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Evaporative Light-Scattering Analysis of Sulforaphane in Broccoli Samples: Quality of Broccoli Products Regarding Sulforaphane Contents

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Broccoli sulforaphane has received attention as a possible anticarcinogen. Sulforaphane analysis is difficult due to the lack of a chromophore for spectrometric detection. Hence, we developed a method for determining sulforaphane by using high-performance liquid chromatography (HPLC) coupled with an evaporative light-scattering detector (ELSD). Sulforaphane was extracted from acid-hydrolyzed broccoli samples, followed by solid-phase extraction and reversed-phase HPLC. Sulforaphane was detected by ELSD and concurrently identified by electrospray ionization time-of-flight mass spectrometry. The recovery of sulforaphane from broccoli samples was above 95%. The detection limit was 0.5 μg. The present method was sensitive enough to determine sulforaphane in mature broccoli, broccoli sprouts, and commercial broccoli products. Sulforaphane concentration in broccoli sprout (1153 mg/100 g dry weight) was about 10 times higher than that of mature broccoli (44−171 mg/100 g dry weight). Therefore, the broccoli sprout is recommended as a source of sulforaphane-rich products. In contrast, we found that sulforaphane could not be detected in most of broccoli products, suggesting present commercial broccoli products having low quality.

KEYWORDS: Broccoli; broccoli sprouts; evaporative light-scattering detector (ELSD); sulforaphane

INTRODUCTION

Sulforaphane (4-methylsulfinylbutyl isothiocyanate) is a naturally occurring sulfur-containing isothiocyanate (**Figure 1**). Sulforaphane is abundant in cruciferous vegetables such as broccoli, cauliflower, cabbage, and kale, of which the highest concentration is in broccoli (280 mg/100 g dry weight) (*1*). Sulforaphane is formed from its glucosinolate (glucoraphanin) by myrosinase, when broccoli tissue is crushed or chewed.

In 1992, Zhang et al. (*1*) initially identified broccoli sulforaphane as an inducer of quinone reductase, a phase II detoxification enzyme. Subsequently, these authors reported that sulforaphane administration prevented dimethylbenz(*a*)-induced mammary tumors in rats (*2*). Other recent studies revealed that sulforaphane induced apoptosis in several cancer cell lines (*3*−*5*). These findings (*1*−*5*) suggested that broccoli sulforaphane may be beneficial for cancer prevention.

Currently, various broccoli products (i.e., powder and tablets) are commercially supplied. Their labels have shown the bioavailability of sulforaphane; however, the majority of products do not display the exact sulforaphane concentration. This is due to lack of a suitable analysis method for sulforaphane. In general, high-performance liquid chromatography (HPLC) with UV detection (201 nm) is used for sulforaphane analysis (*6*, *7*).

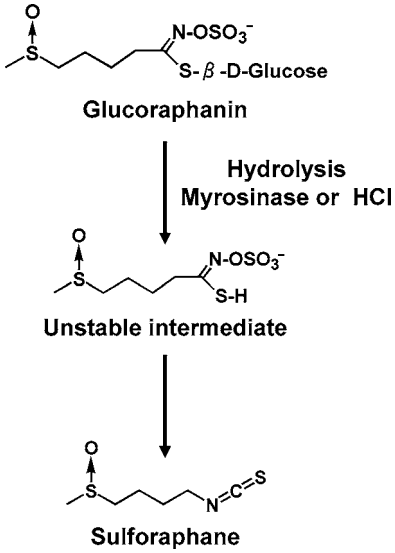

Figure 1. Sulforaphane is the aglycone breakdown product of the glucosinolate (glucoraphanin).

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However, the HPLC-UV method is insensitive, because sulforaphane has no UV chromophore. Thus, development of an alternative method is desired. The evaporative light-scattering detector (ELSD) is based on mass detection, irrespective of the UV properties of analytes. Therefore, ELSD might be possible for sulforaphane assay.

In this study, we optimized ELSD parameters and established a new HPLC-ELSD method of sulforaphane determination. By using this method, we succeeded in determining sulforaphane in mature broccoli and broccoli sprouts. The method is rapid, accurate, and reproducible, which may be applicable to the official method to measure sulforaphane.

MATERIALS AND METHODS

Chemicals. Sulforaphane was purchased from LKT laboratories (St. Paul, MN). Acetonitrile, *tert*-butyl alcohol, and distilled water were obtained from Wako (Osaka, Japan). All the other reagents were of analytical grade.

Materials. Mature broccoli (*Brassica oleracea* L.) and broccoli sprouts were purchased from the general market in Sendai, Japan. Broccoli was cut into two parts: flower head and stalk. Broccoli samples were cleaned with water and lyophilized for 24 h. Dried samples were disintegrated and then stored at −30 °C.

Instrumentation and Chromatography. The HPLC system consisted of a JASCO PU-980 pump (Japan Spectroscopic Co., Tokyo, Japan), a JASCO CO-965 column oven, and a JASCO AS-851 intelligent autosampler. An ODS column (YMC-Pack Pro C18, 4.6 mm × 150 mm; YMC, Kyoto, Japan) was used with a mixture of distilled water, acetonitrile, and *tert*-butyl alcohol (8:1:1) containing 5 mM ammonium acetate at a flow rate of 1 mL/min. The column temperature was maintained at 40 °C. The eluent was split at the post column. One of the split eluents (flow rate, 0.95 mL/min) was sent to a SEDEX 55 ELSD (Sedere, Alfortville, France). The temperature of the drift tube was set at 40 °C. Nitrogen gas was used as nebulizer gas at a pressure of 2.0 bar. The gain was usually set at 10. Peak areas were recorded using a Chromatocorder 12 (System Instruments, Tokyo, Japan). The other split eluent (flow rate, 0.05 mL/min) was sent to a Mariner electrospray ionization time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA). Mass spectrometry was carried out in the positive ion measurement mode with a spray voltage of 2900 V, a nozzle potential of 100 V, and a nozzle temperature of 140 °C. The flow rate of the nebulizer gas was 0.3 mL/min. Full scan spectra were obtained by scanning masses between *m*/z 100 and 800 at 3 s/scan.

Quantification of Sulforaphane. Standard sulforaphane was dissolved in methanol for preparing standard solutions (50−500 μg/mL). A 20 μL portion of each solution (1−10 μg of sulforaphane) was subjected to HPLC-ELSD, and a cali1bration curve was made.

Sulforaphane was extracted from broccoli sample by the methods of Bertelli et al. (*6*) and Vaughn and Berhow (*8*) with slight modification. Initially, the lyophilized broccoli sample (1 g) was suspended in 30 mL of 0.1 M HCl and incubated at 30 °C for 24 h in order to release sulforaphane from glucosinolates. After that, the incubation mixture was relyophilized and disintegrated. For the extraction of sulforaphane, the lyophilized sample (100 mg) was suspended in 6 mL of dichloromethane and incubated at 30 °C for 24 h. After filtration with a Minisart RC 15 filter (0.45 μm; Sartorius, Hannover, Germany), the resultant incubation mixture was loaded onto a SepPak silica cartridge (Waters, Milford, MA). The cartridge was rinsed with 2 mL of ethyl acetate, and the eluent was discarded. Sulforaphane was recovered with 3 mL of methanol. The methanol extract was evaporated and redissolved in 100 μL of methanol. A 20 μL portion was subjected to HPLC-ELSD/MS. The sulforaphane concentration was calculated using the equation of the calibration curve. Determinations were performed within 1 month after the purchasing of the broccoli and broccoli sprouts.

Determination of Sulforaphane in Broccoli Products. Seven different kinds of broccoli products commercially available in Japan (i.e., two kinds of powder and five kinds of tablets) were purchased from the general market. Powder was used intact, and tablets were ground to powder, then used for analysis. Sample treatment and measurement was the same as that of the broccoli and broccoli sprouts described above.

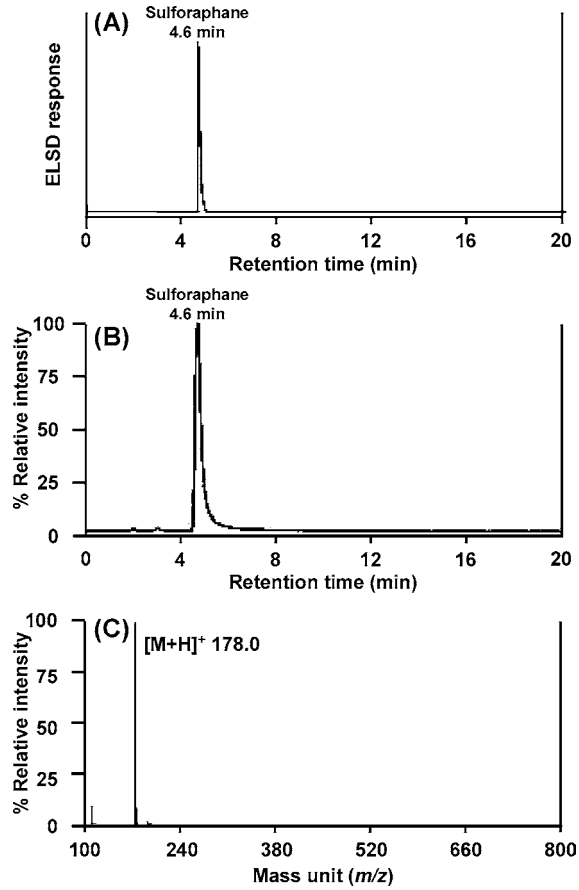

Figure 2. HPLC-ELSD/MS chromatograms of standard sulforaphane: (**A**) ELSD chromatogram; (**B**) total ion chromatogram; (**C**) MS spectrum of the peak ascribed to sulforaphane (4.6 min), showing a molecular ion $[M + H]^+$ at *m*/*z* 178.0. Standard sulforaphane (10 μg) was analyzed by HPLC-ELSD/MS following optimal conditions: column, YMC-Pack Pro C18 (4.6 mm × 150 mm); mobile phase, a mixture of distilled water, acetonitrile, and *tert*-butyl alcohol (8:1:1) containing 5 mM ammonium acetate; flow rate, 1 mL/min; column temperature, 40 °C.

RESULTS

Initially, HPLC-ELSD parameters (mobile phase composition for HPLC and drift tube temperature and nebulizer gas pressure for ELSD) were optimized to permit standard sulforaphane analysis. **Figure 2A** shows an ELSD chromatogram of standard sulforaphane (10 μg) under the optimal condition. A peak ascribed to sulforaphane was detected at a retention time of 4.6 min. This peak component gave a molecular ion $[M + H]^+$ at *m*/*z* 178.0, which was identical to that of sulforaphane (**Figure 2B**,**C**). **Figure 3** is a standard curve of sulforaphane. The correlation between the ELSD response (peak area) and sulforaphane concentration was described by the following equation; $y = (7.8 \times 10^4)x^{2.1}$ (y, peak area; x, sulforaphane amount (μg)); $r^2 = 0.99$. The detection limit for sulforaphane was 0.5 μg (2.8 nmol) at a signal-to-noise ratio of 3.

Next, the present HPLC-ELSD method was applied for broccoli sulforaphane determination. **Figure 4A** shows an ELSD chromatogram of mature broccoli extract. A sulforaphane peak appeared at a retention time of 4.6 min, which could be identified as sulforaphane based on the MS profiles (**Figure 4B**,**C**). For the recovery study, standard sulforaphane (5−10

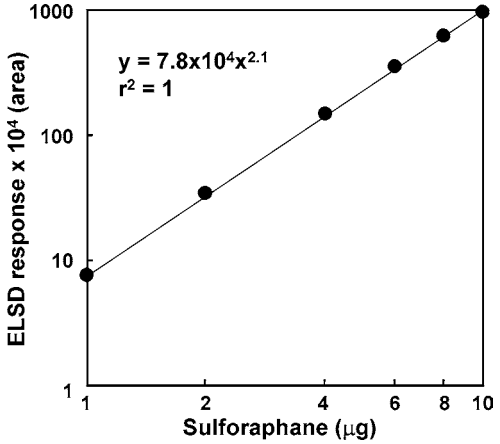

Figure 3. Standard curve for sulforaphane using the ELSD detector.

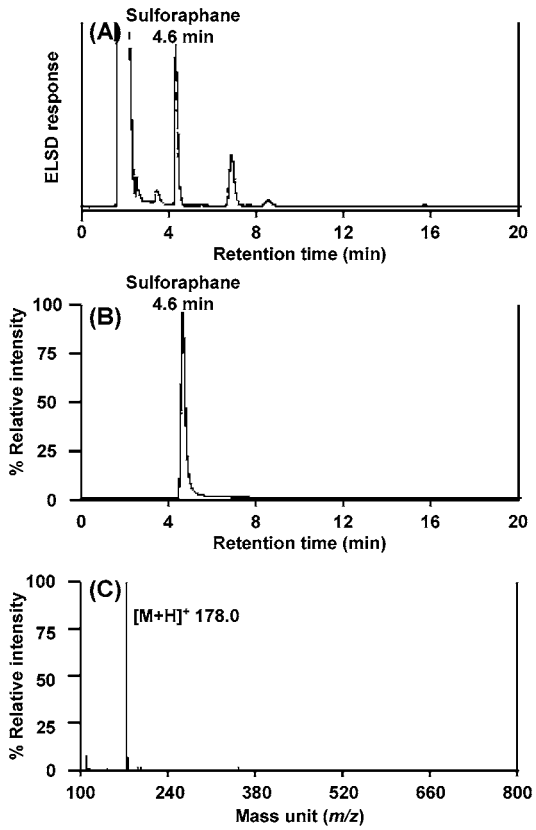

Figure 4. HPLC-ELSD/MS analysis of broccoli sulforaphane: (**A**) ELSD chromatogram; (**B**) single ion plot of the mass corresponding to the $[M + H]^+$ ion of sulforaphane (*m*/*z* 178.0); (**C**) MS spectrum of the peak detected at 4.6 min in chromatograms **A** and **B**. Sulforaphane was extracted from mature broccoli (floret) and analyzed by HPLC-ELSD/MS.

μg) was added to 100 mg of mature broccoli powder, and the extract was analyzed by HPLC-ELSD. The recovery of sulforaphane was calculated for above 95%. **Table 1** shows the sulforaphane concentration in broccoli samples, which were purchased from the general market. The sulforaphane concentration in broccoli and broccoli sprouts was defined as 44−1153 mg/100 g (0.044−1.153%) dry weight. The highest concentration of sulforaphane (1153 mg/100 g dry weight) was found in broccoli sprout. In contrast, sulforaphane could not be detected in most of broccoli products, but a little amount of sulforaphane (20.1 mg/100 g dry weight) was found in broccoli sample C (**Table 1**).

Table 1. Sulforaphane Concentration of Broccoli Samples on the Market

samples	part or form	sulforaphane concn[a] (mg/100 g dry weight)
mature broccoli A	floret	171.3 ± 4.7
	stem	89.3 ± 2.8
mature broccoli B	floret	69.1 ± 4.5
	stem	44.3 ± 0.3
broccoli sprouts	whole	1153.0 ± 32.7
broccoli product A	tablet	<1
broccoli product B	powder	<1
broccoli product C	tablet	20.1 ± 3.2
broccoli product D	powder	<1
broccoli product E	tablet	<1
broccoli product F	tablet	<1
broccoli product G	tablet	<1

[a] Values are means ± SD, $n = 3$.

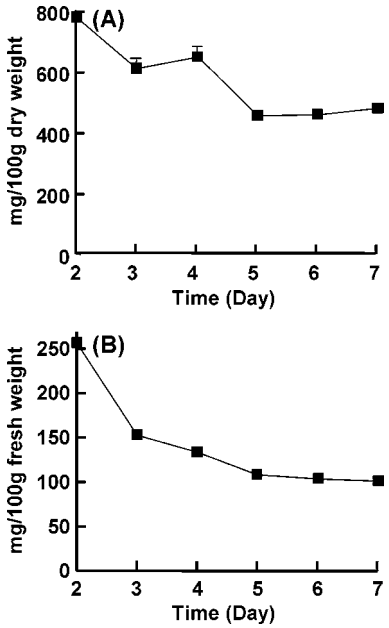

Figure 5. Decrease of sulforaphane concentration during the growth of broccoli sprouts. Broccoli sprouts were grown with a rotary cultivator and harvested on days 2–7 after planting. Sulforaphane in these broccoli sprouts was determined by HPLC-ELSD. The concentration unit was expressed as mg/100 g dry weight (**A**) and mg/100 g fresh weight (**B**).

Finally, the effect of growth for the broccoli sprout on sulforaphane concentration was investigated. Broccoli sprouts were grown from seeds with a rotary cultivator at Takamori Farm (Tsukidate-cho, Miyagi, Japan) and harvested on days 2−7 after planting. Sulforaphane concentration tended to decrease during the growth of the sprouts (**Figure 5**).

DISCUSSION

ELSD has been used in the HPLC field since the late 1970s. ELSD is a semiuniversal detector that can detect any nonvolatile analyte (*9*). During the past decade, the HPLC-ELSD method had proven advantageous for determining several lipids (*10*−*13*), carbohydrates (*14*), amino acids (*15*), and alkaloids (*16*, *17*) bearing weak or no chromophores. Hence, ELSD might be applicable to sulforaphane analysis. As we expected, ELSD is suitable for detection of standard sulforaphane (**Figure 2**). The best HPLC separation was achieved under the following condition: mobile phase, a mixture of distilled water, acetonitrile, and *tert*-butyl alcohol (8:1:1) containing 5 mM ammonium

acetate; flow rate, 1 mL/min; column temperature, 40 °C. The standard curve of sulforaphane using ELSD was not linear, but linearization was possible by using double-logarithmic coordinates (**Figure 3**). This is a common feature of the ELSD detector (*10*−*17*). The detection limit of sulforaphane was 0.5 μg (2.8 nmol). Generally, the HPLC-ELSD detection limit of analytes (i.e., lipids and carbohydrates) is around 1 μg (*10*−*17*). Hence, the sensitivity of the present method for sulforaphane is relatively high.

In preliminary experiments, when acid-hydrolyzed broccoli extract was directly subjected to HPLC-ELSD, several unknown peaks were detected near sulforaphane (data not shown). These impurities hindered precise sulforaphane determination. Thus, to remove the impurities, the broccoli extract was applied to a solid-phase extraction. This procedure was useful in the next HPLC analysis (**Figure 4**). Broccoli sulforaphane was eluted as a defined peak on the ELSD chromatogram. No interference peaks appeared. The sulforaphane peak was intense enough to determine the concentration. Recovery of sulforaphane was above 95%. Therefore, sulforaphane could be efficiently and quantitatively extracted from broccoli samples. These results indicated that the present method is highly accurate and precise. On the other hand, unless our developed method provided a high recovery rate of sulforaphane, it needed about 2 days for pretreatment (with HCl) of the broccoli sample and extraction of sulforaphane. As an extraction method, we had tried to prepare sulforaphane by many simple extraction methods, but the recovery rates were very low. In contrast, unless 2 days is importantly needed, by using our present extraction method, the recovery rate was very high (97%). The modification of our extraction method will be further developed.

Substantial amounts of sulforaphane (44−1153 mg/100 g dry weight) were found in mature broccoli (florets and stem) and broccoli sprouts (**Table 1**). Broccoli sprouts contained about 10 times the amount of sulforaphane found in mature broccoli, as previously reported in several studies (*18*, *19*). Our determined values are almost consistent with those of a previous HPLC-UV method (*6*). From these results, we confirmed that the present HPLC-ELSD method is sensitive enough to determine broccoli sulforaphane concentration. In contrast, unexpectedly, sulforaphane could not be detected in most of broccoli products, but a little amount of sulforaphane was found in broccoli sample C (**Table 1**). It is therefore likely that sulforaphane or its precursor (glucoraphanin) is degraded during the manufacturing and/or storing processes of broccoli products. Currently, various broccoli products are available to consumers. Nevertheless, their sulforaphane concentrations are not ordinarily specified. As there is no official method to measure sulforaphane, the present method may be applicable to the assay of sulforaphane present in various tablets, supplements, and other related products. In addition, according to the results of the sulforaphane contents in broccoli products (**Table 1**), the sulforaphane contents in commercialized products were lower than that stated on the product labeling, suggesting present commercialized sulforaphane products have low quality (*20*). By using our method as reference, it is possible to develop sulforaphane-rich products, which is now being studied as our further objective.

Recently, Matusheski et al. (*7*) reported the determination of sulforaphane by gas chromatography (GC). The sensitivity of the GC method is high (about 0.5 μg sulforaphane), but the analytical time is more than 30 min. This is a major disadvantage in case of analyzing multiple samples. In contrast, our HPLC-ELSD method enables the rapid determination of sulforaphane. Note that the analytical time of the present method is less than 10 min.

In this study, we confirmed that broccoli sprouts are enriched in sulforaphane (**Table 1**). However, little is known about the relationship between sprouts growth and sulforaphane concentration. Interestingly, sulforaphane concentration tended to decrease during the growth of sprouts (**Figure 5**). Since the 2-day-old broccoli sprouts showed the highest amount of sulforaphane, such young broccoli sprouts can be recommended as a source of sulforaphane-rich products. Most recently, Matusheski et al. (*18*) reported that preheating broccoli and broccoli sprouts to 60 °C increased the myrosinase-catalyzed formation of sulforaphane. They also found that sulforaphane formation was associated with epithiospecifier protein (ESP) activity. Therefore, conversion of glucoraphanin to sulforaphane in broccoli may be achieved by processing methods (e.g., temperature and pressure treatment).

According to the health benefit of broccoli sulforaphane, this compound was identified as a likely chemopreventive agent based on its ability to induce phase II detoxification enzymes (*1*). Recent studies suggested that sulforaphane induces cell cycle arrest and apoptosis in cancer cells (*3*−*5*), inhibits tubulin polymerization (*21*), activates checkpoint 2 kinase (*22*), and inhibits histone deacetylase activity (*23*). Theses findings suggested that sulforaphane may be effective during the postinitiation stages of carcinogenesis. In vivo studies are needed to further clarify the anticarcinogenic effect of broccoli sulforaphane.

In conclusion, we newly established the HPLC-ELSD method to determine broccoli sulforaphane with high selectivity and sensitivity. The method would also be applicable for the measurement of isothiocyanates rather than sulforaphane. This method may also be used as an investigation tool of the metabolic fate and bioavailability of sulforaphane.

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