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Development and Validation of a Spectrophotometric Method for Quantification of Total Glucosinolates in Cruciferous Vegetables

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ABSTRACT: Given their putative role in chemoprevention, validated methods are needed for quantification of total glucosinolates. Based on the colorimetric reaction of ferricyanide with 1-thioglucose, released by alkaline hydrolysis of glucosinolates, we developed a simple and sensitive method for spectrophotometric quantification of total glucosinolates in cruciferous vegetables. Lyophilized and ground vegetables are extracted with 80% boiling methanol. Extracted glucosinolates are isolated using a strong anion exchange column and then hydrolyzed with 2 N NaOH to release 1-thioglucose. Ferricyanide is added, and the decrease in absorbance is measured at 420 nm, with final values adjusted for background. Recovery of internal standard (sinigrin) was 107%. Intra- and interassay coefficients of variation were 5.4% and 15.8%, respectively. Dose response was linear with sinigrin and amount of plant material extracted ($R^2 \geq 0.99$). Using sinigrin, the lower limit of quantification was 0.6 mg. This straightforward method may be an alternative to time-consuming and costly chromatographic methods.

KEYWORDS: *broccoli, cabbage, watercress, Brassica, analysis*

INTRODUCTION

Primarily found in plants of the Brassicales order, glucosinolates are anionic secondary metabolites that are rich in sulfur. Upon disruption of the plant material through cutting, chewing, chopping, etc., myrosinase (thioglucoside glucohydrolase, EC 3.2.1.147) is released from the plant tissue and acts on glucosinolates to yield a number of bioactive metabolites (e.g., isothiocyanates, indoles). Glucosinolates have been investigated for their effects on insect resistance in plants (reviewed in ref 1) and for the putative chemopreventive activity of their metabolites in humans (reviewed in ref 2). Hence, there is interest in widely applicable and validated methods for analysis and quantification of glucosinolates.[3]

Commonly used methods of analysis are high performance liquid chromatography (HPLC) and liquid chromatography coupled with mass spectrometry (LC−MS). However, these methods have several significant limitations. Most challenging is the lack of availability of individual pure standards for most of the over 120 glucosinolates identified to date. LC−MS is dependent on having pure standards of each analyte of interest for accurate quantification.[3] Use of relative response or proportionality factors for analysis has inherent drawbacks such as nonuniform approaches in determination of the response factors, the limited number of glucosinolates (28) for which response factors are available due to the lack of standards, inaccuracies generated from assigning default values for glucosinolates with unassigned response factors, and failure of researchers to report specifically which factor was used for each analyte.[3] Additional challenges with HPLC and LC−MS approaches include access to necessary equipment and the high cost of equipment acquisition and maintenance (particularly for LC−MS). Further, validation of glucosinolate extraction and analytical methods has not always been reported; when it has, it has usually been carried out with rapeseed instead of mature vegetables commonly consumed that have differing tissue types and profiles of secondary metabolites. Many of these methods also involve time-intensive sample preparation, particularly those involving desulfation steps (e.g., ISO 9167-1). Moreover, desulfation is problematic due to some glucosinolates not being readily hydrolyzed by sulfatase.[3]

Jezek et al.[4] described their preliminary work with rapeseed and a ferricyanide assay as a promising method of glucosinolate analysis for further development and application to mature plant material. The method is based on alkaline hydrolysis of glucosinolates to yield 1-thioglucose. Ferricyanide oxidizes 1-thioglucose and the loss of the chromogenic ferricyanide can be assessed spectrophotometrically and used to determine total glucosinolate content (Figure 1).

$$2C_6H_{11}O_5S^- + 2Fe(CN)_6^{3-} \rightarrow \underset{\text{(Colored)}}{} \quad C_6H_{11}O_5SSO_5H_{11}C_6 + \underset{\text{(Colorless)}}{2Fe(CN)_6^{4-}}$$

Figure 1. Alkaline hydrolysis of glucosinolates yields 1-thioglucose, which is oxidized by ferricyanide.

We describe here a novel and streamlined modification, validation, and application of a ferricyanide assay for use with mature plant material. Furthermore, we developed and validated a novel method for extraction of glucosinolates from mature plants. The result is a simple, relatively time efficient, and accessible method for quantification of total glucosinolates in cruciferous vegetables.

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MATERIALS AND METHODS

Materials. Sinigrin hydrate from horseradish was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were reagent grade. Strong anion exchange solid phase extraction (SPE) columns (LC-SAX, 500 mg) were purchased from Supelco (Park Bellefonte, PA).

Instrumentation. Absorbance was measured using a Varian Cary 50 Scan UV−visible spectrophotometer. A heating block was used for sample extraction, a PRO 200 homogenizer was used for homogenizing, and a SPE column processor with a vacuum pump was used for partial purification of the samples on the strong anion exchange columns.

Sample Source and Preparation. In addition to sinigrin, fresh broccoli, green cabbage, and hydroponically grown watercress were used in the method development. The vegetables were organically grown varieties purchased from a local supermarket. The broccoli head was divided, and the receptacle was cut to a length of 5−7.5 cm. For cabbage, the outer leaves were removed and the head was cut into 4 wedges. The petiole of the watercress leaves was cut about 2.5 cm from the hydroponic root mass. Vegetable samples were individually contained in finely woven netted bags, weighed and placed into liquid nitrogen to shock freeze the sample. The samples were held on dry ice until being lyophilized. Dry weights were recorded, and the dried vegetable was ground using a food processor. All samples were stored at −80 °C until extraction.

On the day of extraction, bags containing the lyophilized ground vegetables were retrieved from the −80 °C freezer and brought to room temperature before opening to prevent condensation forming on the plant material from ambient moisture. The sample weights for extraction were 100 mg for watercress and 200 mg for broccoli and cabbage. A minimum of 6 replicates of each vegetable were weighed and placed in 15 mL glass screw cap tubes.

Myrosinase Activation. To differentiate the reaction of potentially interfering compounds identified by Jezek et al.,[4] such as ascorbic acid and cysteine, from 1-thioglucose derived from the base hydrolysis of glucosinolates, endogenous glucosinolates were degraded by the activation of myrosinase. In these samples, any oxidation of ferricyanide would be due to interfering substances. Activation was accomplished by the addition of 1 mL of H_2O to half of the weighed samples. Tubes were held at room temperature for 30 min with intermittent vortexing. After 30 min, 4.5 mL of methanol was added and tubes were vortexed and held at room temperature throughout the extraction.

Glucosinolate Extraction and Purification. Myrosinase was inactivated in the remaining three weighed samples of each vegetable, and the glucosinolates were extracted and purified in three steps. First, tubes containing the dried vegetable were preheated to 80 °C in a heating block for 10 min. Simultaneously, a parallel set of tubes containing only 4.5 mL of methanol were heated in loosely capped tubes. The hot methanol was then transferred to the vegetable-containing tubes through a small glass funnel. This circumvented the need to pipet hot methanol. The vegetable tubes were capped loosely, vortexed, and heated for 20 min at 80 °C. One milliliter of boiling H_2O was added using a positive displacement pipet, and the tubes were capped tightly and then refluxed for 30 min at 80 °C. Sinigrin recovery was used to confirm the stability of glucosinolates at 80 °C. Samples were then removed from the heating block and brought to room temperature.

Samples were homogenized using a stainless-steel generator in the water−methanol mix for 30 strokes at a power setting of 2.5. The homogenate was transferred to a high speed polypropylene centrifuge tube and centrifuged for 20 min at 18000*g*. The supernatant was transferred to a 12 mL conical tube and the pellet resuspended in 2.5 mL of 90% methanol. Samples were spun again for 20 min at 18000*g*, and the supernatant was pooled with the first supernatant. The pellet was resuspended once again with 2.5 mL of 90% methanol and the supernatant pooled with the other supernatants. The pooled supernatant was dried under nitrogen gas in a water bath at 45 °C and the extract stored at −20 °C until further processing. Preliminary testing demonstrated that the extracts were stable for at least 48 h under these conditions.

A strong anion exchange column was used for partial purification of the glucosinolates from the vegetable extracts. Columns were activated with 3 mL of methanol, followed by 3 mL of water, 2.5 mL of 0.5 M sodium acetate (pH 4.6), and 2.5 mL of water. Samples were resuspended in 3 mL of water, filtered through a 6 mL syringe containing a plug of glass wool packing, and applied to the activated column. The sample tubes and glass wool filters were rinsed with 2.5 mL of water, the rinse was applied to the column, and another 2.5 mL of water was applied to the column. Glucosinolates were eluted with 3 mL of 0.5 M sodium chloride. The eluate was collected into a graduated conical tube and the volume of the eluate recorded. Sample eluates were stored at −20 °C until analyzed. Comparison of sample eluates that were assayed immediately with aliquots of the same sample eluates stored at −20 °C confirmed the stability of the purified glucosinolates during storage at this temperature.

Glucosinolate Analysis. Glucosinolates were hydrolyzed under alkaline conditions to release 1-thioglucose by addition of 2 mL of freshly prepared 2 M sodium hydroxide. Samples were neutralized after 30 min with 310 μL of concentrated hydrochloric acid. The sinigrin standard curve was prepared and serially diluted using freshly made 1 M sodium hydroxide as the diluent. The final concentrations of the standards in the reaction mix ranged from 0.03125 mM to 1 mM sinigrin. Standards were also neutralized with 7.2% of total final volume concentrated HCl. The reaction was initiated by addition of 1.5 mL of a solution of 2 mM potassium ferricyanide in 0.4 M phosphate buffer (pH 7.0) to 1.5 mL of the standard solution or sample extract. Absorbance was read at 420 nm at 2 min after addition of the ferricyanide solution (see Figure 2).

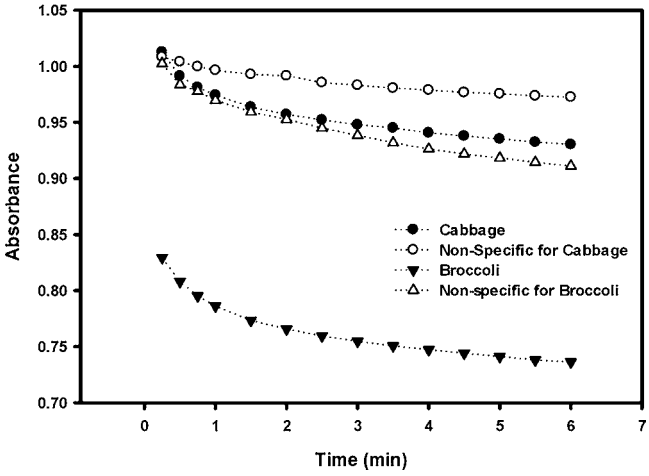

Figure 2. Varying reaction times with ferricyanide in extracts prepared at 80 °C and room temperature. A 2 min reaction time is optimal for complete reaction of thioglucose. Absorbance from room temperature extracts (active myrosinase) can be used to adjust for nonspecific reactions with ferricyanide.

Calculation of Total Glucosinolate Concentration. Absorbance is inversely related to the 1-thioglucose concentration. Therefore, absorbance was transformed to give a positive slope by subtracting the standard and sample absorbances from 1. Regression of the transformed standard absorbance with concentration typically resulted in an $R^2 \geq 0.99$ with a slope between 0.80 and 0.88. Sample glucosinolate concentrations were determined from the standard curve and expressed as μmol of glucosinolates/g dry weight of vegetable. The reduction of ferricyanide in the samples extracted at room temperature gives an estimate of interference. Calculated sample amounts were corrected by subtracting this estimate of interference. Thus, the corrected estimate represents only the reaction of 1-thioglucose with ferricyanide. See Figure 3 for a summary of the entire method.

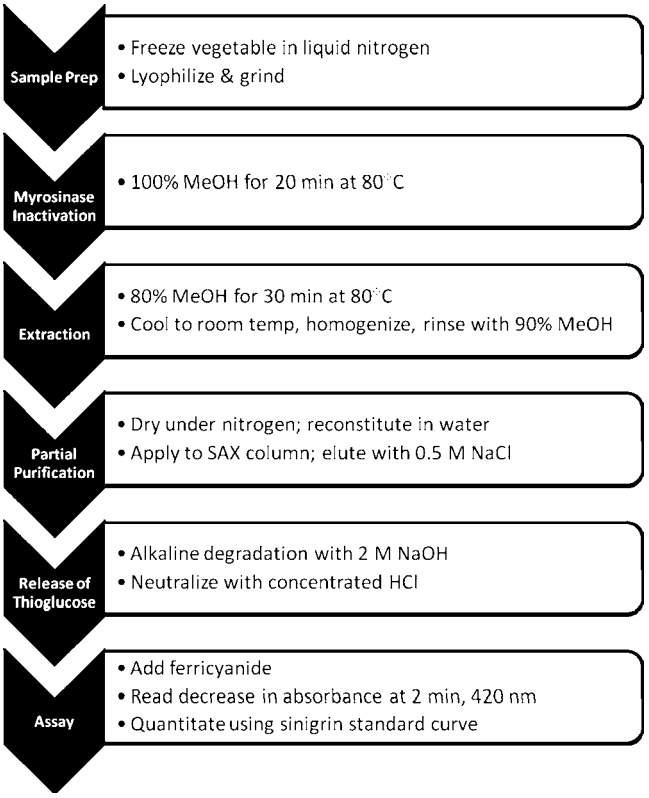

Figure 3. Summary of sample preparation and assay. Abbreviation: SAX, strong anionic exchange.

Method Validation. Linearity was tested using four sinigrin standard concentrations handled similarly to vegetable samples. Linearity was also confirmed with three different amounts of watercress (150−250 mg). Recovery was determined by spiking watercress samples with sinigrin (0.5 mg) and comparing with nonspiked samples. Stability of glucosinolates to the high temperature used in the extraction process was determined by comparing sinigrin in water held at room temperature to sinigrin held in near boiling water for 20 min, then added to methanol at 80 °C and held for 30 min. Watercress was used for determination of the intra- and interassay coefficients of variation. The intra-assay coefficient of variation was calculated using four replicates of 150, 200, and 250 mg of watercress. The interassay coefficient of variation was calculated from four replicates of extraction, purification, and assay of four samples of 100 mg of watercress. To determine specificity, 100 mg each of watercress and spinach (void of glucosinolates) were extracted, purified, and assayed as described above. The limit of detection was determined using decreasing amounts of sinigrin. Different sources of watercress, broccoli, and cabbage were analyzed for total glucosinolates using the above methods and compared to published values of total glucosinolates.

RESULTS AND DISCUSSION

Extraction and Partial Purification. Using pressurized liquid extraction, Mohn et al.[5] reported thermal degradation of glucosinolates at temperatures above 50 °C, and losses greater than 60% at 100 °C. Consequently, we verified glucosinolate stability at the temperatures and solvent concentrations employed during myrosinase deactivation and glucosinolate extraction. Sinigrin concentration processed through our extraction procedure using 80 °C methanol did not differ from an equal concentration of sinigrin held only at room temperature in water (1.05 ± 0.06 vs 0.96 ± 0.10 mg/mL, respectively, $p = 0.2$, $n = 4$), indicating no thermal degradation of sinigrin under our extraction conditions. Due to the potential presence of additional compounds that may react with ferricyanide (phenols, ascorbic acid[4]), we utilized strong anion-exchange SPE columns for simple and quick partial purification of vegetable extracts. We initially observed that extraction weight versus estimated glucosinolate concentration for watercress was nonlinear at higher tissue weights when using the same range of extraction weights as broccoli and cabbage. We questioned whether the SPE columns may become overloaded, leading to incomplete binding of glucosinolates, when the extraction weight is too high for vegetable types highly concentrated in glucosinolates as is watercress. Hence, we tested the binding capacity of the SPE column using the sinigrin standard. To estimate the maximum amount of glucosinolates that can bind, we applied to strong anion exchange columns 0.125−16 mg of sinigrin in water (3 mL application volume) and completed the partial purification steps. These samples, plus identical samples that were not column purified, were then assayed as described in Materials and Methods. We anticipated a linear relationship between measured glucosinolates and amount of sinigrin applied that would become nonlinear at some application amount for the column purified samples but not the nonpurified samples if column overload was occurring. There was no difference in glucosinolates between column purified samples and their corresponding nonpurified samples at any amount assayed, indicating no saturation of the column with glucosinolates at any amount applied. However, in both column purified and nonpurified samples, the linear relationship between measured glucosinolates and the applied amount of sinigrin became nonlinear at concentrations greater than 4 mg/mL and was nonexistent after 8 mg/mL. We conclude that the ferricyanide becomes limiting above 4 mg of glucosinolates/3 mL application volume and that the binding capacity of the strong anion column is not saturated at least up to 4 mg of glucosinolates. For application of the method to vegetables not tested here, we recommend determining extraction weights from initial examination of the linearity of a range of extraction weights versus estimated glucosinolate concentration for each vegetable, as we did for watercress, broccoli, and cabbage.

Quantification. At room temperature, myrosinase is active and converts glucosinolates to metabolites that do not react with ferricyanide. Hence, we included room temperature extracted plant samples in each assay in order to control for any nonspecific reactions with ferricyanide. We compared room temperature incubation times of 20 min, 40 min, and 60 min. There was no further loss of glucosinolates after 20 min of incubating plant samples at room temperature, indicating complete hydrolysis of all glucosinolates within 20 min. Therefore, the measured absorbance of samples incubated at room temperature for 20 min was used to adjust the results for background. The greater decrease in absorbance with boiled extracts of cruciferous vegetables (myrosinase inactive) compared to room temperature extracts (myrosinase active) indicates the specificity of the reaction for glucosinolates (Figure 2). This specificity was further confirmed by comparing extracts of spinach (void of glucosinolates) with extracts of watercress. Loss of chromogenic ferricyanide was not detected in spinach extracts whereas extracts of watercress showed the expected decrease in absorbance.

Most existing glucosinolate quantification methods, including the method by Jezek et al.,[4] were validated using only rapeseed, which has a different tissue structure and different profiles of secondary metabolites and interfering compounds compared to mature cruciferous plants or vegetables. Hence, while Jezek et al. used a 15 s reaction time with ferricyanide for extracts of

rapeseed,[4] we tested and determined that a 2 min reaction time for vegetable extracts is optimal for complete reaction of thioglucose with ferricyanide but prior to any significant nonspecific reactions (Figure 2).

Recovery was determined by comparing extraction and assay of sinigrin alone or added to vegetable samples. Recovery of sinigrin alone was 100% and was 107% when added to watercress samples. However, since sinigrin is not present within the vegetable, it may not truly represent extraction of the glucosinolates within the plant matrix.

Precision of the assay was determined over a four-day period. Intra- and interassay coefficients of variation (CV) were 5.4% and 15.8%, respectively, using watercress. The interassay CV using sinigrin was 5.8%. This suggests that the complexity of the plant matrix may be contributing to variability and could account for the higher interassay CV given that we use two measurements to arrive at total glucosinolates (absorbance of myrosinase-inactive samples and absorbance of myrosinase-active samples to adjust for nonspecific interfering compounds). Nonetheless, a CV of 15.8% is comparable to general expectations.[6] Inclusion of a plant matrix positive control in each assay throughout a project would aid interpretation of data. The method was linear over a range of sinigrin concentrations from 0.03125 mM to 1 mM and three amounts of watercress extracted (correlation coefficients ≥0.99; Figure 4). Using sinigrin, the lower limit of quantification was determined as 0.6 mg.

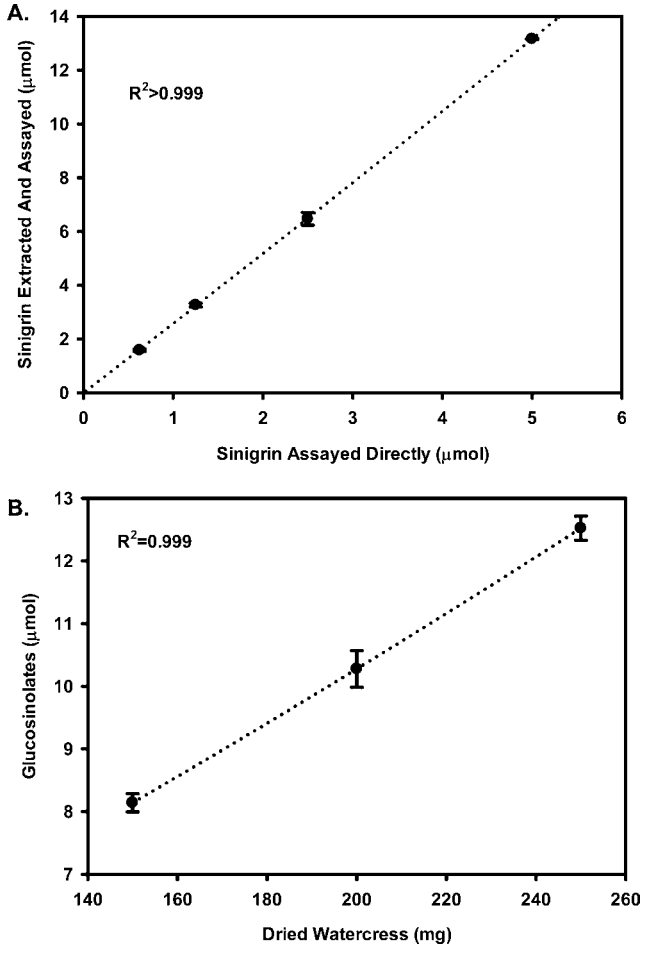

Figure 4. Linearity of the method over increasing concentrations of sinigrin (A) and increasing dry weights of watercress that were extracted (B).

We applied our method to the quantification of total glucosinolates in not only watercress but broccoli and cabbage as well. Our results with different sources of each type of vegetable (purchased on different days) were similar to previously published values determined by varying methods (Table 1). Generally, our results were higher than previously reported data on total glucosinolate content using different methods that typically rely on summing individual glucosinolates for which standards are available. Given that our values are not lower than previously published reports, the data in Table 1 also corroborate our validation data that indicate glucosinolates are not degraded during our sample preparation and extraction process.

Table 1. Total Glucosinolates in Cruciferous Vegetables Determined Spectrophotometrically Compared to Published Values Using Other Methods

vegetable	method of anal.[a] (citation)	glucosinolate estimate μmol/100 g fresh wt	glucosinolate estimate μmol/g dry wt
watercress	spec[b]	340.5	14.9
	spec[c]	476.3	28.7
	spec[d]	512.4	37.5
	spec[e]	557.1	42.8
	spec[f]	616.8	44.1
	HPLC, UV detection of total ITC[7]	17.1−144.6	data not available
	desulfation, HPLC, PDA detection[8]	data not available	7.96−12.56
	HPLC, UV detection of gluconasturtiin[9]	202[g]	data not available
broccoli	spec[b]	252.8	10.8
	spec[c]	244.7	21.6
	spec[d]	262.4	21.0
	spec[e]	222.6	20.6
	spec[f]	303.4	23.0
	desulfation, HPLC, UV detection[10]	144.5[g]	data not available
	LC−MS/MS[11]	62.4	data not available
	desulfation, HPLC-MS/MS[12]	data not available	26.6
	desulfation, HPLC-MS/MS[13]	data not available	71.4
cabbage	spec[c]	35.4	2.6
	spec[d]	176.2	16.6
	spec[e]	235.6	26.0
	spec[f]	70.9	6.6
	HPLC, UV detection of total ITC[7]	11.9−62	data not available
	LC−MS/MS[11]	10.3	data not available
	desulfation, HPLC[14]	data not available	24.0

[a]Abbreviations: Anal., analysis; Spec, spectrophotometric (the method presented in this paper); HPLC, high performance liquid chromatography; UV, ultraviolet; ITC, isothiocyanate; PDA, photodiode array; LC, liquid chromatography; MS, mass spectrometry. [b]Using vegetables purchased on 01/18/2010. [c]Using vegetables purchased on 02/19/2010. [d]Using vegeatbles purchased on 03/03/2010. [e]Using vegetables purchased on 03/11/2010. [f]Using vegetables purchased on 04/01/2010. [g]Converted to μm/100 g fresh weight based on information provided in original article.

Over 120 glucosinolates have been described. Methods that seek to quantitate total glucosinolates in cruciferous vegetables

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by summing the individual glucosinolates are severely limited by the lack of availability of authentic standards for most of the known glucosinolates. Thus, such methods inherently cannot assess total glucosinolates present in the plant matrix. Since evidence that any single glucosinolate has a greater health benefit than all of the other glucosinolates in human or animal studies is currently lacking, a measure of total glucosinolates within the diet will be preferable to identifying a small number of individual glucosinolates for many studies. Therefore, the quick and reliable method reported here for extracting and quantifying total glucosinolates can have broad application. Although an alternative approach to total glucosinolate analysis is to assess the metabolites of glucosinolates produced in the plant when myrosinase is released,[7,15] these methods have mostly focused on only one of the many groups of metabolites, isothiocyanates, and thus underestimate the total glucosinolate content. Furthermore, some glucosinolates, such as indole glucosinolates, produce unstable metabolites (see review in ref 16) which could also lead to underestimation of total glucosinolates using metabolite approaches. The extraction and spectrophotometric assessment method presented here is a time- and cost-efficient alternative for total glucosinolate assessment. It was validated using commonly consumed cruciferous vegetables of broad research appeal and overcomes the challenges and limitations inherent to chromatographic methods. This method may be suitable for developing a database of the total glucosinolate content of cruciferous vegetables.

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Notes

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ABBREVIATIONS USED

Spec, spectrophotometric (the method presented in this paper); HPLC, high performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; SAX, strong anion exchange; SPE, solid phase extraction; UV, ultraviolet; ITC, isothiocyanate; PDA, photodiode array

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