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Simultaneous Quantification of 22 Glucosinolates in 12 *Brassicaceae* Vegetables by Hydrophilic Interaction Chromatography−Tandem Mass Spectrometry

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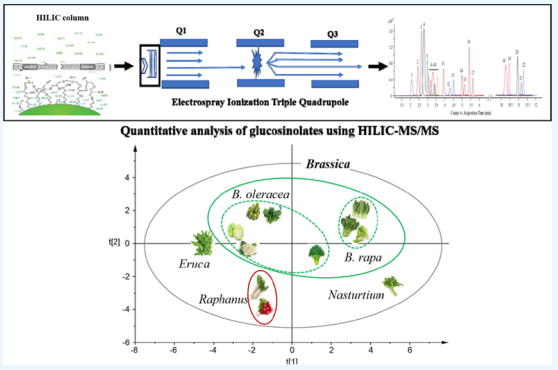
Supporting Information

ABSTRACT: Glucosinolates, which are unique to *Brassicaceae* vegetables, have diverse biological activities, including antimicrobial, antioxidant, and anticancer actions. In this study, we applied hydrophilic interaction chromatography−tandem mass spectrometry (HILIC−MS/MS) to the simultaneous quantification of 22 glucosinolates in 12 *Brassicaceae* vegetables, including pak choi, choy sum, Chinese cabbage, cauliflower, cabbage, broccoli, Kai Lan, Brussels sprouts, rocket salad, daikon radish, red cherry radish, and watercress. Significant differences in concentration and composition of glucosinolates were observed among these vegetables. Cabbage had the highest level of total glucosinolates (μg/g dry weight: 19 551.2 ± 1317.7), whereas Kai Lan had the lowest level (7611.3 ± 868.4). Aliphatic and indole glucosinolates were the major components in the 12 vegetables ranging from 76 to 100%, except watercress (37%). On the basis of the content of glucosinolates, the 12 vegetables were well distinguishable and classified according to their morphological taxonomy. This study presents a HILIC−MS/MS approach for quantification of glucosinolates, and demonstrates the potential of glucosinolate profiles for *Brassicaceae* species identification.



INTRODUCTION

Glucosinolates are secondary plant metabolites that occur naturally in the family *Brassicaceae*,[1] which are a group of sulfur-containing glucosides having a common core structure with a β-D-thioglucose group, a sulfonated aldoxime moiety, and a variable side chain (=R) derived from amino acids. Depending on the variable R group, glucosinolates can be categorized into three major classes: aliphatic, indole, and aromatic glucosinolates.[2] To defend against attacks by harmful organisms, glucosinolates can be hydrolyzed by the endogenous enzyme myrosinase into various breakdown products, including isothiocyanates, nitriles, thiocyanates, epithionitriles, and oxazolidinethiones.[3] Because of its characteristic aroma, glucosinolate-containing plant material is commonly used in the biofumigation for plant protection against soil-borne pathogens in agriculture and horticulture.[4] Recently, glucosinolates have been of interest for their diverse biological activities, ranging from antimicrobial, antioxidant, and anticancer actions.[1,5−7]

Brassicaceae vegetables are widely distributed and consumed in Asia, and include species such as *Brassica oleracea* (e.g., cauliflower, Brussels sprouts, cabbage, broccoli, and Kai Lan), *Brassica rapa* (e.g., pak choi, choy sum, and Chinese cabbage), *Nasturtium officinale* (e.g., watercress), *Raphanus sativus* (e.g., daikon radish and red cherry radish), and *Eruca sativa* (e.g., rocket salad).[8] Evidence indicates that the concentration and composition of glucosinolates may vary among and within individual *Brassicaceae* species.[9] Considering the enormous potential of biodiversity, screening of glucosinolate-enriched *Brassicaceae* species is of particular interest for nutritionists, agribusiness, and consumers at large. Moreover, recent studies indicate that species classification based on the secondary metabolites has emerged as an effective chemotaxonomic tool, providing detailed biochemical information about the differences and similarities among plant species.[10] So far, there are only a handful of studies on quantification of glucosinolates in cruciferous or *Brassica* species; thus, it is interesting to explore the potential of use of quantitative glucosinolate profiles for the chemotaxonomic classification of *Brassicaceae* species.

To date, more than 130 glucosinolates have been reported in *Brassicaceae* vegetables;[11] however, only a small portion of them (<10%) have been quantified because of the difficulties

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	Vegetables	Latin name	Species	Glucosinolates (%)		
				Aliphatic	Indole	Aromatic
	Pak choi	*Brassica rapa* subsp. *chinensis*	*Brassica rapa*	33%	65%	2%
	Choy sum	*Brassica rapa* var. *parachinensis*	*Brassica rapa*	25%	74%	1%
	Chinese cabbage	*Brassica rapa* subsp. *pekinensis*	*Brassica rapa*	16%	82%	2%
	Cauliflower	*Brassica oleracea* var. *botrytis*	*Brassica oleracea*	27%	73%	0%
	Cabbage	*Brassica oleracea* var. *capitata*	*Brassica oleracea*	27%	65%	8%
	Broccoli	*Brassica olearacea* var. *italica*	*Brassica oleracea*	13%	67%	20%
	Kai lan	*Brassica olearacea* var. *alboglabra*	*Brassica oleracea*	64%	28%	8%
	Brussels sprouts	*Brassica oleracea* var. *gemmifera*	*Brassica oleracea*	43%	55%	2%
	Rocket salad	*Eruca sativa*	*Eruca*	61%	16%	24%
	Daikon radish	*Raphanus sativus*	*Raphanus*	59%	40%	1%
	Red cherry radish	*Raphanus raphanistrum* subsp. *sativus*	*Raphanus*	50%	49%	1%
	Watercress	*Nasturtium officinale*	*Nasturtium*	1%	36%	63%

Figure 1. Twelve commercial *Brassicaceae* vegetables and their glucosinolate composition.

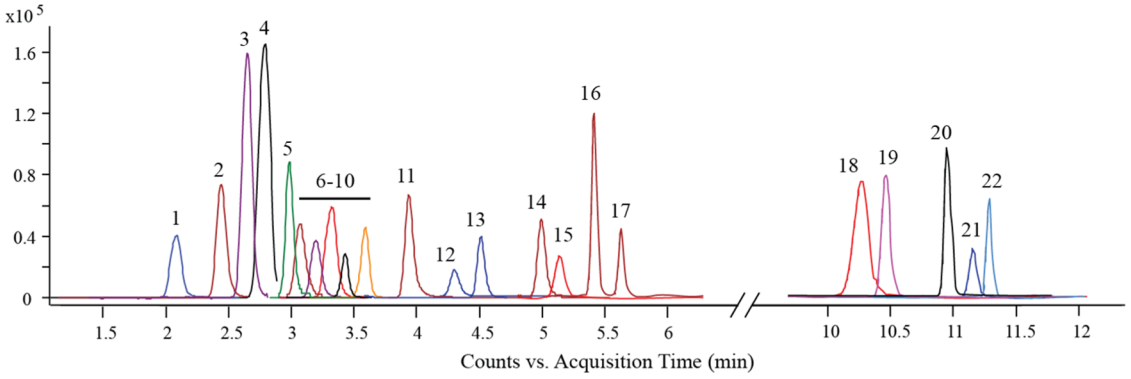

Figure 2. Representative HILIC−MS/MS chromatogram of 22 glucosinolates.

in trace-level determination of most glucosinolates using routine analytical methods. In general, reversed-phase liquid chromatography (RPLC) coupled to diode-array detector (DAD) or mass spectrometry (MS) is the most frequently used method for quantification of glucosinolates.[12,13] In a recent study,[14] a total of 12 glucosinolates were measured in 9 *Brassica* crops using RPLC−DAD, which is the largest number of glucosinolates quantified in an individual study so far. Owing to their biochemical properties, high volume of consumption, and health effects, it is thus time to develop more sensitive and selective methods for quantification of glucosinolates in vegetables. In 2007, Wade et al. for the first time applied hydrophilic interaction liquid chromatography (HILIC) for quantification of glucosinolates, but only three glucosinolates were measured in their study including glucoraphanin, glucoriberin, and sinigrin.[15] HILIC utilizes a gradient of increasing aqueous content to elute analytes from a hydrophilic stationary phase, which is a more appropriate analytical method for analysis of polar components.[16,17] Thus, it is a more effective technique than RPLC for separation of glucosinolates because of their highly polar nature.

In this study, we established an analytical method based on HILIC−MS/MS to quantify and compare the glucosinolate profiles across 12 commonly consumed *Brassicaceae* vegetables in Asia, including cauliflower, Brussels sprouts, cabbage, broccoli, Kai Lan, pak choi, choy sum, Chinese cabbage, watercress, daikon radish, red cherry radish, and rocket salad. Moreover, the potential use of glucosinolate profiles for *Brassicaceae* species classification was explored (Figure 1).

RESULTS AND DISCUSSION

Method Validation. In this study, an analytical method based on HILIC−MS/MS was developed for the quantitative analysis of glucosinolates. Compared to the previously reported HILIC method for determination of limited glucosinolates,[15] our present method allowed simultaneous quantification of a panel of 22 glucosinolates using the multiple reaction monitoring (MRM) mode, with a powerful separation in a relatively short analysis time (Figure 2). The optimized MRM conditions of 22 glucosinolates are shown in Table 1. In the validation process for the new method, we examined its limit of detection (LOD), limit of quantification (LOQ),

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Table 1. Multiple Reaction Monitoring (MRM) Conditions of 22 Glucosinolates in HILIC−MS/MS Analysis[a]

glucosinolates	RT (min)	precursor ion	product ion	CE (V)	internal standard	calibration curve
aliphatic						
sinigrin[b]	3.94	358.0	97, 195	24	glucosinalbin	sinigrin
glucoiberin[b]	11.30	422.0	259, 358	26	glucosinalbin	glucoiberin
glucocheirolin[b]	5.19	438.0	97, 259	26	glucosinalbin	glucocheirolin
glucoraphasatin	2.79	418.0	97, 259	26	glucosinalbin	glucoerucin
glucosativin	3.45	406.0	97, 259	26	glucosinalbin	glucoerucin
glucoerucin[b]	3.00	420.0	97, 259	26	glucosinalbin	glucoerucin
glucoraphenin[b]	10.30	434.0	97, 259	24	glucosinalbin	glucoraphenin
glucoraphanin[b]	11.20	436.0	97, 372	26	glucosinalbin	glucoraphanin
gluconapin[b]	3.60	372.0	97, 259	26	glucosinalbin	gluconapin
epiprogoitrin[b]	5.42	388.0	97, 259	24	glucosinalbin	epiprogoitrin
progoitrin[b]	5.72	388.0	97, 259	24	glucosinalbin	progoitrin
glucoalyssin[b]	10.46	450.1	97, 259	26	glucosinalbin	glucoalyssin
glucobrassicanapin[b]	3.10	386.1	97, 259	26	glucosinalbin	glucobrassicanapin
gluconapoleiferin	5.00	402.1	97, 259	26	glucosinalbin	glucobrassicanapin
indole						
glucobrassicin[b]	3.23	447.1	97, 259	26	glucosinalbin	glucobrassicin
neoglucobrassicin	2.21	477.1	97, 259	24	glucosinalbin	glucobrassicin
4-methoxyglucobrassicin	3.29	477.1	97, 259	26	glucosinalbin	glucobrassicin
4-hydroxyglucobrassicin[b]	4.52	463.0	97, 259	26	glucosinalbin	4-hydroxyglucobrassicin
aromatic						
glucotropaeolin[b]	2.65	408.0	97, 259	24	glucosinalbin	glucotropaeolin
gluconasturtiin[b]	2.49	422.1	97, 259	24	glucosinalbin	gluconasturtiin
glucobarbarin[b]	10.96	438.1	97, 259	26	glucosinalbin	glucobarbarin
glucosinalbin[b,c]	4.30	424.0	97, 259	26	glucoraphenin	glucosinalbin

[a]RT, retention time; CE, collision energy. [b]These 17 glucosinolates have available standards. [c]Glucosinalbin is present only in rocket salad and does not exist in other 11 examined vegetables. In this study, it was used as an internal standard for quantitative analysis of glucosinolates in the 12 vegetables, except rocket salad, in which glucoraphenin was used as the internal standard for quantitative analysis of glucosinolates, as it is absent in rocket salad.

Table 2. Method Validation of HILIC−MS/MS for 17 Glucosinolates with Available Standards[a]

	calibration curve					accuracy (%)		recovery (%)	
glucosinolates	formulation	R^2	concentration (ng/mL)	LOD (μg/g dry weight)	LOQ	intrabatch	interbatch	low	high
sinigrin	$y = 0.037x - 0.106$	0.9997	1−500	0.007	0.023	4.35	5.96	79.44	103.06
glucoiberin	$y = 0.006x + 0.012$	0.9994	1−500	0.003	0.010	4.82	6.32	112.10	84.36
glucocheirolin	$y = 0.038x - 0.036$	0.9998	1−500	0.004	0.013	4.17	3.34	95.74	106.37
glucoerucin	$y = 0.073x - 0.725$	0.9991	1−1000	0.009	0.030	7.21	6.76	84.04	110.19
glucoraphenin	$y = 0.47x - 1.149$	0.9985	1−500	0.001	0.003	2.00	8.85	90.45	84.25
glucoraphanin	$y = 0.018x - 0.020$	0.9998	1−500	0.013	0.043	4.06	7.67	85.26	98.40
gluconapin	$y = 0.054x - 0.012$	0.9999	1−250	0.003	0.010	8.61	5.96	78.36	87.43
epiprogoitrin	$y = 0.033x - 0.068$	0.9994	1−250	0.007	0.023	6.10	5.70	117.01	96.59
progoitrin	$y = 0.015x - 0.061$	0.9997	1−1000	0.028	0.093	4.23	5.35	84.69	95.64
glucoalyssin	$y = 0.017x + 0.029$	0.9991	1−500	0.013	0.043	6.74	9.04	95.26	114.17
glucobrassicanapin	$y = 0.079x - 0.936$	0.9987	1−1000	0.008	0.027	9.24	9.95	81.67	91.45
glucobrassicin	$y = 0.042x - 0.133$	0.9995	1−500	0.019	0.063	5.51	8.75	92.30	120.31
4-hydroxyglucobrassicin	$y = 0.004x - 0.016$	0.9996	1−1000	0.021	0.070	2.43	9.02	76.46	107.01
glucotropaeolin	$y = 0.082x - 0.265$	0.9998	1−500	0.004	0.013	5.58	7.29	120.14	106.33
gluconasturtiin	$y = 0.052x - 0.129$	0.9990	1−500	0.006	0.020	4.29	7.16	105.76	102.56
glucobarbarin	$y = 0.002x + 0.003$	0.9979	5−500	0.023	0.077	6.54	3.33	114.79	99.05
glucosinalbin	$y = 0.003x + 0.008$	0.9991	5−500	0.007	0.023	2.02	8.99	95.01	101.22

[a]LOD, limit of detection; LOQ, limit of quantification.

linearity, accuracy, precision, and recovery (Table 2). LODs under the present chromatographic conditions, measured with a signal-to-noise ratio (S/N) of 3, ranged from 0.001 to 0.028 μg/g dry weight. LOQs, measured with a signal-to-noise ratio (S/N) of 10, ranged from 0.003 to 0.093 μg/g dry weight. The calibration curves and correlation coefficients were determined using a linear regression model. Good linear regression with $r^2 > 0.997$ was obtained in all relevant ranges. For intrabatch and interbatch precision and accuracy, the relative standard deviation (RSD) values ranged from 2.00 to 9.24 and 3.33 to 9.95%, respectively. The mean recoveries were from 76.46 to 120.14% with RSD less than 9.07%. These results indicated that the newly developed method was satisfactory with acceptable precision, accuracy, reproducibility, and recovery.

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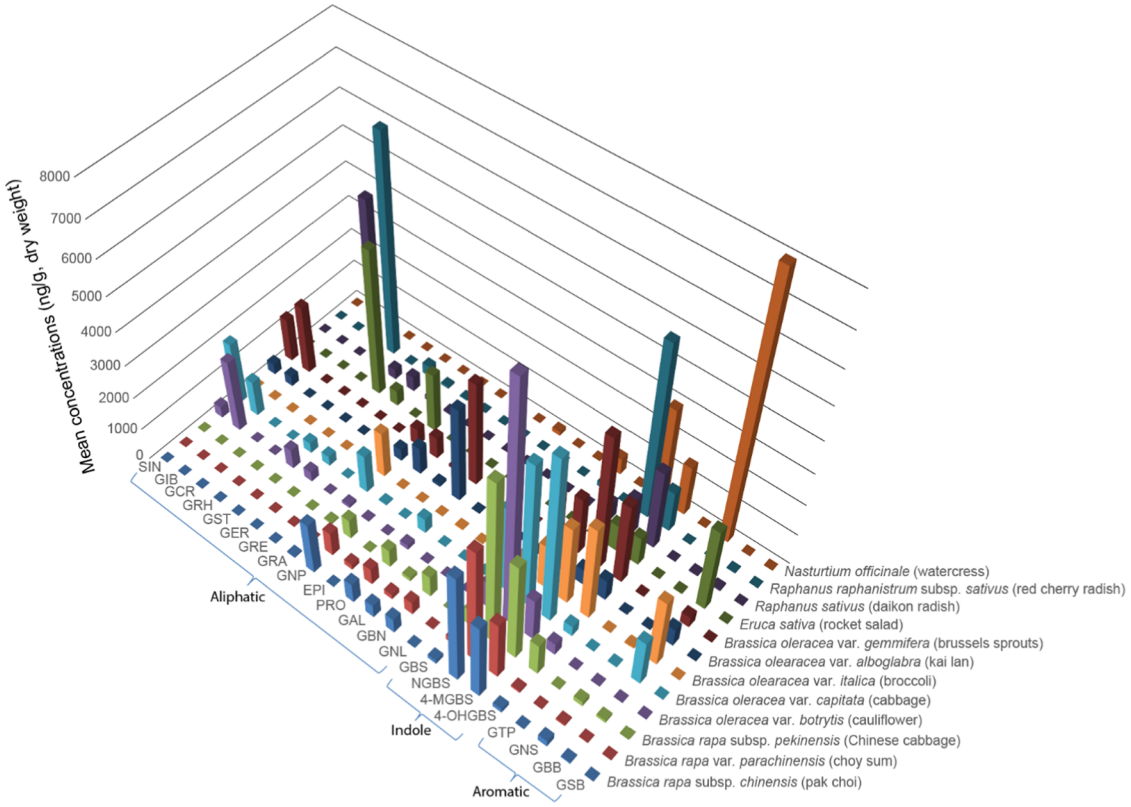

Figure 3. Mean concentrations of 22 glucosinolates in 12 *Brassicaceae* vegetables. SIN, sinigrin; GIB, glucoiberin; GCR, glucocheirolin; GRH, glucoraphasatin; GST, glucosativin; GER, glucoerucin; GRE, glucoraphenin; GRA, glucoraphanin; GNP, gluconapin; EPI, epiprogoitrin; PRO, progoitrin; GAL, glucoalyssin; GBN, glucobrassicanapin; GNL, gluconapoleiferin; GBS, glucobrassicin; NGBS, neoglucobrassicin; 4-MGBS, 4-methoxyglucobrassicin; 4-OHGBS, 4-hydroxyglucobrassicin; GTP, glucotropaeolin; GNS, gluconasturtiin; GBB, glucobarbarin; and GSB, glucosinalbin.

Glucosinolate Profiles. A total of 22 glucosinolates were quantified in this study, including 14 aliphatic glucosinolates (sinigrin, glucoiberin, glucocheirolin, glucoraphasatin, glucosativin, glucoerucin, glucoraphenin, glucoraphanin, gluconapin, progoitrin, epiprogoitrin, glucoalyssin, glucobrassicanapin, and gluconapoleiferin), 4 indolic glucosinolates (glucobrassicin, neoglucobrassicin, 4-methoxyglucobrassicin, and 4-hydroxyglucobrassicin), and 4 aromatic glucosinolates (glucotropaeolin, gluconasturtiin, glucobarbarin, and glucosinalbin) (Table 1). Of these, 17 glucosinolates with available standards were absolutely quantified in the 12 vegetables examined and 5 glucosinolates without available standards were semiquantified. In this study, the five glucosinolates without available standards were tentatively identified on the basis of the specific MS^n patterns of glucosinolates (Supporting Information Table S1). Briefly, glucosinolates can produce specific MS^2 fragmentations at m/z 195, 241, 259, and 275, and furthermore, the most abundant MS^2 fragmentation of m/z 259 gives rise to MS^3 fragmentations at m/z 139 and 97.[18] Two isomers, neoglucobrassicin and 4-methoxyglucobrassicin, which exhibit identical molecular masses, were differentiated by comparison with reported MS^2 fragmentation ions ratio and elution sequence during reversed-phase high-performance liquid chromatography.[19] The concentrations of glucosinolates were determined from the experimental peak area by analytical extrapolation in a standard calibration curve, and expressed as μg/g of dry weight. In the semiquantified analysis, glucoraphasatin and glucosativin were quantified as glucoerucin equivalents, gluconapoleiferin was quantified as glucobrassicanapin equivalent, and neoglucobrassicin and 4-methoxyglucobrassicin were quantified as glucobrassicin equivalents (Table 1).

Different Glucosinolates among 12 *Brassicaceae* Vegetables. The expression of 22 glucosinolates in the 12 *Brassicaceae* vegetables is shown in Figure 3 and Supporting Information Table S2. As shown in Figure 3, we found that the concentration and composition of glucosinolates varied significantly among 12 vegetables. Of the 12 vegetables examined, cabbage had the highest level of total glucosinolates (in μg/g dry weight: 19 551.2 ± 1317.7), while Kai Lan had the lowest level of total glucosinolates (in μg/g dry weight: 7611.3 ± 868.4) (Supporting Information Table S2). This agrees with the concentrations of total glucosinolates in previous reports.[14,20,21] Moreover, we noted that aliphatic and indolic glucosinolates were the major components in the 12 vegetables representing 76−100% of total glucosinolates, except watercress (37%) (Figure 1). Specifically, aliphatic glucosinolates constituted 61% of total glucosinolates in rocket salad. The indolic glucosinolates accounted for the major components in *Brassica* vegetables, including pak choi, choy sum, Chinese cabbage, cauliflower, Brussels sprouts, cabbage, and broccoli, ranging from 54 to 82%. For daikon radish and red cherry radish, subspecies of *R. sativus*, the contents of aliphatic and indole glucosinolates were almost equal (40−59%). These results are in accordance with a recent finding that aliphatic and indolic glucosinolates are the major components in *Brassicaceae* vegetables,[1] although our study

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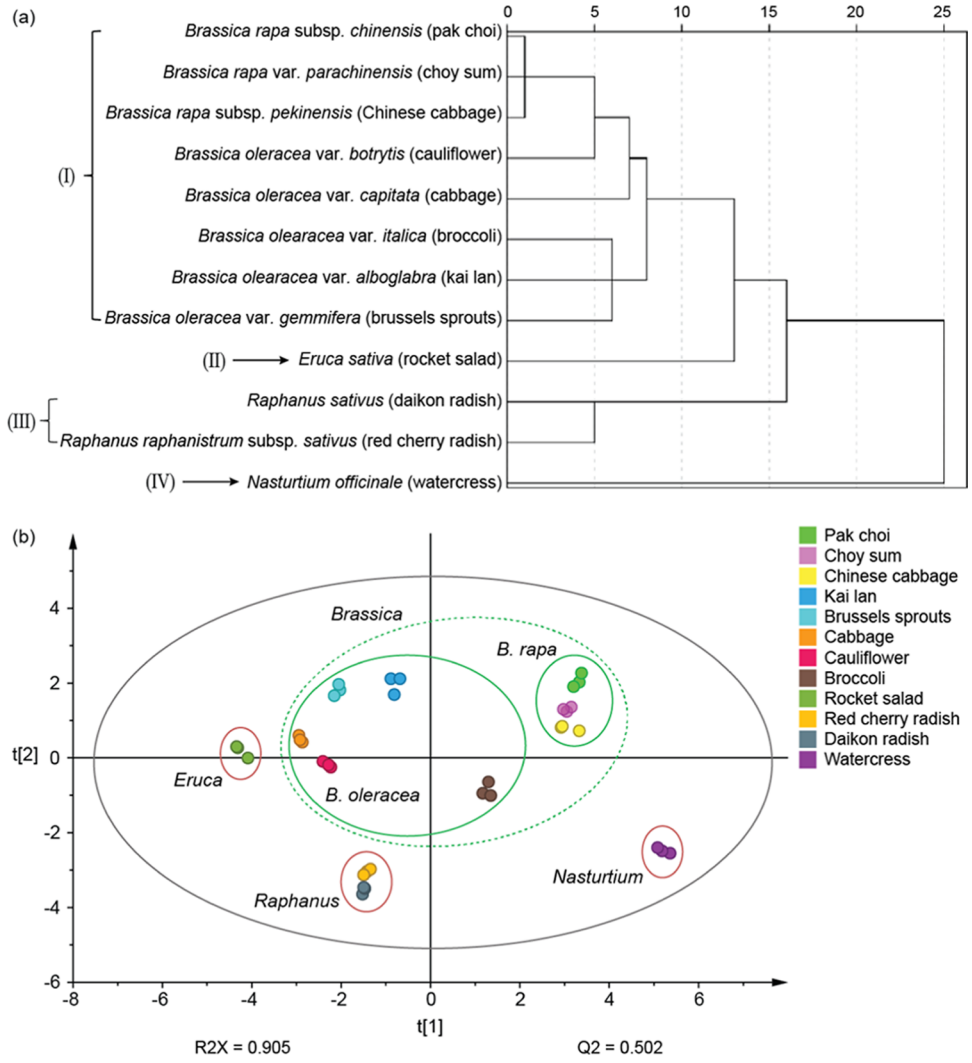

Figure 4. Cluster analysis of 12 *Brassicaceae* vegetables based on the content of 22 glucosinolates. (a) Hierarchical cluster analysis. (b) Principal component analysis.

investigated a more comprehensive range of *Brassicaceae* vegetables and a wider range of glucosinolates.

As shown in the more detailed data in Supporting Information Table S2, glucoraphanin, gluconapin, 4-methoxyglucobrassicin, 4-hydroxyglucobrassicin, and gluconasturtiin were the 5 most common glucosinolates found in all 12 vegetables. Sinigrin and glucoiberin were the most predominant glucosinolates in Kai Lan, Brussels sprouts, cabbage, and cauliflower, whereas glucobrassicanapin and glucoalyssin were the most predominant glucosinolates in choy sum, pak choi, and Chinese cabbage. It was also noted that glucocheirolin was detected only in cabbage and Kai Lan. Glucoerucin was a major glucosinolate in rocket salad but was also detected in white radish, red cherry radish, cabbage, and cauliflower. The highest concentration of glucoraphenin and glucoraphasatin was observed in white radish and red cherry radish, whereas notably glucoraphenin was found only in white radish and red cherry radish. The concentrations of glucosativin, glucoerucin, glucoraphanin, glucobarbarin, and glucosinalbin were detected in relatively higher quantities in rocket salad as compared to those in other vegetables. Furthermore, glucosinalbin was present only in rocket salad. In contrast to other *Brassicaceae* vegetables, gluconasturtiin was the predominant glucosinolate in watercress. In brief, the major profiles of the 22 glucosinolates quantified in the 12 vegetables were consistent with previous studies that focused on these vegetables.[14,20,22]

In addition to dry weight, we also determined fresh weight concentrations of glucosinolates in μg/g (Supporting Information Table S3). As noted, dry and fresh weights of glucosinolates showed similar glucosinolate patterns in the 12 vegetables.

Chemotaxonomic Classification. The botanical taxonomic status of the family *Brassicaceae* is quite complex as different concepts for the classification and status of the *Brassicaceae* species and subspecies exist.[23] With the development of DNA sequencing methods, biological systematic analysis has increasingly been based on DNA sequence analysis.[24] In addition to genetic taxonomy, phytochemical taxonomy has been shown to be able to provide supplementary information in species identification in the last decade.[25] Following a previously reported protocol,[26] we investigated the potential use of glucosinolate profiles as reference markers for chemotaxonomic classification of the 12 *Brassicaceae* vegetables using both hierarchical cluster analysis (HCA) and principal component analysis (PCA). HCA dendrogram showed the division of the 12 vegetables into 4 major branches on the basis

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of the average concentrations of glucosinolates in each individual vegetable (Figure 4a). Vegetables in branch I belong to the *Brassica* genus and were further divided into two species, *B. rapa* and *B. oleracea*. As shown here, the results of glucosinolate-based chemotaxonomic classification were closely aligned with the morphology-based classification. To further explore the relationship among the 12 vegetables across 3 different batches, PCA, an unsupervised pattern recognition method, was performed using the same data. A two-dimensional PCA score plot was utilized to depict the general variation of glucosinolate profiles among the 36 samples (12 vegetables × 3 batches). As shown in Figure 4b, the samples were primarily divided into four clusters according to their species. The clustering pattern is consistent with the classification from the HCA tree. These findings demonstrate that glucosinolate-based chemotaxonomic classification can be used to distinguish different *Brassicaceae* species.

In summary, in this study, we established a new, reliable, and precise analytical method for the quantitative analysis of glucosinolates using HILIC−MS/MS, and described the accumulation patterns of 22 glucosinolates in 12 *Brassicaceae* vegetables. Significant variations in the concentrations and compositions of glucosinolates were observed among different *Brassicaceae* vegetables. On the basis of the content of 22 glucosinolates, the 12 vegetables can be divided into 4 clusters, which is consistent with morphological taxonomy. The findings demonstrate that phytochemical taxonomy has the potential to be used for distinguishing among *Brassicaceae* species by identifying variants of chemotaxonomic markers like glucosinolates.

MATERIALS AND METHODS

Chemicals and Reagents. Acetonitrile, methanol, formic acid, and ammonium formate were purchased from Sigma-Aldrich (St. Louis, MO). The 10 glucosinolate standards including glucocheirolin, progoitrin, glucoraphenin, epiprogoitrin, glucobrassicanapin, glucoalyssin, glucobrassicin, gluconasturtiin, 4-hydroxyglucobrassicin, and glucobarbarin were purchased from Cfm Oskar Tropitzsch GmbH (Marktredwitz, Germany). Another seven glucosinolate standards including sinigrin, glucoiberin, glucotropaeolin, glucoraphanin, gluconapin, glucoerucin, and glucosinalbin were purchased from ChromaDex (Santa Ana, CA). Distilled water was purified "in-house" using a Milli-Q purification system (Bedford, MA).

Sample Collection and Preparation. On the basis of earlier studies in Singapore[27,28] and vegetable-consumption patterns in the Asian region, 12 commonly consumed *Brassicaceae* vegetables, including *B. rapa* subsp. *chinensis* (pak choi), *B. rapa* var. *parachinensis* (choy sum), *B. rapa* subsp. *pekinensis* (Chinese cabbage), *B. oleracea* var. *botrytis* (cauliflower), *B. oleracea* var. *capitata* (cabbage), *Brassica olearacea* var. *italica* (broccoli), *Brassica olearacea* var. *alboglabra* (Kai Lan), *B. oleracea* var. *gemmifera* (Brussels sprouts), *E. sativa* (rocket salad), *R. sativus* (daikon radish), *Raphanus raphanistrum* subsp. *sativus* (red cherry radish), and *N. officinale* (watercress), were included in this study (Figure 1). Three batches of these 12 vegetables were purchased from three local supermarkets in Singapore. The fresh vegetables (whole tissue) were washed using tap water and then snap-frozen in liquid nitrogen and freeze-dried. The freeze-dried vegetables were ground into a fine powder and stored at −80 °C before analysis. For glucosinolates extraction, 100 mg of freeze-dried samples was treated with 1 mL of 70% methanol (v/v) containing 150 ng/mL of internal standard (glucosinalbin or glucoraphenin) and incubated at 70 °C for 10 min. After being cooled in an ice bath, the extracts were centrifuged at 15 000*g* for 15 min, and the supernatants were collected. The extraction procedure was repeated twice with 70% methanol (v/v) to give a final extract containing 50 ng/mL of internal standard. The supernatants were then combined and evaporated until dry at 40 °C. The dried samples were reconstituted in 3 mL of 70% acetonitrile (v/v) and filtered through a 0.22 μm nylon filter for analysis. Samples were extracted and analyzed in triplicates, and data were represented as mean ± SD (n = 3).

Glucosinolate Profiling and Identification. Agilent 1290 ultra-high-performance liquid chromatography system (Waldbronn, Germany) coupled to a 6540 quadrupole time-of-flight (Q-ToF) mass detector (Agilent, Santa Clara, CA) equipped with an electrospray ionization source was used for glucosinolate profiling and identification. The separation of glucosinolates was achieved on a Waters ACQUITY UPLC BEH HILIC column (2.1 × 100 mm^2, 1.7 μm). The mobile phase consisted of A (30% acetonitrile containing 10 mM ammonium formate, 0.1% formic acid) and B (95% acetonitrile containing 10 mM ammonium formate, 0.1% formic acid). The linear gradient was as follows: 0−1 min, 100−100% B; 1−5 min, 100−95% B; 5−8 min, 95−80% B; 8−12 min, 80−15% B at a column temperature of 35 °C with a flow rate of 0.4 mL/min. The autosampler was cooled at 4 °C, and 5 μL of the extract was injected. Electrospray ionization was performed in negative ion mode with the following source parameters: drying gas (N_2) temperature 200 °C with a flow rate of 14 L/min, nebulizer gas pressure 30 psi, sheath gas temperature 400 °C with a flow rate of 11 L/min, capillary voltage 3000 V, and nozzle voltage 800 V. The MS/MS analysis was carried out to study the structures of glucosinolates. Moreover, the commercial standards were analyzed to support the identification of glucosinolates.

Glucosinolate Quantification. Quantitative analysis of glucosinolates was performed on an Agilent 1290 ultra-high-performance liquid chromatography system (Waldbronn, Germany) coupled to a 6490 Triple Quadrupole (QQQ) mass detector (Agilent, Santa Clara, CA) equipped with iFunnel Technology and an electrospray ionization source. Separation of glucosinolates was achieved using the same method as in Q-ToF analysis. Mass spectra were recorded in the multiple reaction monitoring (MRM) mode. Data acquisition and processing were performed using MassHunter software version B.05.00 (Agilent Technologies, CA).

Method Validation. The proposed quantitative method was validated for its limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy, precision, and recovery, according to Food and Drug Administration guidelines for biological methods as in our previously published report.[29] Briefly, accurately weighed standards were dissolved in 70% acetonitrile separately and diluted to provide a series of standard solutions with gradient concentration to make the calibration curves. All of the solutions were stored at −20 °C. Method precision was studied by injecting the same mixed sample solution six times consecutively, both on the same day for intraday variation and on three consecutive days for interday variation. To check the reproducibility, six independently prepared samples from the mixed vegetable powder were analyzed. The recoveries were evaluated by spiking three defined amounts of

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glucosinolate standards (approximately equivalent to 0.8, 1.0, and 1.2 times the concentration of the matrix) into the mixed vegetable sample in triplicates and were extracted and quantified as described earlier.

Statistical Analysis. Independent measurements in triplicates were used for each sample in all statistical analyses. Values below the LOD were assigned as a proxy value of an LOD/2 as usual.[30] The data were diagnosed in histogram plots to be skewed and were therefore transformed into log 2-scale to meet the assumption of normality before statistical analysis. The differences in glucosinolate concentrations among the 12 vegetables were examined using one-way analysis of variance. Both hierarchical cluster analysis (HCA) and principal component analysis (PCA) were applied to identify similarities of glucosinolate profiles in 12 examined vegetables. In HCA, the between-groups linkage method as the amalgamation rule and the squared Euclidean distance as the metric were applied to establish clusters. Statistical analyses were performed using IBM SPSS Statistics 24 and SIMCA 14. A two-sided $p < 0.05$ was considered statistically significant.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b01668.

MS and MS^n information of five glucosinolates (Table S1); mean levels (μg/g dry weight, mean ± SD) of glucosinolates in 12 vegetables (Table S2); mean levels (μg/g fresh weight) of glucosinolates in 12 vegetables (Table S3) (PDF)

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Notes

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■ ABBREVIATIONS

RPLC, reversed-phase liquid chromatography; DAD, diode-array detector; MS, mass spectrometry; HILIC, hydrophilic interaction liquid chromatography; QQQ, triple quadrupole; MRM, multiple reaction monitoring; LOD, limit of detection; HCA, hierarchical cluster analysis; PCA, principal component analysis; RSD, relative standard deviation

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