The Isolation and Determination of Sulforaphane from Broccoli Tissues by Reverse Phase-High Performance Liquid Chromatography

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Sulforaphane is a cognate isothiocyanate of glucoraphanin, released by degradation of glucosinolate through myrosinase enzyme, when Brassica tissues are crushed or chewed. C_8 analytical column and mixture of water/acetonitrile 65/35 (*v/v*) as mobile phase were used to separate sulforaphane in broccoli tissues (e.g., 7-day-seedling, seed, floret and leave). Confirmation of sulforaphane in crude extract of broccoli tissues was monitored using gas chromatography-mass spectroscopy (GC-MS). The highest amount of sulforaphane (1216 μg/g dry weight) found in 7-day-seedling. This method is suitable for routine screening of plant materials and proposes a low cost and robust technique for the analysis of sulforaphane.

Keywords: Sulforaphane; Broccoli; Myrosinase; RP-HPLC.

INTRODUCTION

Sulforaphane [1-isothiocyanato-4-(methylsulfinyl)-butane], a degraded glucosinolate glucoraphanin [4-(methylsulfinyl)butyl glucosinolate] is a very potent inducer of phase II detoxification enzymes.[1,2] This compound plays an important role to control prevention or block any of the multiple stages of the carcinogenic process.[3,4] A large number of epidemiological and clinical studies have shown that increase in the consumption of *Brassica oleracea* (e.g., broccoli, Brussels sprouts, cauliflower, cabbage and kale) can reduce the risk of developing pancreatic,[5] lung,[6] colorectal[7] and prostate cancer.[8] All these varieties contain a group of secondary metabolites called glucosinolates (GLSs).[9] Upon Brassica tissues disruption, glucosinolates are rapidly hydrolyzed by myrosinase enzyme (β-thioglucoside glucohydrolase, EC3.2.3.1) to unstable intermediate that undergo non-enzymatic, intramolecular rearrangement to yield isothiocyanates, nitriles and thiocyanates (Fig. 1). The formation of specific hydrolysis products is dependent on a variety of factors including side chain, pH, metal ions, and the presence of protein cofactors.[10,11]

Sulforaphane was synthesized as described previously.[12,13] Chemical synthesis of sulforaphane is costly and time-consuming, and requires several highly toxic substances and final products from these reactions require further purification. These disadvantages limit synthesized sulforaphane to be used as food additives. Thus, natural sulforaphane is more favorable for common consumer. Furthermore, several methods have been published generally based on LC techniques with UV,[14-18] ELSD[19] and other chromatographic techniques like GC–MS[20] have been applied to sulforaphane analyses.

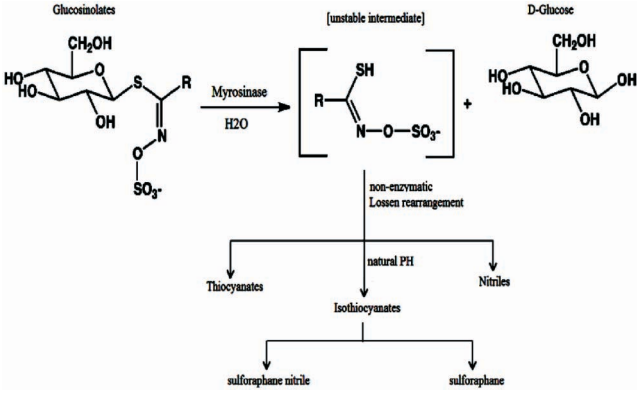

Fig. 1. Enzymatic conversion of a glucosinolate to an unstable intermediate by myrosinase, and subsequent conversion to an isothiocyanate, nitrile, thiocyanates and other product.

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In this study broccoli tissues (*Brassica oleracea L.* var. *botrytis, cv. Calabrese*) have been investigated because of their high content of glucoraphanin and abundant phase II enzyme inducer activity.[2,9] The aim of this study was to develop a low cost, rapid and reliable analytical method to separation and identification of sulforaphane in broccoli tissues by solvent extraction, silica gel column chromatography and high performance liquid chromatography. The analytical column used was C_8 analytical column and the chromatographic analysis was carried out at room temperature and the mobile phase of water/acetonitrile 65/35 (*v/v*). Furthermore, GC-MS was used to confirm the presence of sulforaphane in the crude extract of broccoli tissues.

RESULTS AND DISCUSSION

Subheading

Some authors, previously, were investigated amounts of sulforaphane in broccoli seedling,[16] seed,[15] stalk and leave[17] and package production.[19] While, this study investigate broccoli sulforaphane from seed until mature plant. A simple method was used to isolate the sulforaphane from water-extracted fraction of broccoli tissues. Although, acid hydrolysis performed before extraction consistently enhances the yield of sulforaphane.[14] In order to remove the non-polar impurities, the broccoli seed meal has to be defatted. There are several methods to reach to this objective including hexane defatting with soxhlet method[21] and hexane defatting method at room temperature.[22] Soxhlet method uses high temperature for long period of time, so it can show some destructive effect of myrosinase enzyme. In order to prevent this effect, the defatted seed was used with hexane over night at room temperature. The solvent used in extraction steps was methylene chloride as described in literature[17,21,22] that can be alternated with ethyl acetate.[15] One practical method to enhance the purification of sulforaphane is column chromatography[15,18] which is performed with methylene chloride phase containing compounds of interest. When a methylene chloride extracted fraction is analyzed on a reverse phase C_8 analytical column, several peaks are often eluted between solvent and analyte peaks, which attract our interest to purification steps. Thus, to remove the impurities, the sulforaphane fraction obtained from the methylene chloride extract was purified by silica gel column chromatography according to the steps described in extraction and purification section.

HPLC

A large number of previous studies for separation of sulforaphane in real samples have used C_{18} analytical column and linear gradient elution by mixture of water-acetonitrile[15,17] or some special isocratic methods by different mobile phases comprising mixture of water-acetonitrile[16] or water/tetrahydrofuran;[14] while in this study a C_8 analytical column and mixture of water/acetonitrile 65/35 (*v/v*) as mobile phase was used. Although, newly introduced HPLC-ELSD[19] method to determine broccoli sulforaphane with high selectivity and sensitivity. Furthermore, to avoid ghost peak effects and expensive instrument in gradient elution, an isocratic system was offered due to the advantages of simple, more accessible equipment, economy of solvent use due to the need for only a single degassing of the solvent. Under conditions described in HPLC section, retention time of sulforaphane was 5.44 min. Sulforaphane peak identity was assessed by comparing the retention time of the peak in the specimen with the retention time of a standard. Fig. 2A and 2B show the presence of sulfora-

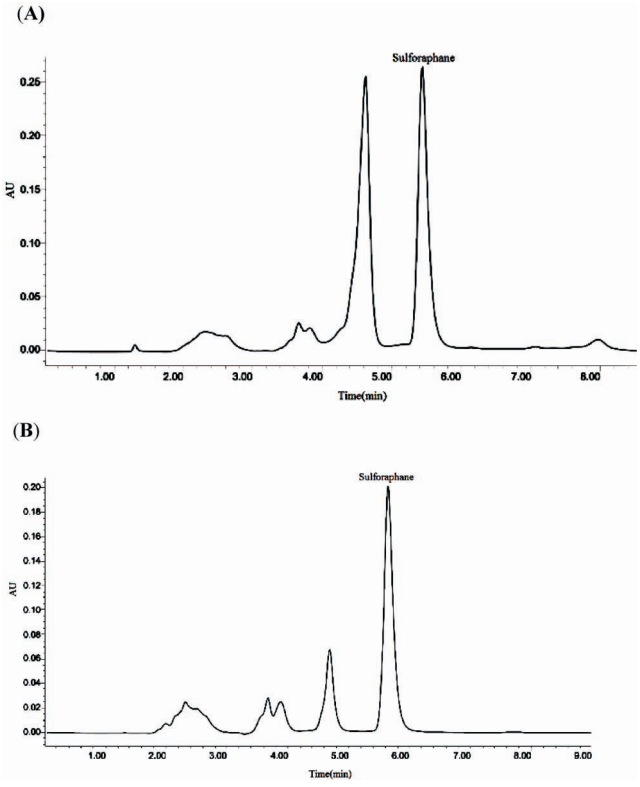

Fig. 2. Partial HPLC chromatogram shows the presence of sulforaphane in methanol phase of crude extract of broccoli seed meal (A) and after passing silica gel column chromatography (B), conditions described in HPLC section.

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Linearity of standard, precision and recovery

Quantification was carried out on the basis of the external standard method. Linear regression of peak area responses (y) vs. concentration (x) gave the following equation: $Y = 9041.6\ X + 23408,\ R^2 = 0.9998$. All injection of standard and samples were carried out in five replicates. The correlation coefficient demonstrates linearity of the method over the concentration range that was analyzed. For the determination of the reproducibility, a randomly selected sample of lyophilized broccoli tissue was extracted and analyzed five times. Limits of detection (LODs), and relative standard deviations (RSD%) were obtained in terms of peak area for sulforaphane in broccoli tissues. For the recovery study, standard sulforaphane 50 μg/L was added to silica column and eluted under condition described in purification step. The recovery of sulforaphane was calculated by use of the equation:

$$\text{Recovery\%} = A_2 / A_1 \times 100$$

That, A_2 is the peak area of sulforaphane obtained from plant material after passing silica column, A_1 is the peak area obtained from standard solution after passing silica column. The recovery of sulforaphane was calculated for above 96%.

Quantification of sulforaphane in broccoli tissues

Table 1 shows the contents (μg/g dry weight) of sulforaphane in broccoli tissues. It is very interesting that there were significant differences, 1.5-300 times, among the broccoli tissues that can be comparable with the other results.[15-17] The highest content of sulforaphane was found in broccoli 7-day-seedling, whereas leaves recorded the lowest. However, differences in sulforaphane contents between the 7-day-old seedling and seed were relatively slight. Results shown sulforaphane concentration tended to decrease during the growth of broccoli seedlings until mature plant. This was also noted by other workers.[9,16] This change may be related to several different myrosinase isoenzymes have been characterized in seeds, seedlings, and vegetative tissues.[23] Thus, the source of myrosinase activity should be carefully considered when studying the products derived from glucosinolate hydrolysis. The high desired compound contents indicate that 7-day-seedling broccoli is good raw materials for preparing sulforaphane.[9]

Table 1. Sulforaphane content in the tissues of Broccoli Ramose Calabrese (n = 5)

Part of plant	Sulforaphane content (μg/g ± SD dry weight)
7-day-seedling	1216 ± 35
Seed	849 ± 4
Floret	207 ± 9
Leaves	4 ± 0.3

GC-MS confirmations

The GC-MS technique was used to confirm the presence of sulforaphane in crude extract from broccoli seed meal. Under conditions described in GC-MS section, retention time of sulforaphane was found to be 21.437 min. The representative chromatogram of the GC profile of crude extract of broccoli seed meal is shown in Fig. 3. Sulforaphane peak identity was assessed by comparing the retention time of the peak in the specimen with the retention time of a standard. The mass spectrum obtained for sulforaphane extracted from broccoli seed meal were, *m/z* (%): 39 (12); 55 (32.26); 72 (100); 85 (7.8); 114 (10.1); 160 (90.84); M = 177 (M^+). These data were in agreement with previously

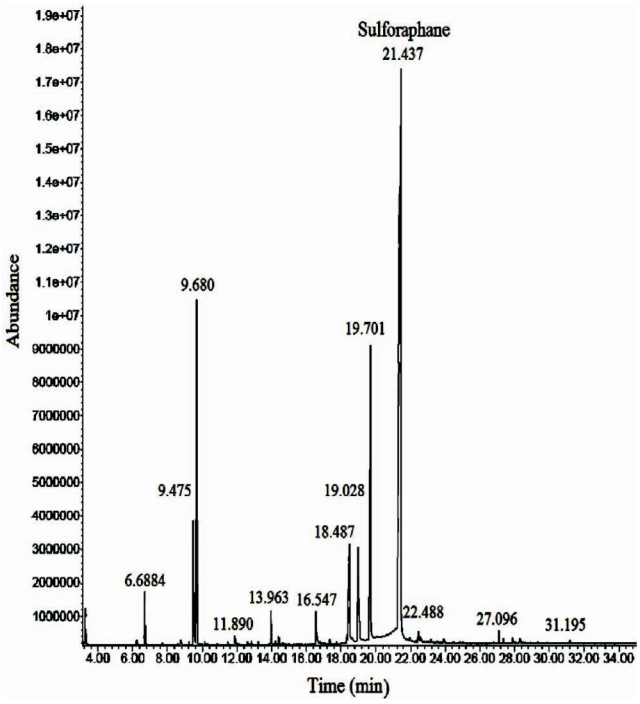

Fig. 3. Gas chromatogram of crude sulforaphane extracted from broccoli seed meal, conditions described in GC-MS section.

published findings.[15,20]

EXPERIMENTAL

Subheading

Materials, reagents and standard

Broccoli Ramose Calabrese seed, floret and leave were provided by Vegetables and Flowers Institute of Sari Academy of Agricultural Science, Mazandaran, Iran. All chemicals were analytical grade. Sulforaphane standard was purchased from Sigma-Aldrich (St. Louis, MO.). HPLC grade acetonitrile and methanol (Merck, Darmstadt, Germany) were used. HPLC grade water was obtained from Milli-Q UF system (Millipore Co, MA, Bedford, USA).

Preparation of seedling

Seeds (20 g) were soaked in 100 mL of 1% sodium hypochlorite for 60 min and then drained and washed three times with distilled water. Afterwards, seeds were soaked in 100 mL distilled water overnight. The imbibed seeds were germinated at pilot scale by layering seeds over moist filter paper in a germination tray. The tray was placed in a refrigerator and incubator seed germinator (FOC 225I, VELPO® Scientifica, Europe) and seeds were continuously watered by spray every 3 hrs. Germination of broccoli seeds was carried out at 25 °C under photoperiod of 16 hrs light and 8 hrs darkness cycle (40 W incandescent bulbs). Sprouted seeds were collected after seven days.

Preparation of standard

A stock solution was prepared with 5 mg of sulforaphane reference standard, which dissolved and diluted in 10 mL acetonitrile. Stoke solution was stored at -80 °C. The desired volumes of the standard stock solution of sulforaphane were pipetted into 1.5 mL Eppendorf Tube and diluted with 1 mL acetonitrile. The final concentrations of sulforaphane were in the range of 50–400 μg/mL. A 10 μL portion of each solution was subjected to HPLC-UV in five replicates, and a cali1bration curve was made. Quantification was carried out on the basis of the external standard method.

Sulforaphane extraction and purification

Sulforaphane was extracted from Broccoli seed meal. Briefly, seed was immediately frozen in liquid nitrogen, lyophilized to dryness and ground to a fine powder with a mortar and pestle. 10 g of this powder defatted with excess hexane overnight at room temperature. Following these, hydrolyzed by adding 100 mL of 20 mM potassium phosphate buffer pH = 7.2 and incubated at room temperature for 8 hrs on shaking set (GFL 3017, Germany). This mixture was extracted three times with equal volumes of methylene chloride which combined and dried at 33 °C under vacuum in a rotary evaporator (Eyela N-1000, Rikakikal, Japan). This residue was dissolved in 50 mL of 5% acetonitrile in water (*v/v*) and washed two times with equal volume of hexane. After that, the aqueous phase was extracted three times with equal volumes of excess methylene chloride. The methylene chloride layers were pooled, dried over anhydrous sodium sulfate, and filtered through filter paper (Whatman No.1). The filtrate was dried at 33 °C under vacuum in a rotary evaporator. The dry extract was dissolved in 3 mL of methanol and filtered through 0.45 μm membrane filter (Acrodisc® GHP 13 mm, USA) and then was stored at -20 °C.

For the purification of sulforaphane, a glassy column (300 × 5 mm) is packed manually with 1 g silica gel 60 (230-400 mesh ASTM, Merck) activated for 6 hrs in 80 °C. Then it was equilibrated through 4 mL methylene chloride. 2 mL of the crude extract gained from methylene chloride phase in solvent extraction procedure was added to the column. Afterward, washing step was performed by 4 mL of 5% methanol in ethyl acetate (*v/v*) and was discarded. Sulforaphane was eluted by passing 4 mL of methanol. Finally, the methanol extract was reduced to 2 mL by the steam of nitrogen at room temperature and filtered through a 0.45 μm membrane filter. The extracts were stored at -20 °C until analysis. The above-mentioned methods were used for the other broccoli tissues.

Apparatus and operating conditions

HPLC

The samples were analyzed with a Waters® HPLC Breeze™ System equipped with Waters model 1525 Binary HPLC pump, Waters 2487 Dual λ absorbance detector, and reversed-phase C_8 column (250 × 4.6 mm i.d., 5 μm, Spherisorb). The injection system used was a Rehodyne (7725i, USA) manual injector. The Waters Breeze system software (version 3.3) provides a single user interface for acquiring, processing and reporting chromatographic data. Analysis were carried out with an acetonitrile/water 35/65 (*v/v*) isocratic elution, both eluents were filtered and degassed using vacuum prior to use. The column temperature was ambient, with a flow rate 1 mL/min, and 10 μL portions were injected into the column. Sulforaphane was detected by UV absorbance detector at 254

nm.

GC-MS

The gas chromatography-mass spectroscopy method was done according to Chaing et al.[20] A Hewlett-Packard (HP) 6890 N gas chromatograph with electronic pressure control (EPC 500) which was connected to an HP 5975B inert MSD was used for the identification of sulforaphane from the crude extract of broccoli seed. An HP-5MS fused silica capillary column (Hewlett-Packard, 30 m, 0.25 mm i.d., 0.25 μm film Thickness, cross-linked to 5% phenyl methyl siloxane stationary phase) was used. The entire system was controlled by MS ChemStation software (Hewlett-Packard, version B.05.05). Electron impact mass spectra were recorded at 70 eV. Ultra-high purity helium (99.999%) was used as the carrier gas at flow rate of 1 mL/min. The injection volume was 1 μL and all the injections were performed in a splitless mode. Injector and detector temperatures were 270 and 280 °C, respectively. Column oven temperature was initially set at 50 °C for 5 min, then increased to 270 °C (ramp, 10 °C/ min), and held for 5 min.

REFERENCES

1. Zhang, Y.; Talalay, P.; Cho, C.-G.; Posner, G.-H. *Proc. Natl. Acad. Sci. USA*. **1992**, *89*, 2399.
2. Fahey, J.-W.; Talalay, P. *Food. Chem. Toxicol*. **1999**, *37*, 973.
3. Maheo, K.; Morel, F.; Langouet, S.; Kramer, H.; Le Ferrec, E.; Ketterer, B.; Guillouzo, A. *Cancer Res*. **1997**, *57*, 3649.
4. Parnaud, G.; Li, P.; Cassar, G.; Rouimi, P.; Tulliez, J.; Combaret, L.; Gamet-Payrastre, L. *Nutr. Cancer* **2004**, *48*, 198.
5. Olsen, G.-W.; Mandel, J.-S.; Gibson, R.-W.; Wattenberg, L.-W.; Schuman, L.-M. *Am. J. Publ. Health* **1989**, *79*, 1016.
6. Le, M.-L.; Yoshizawa, C.-N.; Kolonel, L.-N.; Hankin, J.-M.; Goodman, M.-T. *J. Natl. Cancer Inst*. **1989**, *81*, 1158.
7. Benito, E.; Obrador, A.; Stiggelbout, A.; Bosch, F.-X.; Mulet, M.; Munoz, N.; Kaldor, A. *Int. J. Cancer* **1990**, *45*, 69.
8. Cohen, J.-H.; Kristal, A.-R.; Stanford, J.-L. *J. Natl. Cancer Inst*. **2000**, *92*, 61.
9. Fahey, J.-W.; Zhang, Y.; Talalay, P. *Proc. Natl. Acad. Sci. USA*. **1997**, *94*, 10367.
10. Cole, R.-A. *Phytochemistry* **1978**, *17*, 1563.
11. Uda, Y.; Kurata, T.; Arakawa, N. *Agric. Biol. Chem*. **1986**, *50*, 2735.
12. Schmid, H.; Karrer, P. *Helv. Chim. Acta*. **1948**, *31*, 1497.
13. Schenk, W.-A.; Durr, M. *Chem. Eur. J*. **1997**, *3*, 713.
14. Bertelli, D.; Plessi, M.; Braghiroli, D.; Monzani, A. *Food. Chem*. **1998**, *63*, 417.
15. Liang, H.; Yuan, Q.; Xiao, Q. *J. Chromatogr., B* **2005**, *828*, 91.
16. Sivakumar, G.; Aliboni, A.; Bacchetta, L. *Food. Chem*. **2007**, *104*, 1761.
17. Liang, H.; Yuan, Q.-P.; Dong, H.-R.; Liu, Y.-M. *J. Food. Compos. Anal*. **2006**, *19*, 473.
18. Liang, H.; Li, C.; Yuan, Q.; Vriesekoop, F. *J. Agric. Food. Chem*. **2007**, *55*, 8047.
19. Nagakawa, K.; Umeda, T.; Higushi, O.; Tsuzuki, T.; Suzuki, T.; Miyazawa, T. *J. Agric. Food. Chem*. **2006**, *54*, 2479.
20. Chaing, W.-C.; Pusateri, D.-J.; Leitz, R.-E.; *J. Agric. Food. Chem*. **1998**, *46*, 1018.
21. Vaughn, S.-F.; Berhow, M.-A. *Ind. Crop. Prod*. **2005**, *21*, 193.
22. Matusheski, N.-V.; Walling, M.-A.; Juvik, J.-A.; Klein, B.-P.; Kushad, M.-M.; Jeffery, E.-H. *J. Agric. Food. Chem*. **2001**, *49*, 1867.
23. Lenman, M.; Falk, A.; Rodin, J.; Hoglund, A.-S.; Ek, B.; Rask, L. *Plant. Physiol*. **1993**, *103*, 703.