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HPLC Separation of Sulforaphane Enantiomers in Broccoli and Its Sprouts by Transformation into Diastereoisomers Using Derivatization with (*S*)-Leucine

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ABSTRACT

Racemic sulforaphane, which was derivatized with (*S*)-leucine (L-leucine), was resolved by a reversed phase HPLC with UV detection. The optimum mobile phase conditions were found to be 10 mM citric acid (pH 2.8) containing 22% methanol at 35 °C using detection at 254 nm. Sulforaphane enantiomers in florets and stems of five brands of broccoli and leaves and stems of three brands of broccoli sprouts were analyzed by the proposed HPLC method. Both sulforaphane enantiomers were detected in all the samples. The S/R ratios of sulforaphane in broccoli samples were 1.5–2.6/97.4–98.5% for florets and 5.0–12.1/87.9–95.0% for stems. The S/R ratios in broccoli sprout samples were higher than those in broccoli samples and were found to be 8.3–19.7/80.3–91.7% for leaves and 37.0–41.8/58.2–63.0% for stems. (*S*)-Sulforaphane detected in the broccoli and its sprout samples was doubtlessly identified by separately using an HPLC with a chiral column (Chiralpak AD-RH) and mass spectrometry.

KEYWORDS: Enantioseparation; sulforaphane; diastereomer; broccoli; HPLC.

INTRODUCTION

Sulforaphane (4-methylsulfinylbutyl isothiocyanate, Figure 1-A) is produced by myrosinase-catalyzed hydrolysis of the thioglucosidic bond in glucoraphanin molecule.[1-5] Glucoraphanin, a member of glucosinolates, is abundant in cruciferous vegetables such as broccoli, cabbage, and kale.[6] Glucoraphanin and myrosinase are separately located in vacuoles and myrosin cells, respectively.[1,7] When the vegetables are injured, the enzymatic hydrolysis leads to glucose and an unstable

thiohydroximate-*O*-sulfate intermediate that rearrange non-enzymatically to form primarily sulforaphane and sulforaphane nitrile.[1,8,9] The rearrangement is affected by pH,[10] temperature,[11] metal ion concentration,[10,12] and the concentration of epithiospecifire protein[12] that is a myrosinase co-factor. Glucosinolates could be hydrolyzed by microflora to corresponding isothiocyanates, but the rate of transformation in the human body was shown to be very low (10-20%) in clinical trials.[13]

Sulforaphane has been largely addressed for its chemoprevention mediated through several mechanisms including cell cycle arrest, induction of apoptosis, and phase II detoxifying enzymes. Recent data have suggested that sulforaphane could show its preventive activities on prostate, leukemic, colon, pancreatic, and gastric carcinomas and was identified as an inducer of cancer cell apoptosis.[3,14-16] Recent studies have suggested that sulforaphane may have beneficial effects for cardiovascular, diabetic, and neurodegenerative diseases other than cancers via nuclear factor erythroid-derived 2-related factor 2 signaling pathway.[4,17-19]

Sulforaphane and glucoraphanin have an asymmetric sulfur atom. Vergara *et al.* reported that glucoraphanin isolated from broccoli and *Arabidopsis thaliana* was a pure epimer by NMR methods using a chiral lanthanide shift reagent and that its sulfoxide group had the *R* configuration, suggesting that the configuration retained in the hydrolysis product (*R*)-sulforaphane by myrosinase.[20] Nevertheless, only a few studies have been reported on the difference between sulforaphane enantiomers in physiological activities.[21-23]

Sulforaphane in cruciferous vegetables have been analyzed by GC with mass spectrometry,[24-26] HPLC with ultraviolet detector,[1,27-33] evaporative light scattering detector,[34] and ultra-performance liquid chromatography with mass spectrometry.[35] It

has been reported that sulforaphane levels varied according to cultivars[28-30, 36] and parts (florets, stems, and leaves)[24,28,36] of broccoli. Since the degree of conversion of glucoraphanin to sulforaphane could vary depending on the broccoli matrices, Ares *et al.* studied the optimal conversion and extraction conditions in each broccoli part.[36]

Considering that it may be difficult to show existence of a small amount of (*S*)-glucoraphanin in broccoli samples by the above-mentioned NMR methods using a chiral lanthanide shift reagent,[20] we cannot rule out the possibility that a small amount of (*S*)-sulforaphane exist in cruciferous vegetable samples. To our knowledge, the chiral chromatography of sulforaphane in cruciferous vegetables has not previously been studied. The main strategies have evolved the separation of analyte enantiomers: a direct method and an indirect method.[37-39] A direct method, which does not require chemical derivatization, is based on a chiral stationary phase or with a chiral selector on an achiral stationary phase in a mobile phase. Separation is possible through reversible diastereomeric association between the chromatographic chiral environment and the analyte enantiomers. Use of chiral stationary phases may not be straightforward as a result of interferences from matrix and/or endogeneous compounds. So, a direct method with chiral stationary phases has been used for the separation of purified enantiomers from real samples. An indirect method is based on the formation of diastereomers by the reaction of analyte enantiomers with a chiral derivatization reagent that introduce a second asymmetric center into a chiral analyte. Since diastereomers, which are formed by derivatization of enantiomers with a chiral compound, are no longer enantiomers, diastereomers can be separated by chromatography with achiral stationary phases. The advantage of indirect method include the commercially availability of a large number of chiral derivatizing reagents and a greater choice of chromatographic conditions. In this study, we developed a

chiral HPLC method to analyze sulforaphane enantiomers derivatized with (*S*)-amino acid and performed separation of the enantiomers in broccoli and broccoli sprout samples using the proposed method. (*S*)-Sulforaphane along with (*R*)-sulforaphane was detected in all the samples. The S/R ratios in broccoli samples were found to vary according to parts of the samples.

MATERIALS AND METHODS

Chemicals. Racemic sulforaphane, (*R*)-sulforaphane, (*S*)-sulforaphene, and racemic iberin were obtained from LKT laboratories (St. Paul, MN, USA). 2-Propanol was from Sigma (St. Louis, MO, USA). Triethylamine, ethyl acetate, ethanol, methanol, and citric acid monohydrate were from Kanto chemicals (Tokyo, Japan). (*S*)-Leucine and other chemicals (analytical grade) were obtained from Wako (Osaka, Japan).

Apparatus for HPLC and Mobile phase Conditions. The HPLC system consisted of a Jasco (Hachioji, Japan) model PU-2080 pump, a Jasco Model UV-2075 detector, a Rheodyne (Cotati, CA, USA) manual injector, a Shimadzu (Kyoto, Japan) column oven Model CTO-6A, and a Flom (Ome, Japan) degasser Model AG-14. InertSustainSwift C18 column (5 μm, 4.6 mm i.d. x 150 mm, GL Sciences, Tokyo, Japan) was used. A mobile phase consisted of 22% methanol and 10 mM citric acid (pH 2.8). Elution was carried out at a flow rate of 1.0 mL/min at 35 °C. Analytes were detected at 254 nm. Data acquisition and processing were conducted with a Chromato-PRO (Runtime Instrument, Kanagawa, Japan). For direct chiral analysis, the HPLC system consisted of a Shimadzu column oven Model CTO-10A, a Shimadzu model LC-10AD pump, a Shimadzu degasser Model DGU-14A, an Agilent (Waldbronn, Germany) Model G412 detector, and a Rheodyne manual injector.

Chiralpak AD-RH column (4.6 mm i.d. x 150 mm, Daicel Chemical Industries, Tokyo, Japan) was used and a mobile phase contained 25% acetonitrile. Elution was carried out at a flow rate of 1 mL/min at 35 °C. Analytes were detected at 254 nm. For LC/MS analysis, an LC 7400 series (GL Sciences) equipped with an Agilent 6140 quadrupole mass spectrometer was used. LC/MS separation was performed on a InertSustain C18 column (3 μm, 2.1 mm x 250 mm, GL Sciences) with a mobile phase consisting of 10 mM formic acid/acetonitrile (65/35, v/v) at a flow rate of 0.1 mL/min at 40 °C. ESI conditions (positive ion mode) were as follows: drying gas temperature, 250 °C; drying gas flow, 10 L/min; capillary voltage, 4 kV.

Preparation of (*R*)-sulforaphane (*R*)-Sulforaphane was separated by an HPLC using a chiral column (Chiralpak AD-RH) thermostated at 35 °C with a mobile phase containing 25% acetonitrile at a flow rate of 0.8 mL/min. Two fractions, corresponding to the (*S*)- and (*R*)-enantiomers of sulforaphane, were separated and only the fraction of (*R*)-sulforaphane was chromatographed again under the same conditions. NaCl (2 g) and 5 mL of ethyl acetate were added to the rechromatographed fraction. After shaking vigorously, the mixture was centrifuged at 3,000 rpm for 5 min. The residual mixture was extracted additional two times with 10 mL of ethyl acetate and all the extracts were combined and evaporated at 40 °C to dryness. The residue was dissolved in 1 mL of 2-propanol. When the enantiomer excess (ee) is defined as the difference between the amounts of the two enantiomers in a mixture divided by their total, the purity of the (*R*)-sulforaphane collected was more than 99.9% ee.

Preparation of racemic sulforaphane derivatized with (*S*)-leucine. Stock solution of racemic sulforaphane (2,500 ppm) was prepared with 2-propanol, and

stored at −15 °C. (*S*)-Leucine (40 mM) solution was prepared with 20% ethanol. Triethylamine (1%) solution was prepared with purified water before use. Solutions of sulforaphane (35 μL), (*S*)-leucine (250 μL), and triethylamine (200 μL) and purified water (15 μL) were mixed. When racemic sulforaphane was derivatized with other amino acids, (*S*)-alanine, (*S*)-valine, (*S*)-leucine, (*S*)-methionine, (*S*)-phenylalanine and (*S*)-tryptophan were separately prepared with 20% ethanol at the concentration of 40 mM. The mixture was incubated at 40 °C for 1 h and then sulforaphane derivative was analyzed by HPLC. When sulforaphane concentration in sample solution is low, 100-300 μL of sample solution was evaporated at 40 °C to dryness. The residue was dissolved in 35μL of 2-propanol, and to this was added 250 μL of (*S*)-leucine and 200 μL of trimethylamine solutions and 15 μL of purified water, and then incubated.

Sample extraction and preparation. Five brands of broccoli cultivated in different prefectures in Japan and three brands of broccoli sprouts cultivated in different companies were purchased from local markets. Broccoli (florets and stems) and broccoli sprouts (leaves and stems) were cut into small pieces with scissors. Each broccoli sample (3 g) was added to 9 mL of purified water and was homogenized using a Microtec (Funabashi, Japan) Physcotron homogenizer model NS-52 and was incubated at 30 °C for 2 h. NaCl (6 g) and 30 mL of ethyl acetate was added to the reaction mixture. After shaking vigorously, the mixture was centrifuged at 3,000 rpm for 5 min. The residual mixture was extracted additional two times with 30 mL of ethyl acetate. All the extracts were combined and dried at 40 °C under vacuum in a rotary evaporator. The dry residue was dissolved in 5mL of ethyl acetate. After passing Sep-Pak Plus Silica Cartridges (Nihon Waters, Tokyo, Japan), the cartridge was washed with 5 mL of ethyl acetate and the adsorbed materials were eluted with 5 mL of

methanol. The eluate was dried at 40 °C under vacuum with a rotary evaporator and the dry residue was dissolved in 1mL of 2-propanol. After the solution was centrifuged at 3,000 rpm for 5 min, the supernatant was used as sample solution for derivatization with (*S*)-leucine. For analysis with HPLC with chiral column, aliquot of the supernatant was applied to InertSustainSwift C18 column with a mobile phase consisted of 22% methanol and 10 mM citric acid (pH 2.8). The fraction of native sulforaphane (retention time is approximately 8 min) was collected. Sulforaphane in the fraction was extracted with ethyl acetate. The extracted solution was dried at 40 °C under vacuum with a rotary evaporator and the dry residue was dissolved in 2-propanol.

Water content. Approximately 5 g of broccoli (florets and stems) and broccoli sprouts (leaves and stems) in aluminum dishes were weighed exactly and heated at 80 °C for 4 h. After cooling, the dried samples were weighed.

RESULTS AND DISCUSSION

Factors Affecting Chiral Separation. Budnowski *et al.* reported an analytical method of sulforaphane in biological samples.[40] Although racemic sulforaphane was derivatized with N-(*tert*-butoxycarbonyl)-L-cysteine methyl ester to form a stable dithiocarbamate ester for HPLC analysis with UV detector, the sulforaphane derivative was not resolved by the methods. Phenylisothiocyanate, which is well known as the primary reagent in the Edman degradation method, reacts with amino group of amino acids to form stable phenylthiocarbamyl derivatives that can be detected at 254 nm.[41,42] Using the above reaction, (*R*)- and (*S*)-sulforaphanes can be also converted to the stable diastereomers with (*S*)-amino acid. Amino acids are not expensive and versatile reagents, and enantiomerically pure (S)- and (R)-amino acids (more than 99.9%) are

commercially available. Racemic sulforaphane was derivatized with three aliphatic ((*S*)-alanine, (*S*)-valine, and (*S*)-leucine), one sulfur containing ((*S*)-methionine), and two aromatic ((*S*)-phenylalanine and (*S*)-tryptophan) amino acids (Figure 1-B) under alkaline conditions at 30 °C for 1 h. The obtained sulforaphane derivatives were analyzed by reversed phase HPLC with InertSustainSwift C18 column using a mobile phase consisting of 22% methanol and 10 mM citric acid (pH 2.8). Log P values of L-amino acids are –0.574 for alanine, 0.289 for valine, 0.799 for leucine, 0.213 for methionine, 0.235 for phenylalanine, and 0.704 for tryptophan.[43] An increase in log P value of the aliphatic amino acids as well as the aromatic amino acids used as derivatization reagents brought about an increase in the retention time of the corresponding diastereomers. Sulforaphane was enantioseparated by using all these amino acids except (*S*)-alanine. (*S*)-Leucine was used as the derivatization reagent for further experiments due to the higher separation, a moderately short retention time of sulforaphane, and the better separation of contaminants contained in broccoli and broccoli sprout samples.

In our preliminary study, 0.1% phosphoric acid or 0.1% trifluoroacetic acid was added to the mobile phase to suppress the acid dissociation of a carboxyl group of the derivatized sulforaphane. Though the racemic sulforaphane derivative was fully resolved, peak tailing was observed gradually during repetition of the analysis. To increase the pH of mobile phase, we selected citric acid, which pKa is 2.93,[43] as a modifier of mobile phase. The effect of pH (2.6–4.0) of the mobile phase with 10 mM citric acid on the retention time and the separation of the sulforaphane derivative was evaluated. Raising the pH resulted in gradual decreases in both the retention time and the separation. The racemic sulforaphane derivative was fully resolved (Rs > 1.5) at pH values below 3.4. Although the highest separation was shown with the mobile phase of

pH 2.6, peak tailing was observed at that acidic pH during analyzing repeatedly. Thus, pH of the mobile phase was determined at 2.8 to suppress the peak tailing. The effect of the methanol concentration (20–26%) in the mobile phase on the separation of the sulforaphane derivative was also examined. Both the retention time and the separation increased with decreasing the methanol concentration. The racemic sulforaphane derivative was found to be fully separated at the methanol concentrations below 24%. When acetonitrile was used as a modifier of mobile phase instead of methanol, the sulforaphane derivative was not fully resolved. The 22% methanol concentration was adopted for higher separation and a moderate retention time.

Since amino acids have been derivatized with phenylisothiocyanate in the presence of trimethylamine,[42,44,45] we examined the effect of the concentration (0.05–2%) of trimethylamine in the reaction mixture on the derivatization of sulforaphane with (*S*)-leucine. When 1 mM racemic sulforaphane and 20 mM (*S*)-leucine was incubated in the presence of various concentrations of trimethylamine at 40 °C for 1 h, an increase in the concentration of trimethylamine of the reaction mixture up to 0.4% increased the peak areas of the (*S*)- and (*R*)-sulforaphanes derivatives and then decreased. Thus, the optimum concentration of trimethylamine of the reaction mixture was determined to be 0.4%. The effect of reaction time (0–120 min) on the derivatization of sulforaphane with (*S*)-leucine was examined using a reaction mixture containing 1 mM racemic sulforaphane, 20 mM (*S*)-leucine, and 0.4% trimethylamine at 30 °C. Underivatized sulforaphane decreased substantially with an increase in incubation time up to 60 min but the succeeding decrease was retarded. Peak area of remaining sulforaphane after incubation at 60 min was less than 5% of that without incubation, where the increases in the peak area of both (*S*)- and (*R*)-sulforaphane derivatives correlated well with the decrease in the peak area of sulforaphane.

Therefore, the incubation time was determined to be 60 min.

(*R*)-Sulforaphane, which is commercially available, was derivatized with (*S*)-leucine and the (*R*)-sulforaphane derivative was analyzed by the proposed HPLC method. As a result, the (*R*)-sulforaphane derivative was found to correspond to the latter separated peak. The *S*/*R* ratio of sulforaphane was 2.8/97.2, which was the same ratio as that obtained by chiral HPLC with Chiralpak AD-RH column. Thus, (*R*)-sulforaphane was purified by the chiral HPLC. The purified (*R*)-sulforaphane was derivatized with (*S*)-leucine, and the (*R*)-sulforaphane derivative was analyzed by the proposed method. Since peak corresponding the (*S*)-sulforaphane derivative was not detected, it was found that any racemization of (*R*)-sulforaphane or (*S*)-leucine did not occur. (*R*)-Sulforaphane was also derivatized with (*R*)-leucine instead of (*S*)-leucine (Figure S2-C, Supporting Information). The peak of (*R*)-sulforaphane–(*R*)-leucine was detected at the retention time of (*S*)-Sulforaphane–(*S*)-leucine, suggesting that this result can be used for peak confirmation of (*S*)-sulforaphane in broccoli samples.[46,47] Racemic iberin and (*S*)-sulforaphene (Figure 1-A), which are sulforaphane related compounds, were derivatized with (*S*)-leucine and the iberin derivative was successfully resolved. Because racemic or (*R*)-sulforaphene were not commercially available, the resolvability of the sulforaphene derivative was not confirmed. But the retention times of these two compounds were found to be appreciably different from those of the (*S*)- and (*R*)-sulforaphane derivatives (Figure S2-D and E, Supporting Information).

Separation of sulforaphane enantiomers in broccoli and broccoli sprout samples

Sulforaphane was subjected to the proposed HPLC method using the above optimum conditions. The limit of detection (LOD) of each sulforaphane enantiomer defined as a

signal-to-noise ratio of 3 was 0.001 mM (0.177 mg/L) and the limit of quantification (LOQ) of each enantiomer defined as a signal-to-noise ratio of 10 was 0.003 mM (0.531 mg/L). Linearity ($r^2 > 0.999$) was demonstrated in the concentration range of 0.001−1 mM by each standard curve (12 points) for (*S*)- and (*R*)-sulforaphane. The reproducibility of five consecutive determinations was evaluated at 0.05 mM and 0.5 mM for (*S*)- and (*R*)-sulforaphanes. Good reproducibilities of peak areas (RSD < 4.4%) and retention times (RSD < 0.2%) were obtained for both enantiomers. After the myrosinase-catalyzed hydrolysis of broccoli samples, dichloromethane,[10,24,27,29,30,34] ethyl acetate,[28,48,49] and methyl *t*-butyl ether[36] have been used as sulforaphane-extracting solvents. Since good recoveries were obtained in these solvents, we used ethyl acetate as a versatile solvent. When a standard solution (0.05 mL, 0.1 mL and 0.2 mL) containing 1,250 mg/ L each enantiomer of sulforaphane (final concentration: 20.8 μg, 41.6 μg, and 83.3 μg each enantiomer per g broccoli florets, respectively) was added to broccoli floret samples (3 g), recoveries of (*S*)- and (*R*)-sulforaphanes were between 94 and 104%.

The (*S*)- and (*R*)-sulforaphane contents in broccoli and its sprout samples were analyzed by the proposed HPLC method (Table 1). The representative chromatograms were shown in Figure 2. The (*R*)-sulforaphane levels in florets and stems of broccoli samples and in leaves and stems of broccoli sprout samples ranged from 8 to 54 μg/g, 10 to 14 μg/g, 29 to 96 μg/g, and 7 to 27 μg/g, respectively, and (*S*)-sulforaphane was also detected in all samples, ranging from 0.1 to 1 μg/g, 0.6 to 1.7 μg/g, 4.3 to 20 μg/g, and 4.5 to 16 μg/g, respectively. The ratios of (*S*)-sulforaphane to total sulforaphane extended over 1.5–2.6%, 5.0–12.1%, 8.3–19.7, and 37.0–41.8%, respectively. These results were almost the same as those obtained by the HPLC method using sulforaphane derivatized with (*R*)-leucine (Table 1), suggesting that the peak assigned

to the (*S*)-sulforaphane derivative is authentic and that no co-eluting interfering substances were present. The concentrations of total sulforaphane in the present samples were similar to those reported by Liang *et al.*, who analyzed sulforaphane in fresh broccoli samples.[29] Li *et al.* reported sulforaphane concentrations in lyophilized broccoli and its sprout samples,[28] where the concentrations were 10-20 times higher than those shown in Table 1. Since water contents of florets and stems of broccoli sample (a) and leaves and stems of broccoli sprout sample (f) were 88.8%, 93.7%, 90.3%, and 96.6%, respectively, the total sulforaphane concentrations of the dry samples (μg/g dry weight) correspond well with those reported by Li *et al.*[28]

Confirmation of existence of (*S*)-sulforaphane in broccoli sprout samples. Since the highest ratio of (*S*)-sulforaphane to total sulforaphane was observed for stems of broccoli sprout samples, the existence of (*S*)-sulforaphane was confirmed by using HPLC with a chiral column and LC/MS. Sulforaphane was extracted from broccoli sprouts (another lot of brand (f)) and an aliquot of the extract was analyzed by the proposed HPLC method (Figure 3-B). The ratio of (*S*)- and (*R*)-sulforaphanes was 42.4:57.6. Another aliquot of the extract without derivatization was applied to the proposed HPLC. Native sulforaphane was detected approximately 8 min and the fraction was analyzed by an HPLC using a Chiralpak AD-RH column and 25% acetonitrile as a mobile phase at 35 °C (Figure 3-D). The ratio of (*S*)- and (*R*)-sulforaphanes (42.9:57.1) was almost the same as that obtained by the proposed HPLC method and absorption spectra of both native enantiomers were the same as that for the standard sulforaphane sample. Finally, the two fractions were separately collected and were applied to LC(ESI+)-MS. Both ingredients gave peaks with same retention time and the correct mass with protonation at m/z = 178 (Figure 3-D).

Therefore, existence of (*S*)-sulforaphane and its ratio in the broccoli sprout sample was confirmed.

It is surprising that the ratio of (*S*)-sulforaphane to total sulforaphane varied according to parts of broccoli and its sprouts. It has been reported that biosynthesis of glucosinolates including glucoraphanin proceeds in three stages.[1,20] The first is side-chain elongation of amino acids (methionine for glucoraphanin), the second is development of the core structure, and the third is secondary side-chain modifications, where a sulfide group of glucoraphanin is converted to a sulfoxide group by flavin-monooxygenase in Arabidopsis.[50] The following three mechanisms are possible to explain that the R/S ratio of sulforaphane varied depending on parts of broccoli. (1) Two or more oxidizing enzymes including flavin-monooxygenase, which give different S/R ratios of glucoraphanin, may participate in oxidation of the sulfide group. (2) Difference in decomposition rate (or use in plant tissues) between (*R*)- and (*S*)-glucoraphanin or the racemization rate may vary in parts of broccoli, while there is no report on racemization of glucoraphanin so far. (3) Change in the S/R ratio may occur during the myrosinase-catalyzed hydrolysis of glucoraphanin to form sulforaphane, depending on the matrices in different parts of broccoli.[36] In order to define the mechanism, it is necessary to investigate the chirality of glucoraphanin.

In conclusion, using a derivatization with (*S*)-leucine, an HPLC method for the separation of the sulforaphane enantiomers was developed. Although naturally occurring sulforaphane was reported to have (*R*)-configuration, (*S*)-sulforaphane was also found to be detected in broccoli and its sprout samples. (*S*)-Sulforaphane was identified by the proposed HPLC method with derivatization with (*R*)-leucine instead

of (*S*)-leucine, HPLC analysis of native sulforaphane using a chiral column, and LC/MS method. The ratio of (*S*)-sulforaphane to total sulforaphane varied according to parts of samples; 1.5–2.6% for florets of broccoli, 5.0–12.1% for stems of broccoli, 8.3–19.7 for leaves of broccoli sprouts, and 37.0–41.8% for stems of broccoli sprouts.

ASSOCIATED CONTENT

*** Supporting Information**

This material is available free of charge via the Internet at http://pubs.acs.org.

Chromatograms of racemic sulforaphane derivatized with (*S*)-amino acids (Figure S1) and chromatograms of racemic sulforaphane, (*R*)-sulforaphane, racemic iberin, and (*S*)-sulforaphene derivatized with (*S*)-leucine (Figure S2) (PDF).

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494 **LEGENDS FOR FIGURES**

495 **Figure 1.** Structural formulas of sulforaphane and its related compounds (A) and
496 principle of sulforaphane derivatization with (*S*)-leucine (B). An asterisk represents
497 an asymmetric atom.

498

499 **Figure 2.** Chromatograms of standard solution (A) and sample solutions of
500 sulforaphane extracted from florets (B,C) and stems (D,E) of broccoli sample (a) and
501 leaves (F,G) and stems (H,I) of broccoli sprout sample (f) in Table 1. Sulforaphane was
502 derivatized with (*S*)-leucine (A, B, D, F, and H) or (*R*)-leucine (C, E, G, and I). HPLC
503 was conducted by using an InertSustainSwift C18 column with a mobile phase
504 consisting of 22% methanol and 10 mM citric acid (pH 2.8) at 35 °C. S and R
505 represent (*S*)-sulforaphane–(*S*)-leucine and (*R*)-sulforaphane–(*S*)-leucine, respectively.

506

507 **Figure 3.** Chromatograms of the sulforaphane standard solution (A) and an extract
508 from stems of broccoli sprouts (B) derivatized (*S*)-leucine analyzed by HPLC using an
509 InertSustainSwift C18 column with a mobile phase consisting of 22% methanol and 10
510 mM citric acid (pH 2.8) at 35 °C and the standard solution (C) and sulforaphane
511 purified by achiral HPLC (D) analyzed by an HPLC using a Chiralpak AD-RH column
512 and 25% acetonitrile as a mobile phase at 35 °C. The insets show the mass spectra of
513 the two fractions corresponding to the (*S*)- and (*R*)-sulforaphane peaks. SS, RS, S, and
514 R represent (*S*)-sulforaphane–(*S*)-leucine, (*R*)-sulforaphane–(*S*)-leucine,
515 (*S*)-sulforaphane, and (*R*)-sulforaphane, respectively.

Table 1. Concentrations of (*S*)- and (*R*)-sulforaphanes in broccoli and broccoli sprout samples

Samples		(*S*)-sulforaphane [a] (μg/g)	(*R*)-sulforaphane [a] (μg/g)	S/R ratios of sulforaphane [a]	S/R ratios of sulforaphane [b]
Broccoli					
Florets	a	0.50 ± 0.01 [c]	32.01 ± 0.46	1.5 / 98.5	1.8 / 98.2
	b	0.50 ± 0.03	25.57 ± 0.44	1.9 / 98.1	1.9 / 98.1
	c	0.91 ± 0.23	53.03 ± 1.04	1.7 / 98.3	1.9 / 98.1
	d	0.51 ± 0.01	19.36 ± 0.15	2.6 / 97.4	2.5 / 97.5
	e	0.14 ± 0.01	8.75 ± 0.58	1.6 / 98.4	1.2 / 98.8
average		0.51 ± 0.27	27.74 ± 16.53	1.9 / 98.1	1.9 / 98.1
Stems	a	1.17 ± 0.06	13.24 ± 0.29	8.1 / 91.9	7.8 / 92.2
	b	0.68 ± 0.02	11.27 ± 0.29	5.7 / 94.3	5.3 / 94.7
	c	1.67 ± 0.05	13.08 ± 0.33	11.3 / 88.7	10.6 / 89.4
	d	1.43 ± 0.04	10.37 ± 0.14	12.1 / 87.9	11.5 / 88.5
	e	0.67 ± 0.01	12.66 ± 0.15	5.0 / 95.0	4.6 / 95.4
average		1.12 ± 0.45	12.12 ± 1.25	8.5 / 91.5	8.0 / 92.0
Broccoli sprouts					
Leaves	f	4.31 ± 0.11	29.32 ± 0.26	12.6 / 87.4	12.1 / 87.9
	g	8.65 ± 0.15	95.21 ± 1.97	8.3 / 91.7	7.9 / 92.1
	h	19.63 ± 0.37	80.12 ± 0.96	19.7 / 80.3	18.6 / 81.4
average		10.86 ± 7.90	68.22 ± 34.52	13.7 / 86.3	12.9 / 87.1
Stems	f	4.56 ± 0.30	7.77 ± 0.44	37.0 / 63.0	37.3 / 62.7
	g	15.69 ± 0.25	26.68 ± 0.47	37.0 / 63.0	36.4 / 63.6
	h	11.06 ± 0.18	15.41 ± 0.39	41.8 / 58.2	41.2 / 58.8
average		10.44 ± 5.59	16.62 ± 9.51	38.6 / 61.4	38.3 / 61.7

[a] Sulforaphane was derivatized with (*S*)-leucine.
[b] Sulforaphane was derivatized with (*R*)-leucine.
[c] mean ± standard deviation, n=3.

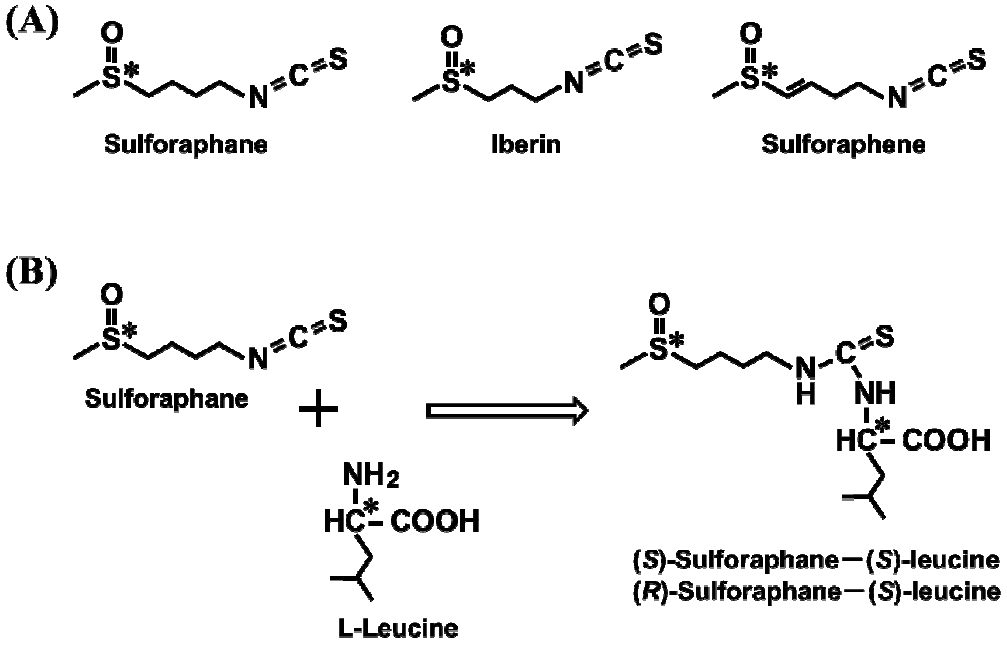

(A)
Sulforaphane
Iberin
Sulforaphene
(B)
Sulforaphane
L-Leucine
(S)-Sulforaphane—(S)-leucine
(R)-Sulforaphane—(S)-leucine

Figure 1

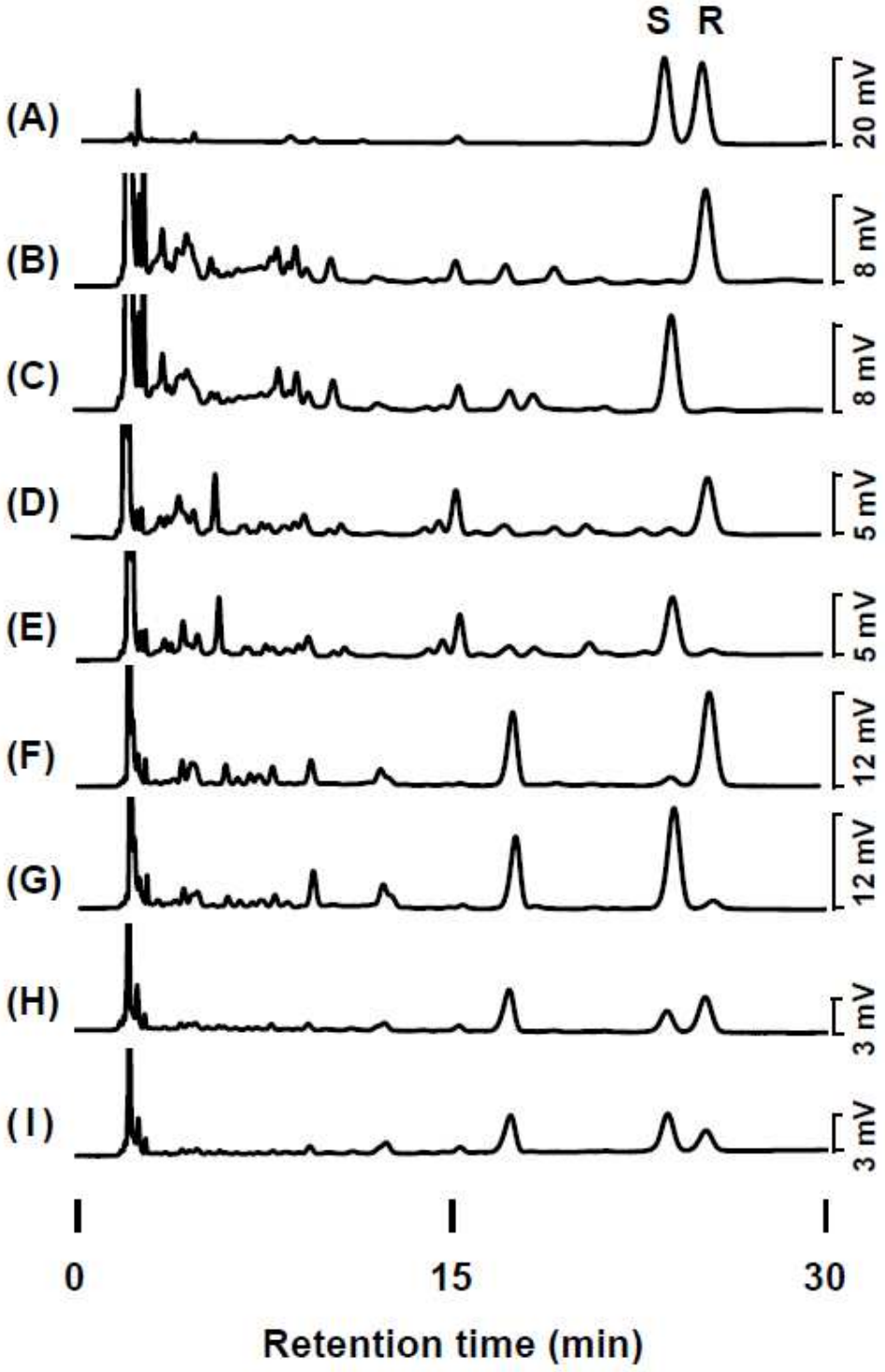

S R
(A)
20 mV
(B)
8 mV
(C)
8 mV
(D)
5 mV
(E)
5 mV
(F)
12 mV
(G)
12 mV
(H)
3 mV
(I)
3 mV
0
15
30
Retention time (min)

Figure 2

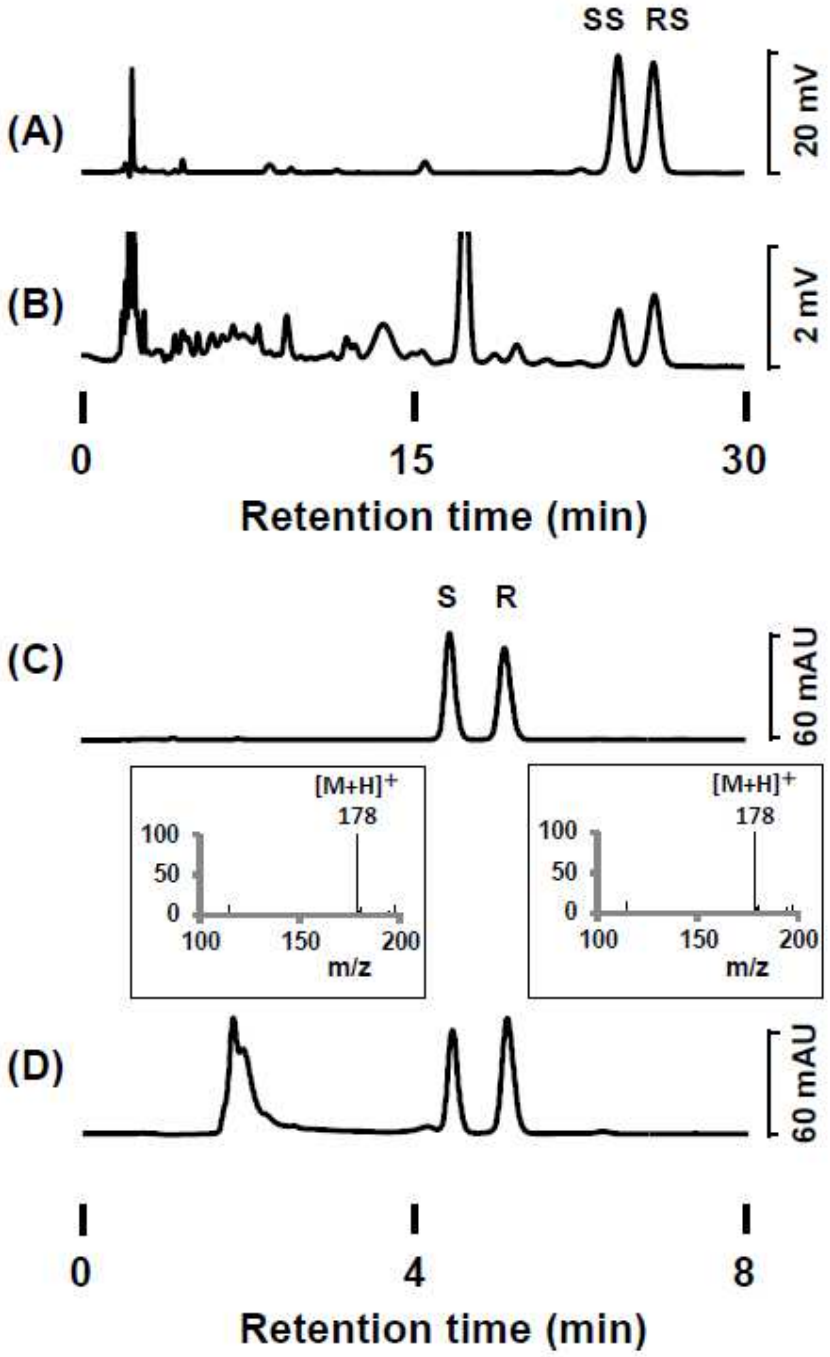
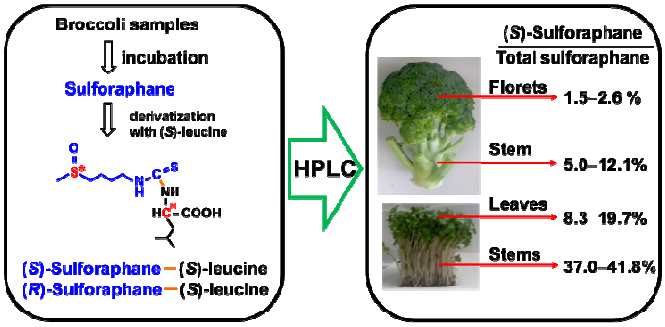

SS RS
(A)
20 mV
(B)
2 mV
0
15
30
Retention time (min)
S R
(C)
60 mAU
[M+H]+
178
100
50
0
100
150
200
m/z
[M+H]+
178
100
50
0
100
150
200
m/z
(D)
60 mAU
0
4
8
Retention time (min)

Figure 3


Broccoli samples
incubation
Sulforaphane
derivatization
with (S)-leucine
(S)-Sulforaphane – (S)-leucine
(R)-Sulforaphane – (S)-leucine
HPLC
(S)-Sulforaphane
Total sulforaphane
Florets
1.5–2.6 %
Stem
5.0–12.1%
Leaves
8.3 19.7%
Stems
37.0–41.8%

TOC graphic