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Short communication

Direct determination of sinigrin in mustard seed without desulfatation by reversed-phase ion-pair liquid chromatography

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Abstract

Reversed-phase ion-pair liquid chromatography has been investigated for directly analyzing sinigrin in mustard seed without desulfatation. After extraction by phosphate buffer (pH 7.0) from the grind-pastes of inactivated-myrosinase mustard seeds, sinigrin was first isolated through deproteinization and centrifugation, followed by filtration and injection into the chromatographic system. A reversed-phase C_{18} column was used to separate the sinigrin with an eluent of acetonitrile (ACN)–water (20:80) containing 0.02 *M* tetrabutylammonium (TBA) as the counter ion at pH 7.0. Detection was carried out with an UV detector operated at 227 nm. Factors affecting the chromatographic separation and quantitative determination, such as concentrations of TBA and ACN, and pH, were studied. The linear dynamic range is larger than three orders of magnitude and the detection limit is 0.045 mg/L. The RSD is around 3% and the recovery is 85% (3% RSD, $n = 3$).

Keywords: Mustard seed; Food analysis; Sinigrin; Glucosinolates; Carbohydrates

1. Introduction

During the past decades, there has been continuous interest in studies of the physiological effects of glucosinolates, as these are suspected to play a role in the pest- and disease-resistance mechanism [1–4]. Among the glucosinolates, sinigrin and its major breakdown product are known as powerful antifungal compounds [5,6]. Thus, a fast and reliable method to quantify sinigrin is required in plant pathology studies.

Glucosinolates in Cruciferae samples are usually extracted and purified according to the method of the European Union prior to HPLC determination [7]. However, this method takes time in the glucosinolate desulfatation step. In order to decrease the analytical time, Feldl et al. [8] developed a capillary electrophoresis method to determine glucosinolates directly without desulfatation. Recently, Paugam et al. [9] modified the CE method to achieve the separation of sinigrin and other glucosinolates by using a micellar electrokinetic capillary chromatographic technique. Because the charge density on glucosinolate is relatively low, it still took about/over 30 min for the glucosinolates to migrate. Thus, an analytical method to complete the determination within a short period of time is worthy of study.

Sinigrin is an ionic compound in an aqueous

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Sinigrin

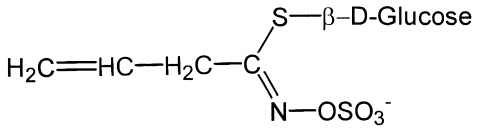

Fig. 1. The chemical structure of sinigrin.

matrix with the chemical structure shown in Fig. 1. With its anionic character, it is not, or slightly, retained on a reversed-phase column. However, it can be analyzed by carrying out modifications to the measuring procedure. In the conventional modification methods, ionic suppression by strong acids to convert sinigrin species into their neutral forms is inappropriate due to the very low pH requirement to neutralize the negative charge on the sulfate group and the pH limit in the application of silica-based C_{18} columns. Thus, in this study, an ion-pair liquid chromatographic method is investigated as an alternative to directly determine sinigrin in mustard seeds without desulfatation.

2. Experimental

2.1. Apparatus

The HPLC system used in this work was a Dynamax SD-200 solvent delivery system (Varian, Walnut Creek, CA, USA), and a Dynamax UV-1 detector, with a 20 μl flow cell. The detection wavelength was set at 227 nm. A reversed-phase Nucleosil C_{18} column (25 cm×4.6 mm I.D., 5 μm particle size) (Supelco, Bellefonte, PA, USA) was used for separation. A Rheodyne 7125 injector (Cotati, CA, USA) with a 20 μL external loop was used for sample introduction. A Varian Star chromatography workstation (system control version 5.3) was used to control the operation of HPLC, obtain the chromatogram, and perform data calculations.

2.2. Chemicals and reagents

Deionized water was produced by a Barnstead Nanopure water system (Thermolyne, Dubuque, IA, USA) for all aqueous solutions. All chemicals and solvents were of ACS reagent grade. A stock solution of 1000 mg/L sinigrin (Sigma, St. Louis, MO, USA) was prepared by dissolving 0.100 g in 90 mL water and diluting to 100 mL. The solution was stored in a brown glass bottle, and kept at 4°C for a maximum of 3 months. Fresh working solutions were prepared daily by appropriate dilution of the stock solution. The HPLC eluent was prepared as 20% (v/v) acetonitrile (ACN; Baker, Phillipburg, NJ, USA) in 0.02 *M* aqueous tetrabutylammonium (TBA) hydrogensulfate (Acros Organics, Belgium). Sodium hydroxide (Riedel-de Haën, Hanover, Germany) and sodium dihydrogenphosphate (Riedel-de Haën) were used to adjust the pH. All eluents were filtered through a 0.45 μm poly(vinylidene difluoride) (PVDF) membrane filter and degassed ultrasonically.

2.3. Sample preparation

Mustard seed was heated in an autoclave at 121°C for 10 min to inactivate the seed myrosinase, and then submitted to an initial grinding in a food processor for 2 min to form seed meal. A 10-g amount of seed meal was collected and weighed into a 100-mL beaker, then heated in a boiling water bath for 1 min. A 50-mL volume of boiling phosphate buffer (20 m*M*, pH 7.0) was added to the preheated sample, mixed and shaken in the boiling water bath at 100°C for 10 min. After cooling on ice, the suspension was centrifuged at 1000 *g*, 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) added. After centrifugation at 1000 *g*, 4°C for 10 min, 2 mL of the supernatant was suspended in 15 mL distilled water, and then filtered through a 0.45 μm PVDF membrane filter. The filtrate was collected for HPLC analysis.

3. Results and discussion

In order to examine the applicability of the proposed ion-pair liquid chromatography for determining sinigrin in mustard seeds, factors affecting the chromatographic behavior and quantitative determination, such as the ion-pair reagent (TBA), the

organic modifier (ACN) and the pH of the eluent, were studied thoroughly.

3.1. Selection of the detection wavelength

In order to obtain the highest detection sensitivity, the detection wavelength is best set at or near λ_{max}. Because the TBA ion has no characteristic absorption above the cut-off wavelength of the eluent, and λ_{max} does not change even in the ion-pairing of sinigrin with TBA, the detection wavelength was set at 227 nm, the λ_{max} of sinigrin.

3.2. Effect of pH

It is well known that the retention behavior of an analyte is influenced by the eluent pH. In ion-pair liquid chromatography, the eluent pH should be controlled carefully to maintain ion-pair formation or interaction. Therefore, the effect of eluent pH on the chromatographic behavior was investigated. Fig. 2a shows the effect of pH on the retention time of sinigrin, and Fig. 2b demonstrates the effect of pH on the quantitative detection of sinigrin. It can be seen that the retention of sinigrin decreases with increasing pH, and that the detection signal is constant in the pH range 3.0 to 7.0, and decreases beyond this range. It is shown that the ion-pair of sinigrin with TBA is stable in the pH range 3.0 to 7.0. Although sinigrin has higher capacity factors at pH values less than 3.0, its detection sensitivity was decreased. By considering the retention time, the detection sensitivity, and the baseline stability, the pH of the eluent was controlled at 7.0.

3.3. Effect of ion-pair reagent

In ion-pair liquid chromatography, the sorption of TBA on the column offers dynamic ion-exchange sites to retain the analytes. Therefore, the retention of sinigrin ion was directly related to the surface charge arising from the adsorbed cation (TBA ion). In studies, an adsorption equilibrium is established between the eluent and the surface of the stationary phase for the sinigrin ion. With the ion-exchange mechanism, retention increases with increasing amount of adsorbed TBA until the surface is saturated. However, Fig. 3a shows that the retention time increases with TBA in the low concentration range, levels off, and then decreases in the higher concentration range. This indicates that another force is present, competing with the ion–ion interaction. As the interaction between analyte (sinigrin ion) and eluent (may be TBA ion in the eluent) is greater than that between the analyte and ion-exchange sites, the retention time decreases. Fig. 3b demonstrates the influence of TBA on quantitative detection. It can be seen that the detection peak area increases with TBA in the low concentration range. As the concentration of TBA increases above 0.005 *M*, the detection signal does not change until 0.02 *M*. By considering the retention behavior and the quantitative sensitivity of sinigrin, 0.02 *M* TBA ion was selected as the counter-ion in the eluent.

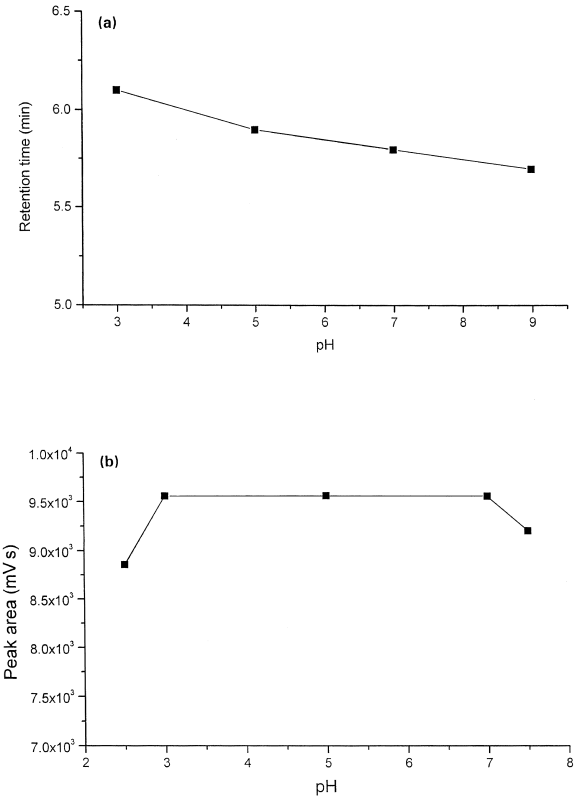

Fig. 2. The effect of pH on (a) the qualitative retention and (b) the quantitative detection of sinigrin. Elution conditions: 20% (v/v) of ACN, 0.02 *M* aqueous TBA, at various pH values, at a flow-rate of 1.0 mL/min.

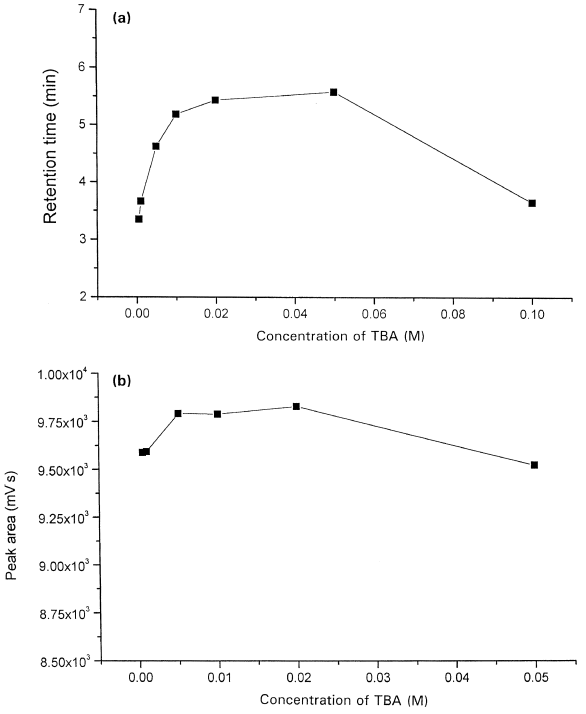

Fig. 3. The effect of TBA on (a) the qualitative retention and (b) the quantitative detection of sinigrin. Elution conditions: 20% (v/v) of ACN, various amounts of TBA, at pH 7.0, at a flow-rate of 1.0 mL/min.

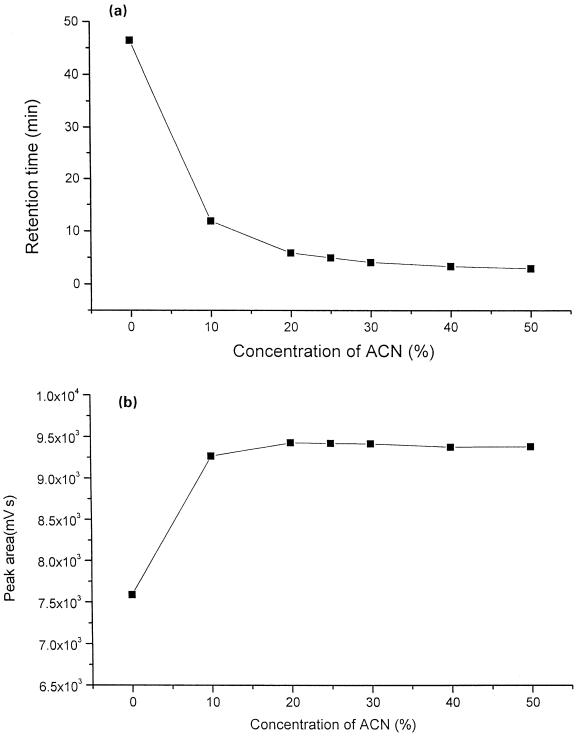

Fig. 4. The effect of organic modifier (acetonitrile) on (a) the qualitative retention and (b) the quantitative detection of sinigrin. Elution conditions: various amounts of ACN, 0.02 *M* aqueous TBA, and pH 7.0, at a flow-rate of 1.0 mL/min.

3.4. Effect of organic modifier

The in situ formed neutral ion-pair will adsorb onto the surface of a reversed-phase column firmly owing to the large size of the ion-pair. Therefore, an organic modifier is often required to add to the eluent to compete with the TBA or ion-pairs to control the elution rate. Taking into consideration the viscosity and the dielectric constant, acetonitrile was selected as the organic modifier (rather than methanol) in the present studies. The effect of ACN addition on the retention behavior and quantitative detection are shown in Fig. 4a and b. In Fig. 4a, it is clear that the retention time decreases rapidly as the acetonitrile content increases. However, in Fig. 4b, the detection sensitivity increases with the addition of ACN until 20% addition, and then levels off. As a compromise between retention and quantitative detection, an eluent of 20% aqueous acetonitrile solution containing 0.02 *M* TBA as the counter-ion at pH 7.0 is recommended to analyze sinigrin species.

3.5. Chromatograms of standard sample and real sample

Using optimal conditions, the chromatogram of a sinigrin standard solution is shown in Fig. 5a. Clearly, the retention time of sinigrin is only 6.5 min. Fig. 5b shows the chromatogram of sinigrin in a real mustard seed sample. It is clear that sinigrin is well separated from other species. We failed to identify peaks with liquid chromatography–mass spectrometry (LC–MS) due to the fact that ion-pairing reagents are unsuitable for use with LC–MS methods [10]. Therefore, the peak in Fig. 5b was re-verified with the standard addition method and

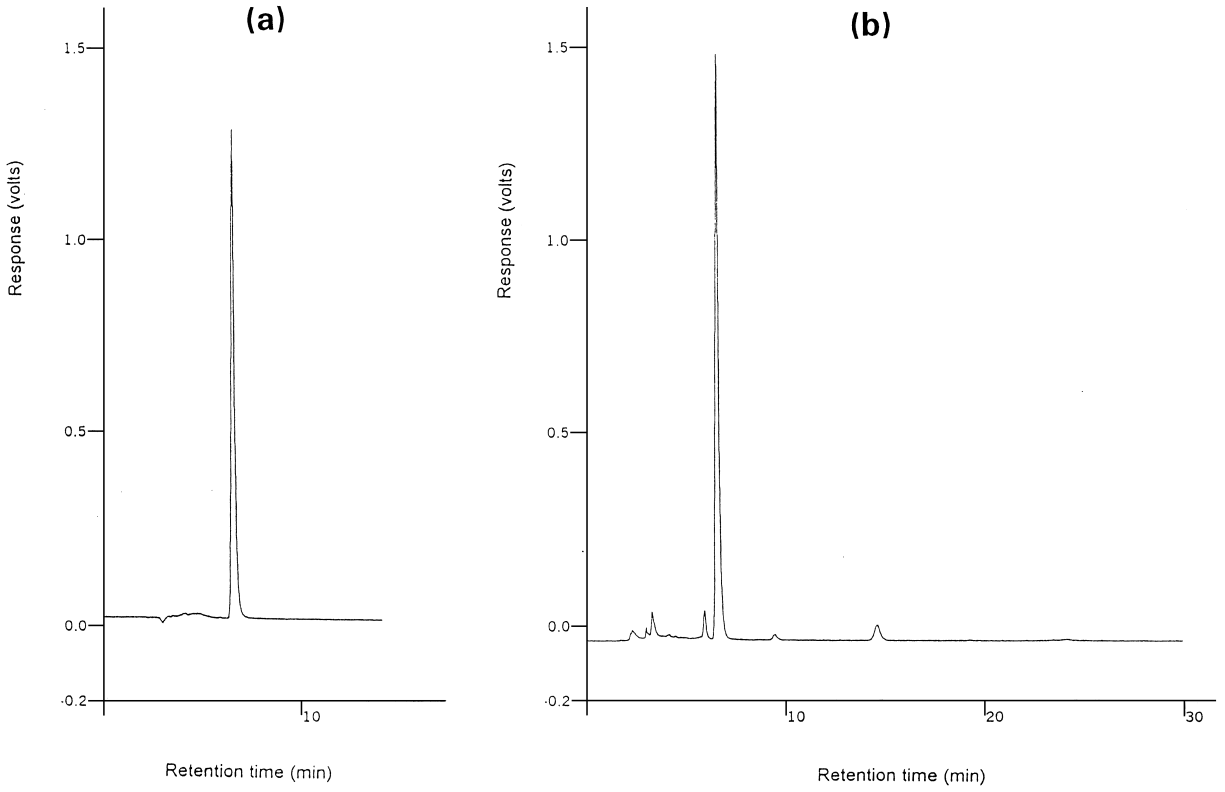

Fig. 5. Chromatogram of sinigrin in (a) standard solution and (b) in a real mustard seed sample. Elution conditions: 20% (v/v) ACN, 0.02 *M* aqueous TBA, pH 7.0, flow-rate 1.0 mL/min.

with UV spectrometry after fractional collection; the peak is definitely sinigrin.

3.6. Calibration graph, detection limit, precision, and accuracy

In order to test the applicability of the method for the determination of sinigrin, a calibration graph was prepared for sinigrin over the range 0.2 to 300 mg/L. The linear relationship ($y = 153x + 17$) between the peak areas and the injected quantities is very good. The correlation coefficient is 0.9998. The reproducibility was examined with five replicate injections of a real sample. Peak areas were measured and the relative standard deviation was calculated. The detection limit was calculated as three times the average background noise level. The value was 0.045 mg/L. The accuracy was evaluated by a spike of 50 mg/L sinigrin in the seed meal of a real sample. After the pretreatment described previously, and chromatographic detection, the recovery was 85% (3% RSD, $n = 3$). The precision was 3.0%.

4. Conclusion

From the results, the method appears to be of interest because it allows the quantification of sinigrin within a short time (6.5 min) and no desulfatation step was required; the total procedure was quite fast. It is also a reliable method in terms of linearity, reproducibility, and limit of detection. In addition, the system is extremely flexible as the concentration of TBA and acetonitrile, the pH, and the detector wavelength can all be adjusted to optimize the conditions for a given situation. This study indicates

that the proposed ion-paired HPLC method can be an alternative to the traditional HPLC and capillary electrophoresis methods in the determination of sinigrin in Cruciferae samples such as mustard seed.

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