 2.0.

Effect of Perfusion Flow Rate and Its pH on the Microdialysis Sampling. A high perfusion flow rate can decrease the detection sensitivity due to a dilution effect and the arising pressure that decreases the diffusion tendency of sample aliquot. To obtain an acceptable diffusion recovery in an acceptable time, the influence of the perfusion flow rate on the diffusion efficiency of sinigrin was examined. As expected, the higher the perfusion flow rate is, the lower the recovery obtained. Although a lower perfusion rate increases analyte recovery, it is limited by the increased collection times to fill the chromatographic sampling loop. As 15 min is required for one analytical run of real samples, a flow rate of 10 μL/min is enough to obtain five times the volume of the sample loop. Therefore, the flow rate of perfusion was selected as 10 μL/min. As the effect of perfusate pH, the recovery increased with the increase of pH. This reveals that a perfusate with a higher pH can ionize the sinigrin and convert it into its anionic form, and it does not back diffuse into the sample solution. Considering the durability of the microdialysis system and the HPLC system, the pH of the perfusate was controlled at pH 12.

Effect of Ion-Pair Reagent in Perfusate on the Microdialysis Sampling. The second chemical equilibrium concept was applied to increase the extraction efficiency in microdialysis sampling by adding TBA ion to form an ion pair with the diffused sinigrin in

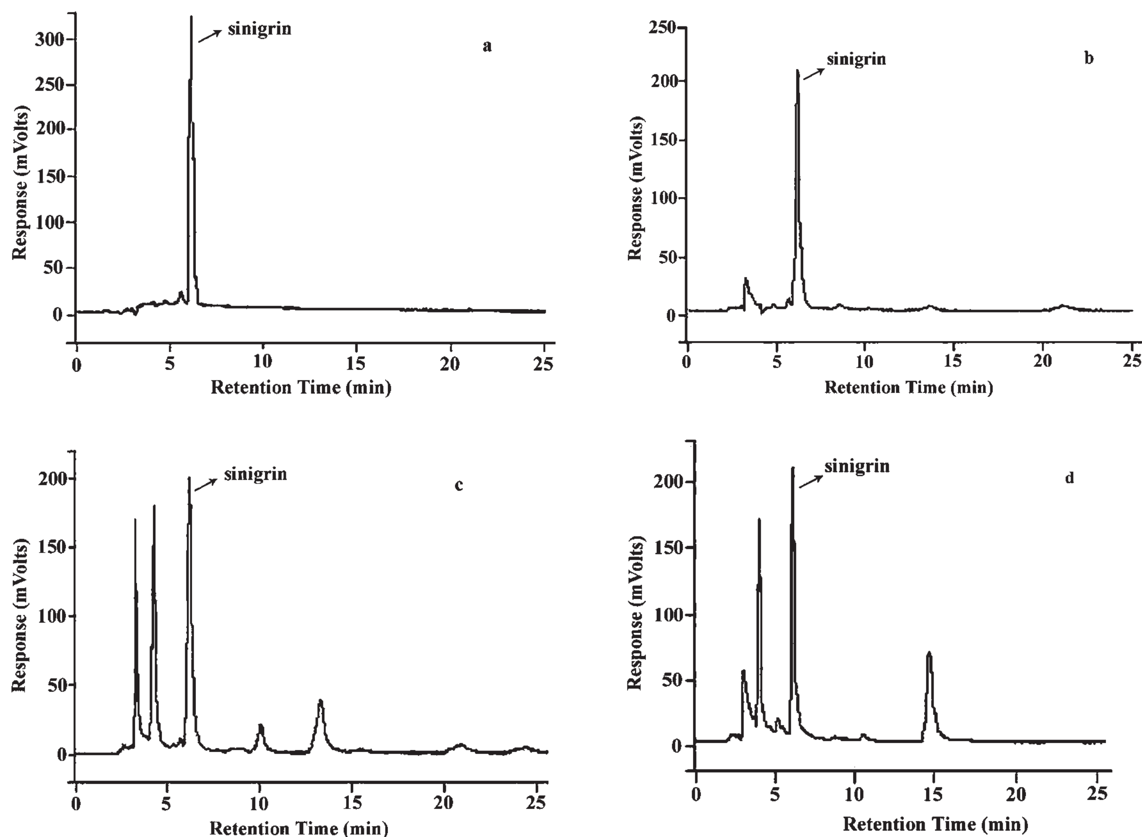

Figure 4. Chromatograms of sinigrin in the seeds of cruciferous vegetables: (**a**) Sinigrin standard, (**b**) Brussels, (**c**) cauliflower, and (**d**) cabbage. Microdialysis sampling: sample solution at pH 2.0 and perfusate at a 10 μL/min flow rate with 0.1 M TBA aqueous solution at pH 12. Chromatographic conditions are as described in the section Sample Preparation and HPLC Analysis.

the perfusion stream. **Figure 3** demonstrates the recovery of sinigrin under various quantities of TBA ion in perfusate. On the basis of the figure, the recovery increases initially with the addition of TBA and then becomes flat after 0.1 M TBA. Therefore, 0.1 M TBA was added into the perfusate for microdialysis sampling.

Validation of the Method. To test the applicability of the proposed method for the determination of sinigrin, a calibration plot was set up for the sinigrin in the sample solutions at a range of 1.0−100 mg/L. The plot was specified by the equation $y = 84500x + 38300$ (y is the peak area, and x is the injection concentration). The linear relationship between the peak area and the concentration was in good agreement with the correlation coefficient of 0.9987. The detection limit was calculated by dividing three times the average background noise by the detection sensitivity (slope of calibration plot), which was 0.3 mg/L. The calibration plot of sinigrin established over a concentration range of 0.5−100 mg/L by direct injection of the standard solutions was specified by the equation $y = 173500x + 19100$ (y is the peak area, and x is the injection concentration). The slope of the calibration plot represents the detection sensitivity of the analyte. Thus, 48.7% of sinigrin concentration was collected in the perfusion flow during the microdialysis sampling from the slope ratio of the linear regression equations (for that via the microdialysis to that by the direct injection of standards). The proposed method was examined by the analyses of sinigrin in three samples of Cruciferae seeds (Brussels, cauliflower, and cabbage). After the procedure described in the Materials and Methods and with appropriate dilution, the sample solution was subjected to online microdialysis under optimum conditions, and the diffusate was analyzed by HPLC for the sinigrin content. Chromatograms are shown in **Figure 4a−d**. The peak with a retention time of 6.12 min in each chromatogram was referred to the sinigrin. It is clear that sinigrin is well-separated from other species. The peak of sinigrin in **Figure 4** was reverified not only by the standard addition method but also by UV spectrometry following fraction collection. The content of sinigrin in per gram of seed sample was calculated from the sinigrin concentration obtained from the calibration plot and the volume of sample solution. The contents of sinigrin in seeds were 49.8 (3.15% RSD), 20.0 (4.43% RSD), and 19.8 (4.22% RSD) mg/g for Brussels, cauliflower, and cabbage, respectively, for three determinations. When spiked with 40 mg/g sinigrin in the seed powder of Brussels, the recovery was 102.4% with 3.15% RSD (n = 3), which was obtained from the division result of the detected quantity of spiked sample minus the detected quantity of original sample by the spiked quantity, after the pretreatment procedure as described previously and the analysis using the proposed method.

Comparison of the Proposed Method with the Conventional Method. The sinigrin content in the seed powder of Brussels was analyzed by the proposed online microdialysis method and by the conventional pretreatment method with ion-pair liquid chromatography. **Table 1** lists the results from both methods. As compared with the conventional method, the proposed method offers a higher content of sinigrin. The accuracy (reflected by the recovery) was 102.4% with 3.15% RSD (n = 3) as compared with that of 94.7% with 4.72% RSD (n = 3) by the conventional method. Results listed in **Table 1** indicate that the detected quantity of sinigrin obtained by the proposed method is 159% of that obtained by the conventional extraction method. A series of tests have revealed that increasing the volume of extraction solution (phosphate buffer) increases the detected quantity of sinigrin in the conventional method, which shows that the volume of extraction buffer should be increased in order to increase the detected quantity of sinigrin in the conventional method. However, the tedious and time-consuming (more than 1 h) procedure is the shortcoming of the conventional method. Through the proposed protocol, the procedure has been simplified, and the time has been shortened.

Table 1. Comparison of the Detected Quantity of Sinigrin in the Seed of Brussels by the Proposed Method and the Conventional Method

	microdialysis	conventional method
detected quantity (μg)	49585	31423
spiked quantity (μg)	40000	20000
total detected quantity (μg)	90821	50380
recovery (%)	102.4	94.7
RSD (n = 3) (%)	3.15	4.72

The results indicate that the proposed method can be successfully applied to analyze sinigrin in vegetable seeds using microdialysis as a sample pretreatment coupled online to the HPLC-UV determination. In addition to having no desulfation with ion-pair liquid chromatography, the tedious and time-consuming pretreatment procedure has been improved by microdialysis in the present method. Experimental results show that the proposed method can be used to determine sinigrin in the seed powders of vegetables with the advantages of easy operation, high recovery, and less time consumption.

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