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Development of a Sensitive Analytical Method for the Determination of Sulforaphane in Vegetables by Automated Online In-Tube SPME/LC–MS/MS

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Abstract

Sulforaphane (SFN), an isothiocyanate abundant in cruciferous vegetables, possesses well-documented anticancer, antioxidant, and anti-inflammatory properties, and has attracted interest as a dietary chemopreventive agent. However, sensitive and fully automated methods for its quantification in foods remain limited. In this study, we developed an automated analytical system coupling in-tube solid-phase microextraction (IT-SPME) with high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) to determine SFN in vegetable samples. Vegetable samples were autohydrolyzed in aqueous solution at 37 °C to generate SFN, which was then extracted with methanol by sonication. The extract was concentrated onto a Carboxen 1006 capillary column by IT-SPME, separated within 5 min on an Inertsil C8 column, and detected in positive electrospray ionization mode using multiple reaction monitoring. The detection limit was 5.6 pg/mL, with good linearity (R^2 = 0.9995) over 0.1–100 ng/mL, intra- and inter-day precisions below 2.9% and 3.9%, respectively, and accuracy of 98–102%. SFN was detected at concentrations of 600 μg/g or higher in broccoli sprouts, florets, and stems, but was found at less than one-tenth of these levels in other vegetables tested. This method provides a simple, sensitive, and solvent-efficient tool for SFN quantification in vegetables, with potential application in food functionality assessment and chemopreventive research.

Keywords: sulforaphane; cruciferous vegetables; in-tube SPME; LC–MS/MS; online automated sample preparation

1. Introduction

Previous studies have shown that consuming cruciferous vegetables such as broccoli, cauliflower, cabbage, kale, Chinese cabbage, and daikon radish can improve normal bodily functions and reduce the risk of developing chronic diseases such as cancer, cardiovascular disease, hyperlipidemia, diabetes, and obesity [1–6]. These health-promoting effects are attributed to isothiocyanates, which are decomposition products of glucosinolates found in cruciferous vegetables [1–6]. In particular, sulforaphane (4-methylsulfinylbutyl isothiocyanate, SFN) (Figure 1), which is a natural isothiocyanate found in the seeds, sprouts, fruits, leaves, roots, and stems of cruciferous plants, exhibits various biological effects such as anticancer [7–9], antioxidant [10–13], anti-inflammatory [10–14], neuroprotective [12–15], anti-obesity [16–18], and antibacterial [19,20], and has attracted considerable attention in functional foods and clinical medicine from a chemoprevention perspective [4–6,21].

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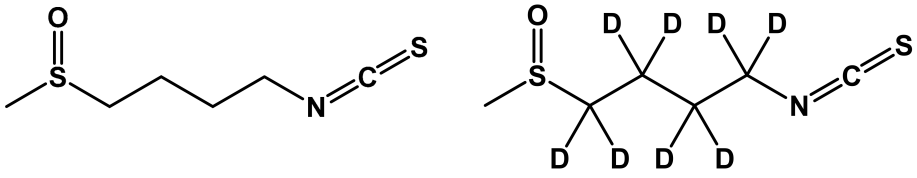

Sulforaphane (SFN) Sulforaphane-d8 (SFN-d8)

Figure 1. Structure of sulforaphane and sulforaphane-d8 (internal standard).

SFN is produced when glucoraphanin (4-methylsulfinylbutylglucosinolate), a precursor abundant in various cruciferous vegetables, is hydrolyzed by the myrosinase enzyme (thioglucoside glucohydrolase, EC 3.2.3.1) [4–6]. When these vegetables are chopped, ground, or chewed, the tissue is broken down, and the endogenous myrosinase that was sequestered within the tissue comes into contact with the glucoraphanin. This causes the β -thioglucoside bond in glucoraphanin to be cleaved, yielding d-glucose and an unstable thiohydroxamate-O-sulfinate, which undergoes non-enzymatic rearrangement (transposition) under neutral pH conditions to produce SFN [4–6,12]. However, under acidic pH conditions or in the presence of epithiospecifier proteins (myrosinase cofactors) and Fe^{2+} ions, glucoraphanin is rearranged to SFN nitrile, which has almost no biological activity [4–6,12,22,23].

To evaluate the biological activity and functional properties of SFN in food, it is necessary to determine the SFN content and intake levels in vegetable samples. However, since the structure of SFN is unstable and prone to degradation, and its production by hydrolysis of glucoraphanin is affected by pH, temperature, metal ions, and cofactor proteins [5,23,24], it is necessary to efficiently produce SFN under optimal hydrolysis conditions. Generally, SFN in vegetable samples is separated and quantified using various chromatographic methods after enzymatic hydrolysis of the sample with myrosinase. However, challenges remain regarding the simplicity and speed of the procedure, sensitivity and selectivity, interference from the sample matrix, and impacts on human health and the environment. Therefore, to accurately measure the SFN content in samples, it is necessary to develop a reliable analytical method capable of overcoming these challenges.

Various methods have been developed for the analysis of SFN, including gas chromatography mass spectrometry (GC–MS) [24], high-performance liquid chromatography (HPLC) [25–31], LC–MS [32,33], and LC–MS/MS [33–37]. However, GC–MS is unsuitable for quantitative analysis [2,4,33] because SFN is prone to thermal decomposition at the injection port [4,5]. HPLC uses UV detectors or diode array detectors, but the separation time is relatively long, and the detection selectivity and sensitivity are low due to the weak UV absorption of SFN, and it is susceptible to strong interference from the sample matrix [33,34,36]. On the other hand, LC–MS and LC–MS/MS methods provide information on molecular weight, resulting in high selectivity and sensitivity, enabling accurate identification and quantification [33,36,37]. However, for all of these methods, sample pretreatment steps are essential to extract SFN from complex sample matrices.

To analyze SFN in vegetable samples, it is first necessary to efficiently convert glucoraphanin in the sample into SFN. Typically, autohydrolysis using endogenous myrosinase is performed [4,25] under acidic (pH 3–6) [25,26] or neutral conditions [24,27–32,34,35]. Methods for extracting the generated SFN include liquid-liquid extraction (LLE) [24,27–29,32–34], dispersed liquid-liquid microextraction (DLLME) [31], QuEChERS [35], ultrasonic-assisted extraction (UAE) [31,32,35,37], solid-phase extraction (SPE) [25,26,30,37], and magnetic-dispersed solid-phase extraction (MSPE) [36]. While the LLE method is simple and versatile, it has several drawbacks, including the need to use large amounts of harmful organic solvents such as dichloromethane, chloroform, and ethyl acetate, which have adverse effects

on the environment and human health, inefficient extraction, and susceptibility to thermal decomposition of heat-sensitive compounds [5]. Therefore, methods such as the DLLME method, QuEChERS method, UAE method, SPE method, and MSPE method have been developed with the aim of providing more environmentally friendly and sustainable extraction methods by minimizing solvent use, improving extraction efficiency, and enabling the gentle handling of heat-sensitive compounds. The DLLME and QuEChERS methods are simple, versatile, environmentally friendly, eliminate matrix effects, and demonstrate excellent recovery rates. Furthermore, the UAE method offers advantages such as high extraction efficiency, rapid extraction rates, excellent handling of heat-sensitive compounds, environmental friendliness, and cost-effectiveness [5,32]. On the other hand, SPE and MSPE methods offer efficient extraction and excellent selectivity, and target compounds can be efficiently concentrated while removing interferents by selecting appropriate solid-phase materials [5,25,26,30,35,36]. However, many of these methods involve multi-step pretreatment processes, making them complicated and time-consuming to operate, and difficult to automate.

We have previously developed a highly sensitive automated analytical method that combines the in-tube solid-phase microextraction (IT-SPME) method [38,39], which uses a capillary column as an extraction device to efficiently extract and concentrate target compounds without the use of organic solvents, with LC–MS/MS, and have reported its applicability to the analysis of various food samples [38–42]. In this study, we aimed to develop an accurate, precise, and highly sensitive analytical method for the simple, rapid, and efficient extraction and concentration of SFN in vegetable samples using the IT-SPME LC–MS/MS method. A key feature of this study is the establishment of an environmentally friendly IT-SPME LC–MS/MS system capable of fully automated processing, from sample introduction to extraction, concentration, separation, and data processing, with almost no use of organic solvents. This system addresses the issues associated with conventional methods, such as cumbersome pretreatment procedures, high solvent consumption, interference from the food matrix, and low sensitivity and reproducibility, and aims to achieve efficient pretreatment and rapid, highly sensitive, and selective analysis for the quantification of SFN in vegetable samples.

2. Materials and Methods

2.1. Reagents and Standard Solutions

SFN was purchased from Cayman Chemical (Ellsworth Road, Ann Arbor, MI, USA). Stable isotope-labeled SFN-d8 (99.9% isotope purity) was purchased from Toronto Research Chemicals Inc. (TRC, North York, ON, Canada) and utilized as the internal standard (Figure 1). Standard SFN and the SFN-d8 were dissolved in LC–MS-grade methanol (Kanto Chemical, Tokyo, Japan) to prepare a 1 mg/mL stock standard solution, and stored in a tightly capped amber glass vial at 4 °C. Prior to use, a working standard solution was prepared by diluting with LC–MS-grade distilled water (Kanto Chemical) to the required concentration. LC–MS-grade methanol, formic acid, and distilled water from Kanto Chemical were used for the mobile phase and sample preparation.

2.2. LC–MS/MS Instrument and Analytical Conditions

LC–MS/MS analysis was performed using a system consisting of LC-1260 Infinity (Agilent Technologies, Santa Clara, CA, USA) coupled with a triple quadrupole mass spectrometer G6460C (Agilent Technologies). An Inertsil C8-3 column (2.1 × 50 mm, particle size 3 µm; GL Science Inc., Tokyo, Japan) was used for LC separation. The mobile phase consisted of 0.1% formic acid in methanol (60:40, *v*/*v*) at a flow rate of 200 µL/min for 5 min. MS/MS analysis was performed in positive electrospray ionization (ESI^+) multiple reaction

monitoring (MRM) mode, monitoring the SFN transition ions m/z 178→114 as the quantifier and m/z 178→72 as the qualifier ion, and monitoring the SFN-d8 transition ions m/z 186→122 as the quantifier. The ion source conditions were set as follows: gas temperature 300 °C, gas flow rate 10 L/min, nebulizer pressure 40 psi, sheath gas temperature 400 °C, sheath gas flow rate 11 L/min, and capillary voltage 3000 V. The measurement conditions were set as follows: fragmentor voltage 135 V, collision energy 9 V, and cell accelerator voltage 4 V. Mass Hunter Workstation Software (Version B.07.00, Agilent Technologies) was used for data processing.

2.3. IT-SPME Operation and Optimization

The IT-SPME [38,39] is a method that automatically performs capillary column washing, adsorption and concentration of the sample solution, and desorption by mobile phase using an autosampler injection program. In this study, a Carboxen 1006 capillary column (60 cm × 0.32 mm I.D.) was used as the extraction device and connected between the autosampler injection loop and injection needle using a 2.5 cm long polyether ether ketone (PEEK) tube, standard 1/16-inch stainless steel nuts, ferrules, and union connectors.

One mL of the sample solution was placed in a 2 mL autosampler vial with a silicon/polytetrafluoroethylene (PTFE) septum and set in the autosampler sample tray. Four additional autosampler vials were also placed in the tray: two containing 1.5 mL methanol, one containing 1.5 mL distilled water, and one blank vial. Prior to sample extraction, the 6-port valve was set to the LOAD position to condition the capillary column. First, the capillary was washed and conditioned at a flow rate of 200 µL/min as follows: drawing/ejecting 40 µL methanol for 2 cycles; next drawing 50 µL of air to remove the mobile phase solvent from the capillary; and then drawing/ejecting 40 µL distilled water for 4 cycles to replace the contents of the capillary with water. Subsequently, with the 6-port valve remaining in the LOAD position, extraction was performed by drawing/ejecting 40 µL of sample 20 times at a flow rate of 200 µL/min to extract analytes onto the stationary phase on the inner surface of the capillary. After extraction, the injection needle tip was washed once by drawing/ejecting 2 µL methanol from another autosampler vial. Finally, analytes extracted onto the capillary column were desorbed from the capillary coating by the mobile phase flow when the 6-port valve was switched to the INJECT position, and was subsequently transferred to the LC–MS/MS system. This series of IT-SPME controlled processes and the LC–MS/MS were fully automated by the autosampler software of the Mass Hunter Workstation. Figure 2 shows an overview of the IT-SPME/LC–MS system.

To optimize the IT-SPME method described above, various factors, including the type of capillary coating, the number of drawing/ejecting cycles, flow rate, pH of the sample solution, and methanol concentration were investigated using a 10 ng/mL SFN standard solution. Various extraction capillaries including Quadrex 007-5 (5% phenyl, 95% methylpolysiloxane, 0.32 mm I.D., 12 µm film thickness, Quadrex Corporation, Woodbridge, CT, USA), CP-Sil 19CB (14% cyanopropylphenyl, 86% polydimethylsiloxane, 0.32 mm I.D., 1.0 µm film thickness, Varian Inc., Lake Forest, CA, USA), Supelco WAX (polyethylene glycol, 0.32 mm I.D., 0.5 µm film thickness, Supelco, Bellefonte, PA, USA), Supel-Q PLOT (divinylbenzene polymer, 0.32 mm I.D., 12 µm film thickness, Supelco), and Carboxen 1006 PLOT (carbon molecular sieve, 0.32 mm I.D., 17 µm film thickness, Supelco) were used to compare extraction efficiencies. The number of drawing/ejecting cycles was examined in the range of 5 to 25 at a flow rate of 200 µL/min, and the flow rate was examined in the range of 50 to 250 µL/min. Additionally, the effects of the pH of the sample solution and the methanol concentration were examined by adding a buffer solution with a final concentration of 20 mM (pH 3–8) and varying the methanol concentration from 0% to 20%. Furthermore, using a 10 ng/mL SFN standard solution, the absolute extraction

rate of SFN into the capillary column was calculated from the peak area counts obtained by direct injection (10 µL injection) and the IT-SPME method (1 mL solution) using the following equation.

$$\text{Absolute extraction rate } (\%) = \frac{\text{B}}{\text{A} \times 100} \times 100$$

A: Peak area count of SFN obtained by direct LC–MS/MS analysis of 10 µL of a 10 ng/mL standard solution.

B: Peak area count of SFN obtained by IT-SPME LC–MS/MS analysis of 1 mL of a 10 ng/mL standard solution.

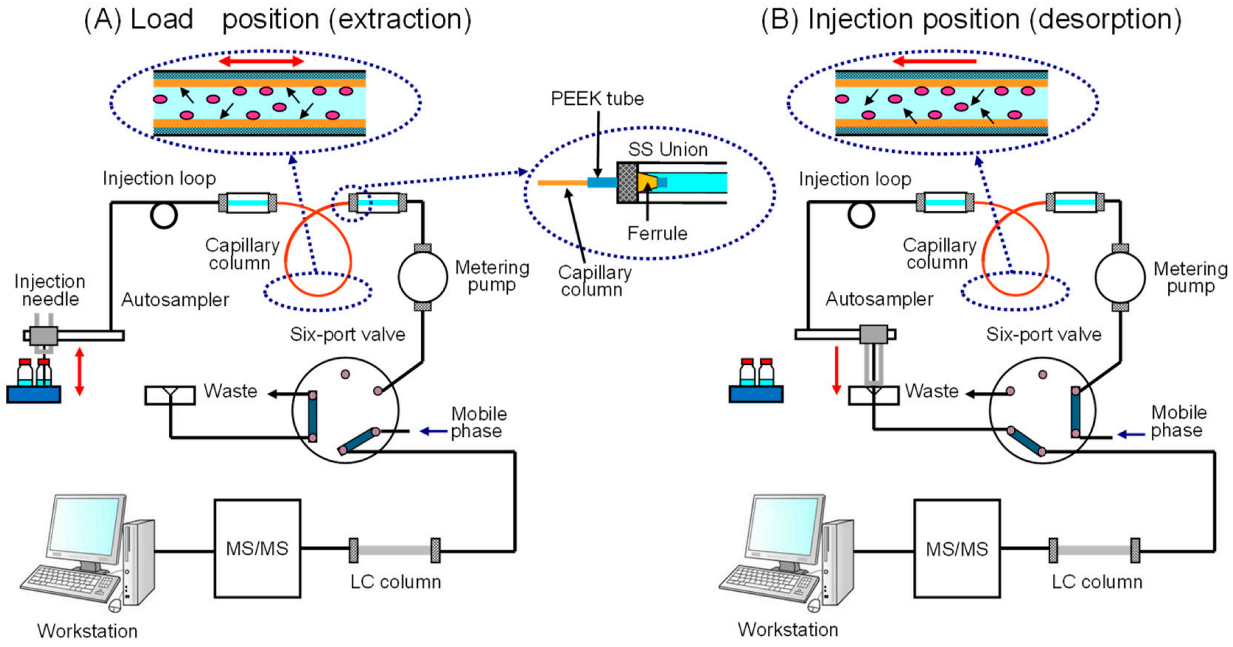

Figure 2. Schematic diagram of the IT-SPME LC–MS/MS system.

2.4. Method Validation Study

The developed analytical method was evaluated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), precision, and accuracy. Linearity was assessed based on the correlation coefficients (n = 24) of calibration curves constructed using the peak area count ratio of SFN to SFN-d8 by triplicate analysis of standard SFN at eight concentrations (0.1, 0.2, 0.5, 1, 5, 10, 50, and 100 ng/mL) in the presence of 20 ng/mL SFN-d8. To evaluate sensitivity, the LOD and LOQ were calculated based on signal-to-noise ratios (S/N) of 3 and 10, respectively. To evaluate precision and accuracy, standard solutions at low (0.1 ng/mL), medium (1 ng/mL), and high (10 ng/mL) concentrations were used, and six replicate measurements (n = 6) were performed for each. Intra-day precision was evaluated by independent measurements on the same day, and inter-day precision was evaluated by performing measurements on six different days. Precision was calculated as the relative standard deviation (RSD, %), and accuracy was evaluated as the percentage (%) based on deviations from the calibration curve. For the spiking recovery test, known amounts of SFN (1.5, 15, and 150 µg/g in food sample) were added to a methanol extract prepared from a vegetable sample (cabbage), and the recovery rate (%) was calculated.

2.5. Sampling and Preparation of Vegetable Samples

As cruciferous vegetable samples, commercially available broccoli sprouts, broccoli florets, broccoli stem, cabbage, lettuce, and mizuna were purchased at a local market. Each

sample was washed and air-dried at room temperature (approximately 25 °C) for 48 h to remove any contaminants and moisture. The parts of each vegetable used were as follows: broccoli (florets and stems), broccoli sprouts, and mizuna (entire above-ground parts), cabbage, and lettuce (leaves).

Each vegetable sample was homogenized using a blender (Ace Homogenizer AM-7, Nissei, Tokyo, Japan). SFN in the vegetable samples was hydrolyzed and extracted following the hydrolysis method described by Jie et al. [36] and the ultrasonic extraction method using methanol described by Yingchao et al. [37], with slight modifications. In brief, approximately 0.5 g of the homogenized sample was weighed into a 50 mL Pyrex centrifuge tube with a stopper, 5 mL of distilled water was added, and the mixture was allowed to stand at 37 °C for 3 h. Then, 5 mL of methanol was added, and extraction was performed by treating the mixture for 15 min at room temperature (approximately 25 °C) using an ultrasonic bath (Bransonic 2510J-DTH, 40 kHz, 70 W, Yamato, Tokyo, Japan). After extraction, the sample extract was centrifuged at 1000× *g* for 5 min, and the residue was further sonicated with 5 mL of methanol and centrifuged. The resulting supernatants were combined and filtered through a syringe filter (0.45 μm, Millipore, Billerica, MA, USA). A portion (50 μL) of the filtered sample solution was transferred to a 2 mL autosampler vial with a silicone/PTFE septum, and 20 μL of 1 μg/mL SFN-d8 was added. Then, the total volume was adjusted to 1 mL with distilled water for IT-SPME/LC–MS/MS analysis. The contents of SFN were determined as amounts per g of vegetable using a calibration curve. For sample A g, the total volume of the extract is 15 mL, of which 0.05 mL is sampled (corresponding to the sample mass of A/300 g). Therefore, the SFN content (ng/g) is calculated by dividing the concentration of the sample solution (ng/mL) obtained from the calibration curve by the sample mass (A/300 g).

2.6. Matrix Effects

As a type of matrix effect, molecules co-eluting the target compound in the sample matrix may interfere with the ionization process of the mass spectrometer. In this study, the matrix effect was quantified as the ratio of the slopes of the calibration curves of the matrix-fitted standard and the solvent standard. If the slope ratio is in the range of 0.8 to 1.2, the matrix effect is not considered. If the ratio is less than 0.8 or greater than 1.2, a signal suppression or enhancement effect is observed. Furthermore, the matrix factor was calculated from the recovery rate of SFN-d8 added to the vegetable extract using the following equation.

$$\text{Matrix factor } (\%) = \frac{\text{B}}{\text{A}} \times 100$$

A: Peak area count of SFN-d8 obtained by IT-SPME LC–MS/MS analysis of 20 ng/mL SFN-d8 in water.

B: Peak area count of SFN-d8 obtained by IT-SPME LC–MS/MS analysis of 20 ng/mL SFN-d8 in vegetable extract.

3. Results and Discussion

3.1. Optimization of IT-SPME Conditions for SFN

The IT-SPME system used in this study is a simple and widely used sample preparation method that automates the online extraction and concentration of target analytes from sample solutions without using organic solvents, enabling the continuous analysis of multiple samples [38,39]. The capillary column used as the extraction device was conditioned immediately prior to the analysis of each sample by washing with methanol and water to remove contaminants from the column. The air gap incorporated in the conditioning program was necessary to prevent mixing of the mobile phase and sample solution, and

to avoid desorption of analytes extracted onto the capillary coating during the extraction step. When using a 40 µL sample, a capillary tube with an inner diameter of 0.32 mm and a length of 60 cm (internal volume: 48 µL) effectively prevented contamination of the sample loop and metering pump. To optimize the IT-SPME conditions, the effects of capillary coating, drawing/ejecting cycles of sample solution, flow rate, sample solution pH, and methanol content in the sample solution were investigated using a 10 ng/mL SFN standard solution. SFN extraction efficiency was evaluated from the peak area obtained by IT-SPME LC–MS/MS.

Since extraction efficiency in IT-SPME highly depends on the type of capillary coating, we compared extraction efficiency using five different commercially available GC capillary columns with different stationary phases: liquid-phase capillaries such as Quadrex 007-5 with a low-polarity stationary phase, CP-Sil 19CB with a medium-polarity stationary phase, and Supelco WAX with a high-polarity stationary phase, as well as Supel-Q PLOT and Carboxen 1006 PLOT, both coated with thick-film porous adsorbents as stationary phases. As shown in Figure 3, it was found that SFN is more easily extracted onto highly polar liquid-phase stationary phases or PLOT-type stationary phases with a large surface area. Among the capillary columns examined, the Carboxen 1006 capillary yielded the highest extraction efficiency and a good peak shape; therefore, the Carboxen 1006 capillary was used as the extraction device in this study. The selection of capillary inner diameter (I.D.) and length depend on sample solution loading volume. Larger I.D.s reduce the contact area between the sample solution and the coating, decreasing extraction efficiency, whereas excessive length increases band broadening and extraction time. A capillary tube 60 cm long with 0.32 mm I.D. was therefore optimal for loading a 40 µL sample [38,39].

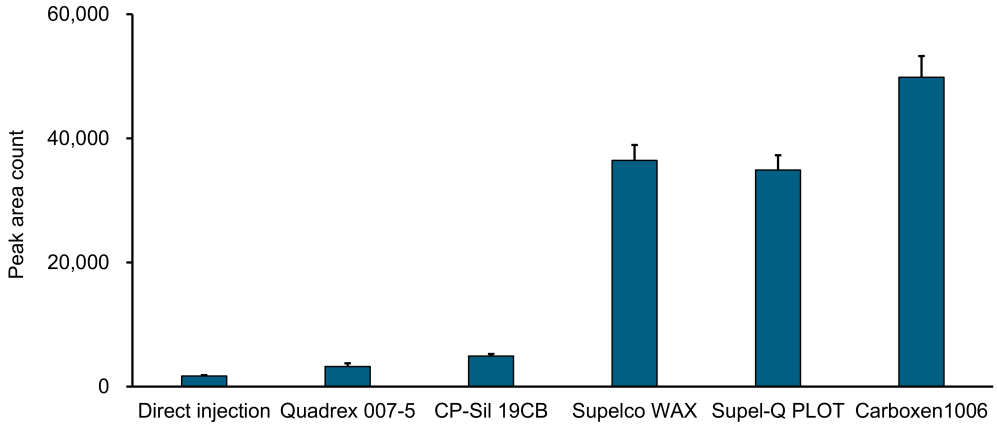

Figure 3. Effects of capillary coatings on IT-SPME of SFN. Extraction was performed by 20 draw/eject cycles of 40 µL of standard solution at a flow rate of 200 µL/min.

In IT-SPME, the number and flow rate of drawing/ejecting cycles affect the extraction efficiency because the analyte is extracted onto the capillary coating by repeatedly drawing/ejecting the sample solution into the capillary. The effect of drawing/ejecting cycles was evaluated at a flow rate of 200 µL/min with 5, 10, 15, 20, and 25 using Carboxen 1006 capillary. As shown in Figure 4A, the peak area counts increased with the number of cycles, but the rate of increase slowed after 20 cycles; therefore, considering the analysis time, the number of drawing/ejecting cycles was set to 20. Furthermore, the effect of flow rate with 20 drawing/ejecting cycles was evaluated at 50, 100, 150, 200, and 250 µL/min. As shown in Figure 4B, the effect of flow rate on extraction efficiency was found to be relatively small. However, since increasing the flow rate can shorten the analysis time, the flow rate of drawing/ejecting was set to 200 µL/min. On the other hand, the pH of

the sample solution and the methanol content may affect the extraction efficiency of the analyte onto the capillary coating. The effects of sample pH were examined using Carboxen 1006 capillary by adjusting the solution pH to 3, 4, 5, 6, 7, and 8. As shown in Figure 4C, no significant changes were observed, and a similar effect was obtained even when using distilled water; therefore, the sample solution was used as is without pH adjustment using buffer solution. Furthermore, since food samples are extracted with methanol, the effects of methanol in sample solution were examined after adjusting the methanol concentration to 0, 2, 5, 10, and 20%. As shown in Figure 4D, the extraction efficiency decreased with increasing methanol content. However, even for samples with low SFN content, diluting them with water more than 20 times reduced the methanol content to about 3%, thus ensuring an extraction rate of over 80%.

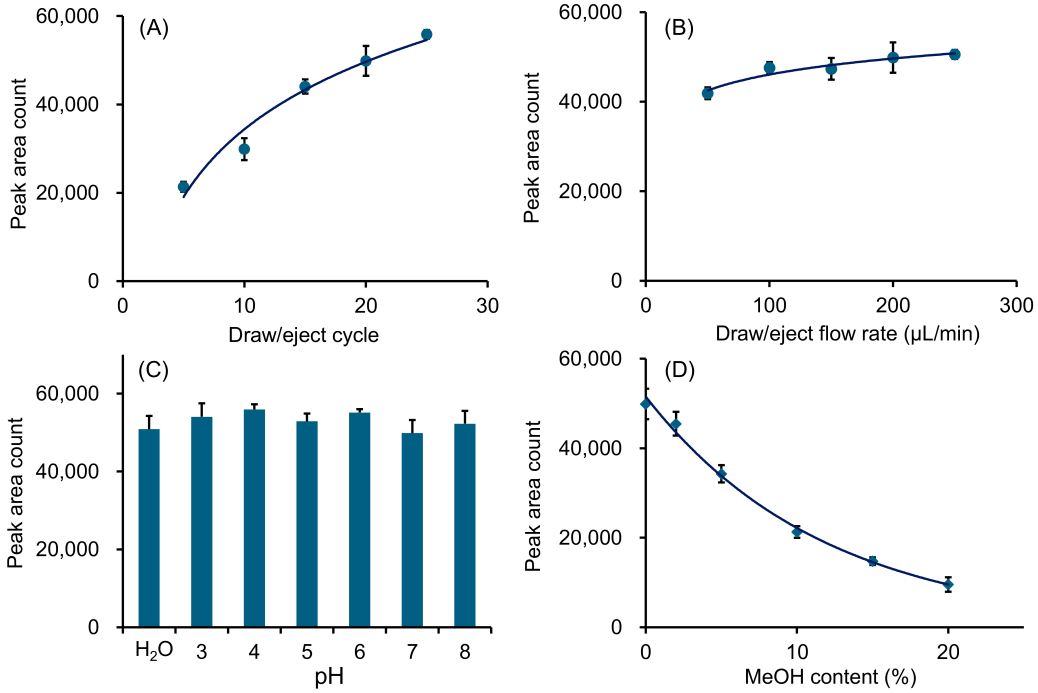

Figure 4. Effects of (**A**) drawing/ejecting cycles, (**B**) flow rate, (**C**) sample pH, and (**D**) MeOH content on IT-SPME of 10 ng/mL SFN. Extraction was performed with a Carboxen 1006 capillary by draw/eject cycles of 40 μL of standard solution.

SFN extracted into the capillary was almost completely desorbed by the mobile phase flow and directly introduced into the LC column, with no carryover observed. The absolute extraction rate of SFN into the capillary column, calculated by comparing the peak area counts obtained using the direct injection method and the optimized IT-SPME method, was low at 15.5 ± 0.7% (n = 6), and similarly, the internal standard SFN-d8 also showed a low value of 15.1 ± 0.7% (n = 6). However, since extraction by IT-SPME was performed using an autosampler, good reproducibility was obtained for both, with an RSD of 4.6%. Furthermore, since the absolute extraction rates of SFN and SFN-d8 were almost the same, the low extraction rates of these compounds were corrected by using the peak area ratio of SFN to SFN-d8, and there were no issues on the quantitative analysis. Figure 5 shows MRM chromatograms obtained using the direct injection method (10 μL injection) and the IT-SPME method (1 mL solution) with a 10 ng/mL SFN standard solution. Despite a low absolute extraction rate, the IT-SPME method was found to detect SFN with higher sensitivity than the direct injection method due to its concentration effect.

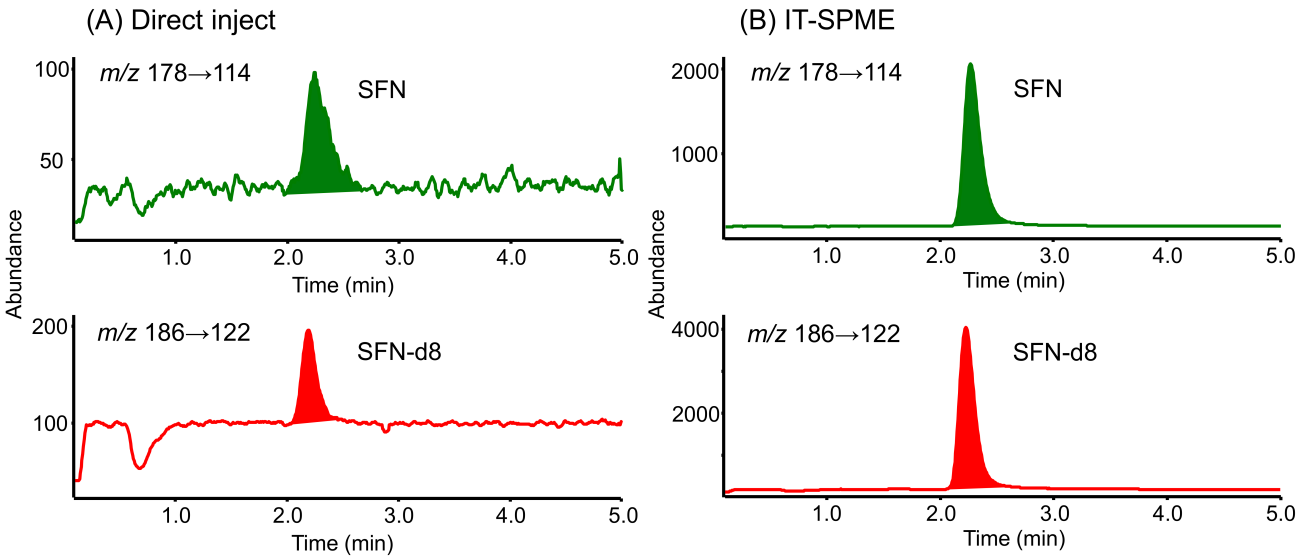

Figure 5. MRM chromatograms of SFN obtained from 10 ng/mL standard solution by (**A**) direct injection and (**B**) IT-SPME LC–MS/MS. Analytical conditions are described in the Experimental section.

3.2. LC–MS/MS Analysis

SFN and SFN-d8 showed good sensitivity in the ESI^+ mode. The protonated molecular ion $[M + H]^+$ and the most intense product ion generated from $[M + H]^+$ were selected as the precursor (Q1) and product (Q3) ions, respectively. In this study, *m*/*z* 178→114 and 178→72 were selected for SFN as the quantifier and qualifier ions MRM measurement, respectively. For SFN-d8, *m*/*z* 186→122 was selected as the quantifier ion. The optimized MS/MS parameters and MRM transitions for SFN were in good agreement with previously reported results [35]. Furthermore, the retention times for the quantifier and qualifier ions of SFN were identical at 2.30 ± 0.02 min (n = 6), and both exhibited good reproducibility with an RSD of less than 1%. Additionally, the ion ratio of the qualifier to the quantifier was 37 ± 2% (n = 6), indicating sufficient detection levels and confirming that SFN can be reliably detected for identification purposes. The chromatography conditions were optimized to minimize matrix effects, maintain good peak shape, and reduce analysis time without carryover through initial capillary conditioning. Among the tested LC columns, the Inertsil C8-3 column (2.1 × 50 mm, 3 µm) provided optimal separation and peak shape. As shown in Figure 5, SFN and SFN-d8 were reproducibly detected as single peaks at retention times of 2.30 ± 0.02 and 2.27 ± 0.01 min (n = 6), respectively, using a mobile phase of 0.1% formic acid/methanol (60:40, *v*/*v*) at a flow rate of 0.2 mL/min. The developed online IT-SPME LC–MS/MS analysis method involved a total analysis time of approximately 20 min per sample, from initial capillary conditioning, extraction and concentration by IT-SPME, online loading into LC–MS/MS, chromatographic separation, and detection. This enabled automated analysis of approximately 72 samples per day during nighttime operation, resulting in substantial labor savings.

3.3. Validation of the Analytical Method

Performance of the developed IT-SPME LC–MS/MS method was evaluated by assessing analytical parameters, including linearity, LOD, precision, and accuracy. The results of the analytical method validation are shown in Tables 1 and 2. The calibration curve showed a linear relationship with the regression equation y = 0.0323x + 0.0317 and a correlation coefficient R^2 = 0.9995 (n = 24) over the concentration range of 0.1–100 ng/mL (8 points, each concentration measured 3 times). The LOD (S/N = 3) of SFN was 5.6 pg/mL, demonstrating approximately 50-fold higher sensitivity compared to the direct injection method.

Furthermore, even the most sensitive LC–MS/MS method reported in the literature has an LOD of approximately 50 pg/mL [35,36], indicating that this method is more than 9 times more sensitive. Thus, the reason the IT-SPME method exhibits higher detection sensitivity is that, whereas the direct injection method is affected by impurities in the sample solution, the IT-SPME method not only concentrates the compounds, but also reduces the noise level because only the compounds extracted onto the capillary stationary phase are introduced into the LC column. On the other hand, when reproducibility was evaluated using low-, medium-, and high-concentration standard solutions, the intra-day and inter-day precisions were 2.9% or less and 3.9% or less, respectively. Furthermore, the accuracy was in the range of 98–102%, indicating that this method demonstrated acceptable precision and accuracy for quantitative analysis of SFN. Therefore, the IT-SPME/LC–MS/MS method developed in this study, by incorporating online extraction and concentration, not only enables operational simplicity and automation, but also allows for selective, highly sensitive, and high-performance analysis.

Table 1. Linearity and sensitivity of the IT-SPME LC–MS/MS method for SFN.

Compound	Mass Transition (*m*/*z*)	Linearity		LOD [2] (pg/mL)		LOQ [3] (ng/g Food)
		Range (ng/mL)	CC [1] (R^2)	Direct Injection	IT-SPME	
SFN	178→114	0.1–100	0.9995	277	5.6	7.8

[1] Correlation coefficient (n = 24); [2] limits of detection (S/N = 3); [3] limits of quantification (S/N = 10).

Table 2. Precision and accuracy of the IT-SPME LC–MS/MS method for SFN.

Compound	Concentration (ng/mL)	Precision (RSD, %), (n = 6)		Accuracy (%), (n = 6)	
		Intra-Day	Inter-Day	Intra-Day	Inter-Day
SFN	0.1	2.8	3.4	101.5	98.4
	1.0	2.9	3.9	99.4	99.5
	10	1.3	2.3	99.8	99.7

3.4. Application to the Analysis of Vegetable Samples

To apply the developed method to food analysis, glucoraphanin in vegetable samples must first be converted to SFN. Since the optimal pH for myrosinase, the hydrolyzing enzyme, is near acidic or neutral, hydrolysis has been performed under conditions of pH 3 [26], pH 6 [25], or in water or at near-neutral pH [24,27,29–36]. However, glucoraphanin also produces SFN nitrile at low pH, and it is hydrolyzed to SFN thiocyanate at pH 8 or higher, and these products are considered undesirable due to lack of bioactivity [32]. Therefore, many of the current methods involve autohydrolysis using pure water, with the samples allowed to stand for 0.5–2 h at room temperature or 30–45 °C [29,30,34–36]. In this study, we investigated the autohydrolysis time in distilled water at 37 °C within a range of 1–3 h, and almost complete hydrolysis was achieved in 3 h; subsequent experiments were performed using autohydrolysis at 37 °C for 3 h (Figure 6A). Furthermore, while chloroform [24,25,31], dichloromethane [26,28,29,32,35], ethyl acetate [27,30,33,34], and methanol [33,37] are used for solvent extraction of SFN after hydrolysis, methanol was chosen considering its introduction into IT-SPME after extraction. Regarding the methanol extraction of SFN obtained by hydrolysis, since SFN is present in vegetable samples, we added SFN-d8 to two types of vegetable samples to evaluate the number of extraction cycles required. As shown in Figure 6B, the extraction efficiency remained virtually unchanged between the second and third cycles, and since two extraction cycles were sufficient to

recover more than 95% of the SFN-d8 in both samples, we decided to combine the extracts from the two cycles.

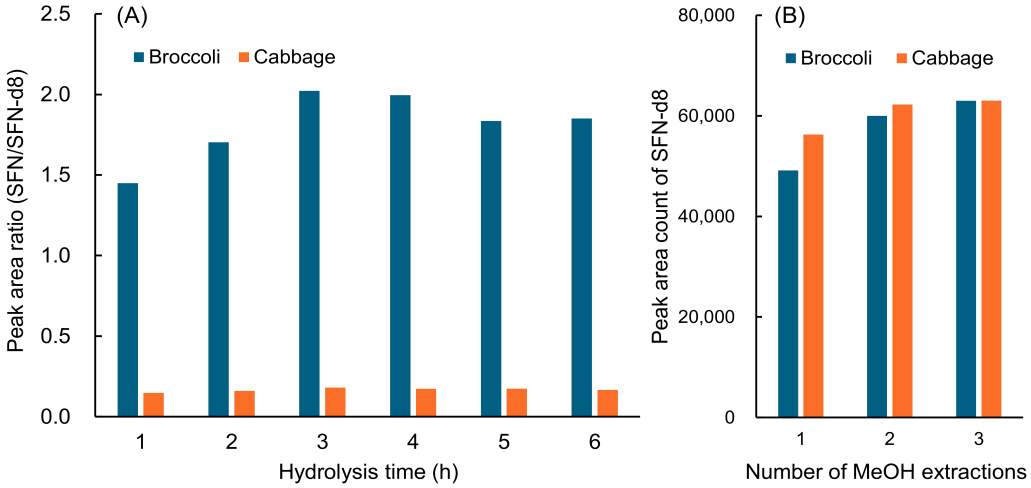

Figure 6. Effect of (**A**) hydrolysis time and (**B**) methanol extraction on vegetable samples.

Food samples contain various matrix components, and matrix effects such as ion suppression or enhancement may occur during LC–MS/MS analysis. Figure 7 shows the MRM chromatogram obtained from cabbage, where almost no contaminating peaks were observed, and SFN could be selectively detected. To evaluate the matrix effect, SFN standard solutions were added to water and cabbage extract at concentrations of 2.5, 25, and 250 ng/mL (corresponding to 1.5, 15, and 150 µg/g per cabbage sample). Absolute calibration curves based on the peak area of SFN and calibration curves using the internal standard method with SFN-d8 were created. As shown in Table 3, the ratio of the slope of the calibration curve obtained from cabbage extract to the slope of the calibration curve obtained from water using the absolute calibration method and the internal standard method was 0.988 and 0.990, respectively, indicating that the matrix had almost no effect in either case. In addition, the matrix factor based on the recovery rate of SFN-d8 from cabbage extract was 99.7%, further demonstrating that analysis could be performed without being affected by the matrix.

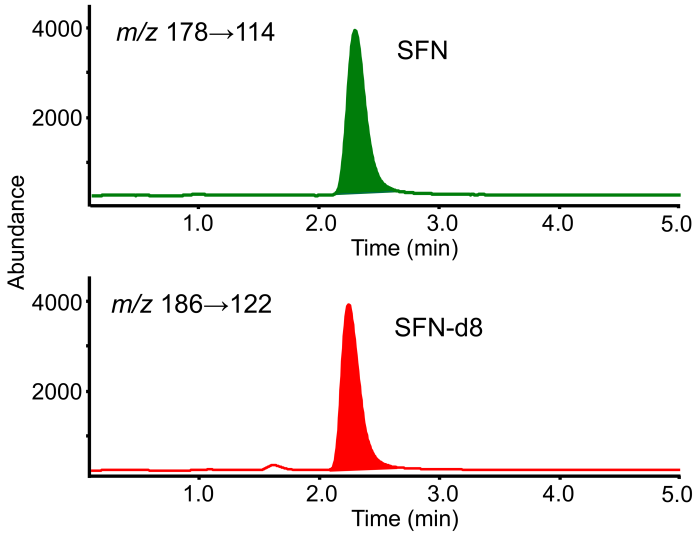

Figure 7. MRM chromatograms of SFN obtained from cabbage sample by IT-SPME LC–MS/MS. Analytical conditions are described in the Section 2.

Table 3. Quantitative assessment of the matrix effects.

	Absolute Calibration Curve [1]		Calibration Curve Using the Internal Standard [2]	
	Water	**Cabbage Extract**	**Water**	**Cabbage Extract**
Slope	2406	2377	0.0804	0.0796
Slope ratio	0.988		0.990	
Matrix factor	99.7 ± 4.3			

[1] Calculated from the peak area of SFN (n = 18). [2] Calculated from the peak area ratio of SFN to SFN-d8 (n = 18).

Furthermore, the LOQ (S/N = 10) for SFN in food samples was 7.8 ng/g (Table 1), and the recovery rate of SFN added to cabbage extract was over 98% (Table 4), demonstrating that this method is fully applicable for the quantitative analysis of trace amounts of SFN in food samples.

Table 4. Recovery of SFN added to cabbage extract.

Concentration (μg/g)		RSD (%)	Recovery (%)
Spiked	**Mean ± SD (n = 6)**		
0	23.55 ± 1.65	7.0	—
1.5	25.03 ± 0.04	0.2	98.7
15	38.41 ± 0.35	0.9	99.1
150	171.65 ± 3.51	2.0	98.7

Table 5 shows the results of measuring the SFN content in commercially available vegetables using this method. Relatively high contents of SFN were detected in broccoli sprouts and broccoli florets. In contrast, vegetables such as cabbage and lettuce showed relatively low SFN content, while levels in mizuna were below the LOQ. Furthermore, it was confirmed that broccoli florets contain more SFN than the stems. The differences in SFN content observed among different parts of the vegetable are thought to be related to variations in the distribution of the precursor glucoraphanin and differences in myrosinase activity. Broccoli florets showed higher SFN contents than stems in the present study. Furthermore, since myrosinase activity and cell structure differ among tissues, there may be variations in hydrolysis efficiency upon tissue disruption. Previous reports have also indicated that while SFN is widely present in cruciferous plants, its content varies significantly depending on the plant species and growth stage [5]. While SFN contents in broccoli sprouts have been reported to range from 273 to 3632 μg/g (dry weight) [2], mature broccoli is known to have relatively low contents [5,7]. Based on these reports, sprouts are known to have higher SFN content compared to mature plants.

Table 5. SFN contents in several vegetable samples and matrix factors.

Vegetable Sample	Sulforaphane Content (μg/g), n = 3	Matrix Factor (%), n = 3
Broccoli sprouts	1847 ± 36	104.7 ± 3.7
Broccoli florets	1253 ± 147	99.4 ± 3.2
Broccoli stem	659 ± 98	102.3 ± 3.6
Cabbage	23.6 ± 1.7	99.2 ± 4.1
Lettuce	3.5 ± 0.3	94.7 ± 4.5
Mizuna	<LOQ	99.7 ± 1.5

4. Conclusions

In this study, we developed an automated online IT-SPME/LC–MS/MS method for the analysis of SFN in vegetable samples using a Carboxen 1006 PLOT capillary. As shown in Table 6, previously reported methods for SFN analysis employ liquid-liquid or solid-phase extraction for sample preparation and concentration, which are relatively complicated and require harmful organic solvents such as chloroform, dichloromethane, and ethyl acetate. This method proposes a green pretreatment approach that is environmentally friendly and safe for human health, minimizing the use of organic solvents, and its novelty lies in the automated online coupling of sample pretreatment and chromatographic analysis. Furthermore, the detection sensitivity is superior to that of conventional methods, and the SFN content in various cruciferous vegetables was accurately determined. Based on these results, the IT-SPME/LC–MS/MS method developed in this study is useful as a selective and sensitive quantitative analytical method for SFN in vegetable samples, and is expected to find application in the evaluation of functional food components and quality control.

Table 6. Comparison of different analytical method for determination of SFN in vegetable samples.

Sample	Sample Preparation	Detection	LOD/LOQ	Precision RSD (%)	Content [1]	Ref.
Broccoli florets, Brussels sprout, Green cabbage, Red cabbage, Chinese kale, Turnip leaves	Autohydrolysis of 10 g plant-derived powder in 10 mL deionized water at room temperature for 12 h. Centrifugation, lyophilization, and redissolution in pH 7 PBS buffer, followed by two extractions with 1 mL $CHCl_3$.	GC–MS	—	—	48–540 μg/g FW	[24]
Broccoli	Autohydrolysis of 1 g fresh or 150 mg lyophilized sample in 4 mL acidic water (pH 6) at 45 ± 2 °C for 2.5 h. Extraction with 20 mL $CHCl_3$, absorption onto silica gel cartridge, elution with 3 mL MeOH, evaporation to dryness in a vacuum oven at 45 °C for 2 h, and redissolution in 2 mL CH_3CN.	HPLC–UV-DAD	LOD: 0.58 μg/mL	2.1	Edible portion of fresh, 202–684 μg/g DW; Lyophilized crop, 98–183 μg/g DW	[25]
Broccoli	Autohydrolysis of 5 g fresh broccoli powder with 20 mL HCl (pH 3) for 2 h. Extraction 3 times with 20 mL CH_2Cl_2, evaporation to dryness at 30 °C under vacuum, redissolution in CH_3CN, absorption onto SPE cartridge (silica, C18 and amino), elution with 4 mL CH_2Cl_2, evaporation to dryness in a vacuum oven at 45 °C for 2 h, and redissolution in 2 mL CH_3CN.	HPLC–UV	LOD: 0.02 μg/mL	3.54	120–327 μg/g DW	[26]
Cabbage, Broccoli	Autohydrolysis of 0.5 g lyophilized powder with 10.0 mL phosphate buffer (pH 7.0) by shaking for 1 h. Extraction 3 times with 30 mL ethyl acetate, centrifugation, evaporation to dryness at 35 °C under vacuum, and redissolution in 10 mL MeOH.	HPLC–UV	—	1.3	Cabbage, 3.91–52 μg/g DW; Broccoli, 6.55–392 μg/g DW	[27]
Broccoli, Red cabbage	Extraction of fresh or cooked samples with 600 mL CH_2Cl_2 at 35 °C under stirring with a magnetic stirrer, evaporation to dryness under vacuum, extraction with 500 mL distilled water, filtration, and lyophilization.	HPLC–DAD	LOD: 29.7 ng/mL; LOQ: 90 ng/mL	Intra-day: 2.22; Inter-day: 3.77	25.2–125.8 ng/mL	[28]

Table 6. *Cont.*

Sample	Sample Preparation	Detection	LOD/LOQ	Precision RSD (%)	Content [1]	Ref.
Raphanus sativus L. var. caudatus Alef	Homogenizing the fresh sample with deionized water at a 1:1 (*w*/*v*) ratio for 30 min and autohydrolysis by standing at room temperature for 2 h. Filtration through double layers of cheesecloth, extraction of the aqueous filtrate with CH_2Cl_2 3 times in a 1:1 (*v*/*v*) ratio, and evaporation to dryness under vacuum.	HPLC–UV	LOD: 0.36 µg/mL; LOQ: 1.08 µg/mL	Intra-day: 0.51; Inter-day: 0.98	111.94 µg/g DW	[29]
Broccoli	Autohydrolysis of 3 g cut sample in 9 mL purified water at 30 °C for 2 h. Extraction 3 times with 30 mL of ethyl acetate, evaporation to dryness at 40 °C under vacuum, redissolution in 5 mL ethyl acetate, absorption onto Sep-Pak Plus silica cartridges, elution with 5 mL MeOH, evaporation to dryness in a vacuum oven at 40 °C, and redissolution in 1 mL of 2-propanol. Derivatization with (S)-leucine.	HPLC–UV Chiral derivatization	—	—	(R)-form, 7–96 µg/g DW; (S)-form, 0.1–20 µg/g DW	[30]
Broccoli, Cabbage, Brussels sprouts, Radishes, Green mustard, Cauliflower, Rocket seeds, Watercress	Autohydrolysis of 10 g fresh vegetables homogenized with 50 mL water at 37 °C for 2 h. Extraction with mixed solvent of 1 mL CH_3CN and 700 µL $CHCl_3$, dryness of extractant phase under a N_2 stream, and redissolution in 500 µL of MeOH.	HPLC—DAD	LOD: 0.1 µg/mL; LOQ: 0.3 µg/mL	Intra-day: 2.3; Inter-day: 6.7	ND–260.4 µg/g DW	[31]
Broccoli-based supplements	Autohydrolysis of 0.5 g samples in 3.0 mL PBS (pH 7.4) by sonication for 90 min at 37 °C. Extraction 3 times with 10 mL CH_2Cl_2, evaporation to dryness at 35 °C under vacuum, redissolution in 0.5 mL pure HPLC grade CH_3CN.	UHPLC–MS	LOD: 1.3 ng/mL LOQ: 3.9 ng/mL	2.9–26.9	8.35–22.46 µg/g	[32]
Broccoli, White cabbages, Chinese cabbage	Extraction of 30 mg ground raw and cooked cruciferous samples with 1 mL 70% hot MeOH by shaking for 20 min at 700 rpm and 70 °C. Centrifugation, and dilution of the supernatant (500 µL) with a 0.1% formic acid solution (for Xbridge C18 analysis) or CH_3CN (for BEH Amide analysis) in volume ratios of 1:0 and 1:40, respectively.	LC HR-MS LC–QQQ	LC HR-MS LOD: 1.0 ng/mL LOQ: 3.1 ng/mL LC–QQQ LOD: 1.7 ng/mL LOQ: 5.7 ng/mL	Intra-day: 1.57–2.38 Inter-day: 1.41–10.6	Broccoli 13 µg/g FW	[33]
Broccoli	Autohydrolysis of lyophilized and finely milled broccoli samples (50 mg) in water (1:10, *w*/*v*) by standing for 30 min at room temperature. Extraction 3 times with 3 mL ethyl acetate, dryness under N_2 gas, and redissolution in 6 mL initial conditions mobile phase (70% ammonium acetate buffer (10 mM, pH 4.5): 30% CH_3CN with 0.1% formic acid).	UPLC–MS/MS	LOD: 0.003 µM (0.53 ng/mL) LOQ: 0.01 µM (1.77 ng/mL)	Intra-day: 0.95–6.69 Inter-day: 6.84–9.94	455 µg/g DW	[34]

Table 6. *Cont.*

Sample	Sample Preparation	Detection	LOD/LOQ	Precision RSD (%)	Content [1]	Ref.
Rapeseed	Autohydrolysis of 0.5 g crushed rapeseed samples in 10 mL water at 45 °C for 2 h. Ultrasound-assisted extraction twice with 5 mL CH_2Cl_2 for 10 min, dryness under N_2 gas, and redissolution in 1 mL MeOH.	UHPLC–MS/MS	LOD: 0.05 ng/g LOQ: 0.15 ng/g	Intra-day: 4.1–5.9 Inter-day: 9.0–11.3	ND–1622 µg/g FW	[35]
Broccoli, Cauliflower, Cabbage, Carrot, Brassica campestris, Chinese cabbage, Red radish, White radish, Green turnip, Pak choi	Autohydrolysis of 5 g homogenized vegetable sample in 20 mL ultrapure water at 45 °C for 2 h. Adsorption onto MB-COFs, magnetically separation, elution with 4 mL MeOH.	HPLC–MS/MS	LOD: 0.052–0.092 ng/mL LOQ: 0.18–0.31 ng/mL	1.8–7.9	0.14–45.62 µg/g FW	[36]
Seed and leaf of non-edible plant	Ultrasonicated extraction of 10 g air-dried plant material with 40 mL MeOH in ice-water bath for 20 min, dryness completely under N_2 gas, and redissolution in 3 mL H_2O/MeOH (1:1, *v*/*v*).	UPLC–MS/MS	LOD: 0.6 ng/mL LOQ: 2.0 ng/mL	Intra-day: 4.4–6.1 Inter-day: 5.2–6.2	18.7–1237.7 µg/mL	[37]
Broccoli sprouts, Broccoli florets, Broccoli stem, Cabbage, Lettuce, Mizuna	Autohydrolysis of 0.5 g homogenized sample in 5 mL distilled water by standing at 37 °C for 3 h. Ultrasonicated extraction twice with 5 mL of MeOH for 15 min at room temperature, and centrifugation.	LC–MS/MS	LOD: 5.6 pg/mL LOQ: 7.8 ng/g	Intra-day: 1.3–2.9 Inter-day: 2.3–3.9	3.5–1847 µg/g FW	This study

[1] FW: fresh weight; DW: dry weight.

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