Preparative HPLC Method for the Purification of Sulforaphane and Sulforaphane Nitrile from *Brassica oleracea*

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An extraction and preparative HPLC method has been devised to simultaneously purify sulforaphane and sulforaphane nitrile from the seed of *Brassica oleracea* var. *italica* cv. Brigadier. The seed was defatted with hexane, dried, and hydrolyzed in deionized water (1:9) for 8 h. The hydrolyzed seed meal was salted and extracted with methylene chloride. The dried residue was redissolved in a 5% acetonitrile solution and washed with excess hexane to remove nonpolar contaminants. The aqueous phase was filtered through a 0.22-μm cellulose filter and separated by HPLC using a Waters Prep Nova-Pak HR C-18 reverse-phase column. Refractive index was used to detect sulforaphane nitrile, and absorbance at 254 nm was used to detect sulforaphane. Peak identification was confirmed using gas chromatography and electron-impact mass spectrometry. Each kilogram of extracted seed yielded approximately 4.8 g of sulforaphane and 3.8 g of sulforaphane nitrile. Standard curves were developed using the purified compounds to allow quantification of sulforaphane and sulforaphane nitrile in broccoli tissue using a rapid GC method. The methodology was used to compare sulforaphane and sulforaphane nitrile content of autolyzed samples of several broccoli varieties.

Keywords: *Brassica oleracea; glucosinolates; broccoli; sulforaphane; sulforaphane nitrile; isothiocyanate; HPLC*

INTRODUCTION

Epidemiological studies have shown that increased consumption of vegetables, especially cruciferous vegetables such as broccoli, Brussels sprouts, and cabbage, can lower the risk of developing pancreatic (*1*), lung (*2*), colorectal (*3*), and prostate (*4*) cancers. It is theorized that much of this chemoprotective effect can be attributed to the cancer-blocking effects of isothiocyanates. These isothiocyanates are derived from a family of compounds called glucosinolates, which are relatively unique to cruciferous vegetables.

When cruciferous vegetables are ground or chopped, myrosinase enzyme (thioglucoside glucohydrolase, EC 3.2.3.1) and the sequestered glucosinolates come into contact. Myrosinase breaks the β-thioglucoside bond of glucosinolate molecules, producing glucose, sulfate, and a diverse group of aglucon products (Figure 1). Depending upon the structure of the specific glucosinolates and the existing reaction conditions, isothiocyanates and nitriles usually constitute the majority of these aglucons (*5*, *6*).

Harvesting practices, storage conditions, and preparation techniques have the potential to impact the hydrolysis of glucosinolates. These factors may change the ratio of isothiocyanates relative to nitriles in a broccoli meal. The pH during hydrolysis has been found to influence both the activity of myrosinase (*7*) and the ratio of isothiocyanates to nitriles formed in the disrupted tissue of cruciferous vegetables. Using a purified mustard seed myrosinase (*Sinapis alba*) and allyl- and 2-phenethyl-glucosinolates, it has been found that a low pH favored the formation of nitriles and that a higher pH favored the formation of isothiocyanates (*8*). Using a semi-purified myrosinase from rape (*Brassica napus*) and a synthetic 2-hydroxy-3-enylglucosinolate, it has also been reported that at low pH, the predominant hydrolysis product was the nitrile, and that at higher pH isothiocyanate formation was favored (*9*). In addition, higher temperature and increased hydration have been found to favor isothiocyanate formation (*5*) whereas the presence of thiol compounds and ferrous ions have been found to favor nitrile formation (*10*−*12*).

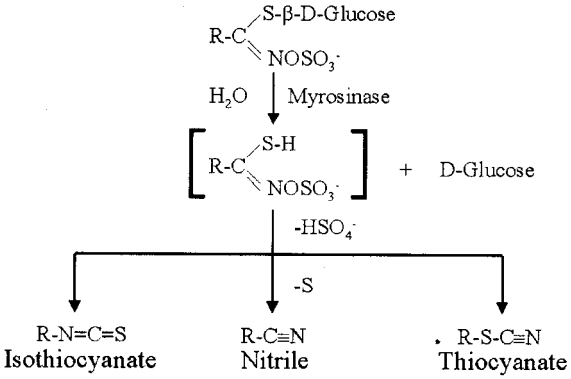

Figure 1. Enzymatic conversion of a glucosinolate to an aglucon intermediate by myrosinase, and subsequent conversion to an isothiocyanate, nitrile, or thiocyanate.

The predominant glucosinolate in most broccoli va-

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rieties is glucoraphanin [4-methylsulfinylbutyl glucosinolate] (*13*). Upon hydrolysis of glucoraphanin by myrosinase, the isothiocyanate sulforaphane [4-methylsulfinylbutyl isothiocyanate] and the nitrile sulforaphane nitrile [5-(methylsulfinyl)pentane nitrile] are formed. Fractionation of broccoli extracts by HPLC has identified sulforaphane as a potent inducer of phase II detoxification enzyme activity in vitro (*14*). In numerous studies, sulforaphane and other isothiocyanates have been shown to inhibit phase I detoxification enzymes, induce mammalian phase II detoxification enzymes, and protect against the initiation of cancer (*14*−*18*). Recently, sulforaphane has been shown to induce apoptosis in human colon cancer cells (*19*), indicating possible chemotherapeutic properties in addition to chemoprotection. Although nitriles have been found to constitute a significant portion of glucosinolate hydrolysis products, research into health benefits has focused almost entirely on the electrophilic isothiocyanate compounds. If the physiological effects of sulforaphane and sulforaphane nitrile do differ substantially, then preferential conversion of glucoraphanin to sulforaphane may be necessary to maximize the health benefit that can be derived from eating broccoli. To study the physiological effects of sulforaphane and sulforaphane nitrile in laboratory animals, gram quantities of these materials are needed.

Purified sulforaphane from broccoli is now commercially available for biological research from several companies, but its high price puts limitations on the amount that can be used. Existing methods for the purification and synthesis of sulforaphane are costly and time-consuming, and are not well suited for large-scale production (*20*−*23*). Sulforaphane nitrile is not commercially available, and existing methods for its purification or synthesis are similarly costly and time-consuming. Therefore, a preparative HPLC method has been developed to allow for the simultaneous large-scale purification of sulforaphane and sulforaphane nitrile from the seed of *Brassica oleracea* var. *italica* cv. Brigadier. Gas chromatography (GC) was used to determine purity, and mass spectrometry was used to confirm the identities of the compounds. Purified sulforaphane was compared to commercially purchased sulforaphane using GC. Purified sulforaphane and sulforaphane nitrile were used to construct a GC standard curve to allow quantification of these compounds in preparations of commercial broccoli varieties.

MATERIALS AND METHODS

Materials. Brigadier broccoli (*Brassica oleracea* var. *italica*) seed was provided by Petoseed, Inc. of Seminis Vegetable Seeds (Oxnard, CA) and stored at 4 °C until use. For quantification of glucoraphanin and its hydrolysis products the broccoli cultivars 'Packman', 'Peto 7', 'Peto 16', and 'Brigadier' were used. Cultivars were grown to commercial harvest maturity at the University of Illinois Vegetable Research Farm (Champaign, IL) according to standard cultural practices developed for cruciferous vegetables in Illinois (*24*). Broccoli heads were harvested and kept on ice for less than 4 h until florets were cut 5 cm from the top of the crown, snap-frozen in liquid nitrogen, and freeze-dried. Solvents and reagents were purchased from Fisher Scientific (Fair Lawn, NJ). White mustard seed thioglucosidase (*Sinapis alba* origin) was purchased from Sigma Chemical Co. (St. Louis, MO). Acetonitrile and methanol were HPLC grade, methylene chloride used in seed and broccoli extraction was reagent grade, and methylene chloride used to extract HPLC peaks was GC/MS grade. Sodium chloride and sodium sulfate were reagent grade. Sulforaphane standard was purchased from LKT Laboratories (St. Paul, MN), and sulforaphane nitrile standard was from the University of Illinois archive (*20*).

Seed Extraction. Seed of the broccoli cultivar 'Brigadier' was chosen on the basis of its high glucoraphanin content (*13*). Seed (500 g) was ground for 10 s in a Tekmar analytical grinder cooled with tap water. Ground seed was defatted 3 times with excess hexane and allowed to dry overnight in a fume hood. Glucosinolate analysis was performed by the method of Kushad and co-workers (*13*) on the defatted seed meal. Following this, glucosinolates were hydrolyzed by adding water in a ratio of 3:1 water/defatted seed (w/w), and the mixture was allowed to autolyse for 8 h at room temperature. Sodium chloride, wet meal, and sodium sulfate were combined in the ratio of 1:1:0.75 (w/w/w) and mixed thoroughly. This paste was extracted 3 times with equal volumes of excess methylene chloride, which was combined and dried at 32 °C under vacuum in a rotary evaporator. The resulting residue was dissolved in 120 mL of 5% acetonitrile in water (v/v) and washed 3 times with excess hexane to remove nonpolar contaminants. The aqueous phase was then filtered through a 0.22-μm membrane filter in preparation for injection onto HPLC.

HPLC Separation. Preparative HPLC separation was performed by injecting 2 mL of filtered aqueous extract onto a Waters Prep Nova-Pak (19 × 300 mm, 60 Å, 6 μm) HR C-18 reversed-phase HPLC column (Waters, Milford, MA). Elution was performed using Waters model 501 pumps to deliver a constant flow rate of 9 mL/min. The solvent system consisted of 10% acetonitrile in water for 3 min, then changed linearly over 2 min to 25% acetonitrile, and maintained isocratically for 15 min. The percentage of acetonitrile was then raised to 100% over 2 min and run isocratically for 2 min to purge the column. Sulforaphane nitrile was detected by refractive index using a Waters R401 differential refractometer and sulforaphane was detected by absorbance at 254 nm using a Waters 486 tunable absorbance detector. Sulforaphane nitrile was collected between 7.5 and 9.5 min and sulforaphane was collected between 17 and 19 min.

Peak Extraction. Fractions of eluate containing compounds of interest were combined and salted with a ratio of 1:3:2.25 eluate/NaCl/Na_2SO_4 (w/w/w), then extracted with excess methylene chloride. The methylene chloride fractions were combined, filtered through a 0.22 μm Teflon filter, and dried at 32 °C under vacuum on a rotary evaporator to obtain purified compound.

Gas Chromatography for Tentative Compound Identification and Quantification. The purified compounds were dissolved in a known volume of methylene chloride and 2-μL portions were injected into a Hewlett-Packard 5890 GC using a Hewlett-Packard 7363A automatic sampler and controller. The column consisted of a 3-m phenyl-methyl deactivated 0.53 mm i.d. guard column (Restek, Bellefonte, PA) followed by a J&W Megabore DB-17 (30 m, 0.53 mm i.d., 1.0 μm film thickness) capillary column (J&W Scientific, Folsom, CA). Injector and detector temperatures were 250 °C and 260 °C, respectively. Column oven temperature began at 80 °C for 2 min, then progressed to 260 °C (ramp 10 °C/min), and was held at 260 °C for 20 min. Helium was used as the carrier gas at a constant flow rate of 30 mL/min. Flame ionization detection was used, with data output to a Spectra Physics SP4200 integrator.

Mass Spectrometry of Purified Compounds. To confirm the identities of purified sulforaphane and sulforaphane nitrile, electron-impact (EI) mass spectra were obtained by the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois. A Micromass 70-VSE mass spectrometer was used with an ion source temperature of 200 °C and a probe temperature of 25 °C. The spectrum was scanned at 70 eV from *m*/*e* 50−200.

Quantification of Glucoraphanin, Sulforaphane, and Sulforaphane Nitrile in the Florets of Commercial Broccoli Cultivars. Packman, Peto 7, Peto 16, and Brigadier broccoli cultivars were analyzed for glucosinolate content (*13*) and for concentrations of sulforaphane and sulforaphane

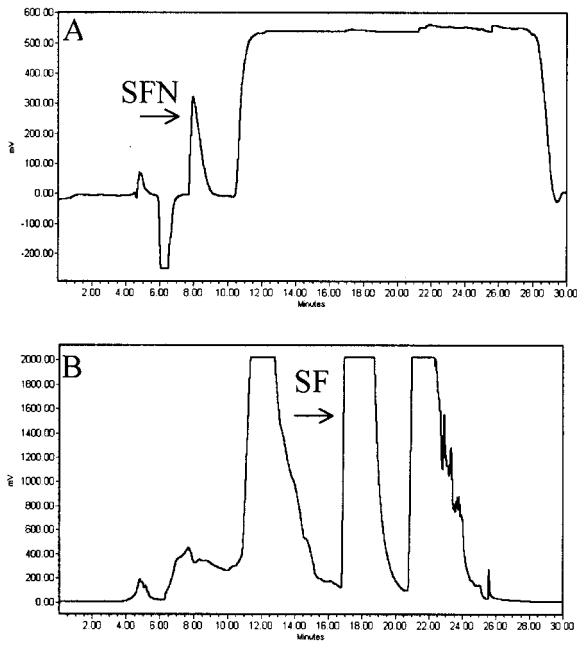

Figure 2. Preparative HPLC separation of (A) sulforaphane nitrile (SFN) using refractive index and (B) sulforaphane (SF) using UV 254 nm.

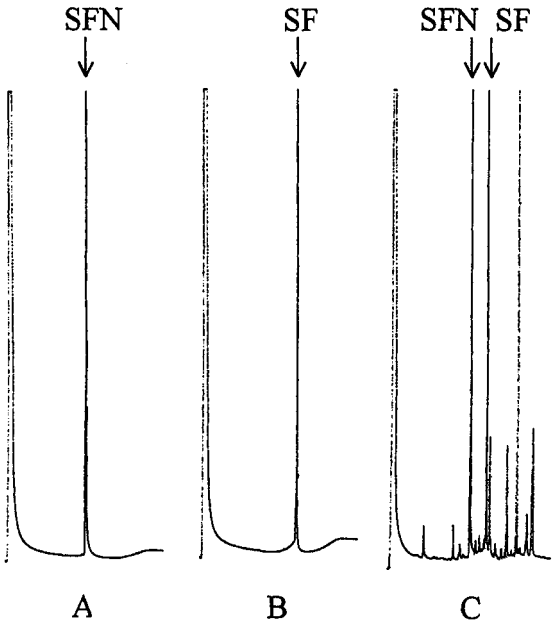

Figure 3. GC chromatographs for (A) purified sulforaphane nitrile (SFN), (B) purified sulforaphane (SF), and (C) extracted broccoli with sulforaphane and sulforaphane nitrile peaks.

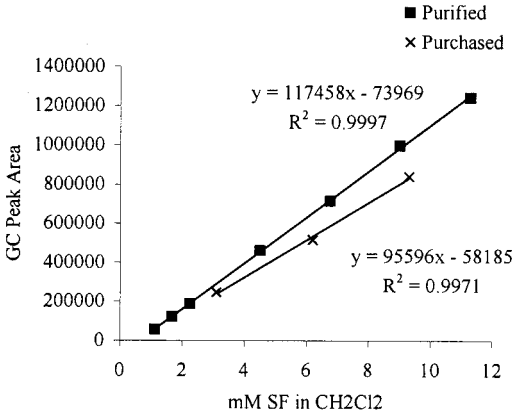

Figure 4. Comparison of sulforaphane purified by preparative HPLC and purchased sulforaphane using GC analysis.

nitrile. Concentrations of sulforaphane and sulforaphane nitrile in broccoli were determined using the method reported by Chiang and co-workers (*25*) with the following modifications. One gram of finely ground, lyophilized vegetative tissue from each broccoli cultivar was rehydrated with distilled deionized water at a ratio of 1:9 ground tissue to water. In addition, Brigadier broccoli was hydrolyzed with exogenous myrosinase enzyme (*Sinapis alba* origin). To heat-inactivate the endogenous myrosinase, dry tissue was heated in a boiling water bath for 10 min, then boiling water was added, and the slurry was boiled for an additional 10 min. After cooling to room temperature (23 °C), the amount of water lost was determined based on weight. Eight units of the myrosinase were dissolved in the amount of lost water and added back to the slurry. All samples were allowed to autolyze for 8 h at room temperature.

Slurries were centrifuged at 10000*g* for 20 min, and the supernatants were collected. A 5% methanol solution (10 mL) was added to each pellet, and they were briefly agitated, centrifuged for another 20 min, and the supernatant was collected. This process was repeated once. Supernatants were pooled and salted with a ratio of 1:1:0.75 (w/w/w) supernatant/$NaCl/Na_2SO_4$. The resulting salt paste was extracted with excess methylene chloride for 1 h. This process was then repeated twice. The methylene chloride extracts were combined and dried under vacuum at 32 °C using a rotary evaporator. The resulting residue was redissolved in 1 mL of methylene chloride and analyzed by gas chromatography as described above. Sulforaphane and sulforaphane nitrile purified using preparative HPLC were used to create standard curves relating concentration of each compound to GC peak area.

RESULTS

Purification Technique. Preparative HPLC with refractive index detection yielded a distinct peak with a retention time of 7.5 min (Figure 2A). GC analysis of this fraction yielded a single peak that corresponded to previously purified and characterized sulforaphane nitrile (*20*) and constituted 99% of the total detectable peak area (Figure 3A). Detection at UV 254 nm yielded a distinct peak with a retention time of 17 min (Figure 2B). GC analysis of this fraction yielded a single peak that corresponded to previously purified and characterized sulforaphane (LKT Labs, St. Louis, MO) and constituted 99% of the total detectable peak area (Figure 3B). The purity of sulforaphane isolated using preparative HPLC was compared to a commercially available sulforaphane using GC analysis. Purified sulforaphane gave a peak area comparable to purchased sulforaphane at several concentrations (Figure 4). Analysis of a broccoli extract revealed peaks corresponding to sulforaphane and sulforaphane nitrile, separated by a return to baseline (Figure 3C).

Mass Spectrometry. Sulforaphane and sulforaphane nitrile were further characterized using mass spectrometry (Figure 5A,B). Mass spectrum (EI) of sulforaphane, *m/z* (%): 55 (38), 59 (11), 63 (31), 64 (19), 72 (100), 114 (9) 144 (1) 160 (43), 178 (M^+, 1). Mass spectrum (EI) of sulforaphane nitrile, *m/z* (%): 52 (15), 53 (14), 54 (33), 55 (100), 61 (12), 63 (61), 64 (54), 82 (34), 145 (M^+, 15).

Recovery of Glucoraphanin Hydrolysis Products from Seed. The HPLC eluate fraction corresponding to each sulforaphane peak yielded 39.8 ± 3.5 mg of purified sulforaphane, and the eluate fraction corresponding to each sulforaphane nitrile peak yielded

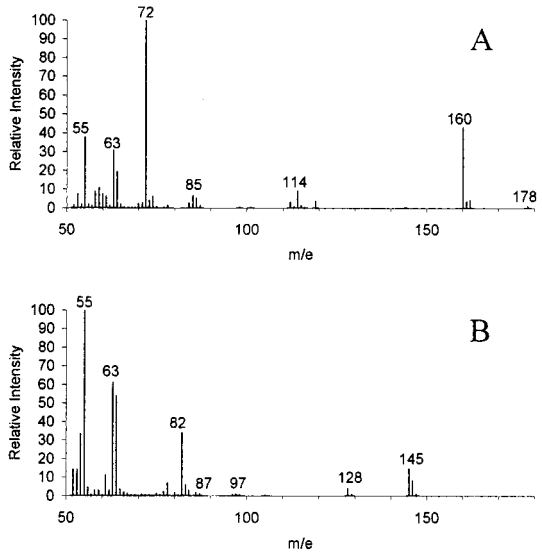

Figure 5. Mass spectra of (A) sulforaphane and (B) sulforaphane nitrile purified using preparative HLPC.

Table 1. Sulforaphane, Sulforaphane Nitrile, and Glucoraphanin Concentration (μmol g^{-1}) in Commercial Broccoli Varieties

cultivar	sulforaphane[a]	sulforaphane nitrile[a]	glucoraphanin[b]
Packman	NQ	5.82 ± 0.08	5.00
Peto 16	NQ	8.57 ± 0.35	NA
Peto 7	NQ	7.60 ± 0.51	5.53
Brigadier	1.89 ± 0.28	8.74 ± 0.25	7.10
Brigadier + exogenous myrosinase	3.40 ± 0.05	3.91 ± 0.41	7.10

[a] Mean ± std. error, $n = 3$. NQ: Not quantifiable (less than 1.12 μmol g^{-1}). [b] Average of 2 determinations prior to hydrolysis. NA: Not analyzed.

32.0 ± 3.3 mg of purified sulforaphane nitrile. On the basis of these results, each kilogram of extracted seed can be expected to yield 4.8 g of sulforaphane and 3.8 g of sulforaphane nitrile.

Glucosinolate analysis of the de-fatted seed meal prior to hydrolysis indicated a glucoraphanin concentration of 98.1 μmol/g DW of defatted seed meal. Each kg of seed yielded 774.8 g of defatted seed meal, and therefore the yield of glucoraphanin (FW 436.5) was 75.9 mmol/kg of whole seed. The yield of sulforaphane (FW 177.3) was 26.9 mmol/kg of whole seed, and the yield of sulforaphane nitrile (FW 145.2) was 26.4 mmol/kg of whole seed. Thus, the total recovered glucoraphanin hydrolysis products were 53.3 mmol/kg whole seed, or a 70% recovery from the parent glucosinolate.

Quantification of Glucoraphanin, Sulforaphane, and Sulforaphane Nitrile in the Florets of Commercial Broccoli Cultivars. Sulforaphane nitrile was the predominant product in all broccoli preparations tested (Table 1). Brigadier broccoli showed the highest level of sulforaphane compared to the other varieties tested, but nitrile was still the predominant glucoraphanin breakdown product. Addition of exogenous myrosinase to the Brigadier preparation caused increased production of sulforaphane and decreased production of sulforaphane nitrile. However, the total amount of sulforaphane and sulforaphane nitrile appeared to be less than that of the naturally hydrolyzed Brigadier broccoli (Table 1). Glucosinolate analysis confirmed that levels of glucoraphanin were in the range of 4.5−7.1 μmol/g DW. The range of combined sulforaphane and sulforaphane nitrile detected by GC was 6.7−10.6 μmol/g DW. Concentrations of sulforaphane in Packman, Peto 7, and Peto 16 were below the linear portion of the standard curve (less than 1.12 μmol/g DW, Figure 4).

DISCUSSION

Several methods for the purification (*20, 21*) or synthesis (*22, 23, 26*) of sulforaphane and its precursor, glucoraphanin (*27*) exist. However, these methods are designed only for the production of milligram quantities of these compounds, and do not provide for the large-scale production required for extensive studies in vivo. Few methods have been reported for the purification or synthesis of sulforaphane nitrile (*20*). Sulforaphane is commercially available, but very expensive, and sulforaphane nitrile is not commercially available at this time.

The purification method we have described yields high purity sulforaphane and sulforaphane nitrile, both obtained from a single HPLC fractionation. The seed of cruciferous vegetables has relatively high levels of glucosinolates per gram compared to vegetative tissue, reducing the amount of starting material necessary to obtain a concentrated aqueous extract suitable for HPLC separation. In addition, the use of seed of the broccoli cultivar Brigadier facilitated the extraction of large quantities of sulforaphane and sulforaphane nitrile because it has been found to contain high levels of glucoraphanin compared to other cultivars (*13*). Although not tested in our system, it is likely that the seed of other broccoli varieties having high glucoraphanin levels will also provide a good source for purification of sulforaphane and sulforaphane nitrile with this method.

The sulforaphane obtained was analyzed semiquantitatively for purity. The purity of the sulforaphane produced in this study was similar to that of the sulforaphane presently commercially available, based on GC analysis (Figure 4). The mass spectrum obtained for sulforaphane matched the spectrum reported by other researchers (Figure 5A; *20, 21, 25, 28*). Because sulforaphane nitrile is not commercially available, purity assessment could not be made by comparison. However, a clear mass spectrum was obtained (Figure 5B) which matched the spectrum reported by other researchers (*20, 25, 28*), and GC analysis showed a single peak that corresponded to previously purified and characterized sulforaphane nitrile (*20*) and constituted 99% of the total detectable peak area (Figure 3A). These results indicate that the extraction and purification methodology yielded highly purified compounds that are well suited for biochemical and toxicological research.

Using an adaptation of a rapid GC method (*25*), several lyophilized broccoli preparations were extracted and analyzed for sulforaphane and sulforaphane nitrile concentrations after hydrolysis using purified compounds for GC standards (Table 1). Interestingly, all varieties that were naturally hydrolyzed produced high levels of sulforaphane nitrile compared to sulforaphane. These results are in conflict with those previously reported (*25*) in which sulforaphane was found to be the predominant product of autolysis in all broccoli varieties tested. A separate study found that in most broccoli preparations tested, sulforaphane was the major autolysis product, but in some, nitrile was predominant depending upon processing conditions (*29*). As the

production of isothiocyanates and nitriles has been found to be responsive to a variety of environmental conditions including pH, hydration, and temperature (*5, 10, 12*), different autolysis conditions may be the cause of this discrepancy. In addition, because different broccoli varieties were used in each study, the formation of one compound over the other may be a characteristic of that variety. Finally, our extraction procedure may provide a more complete recovery of the more polar nitrile compound due to the use of salts in the aqueous phase during methylene chloride extraction.

The predominant production of nitriles from glucosinolates has been previously reported for cruciferous vegetables, including garden cress (*Lepidium sativum*) (*30, 31*), turnip seeds (*Brassica campestris*) (*32*), and raw cabbage (*33*). It has also been documented that different cruciferous vegetables can give unique ratios of glucosinolate autolysis products (*6, 34*), and that seeds of a given species can yield an altered ratio of glucosinolate hydrolysis products compared to the whole vegetable of the same species (*30*). In addition, different varieties of the same turnip species have been found to produce varying ratios of isothiocyanates to nitriles (*32*); therefore, it is possible that different cultivars of broccoli have distinct patterns of hydrolysis product formation. Indeed, we found that the Brigadier broccoli preparation produced a greater ratio of sulforaphane to sulforaphane nitrile than that of the other three varieties tested.

It has been reported that in a system that included an excess of exogenous myrosinase isolated from daikon (*Raphanus sativus*) there was quantitative conversion of glucoraphanin to sulforaphane in sprouts of the broccoli cultivar Saga (*16*). In the study by Chiang and colleagues, in which sulforaphane production predominated over the nitrile, some samples were mixed with exogenous myrosinase prior to hydrolysis (*25*). It is possible that exogenous myrosinase added during hydrolysis may have favored sulforaphane production. Our results suggest that the use of exogenous myrosinase from white mustard (S*inapis alba*) can increase the ratio of sulforaphane to sulforaphane nitrile in Brigadier broccoli (Table 1). It is possible that the myrosinase isolated from certain species favors production of the isothiocyanate because of a structural difference between the enzymes. Alternatively, the presence of an excess amount of myrosinase could drive the reaction faster, thus changing the dynamics of the reaction and thereby the microenvironment of autolysis, to favor the isothiocyanate. There was also a decrease in total glucoraphanin hydrolysis products in the exogenously hydrolyzed broccoli. This could be due to the thermal degradation of a small amount of sulforaphane or sulforaphane nitrile that may have existed prior to hydrolysis or that may have been formed early in the process of heat inactivation of the endogenous myrosinase (*35*).

Until the formation of glucoraphanin autolysis products is better understood, it is difficult to predict the potential health benefit of any cruciferous vegetable solely on glucosinolate content. Although much evidence supports the role of sulforaphane and other isothiocyanates in chemoprotection (*14*−*18*), sulforaphane nitrile has not yet been evaluated for its ability to provide beneficial health effects. It should also be noted that relatively little work has been reported that defines the toxicological properties of sulforaphane nitrile or its effects on the potency of sulforaphane. In a study treating male rats with sulforaphane nitrile, Ringenberg and Wallig suggested that sulforaphane nitrile may detract from the health benefits of sulforaphane due to its depletion of hepatic glutathione (*36*). In contrast, the nitrile compound cyanohydroxybutene, commonly known as crambene, has been shown to induce phase II detoxification enzymes in vivo (*18, 37*). The physiological effects from the nitrile form of sulforaphane are currently undefined, so the chemopreventative effect of whole broccoli as it is eaten is difficult to understand without further research into the biological effects of this compound.

To fully understand the health benefit from broccoli and other cruciferous vegetables, it is necessary to characterize the products of myrosinase-dependent glucosinolate autolysis and the factors controlling their production. It is likely that varying ratios of isothiocyanates and nitriles will impact the chemoprotective properties of these foods. We now report a method that allows the rapid purification of large quantities of sulforaphane and sulforaphane nitrile from broccoli seed, and an effective GC method that allows the easy quantification of glucosinolate autolysis products. We have determined that different broccoli varieties have the potential to produce varying ratios of glucoraphanin autolysis products, and that the addition of excess exogenous myrosinase can change the ratio of these compounds.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; GC, gas chromatography; SF, sulforaphane; SFN, sulforaphane nitrile.

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