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Challenges of developing a valid dietary glucosinolate database☆,☆☆

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ABSTRACT

Glucosinolates are a group of important cancer chemo-preventive sulfur-containing compounds in cruciferous vegetables. To estimate their dietary intake, there is a great need to develop a valid glucosinolate database. The aim of this study was to investigate the key challenges in developing such database. First, three commonly used enzyme deactivation methods (blanching, steaming and microwaving) were compared with samples of raw untreated broccoli and kale. Steaming and microwaving were found to effectively deactivate myrosinase, both led to significantly higher glucosinolate values that blanching (~15–50% higher). Glucosinolates in untreated broccoli was similar to that of blanching broccoli, while glucosinolates were not detected in untreated kale. Heat treatment was also shown to alter the profiles of individual glucosinolate. Quantification of total glucosinolates of four common vegetables was compared by the two most commonly used analytical methods (ISO 9167-1 method and cyclocondensation method). Except for kale, the results from ISO 9167-1 were much higher (~6–8 fold) than that from cyclocondensation method in other three vegetables. In conclusion, the sample preparation procedure, analytical method for quantification and the compounds to be measured must be considered and validated in order to develop a valid dietary glucosinolate database.

1. Introduction

Cruciferous or *Brassica* vegetables refer to a large group of commonly consumed vegetables, including broccoli, kale, cabbages, cauliflower, Brussels sprouts, etc. Epidemiological evidence from prospective cohort studies and retrospective case–control studies have linked consumption of cruciferous vegetables to reduced risk of various cancers, including lung (Wu et al., 2015), gastric (Bosetti et al., 2012), colorectal (Azeem et al., 2015), breast (Bosetti et al., 2012), bladder (Al-Zalabani et al., 2016) and prostate cancer (Chan et al., 2009). Like most other vegetables, cruciferous vegetables are good sources of a variety of nutrients and phytonutrients including vitamins, carotenoids, folate, selenium, glucosinolates and flavonoids. Nonetheless, a large body of evidence accumulated in the past decades suggested that the cancer chemo-preventive effects of cruciferous vegetables may largely be attributed to glucosinolates and their breakdown products (Clarke, 2010).

Glucosinolates are sulfur rich, anionic secondary metabolites found principally in the plant order Brassicales. Over 350 genera and 3000 species in Brassicaceae (older name, Cruciferae), as well as a number of non-cruciferous plants, contain glucosinolates; the genus *Brassica* contain most of the commonly consumed vegetables (Fahey et al., 2001).

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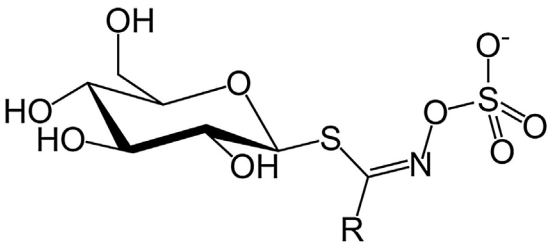

Fig. 1. Chemical structure of glucosinolate.

Chemically, glucosinolates are β-thioglucoside N-hydroxysulfates (also known as (Z)-N-hydroxysulfates or S-glucopyranosyl thiohydroximates) (Fahey et al., 2001) (Fig. 1). The number of reported glucosinolates is now approaching 200, which differ from each other by the side chain R (Clarke, 2010). The glucosinolates are grouped by the structures of their side chain R as three major groups: aliphatic, aromatic and heterocylic (indole) glucosinolates (Fahey et al., 2001).

Types of glucosinolates and their concentration vary enormously among different cruciferous vegetables, both qualitatively and quantitatively, according to species and cultivar, tissue type, growing stage, environmental factors, insect attack and microorganism intrusion (Holst and Williamson, 2004), which complicates the interpretation of epidemiological studies. The total glucosinolate intake in German population was estimated as 14.2 ± 1.1 mg/day for men and 14.8 ± 1.3 mg/day for women (Steinbrecher and Linseisen, 2009); 6.5 mg/day, among which 35% were of indole type in a Spanish adult population (Agudo et al., 2008) and the national mean daily intake is calculated to be 46.1 mg in fresh material, and 29.4 mg in cooked in UK (Sones et al., 1984). However, these crude dietary intake estimates were made based on very limited data or dietary exposure to cruciferous vegetables. There is no dietary intake of glucosinolates estimation in US, due to lack of adequate dietary glucosinolate composition data. To establish the link between the intake of *Brassica* vegetables and the risk of cancer and other chronic diseases, to estimate the dietary intake of glucosinolates and to precisely understand the mechanisms of their health benefits, there is a great need of for a valid database of dietary glucosinolates.

An extensive literature search found only one published study attempted to develop a dietary glucosinolate database (McNaughton and Marks, 2003). This paper was published in 2003 and has since been widely cited in epidemiological studies and used to estimate dietary intake of dietary glucosinolates. However, only 18 references were included in developing this database, and among them 10 studies used the glucose-release methods, 5 used high performance liquid chromatography (HPLC) and 3 used gas chromatography (GC) based methods. Because of the limitation of analytical methods, no data on individual glucosinolates were provided. With significantly more data generated in the last 13 years, and most importantly, the advancement of new analytical methods, it is possible and necessary to develop a new dietary glucosinolate database.

The US Department of Agriculture (USDA)'s Nutrient Data Lab (NDL) has undertaken the development of a Special Interest Database, beginning with a search of the scientific literature. Like the other bioactive databases developed in our lab, such as the flavonoid databases (Bhagwat et al., 2015), the glucosinolate data from the literature must be critically evaluated for 5 quality evaluation categories (sampling plan, sample handling, analytical method, analytical quality control and number of samples), using the data quality evaluation system developed by the USDA (Holden et al., 2005). The data quality evaluation system is modified and made specific for each different group of dietary compounds. Understanding the chemo-physical properties, structure diversity and key influential factors of the bioactive compounds are key to develop valid data quality evaluation criteria. This preliminary study was designed to address the key challenges related to sample preparation and quantitative analysis of glucosinolates in plant foods. The results will be used to develop a data quality evaluation system for dietary glucosinolates.

2. Materials and methods

2.1. Chemicals and reagent

Methanol-HPLC grade, Sodium Acetate, DEAE Sepharose CL–6 B suspension, Imidazole formate and Sinigrin hydrate, Benzene-1,2-dithiol, sulfatase (from *Helix pomatia*) and myrosinase were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1,3-benzodithiole-2-thione was synthesized in the Food Composition and Method Development Lab and purified with thin layer chromatography (TLC). The purity (96.2%) was examined with nuclear magnetic resonance spectroscopy (NMR) (Bruker AVIII–600 MHz, Rheinstetten, Germany).

2.2. Plant materials and processing methods

Selected cruciferous vegetables of the *Brassica oleracea* species (broccoli – Italica Group; kale – Acephala Group; collard greens – Acephala Group; and red cabbage – Capitata Group) were purchased from local grocery stores in Beltsville, MD. The vegetables were cleaned by rinsing with tap water for approximately 60–90 s, followed by rinsing with distilled water. Inedible parts (leaves/scales, hard stems) were cut off and the vegetables were cut into 5 cm × 3 cm pieces. Vegetable pieces were mixed thoroughly prior to further processing. Samples without further processing (control) were freeze-dried immediately after cutting.

Three common enzyme deactivation methods are blanching, steaming and microwaving. The vegetables were cut just before cooking to prevent enzyme reaction and oxidation. For blanching, tap water was added into a stainless steel pot and was heated to boiling. Vegetable pieces were put into the pot for 85 s, then quickly removed from the boiling water and spread on a plate to cool. The cooked vegetables were freeze-dried before analysis. For steaming, the steamer basket was inserted to a stainless steel pot containing approximately 2.5 cm (1 in) of water, with the bottom of steamer basket approximately 5 cm (2 in) above the water surface. The water in the pot was brought to a boil over high heat and the vegetables pieces were scattered over the steamer basket and steamed in medium heat for 5 min. When the vegetables were done, they were quickly removed from the steamer basket and were spread on a plate to cool. For microwaving, vegetable pieces was put in a microwave safe bowl with a teaspoon of water. The bowl was then covered with microwave-safe plastic wrap and microwaved for 1 min at full power (1200W, 2450 MHz) in a microwave oven (Panasonic Model NE-12521, Newark, NJ, USA). The samples were then removed from the microwave and were spread on a plate to cool. After cooling down to room temperature, all cooked vegetables were freeze-dried and stored in a freezer at −20 °C before analysis.

2.3. Extraction, purification and sample preparation

Extraction of glucosinolates was carried out following the ISO9167-1 method (EEC, 1992) with slight modification (Sun et al., 2015). The freeze-dried vegetable samples (both treated and untreated) were ground into powder, and 100 mg of each sample was transferred into a pre-heated test tube at 75 °C for 1 min. Then 5 mL methanol was added and vortexed for 20 s. Next, 200 μL 5 mM sinigrin (internal standard) solution was added to the tube. The extraction solution was kept in the heating block for another 20 min. After the solution cooled down, additional methanol was added back to the liquid level marked. The individual extract was filtered with 0.45 μm syringe filter and filtrate was transferred to HPLC vial for analysis. Each sample was extract three times.

For glucosinolate to be analyzed as desulfated forms, anion exchange resin in mini-columns was used to bind the glucosinolate from

the vegetable extract and then the bound glucosinolate molecules were desulfated with an overnight incubation on the resin. Briefly, a 100 mg vegetable sample was transferred to a test tube and the sample was processed following the previous procedure without adding internal standard. Each sample was extract three times; 10 g of DEAE Sephadex A25 resin was mixed in excess of 2 mol/L acetic acid solution, and was left to settle. Then 2 mol/L acetic acid was added until the volume of the liquid equal to twice the volume of the sediment. The mini-column was placed vertically on a stand and 500 μL ion-exchange of resin was add to each column. Then 1 mL of immidazole formate solution (6 mol/L) was added to each mini-column, followed by rinsing twice with 1 mL of water each time.

Next, 1 mL of the vegetable extract was transferred to a prepared column without disturbing the resin surface and to allow draining. Two 1 mL portions of the sodium acetate buffer (pH = 4) were added, allowing the buffer to drain after each addition. Next, 100 μL of diluted purified sulfatase solution was added to the column and the column was left to react overnight at ambient temperature. A tube was placed under the column to collect the eluate. The desulfo-glucosinolate was obtained with two 1 mL portions of water, allowing the water to drain after each addition and mixing the eluate well (EEC, 1992). The 1,2-benzenedithiol-based cyclocondensation assay was used for quantitation of isothiocyanates (Zhang, 2012; Zhang et al., 1996). Two proportion of lyophilized vegetable powder (0.4 g each) were added with either 200 μL myrosinase IU/mL or 200 μL water (as a control sample, measuring the endogenous isothiocyanates). The sample was mixed thoroughly by vortex and was incubated at 30 °C in the dark overnight for complete hydrolysis. In a 7-mL glass vial, 200 μL ammonium acetate buffer was added, followed by adding 200 μL vegetable extract with and without myrosinase (flushed with nitrogen), and then 400 μL benzene-1,2-dithiol (BDT) solution (flushed with nitrogen). The sample was vortexed for 30 s and then incubated in water bath at 65 °C for 2 h. The sample was left to cool to room temperature and ammonium acetate buffer was added back to the level marked and then centrifuged at 2795*g* for 10 min (Fisher Scientific Centrific Centrifuge, Waltham, MA, USA). The supernatant was filtered through a 0.22 μm PTFE filter, and 2 μL was injected for HPLC analysis.

2.4. Instrument and conditions for cyclocondensation method

An Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA, USA) with G1311 quaternary pump, G1329 B autosampler, G1316A thermostatted column compartment and G1313 diode array detector was used for analysis. Separation was carried out on an Agilent Poreshell C_{18} column (150 × 3.0 mm, 2.7 μm) with an in-line filter (Analytical Scientific Instruments, Richmond, CA, USA). The mobile phase consisted of MeOH (70%) and H_2O (30%) running at a flow rate of 0.4 mL/min with a sample injection volume of 10 μL and a detection wavelength at 365 nm. Column temperature was set at 30 °C. 1,3-benzodithiole-2-thione was used as reference standard and the calibration curve was generated with concentrations at 0.1, 1, 10 and 100 μg/mL.

2.5. Instrumentation for ISO-9167-1 method

Analyses were performed on an Agilent 1290 UHPLC (Agilent Technologies, Palo Alto, CA, USA) equipped with a diode array detector. An Agilent RRHD Eclipseplus C_{18} 2.1 × 150 mm, 1.8 μm column was used for separation. Elution was performed using mobile phase A (0.1% formic acid aqueous solution) and mobile phase B (0.1 formic Acid: acetonitrile, 50:50). The flow rate was 0.4 mL/min, and detection was at 229 nm. Two gradient systems that were used were as follows: 2–10% B, 0–10 min; 10–50% B, 10–25 min; 50–90% B, 25–30 min; 20–30% B. The re-equilibration time is 5 min. An LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was used for the confirmation of each individual glucosinolates. The optimized conditions were set as follows: sheath gas at 70 (arbitrary units), aux and sweep gas at 15 (arbitrary units), spray voltage at 4.8 kV, capillary temp at 270 °C, capillary voltage at 15 V, and tube lens at 70 V. The mass range was from 100 to 1000 amu with a resolution of 30,000, FTMS AGC target at 2e5, FT-MS/MS AGC target at 1e5, isolation width of 1.5 amu, and max ion injection time of 500 ms. Source CID was set at 20%. The most intense ion was selected for the data-dependent scan to offer their MS^2 to MS^3 product ions, respectively, with a normalization collision energy at 35%. Quantification was conducted using desulfated glucosinolates, with desulfo-sinigrin as internal standard and response factors were calculated against internal standard (Table 1) (EEC, 1992).

Table 1
Response factor used in this study.

No.	Desulfo glucosinolate	Response factor
1	desulfosinigrin	1.00
2	desulfoglucoraphanin	1.07
3	Desulfo-4-hydroxyglucobrassicin	0.28
4	Other desulfo- hydroxyglucobrassicin	0.28
5	Desulfo-glucobrassicin	0.20
6	Desulfo-4-methoxyglucobrassicin	0.25
7	Other desulfo methoxyglucobrassicin	0.25
8	Other glucosinolate	1.00

2.6. Statistical analysis

Data were expressed as mean value ± SD (n = 3), or the mean (n = 2). Student's *t*-test was used to analyze differences between groups. A value of $P < 0.05$ was considered as significant difference unless otherwise mentioned. Statistical analyses were performed using SigmaStat statistical software (SigmaStat 3.5).

3. Results and discussion

3.1. Challenge 1–enzyme reaction and sample preparation

Glucosinolates are stored in the plant vacuole, separately from enzyme myrosinase (β-thioglucosidase). Upon cell rupture, myrosinase comes into contact with the glucosinolates and the enzymatic degradation is quickly initiated, resulting in a variety of breakdown products (Hanschen et al., 2014; Redovniković et al., 2008). Breakdown products (isothiocyanates, thiocyanates, nitriles, epithionitriles, oxazolidine-2-thiones) are formed depending upon the side chain structures, reaction conditions and protein factors (e.g. epithiospecifier protein, ESP) (Fahey et al., 2001; Redovniković et al., 2008) (Fig. 2). This phenomenon is described by plant physiologists as glucosinolate-myrosinase defense system against herbivore damage (Hopkins et al., 2009; Textor and Gershenzon, 2009), and it explains why the reaction happens almost immediately after plant tissue damage.

One of the most important principles of developing a database of nutrient or bioactive compounds is to provide accurate descriptions of the nutrient or bioactive compounds included as well as the quantity found in a given food. Furthermore, care must be taken not to alter the sample during preparation, as this could affect the results. In cruciferous vegetables, sample preparation processes like grinding, cutting or even freeze-drying will break the cells and release the myrosinase, which initiate a rapid hydrolysis of the glucosinolates (Mithen et al., 2000). On the other hand, as a result of tissue damage, *de novo* synthesis, especially indole glucosinolates, may balance or indeed exceed losses due to enzymatic hydrolysis (Holst and Williamson, 2004). To avoid the loss or the formation of artificial compounds during sample preparation, it is a necessary first step to deactivate enzymes that are responsible for these reactions.

In this study, three quick and commonly used ways to deactivate enzyme by heat – blanching, steaming and microwaving – were compared with samples of raw untreated broccoli and kale (Fig. 3). It was

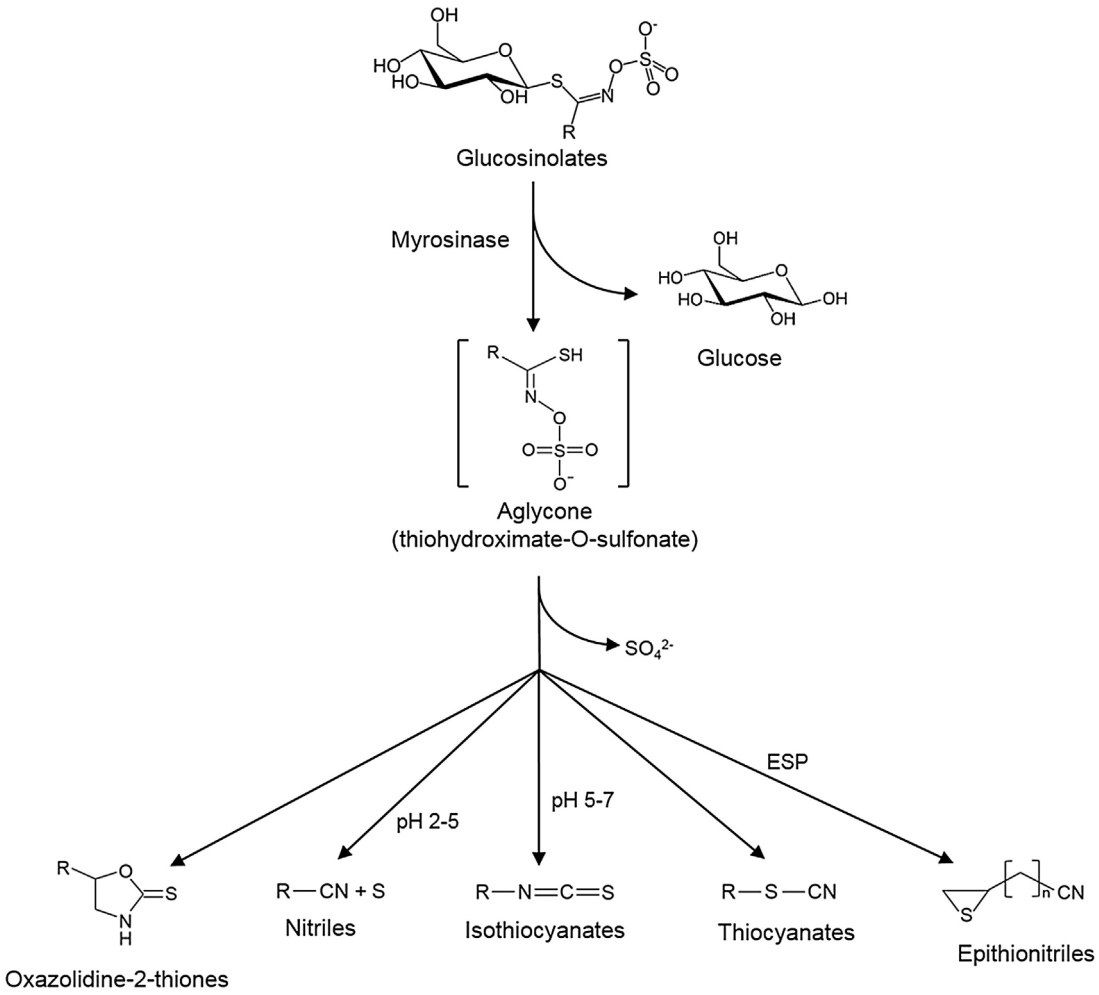

Fig. 2. Formation of different breakdown products from different glucosinolates hydrolyzed by myrosinase under different reaction conditions.

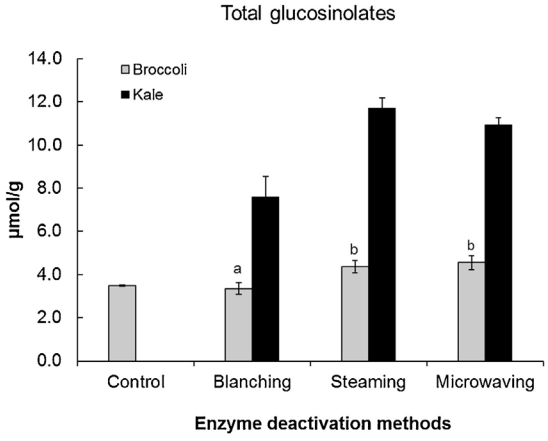

Fig. 3. Total glucosinolate contents in broccoli and kale without and after enzyme deactivation by three commonly used methods (blanching, steaming and microwaving) (mean ± SD, n = 3, different letters indicate statistically different).

shown that for broccoli, steaming and microwaving preserved more glucosinolates than blanching because some glucosinolates may be leached into the cooking water. There was statistically no difference between the values from blanching broccoli and untreated broccoli, both of which suffered minimal loss. The results on kale were surprising as no glucosinolates were detected in untreated kale. Steaming and microwaving were once again shown to be the better methods to preserve glucosinolates, with steaming providing slightly higher values (not significant). Blanching could also preserve glucosinolates, but not as effectively as steaming or microwaving. One possible reason for this dramatic difference between broccoli and kale might be their different enzyme activities. Research has found large variations in myrosinase activity among various cruciferous vegetables. In one study, myrosinase activity could range from 0.3 μmol/min/mg (watercress) to 10.5 μmol/min/mg (radish) (Wilkinson et al., 1984).

While heat treatment can effectively deactivate myrosinase, chemically induced degradation of glucosinolates, usually from heat treatment, may also occur. The formation of chemical breakdown products of glucosinolates is determined by the chemical structure of the glucosinolate, the thermal treatment conditions, and additional factors, such as the surrounding matrix, water content, iron concentration, and the pH value (Bones and Rossiter, 2006; Hanschen et al., 2014). Chemical degradation may not change the total glucosinolates significantly, but would likely change the compound profile of individual glucosinolates. This was indeed shown from this study (Fig. 4). Different enzyme deactivation methods led to different glucosinolate profiles in both broccoli and kale, and it appeared to be determined by the chemical structures of glucosinolate.

Based on discussions above, any glucosinolate analysis of food needs to be preceded by deactivation of enzymes, and they must be handled with great care to avoid degradation during sample preparation. Only by following these procedures can accurate values be determined for inclusion in food composition databases. However, one must also consider that because of the inherent limitations, enzyme deactivation may inevitably change the glucosinolate profile.

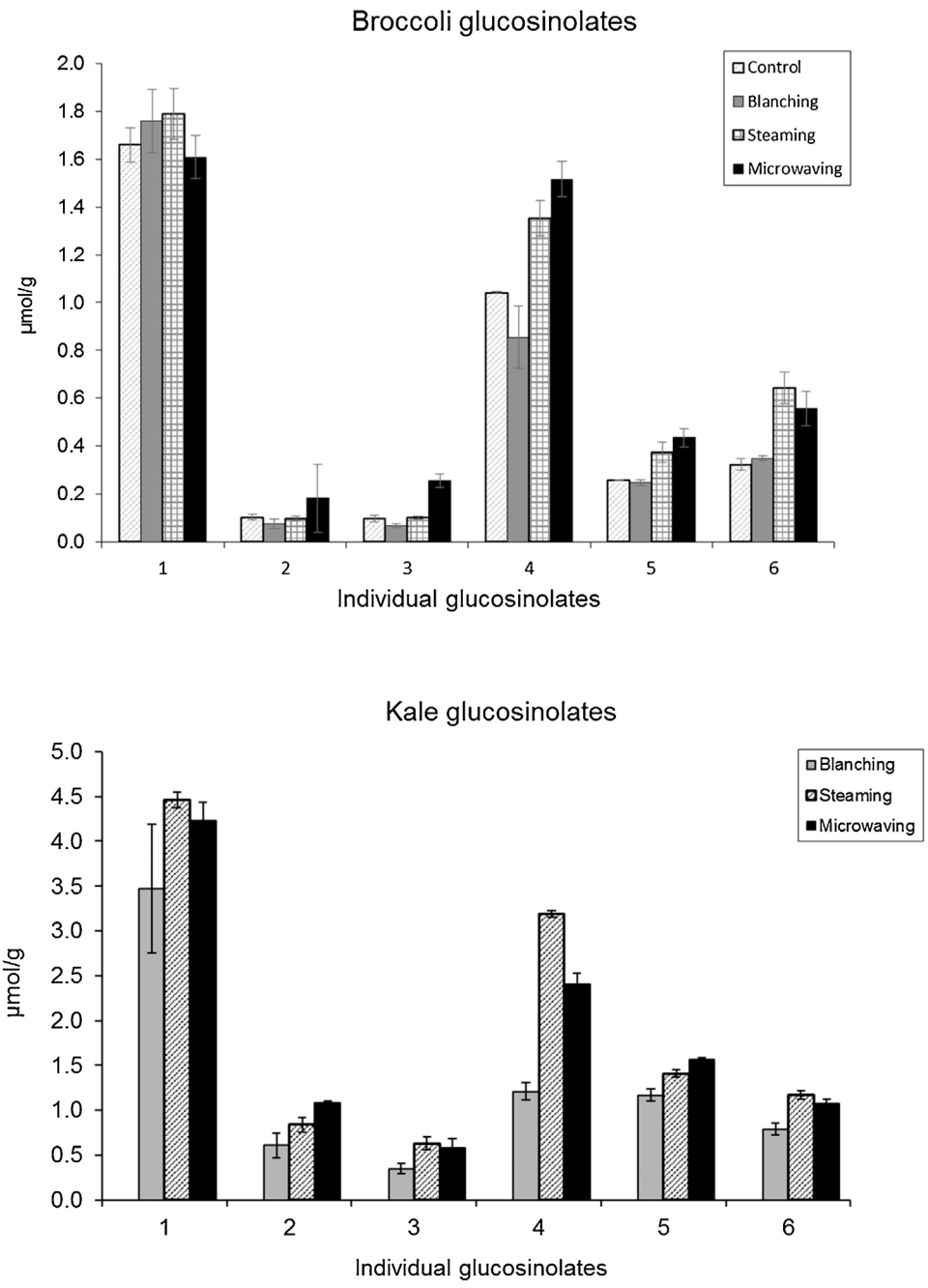

Fig. 4. Contents of individual glucosinolates in broccoli and kale with without and after enzyme deactivation by three commonly used methods (blanching, steaming and microwaving) (mean ± SD, n = 3). Numbers in x-axis represented individual glucosinolates (broccoli: 1. glucoraphanin; 2. 4-hydroxy glucobrassicin; 3. hydroxy glucobrassicin; 4. glucobrassicin; 5. 4-methoxyl-glucobrassicin; 6. neoglucobrassicin; kale: 1. gluconapoleiferin; 2. sinigrin; 3. hydroxyglucobrassicin; 4. glucobrassicin; 5. methoxy-glucobrassicin; 6. neoglucobrassicin).

3.2. Challenge 2–analytical methods

In general, for the analytical data to be considered as high quality for a food composition database, the analytical methods must show specificity, good precision and accuracy, a wide dynamic range, high sensitivity and applicability to different food matrices. Another important principle is to analyze the original/intact compounds as much as possible to avoid losses or formation of artificial compounds during sample processing or derivatization steps. Analytical method has been a challenge for measuring dietary glucosinolates in the past and appears to be a major reason for the lack of valid data for a database. In general, the methods of analyzing glucosinolates can be divided into two categories: 1) chromatography-based methods, such as HPLC–MS and GC, which are capable of measuring individual glucosinolates; and 2) spectroscopic/colorimetric assays, used to determine total glucosinolates, usually by measuring breakdown products of glucosinolates (Clarke, 2010; Mithen et al., 2000) (Fig. 5). Among the commonly used methods illustrated in Fig. 5, HPLC–MS is the most widely used to measure intact glucosinolates, desulfated glucosinolates (ISO 9167-1) (EEC, 1992) and isothiocyanates, providing information on individual compounds.

Glucosinolate data from different quantification methods, as well as the data from different sample preparation steps, could differ from one another significantly. In this study, the quantification of total glucosinolates of four common vegetables (red cabbage, broccoli, kale, and collard) was compared by the two most commonly used methods in the literature: ISO 9167-1 method and 1,2-benzenedithiol-based cyclocondensation assay (Fahey et al., 2001) (Fig. 6). The data showed that except for kale, the results from ISO 9167-1 were much higher than those from cyclocondensation method in all three other vegetables. This could be explained partly by the multiple reaction steps to form 1,3-benzodithiole-2-thione. As is shown in Fig. 2, after hydrolytic cleavage of the glucose by myrosinase, an unstable aglycone thiohydroximate-*O*-sulfonate is formed from aliphatic and aromatic glucosinolates. This

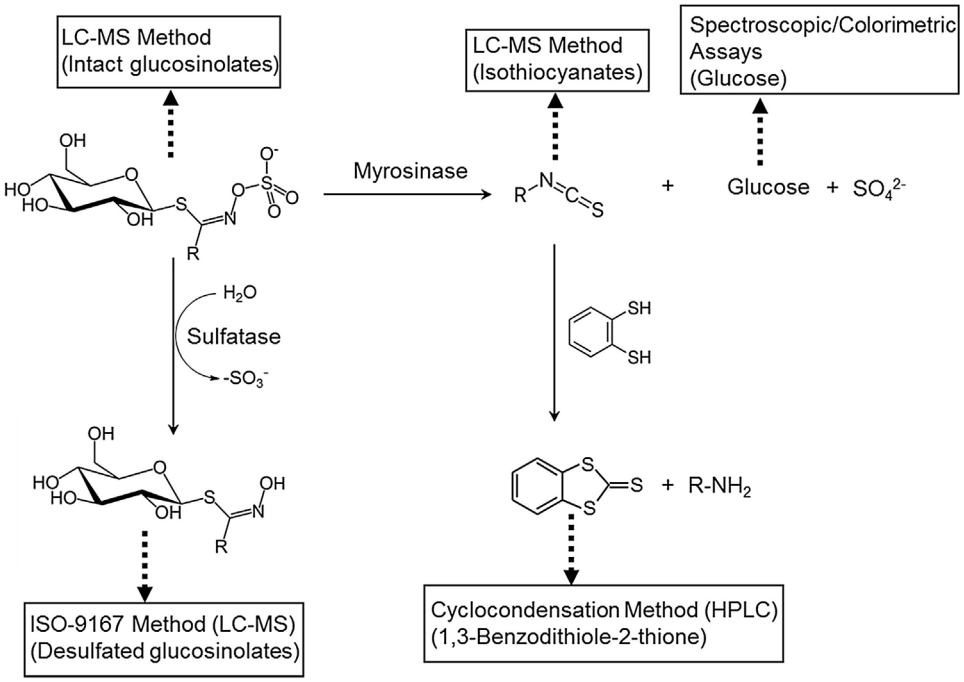

Fig. 5. Brief description of commonly used analytical methods in analyzing and quantifying glucosinolates.

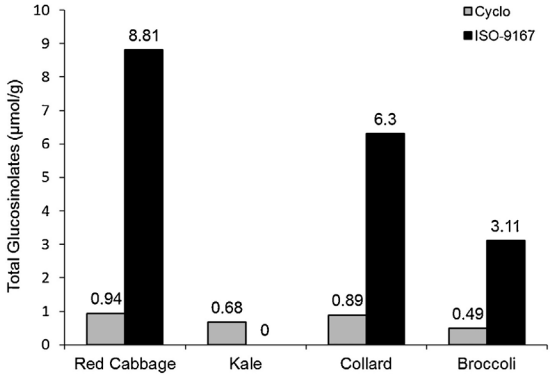

Fig. 6. Comparison of total glucosinolates measured by cyclocondensation method and ISO-9167 method (mean, n = 2).

unstable intermediate then rearranges to form isothiocyanates, or other breakdown products depending upon the side chain structures, reaction conditions and protein factors. The isothiocyanates formed from indole glucosinolates are unstable, and decompose spontaneously to indole-3-carbinol, indole-acetonitrile, thiocyanate and 3,3′-diindolylmethane (Fahey et al., 2001). The isothiocyanates further react with 1,2-benzenedithiol to form 1,3-benzodithiole-2-thione, which can then be measured by HPLC. Therefore, cyclocondensation method actually measures just a portion of total glucosinolates. Only the glucosinolates that form stable nonvolatile isothiocyanates can be measured, which were mostly from aliphatic and aromatic glucosinolates.

Moreover, an increasing number of studies have shown that individual glucosinolates and isothiocyanates have different biological effects (Holst and Williamson, 2004). Therefore, in developing a glucosinolate database, only analytical methods that can provide information on individual glucosinolates will be accepted. In the past, analysis of the intact glucosinolates was not possible because separations of intact glucosinolates were very difficult owing to their chemical properties (high polarity and strong anions) (Clarke, 2010). Recently, the advancement of chromatographic stationary phases, as well as state-of-the-art analytical instrumentation, mainly HPLC coupled with mass spectrometer featuring novel ionization techniques and detector configurations, allowed glucosinolates to be analyzed in their intact forms (Ares et al., 2015, 2014; Franco et al., 2016; Mohn et al., 2007; Tian et al., 2005). Nevertheless, one of the major problems has been the lack of suitable reference standards. There are only a handful of commercially available glucosinolate standards, of which the most commonly used one is 2-propenylglucosinolate (sinigrin). However, sinigrin is not a suitable internal standard because of the presence of this compound in most cruciferous vegetables (Mithen et al., 2000). In addition, as glucosinolates exist naturally as salt, the purity of the standards is often problematic. More data are needed on the absolute purity of standards, especially with respect to water and salt content, stability of standard mixtures in solution, extraction recoveries and myrosinase deactivation (Clarke, 2010). In developing a glucosinolate database, data obtained by measuring intact glucosinolates will be considered optimal if the methods are carefully validated and the individual standards are used in quantification.

The limitation of analyzing intact glucosinolates can be circumvented by using hydrolysis to produce more chromatographically amenable forms via sulfatase to desulfo-glucosinolates, known as the EU official method ISO 9167-1 (1992) (EEC, 1992), which relies on the relative response factors (RRF) of the desulfo-glucosinolates to quantify individual glucosinolates. While response factors may vary up to 10-fold (0.2–2.1), the majority of determinations lie within a very narrow range, indicating that chain elongation and the sulfur oxidation state produce insignificant effects. Hydroxylalkyl groups have the highest values, while indoles, hydroxybenzyl and benzoate esters have the lowest (Clarke, 2010). Since it was developed, ISO 9167-1 remains as a favored method and has been widely used in the quantification of glucosinolates. However, the major drawbacks of this method are that it requires a long, tedious sample preparation procedure, and sometimes it is hard to ensure the specificity and accuracy of degradation products. Based on our preliminary literature review, the majority of analytical data on cruciferous vegetables were obtained by using this method, and it is also considered as an accepted method.

Although measuring isothiocyanate by HPLC-based methods can theoretically be used to quantify individual glucosinolates, this method is rarely used in the literature.

3.3. Challenge 3–what to measure

The purpose for a database of dietary bioactive compounds is to

provide information on their types and contents, which can then be used in various epidemiological studies to evaluate their potential health benefits. Therefore, the compounds to be measured and presented in the database must be related to their health benefits. For glucosinolates, bioavailability studies have found that, in contrast to the evidence obtained from animal studies, intact glucosinolates have by far not been detected in the human body. Glucosinolates are therefore considered as the storage form or precursors of their biologically active aglycones (isothiocyanates). Nearly all of the biological activities of glucosinolates can be attributed to their hydrolytic products, of which the isothiocyanates are prominent forms. Moreover, most cruciferous vegetables are consumed after cooking. Due to the unique feature that glucosinolates coexist with myrosinase, glucosinolates are subject to quick hydrolysis after culinary processes such as chopping, cooking, freezing/thawing and fermentation. So it is likely that when these vegetables are consumed, both glucosinolates and their breakdown compounds exist, and in some cases the breakdown compounds could dominate. As was shown in Fig. 3, no glucosinolates were detected in freeze-dried kale without enzyme deactivation, while the isothiocyanates were found in the same sample. If glucosinolates alone were presented in the database, the contribution of bioactive breakdown compounds would be overlooked. For this reason, we first propose that when considering what to measure, in addition to fresh raw vegetables, samples processed under common culinary and cooking methods should be included in the analysis; and secondly, for processed samples, that both glucosinolates and isothiocyanate should be analyzed and presented in the database.

4. Conclusion

Unlike the databases that have been developed by USDA NDL in the past (e.g., flavonoid databases) there are some unique challenges when developing a glucosinolate database, largely due to their coexistence with myrosinase (β-thioglucosidase) in plant tissues. First, enzyme deactivation is proved to be a necessary step to avoid hydrolytic loss and/or *de novo* synthesis during sample preparation. Nevertheless, the common deactivation methods may inevitably alter the glucosinolate profile. Steaming and microwaving were found to be the most effective mechanisms to deactivate myrosinase. Despite the recent advancement of technology, there remains a need for simple, sensitive, robust and automated methods for the quantification of glucosinolates and/or their breakdown compounds. As studies showing that individual glucosinolates and isothiocyanate have different biological effects, only methods that measure individual glucosinolates and isothiocyanates, including those measuring intact glucosinolates and desulfated method (ISO 9167-1), will be considered acceptable methods for database development. The observation that only isothiocyanates appeared in the human body, and they were demonstrated as actual *in vivo* bioactive compounds, poses an interesting question: what should be measured and included in the database, especially in the processed vegetables? We propose that, in generating data for the database, samples processed using common culinary and cooking methods should be used in the analysis, and that both glucosinolates and isothiocyanate should be analyzed in processed samples and be presented in the database.

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