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Analysis of Isothiocyanate Mercapturic Acids in Urine: A Biomarker for Cruciferous Vegetable Intake

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Cruciferous vegetables contain glucosinolates, which are degraded to isothiocyanates. These are easily absorbed, conjugated to glutathione, and excreted into the urine as their corresponding mercapturic acids. We have developed and validated a solid phase extraction−high-performance liquid chromatography−electrospray ionization mass spectrometry/mass spectrometry method for the specific analysis of individual isothiocyanate mercapturic acids in urine. The range of reliable analysis was 1.0−310 μM in urine. Urine samples fortified with three different levels of isothiocyanate mercapturic acids were measured on six different days by three independent technicians. The relative standard deviation (RSD) of repeatability was 12, 6, and 3%; the RSD of reproducibility was 19, 14, and 8%, and spike recoveries were 103, 104, and 103%, respectively, for 1.04, 10.5, and 313 μM levels. In 24 h urine collected from two volunteers after they consumed broccoli and cauliflower, clearly sulforaphane mercapturic acid (133 μmol) and allyl isothiocyanate mercapturic acid (4.7 μmol) were found. This procedure demonstrates a reliable and efficient method to study the intake and mode of action of isothiocyanates in animal studies and clinical trials.

KEYWORDS: Cruciferous vegetables; glucosinolates; isothiocyanates; mercapturic acids; biomarker

INTRODUCTION

Consumption of fruits and vegetables is associated with a reduced risk on degenerative diseases such as cancer and cardiovascular diseases, as indicated by epidemiological studies (*1*, *2*). In particular, cruciferous vegetables, i.e., cabbages, broccoli, Brussels sprouts, radish, mustard, and cress, appear to have beneficial health potentials (*3*−*5*). In 1978, the daily per capita consumption of standard cruciferous vegetables was reported to be 18 g in the United States and Canada and 45 g in the U.K. (*15*). In The Netherlands, the average daily consumption of cruciferous vegetables in 1992 ranged from 29 (*19*) to 32 g (*5*). Because cruciferous vegetables differ from other vegetables by the presence of glucosinolates, health-promoting effects seem to be attributable to these phytochemicals or their breakdown products, e.g., isothiocyanates (*6*). Isothiocyanates are strong inhibitors of phase I enzymes and inducers of phase II enzymes and are therefore thought to be strong cancer chemopreventors (*7*−*9*). Human intervention trials with large quantities of cruciferous vegetables gave similar effects on phase I and phase II enzymes (*10*−*13*).

Glucosinolates remain intact within the plant cytoplasm until tissue disruption by chewing or cutting, which releases the hydrolysis enzyme myrosinase (see **Figure 1**) (*14*, *15*). Unmetabolized glucosinolates can be degraded, to a lesser degree, by microbes present in the human gut (*16*−*18*). Major breakdown products are isothiocyanates, which are responsible for the pungent taste of mustard. Other breakdown products are indoles and nitriles. Additionally, acid hydrolysis inside the stomach can degrade glucosinolates into isothiocyanates. Glucosinolates may be absorbed without change, to some degree, but the subsequent fate of these compounds in the metabolic scheme remains unknown. Thus, when eating crucifers, both glucosinolates and isothiocyanates are important components to be considered in the metabolic and excretory pathways. Verhoeven et al. (*6*) estimated that an average daily intake in The Netherlands of 21 mg of glucosinolates, corresponding to 50 μmol, can be calculated.

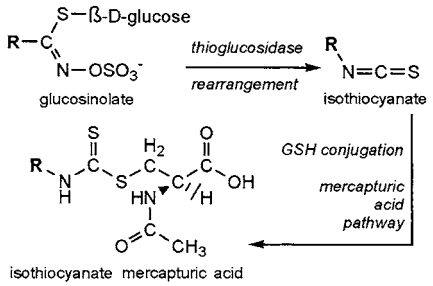

Figure 1. Glucosinolates are enzymatically hydrolyzed into isothiocyanates and are subsequently conjugated in the body to glutathione followed by excretion as mercapturic acids in the urine.

Isothiocyanates are conjugated to glutathione in the body and excreted into the urine as their corresponding mercapturic acids

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(**Figure 1**) as demonstrated in rats (*20*), guinea pigs, rabbits (*21*), and humans (*22*, *23*). Mercapturic acids reflect the uptake of isothiocyanates and thus the intake of glucosinolates present in cruciferous vegetables (*24*−*26*). Therefore, mercapturic acids can be used as a selective biomarker for cruciferous vegetable intake. Different cruciferous vegetables can be differentiated by the variety and amount of glucosinolates (*27*). For example, broccoli is rich in 4-methylthiobutyl glucosinolate, whereas Brussels sprouts are rich in allyl and 2-hydroxy-3-butenyl glucosinolate (progoitrin). On the other hand, garden and water cress are rich in arylaliphatic glucosinolates (*15*).

Isothiocyanates and their corresponding mercapturic acids can be measured in physiological fluids by a sum parameter assay. The assay utilizes 1,2-benzenedithiol as a vicinal dithiol reagent and measures the reaction product, 1,3-benzodithiole-2-thione, spectrophotometrically (*28*, *29*). Chemical specificity of the cyclocondensation reaction is not restricted to isothiocyanates but includes dithiocarbamates and related thiocarbonyl compounds such as carbon disulfide, certain substituted thiourea derivatives, and xanthates (*28*). Besides, the sum parameter does not give any information about which kind of crucifer has been consumed.

We therefore developed an analytical method by which the isothiocyanate mercapturic acids not only can be determined quantitatively but also individually characterized. Mercapturic acids were extracted from urine using solid phase extraction (SPE) followed by separation using reversed phase liquid chromatography. Because specificity by UV detection (absorption maxima in acetonitrile−water at 250 and 270 nm) is too low, mass spectrometric (MS/MS) detection was used. Linearity, repeatability, reproducibility, accuracy, and detection limits of the method were determined. The strength of the application of the current method is demonstrated by the analysis of urine samples collected for 24 h after consumption of cooked broccoli and cauliflower and a salad of mustard, garden cress, water cress, rocket, winter radish, and radish.

MATERIALS AND METHODS

Materials. Urine was collected in clear polyethylene flasks. SPE using Bakerbond octadecyl 100 mg SPE columns (Mallinckrodt Baker B. V., Deventer, The Netherlands) was performed on a vacuum manifold with Teflon fittings. Chromatographic separation was performed using a Waters Alliance 2690 quaternary high-performance liquid chromatograph (HPLC, Waters Chromatography B. V., Etten-Leur, The Netherlands) equipped with a Waters SymmetryShield RP18 3.5 μm guard (2.1 mm × 10 mm cartridge) and analytical (2.1 mm × 150 mm cartridge) column thermostated at 35 °C. This system was equipped with a Finnigan MAT LCQ ion trap mass spectrometer (Thermo Quest B. V., Breda, The Netherlands) and operated by XCalibur software.

Chemicals. Water was demineralized using an ELGA Option 7 Plus water purifier (Salm en Kipp, Breukelen, The Netherlands). All chemicals were from Merck (Merck KGaA, Darmstadt, Germany) and of analytical grade. Phenyl isothiocyanate mercapturic acid (internal standard) and 11 reference compounds (methyl, 4-methylsulfinylbutyl, allyl, isopropyl, 3-butenyl, 1-methylpropyl, butyl, 4-methylthiobutyl, 4-pentenyl, benzyl, and 2-phenylethyl isothiocyanate mercapturic acids) were prepared as described elsewhere (*30*, **Figure 2**). In short, isothiocyanate was dissolved in alcohol and added to a solution of *N*-acetyl-L-cysteine and sodium bicarbonate in water. The corresponding mercapturic acid was obtained with a typical yield of 77%. Because few isothiocyanates are commercially available, others were first synthesized by adding the corresponding alkyl bromide to phthalimide potassium salt. The obtained *N*-alkyl-phthalimide was hydrazinolyzed yielding the alkylamine, which was subsequently substituted with thiophosgene yielding the isothiocyanate with an overall yield of 16%. The internal standard was dissolved in tetrahydrofuran and diluted with 0.25 M hydrochloric acid. Tetrahydrofuran, methanol, and acetonitrile for SPE and chromatography were HPLC grade.

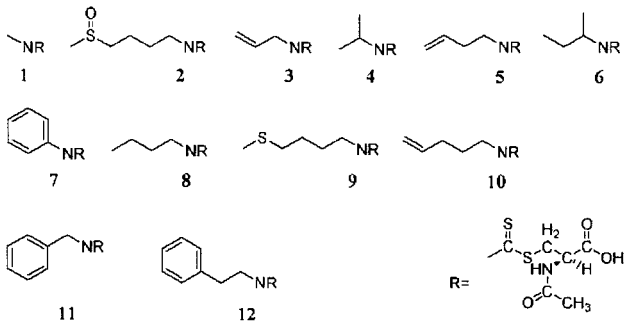

Figure 2. These 12 isothiocyanate mercapturic acids were previously synthesized (*30*) and were used here as reference standards for the analysis of mercapturic acids in urine. Compound **1**, methyl; **2**, 4-methylsulfinylbutyl; **3**, allyl; **4**, isopropyl; **5**, 3-butenyl; **6**, 1-methylpropyl; **7**, phenyl; **8**, butyl; **9**, 4-methylthiobutyl; **10**, 4-pentenyl; **11**, benzyl; **12**, 2-phenylethyl.

Sample Preparation. Twenty microliters of internal standard solution was added to 200 μL of urine in a 0.5 mL Eppendorf tube followed by vortexing. Next, SPE columns were washed with methanol and hydrochloric acid buffer (pH 2), and subsequently, the samples were applied. SPE columns were washed with water and aspirated until dryness. Mercapturic acids were eluted from the column with phosphate buffer (pH 8):acetonitrile (v:v, 1:1) into a vial containing hydrochloric acid buffer (pH 2). From the mixed eluate, 5 μL was analyzed on an HPLC-MS/MS system.

HPLC-MS/MS Analysis. Chromatography was performed using a SymmetryShield column and an acetonitrile gradient. The flow rate of mobile phase was 0.25 mL/min using a linear gradient of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The amount of B was raised from 0 to 30% in 55 min. Electrospray ionization (ESI) in positive mode was found optimal with the following ESI source settings: sheath gas flow, 80%; auxiliary gas flow, 20%; capillary temperature, 225 °C; source voltage, 4.5 kV. Parent masses $[M + H]^+$ were isolated with a *m*/*z* window of 3.0 and fragmented using 30% collision energy, which resulted in 12 different scan filters, one for each mercapturic acid. The spectra of fragments in the range *m*/*z* 100−350 were collected, and peak areas were obtained using the appropriate scan filter and the response of the fragment with *m*/*z* 164.0 (*N*-acetyl-L-cysteine) with a *m*/*z* window of 1.0. For 4-methylsulfinylbutyl isothiocyanate mercapturic acid, the fragment 178.0 (4-methylsulfinylbutyl isothiocyanate) instead of 164.0 was used. Urinary concentrations of mercapturic acids were quantitated using an internal standard and external calibration (**Figure 3**). Because phenyl glucosinolate is not a naturally occurring compound, the mercapturic acid of phenyl isothiocyanate was used as the internal standard.

Method Validation. For the purpose of validation and quality control, calibration standards were added to blank urine at three levels: low (on average 1.04 μM), medium (on average 10.5 μM), and high level (on average 313 μM) in order to determine repeatability, recovery, and reproducibility of analysis. Linearity of analysis was checked by preparing two stock solutions containing all mercapturic acids with subsequent dilutions.

RESULTS AND DISCUSSION

SPE using only C18 columns resulted in acceptable reproducibilities (**Table 1**) and a rapid cleanup method. Urine samples and C18 columns were acidified to pH 2.0 to protonate the mercapturic acids. The measured pK_a of allyl isothiocyanate mercapturic acid was 3.3. Sufficient separation of isothiocyanate mercapturic acids was obtained on a SymmetryShield RP18 HPLC column, which has improved water wettability. A linear gradient of acetonitrile and water is used to separate all compounds. Acetonitrile was used as an HPLC modifier because this resulted in better peak shape as compared to a gradient of

Table 1. Validation Results of SPE-HPLC-MS/MS Analysis of Isothiocyanate Mercapturic Acids in Urine[a]

compd nr.[b]	linearity[c] range (μM)	R^2	slope	intercept (95% CI)	repeatability RSD (%) l	repeatability RSD (%) m	repeatability RSD (%) h	recovery (%) l	recovery (%) m	recovery (%) h	reproducibility RSD (%) l	reproducibility RSD (%) m	reproducibility RSD (%) h	LOQ (μM)[d]
1	0.113–113	0.996	29.0	–0.02 (–0.14, 0.09)	15	6	4.8	93	98	92	23	17	11	0.5
2	0.127–125.5	0.998	58.5	–0.07 (–0.13, 0.28)	18	4	2.4	87	100	96	16	9	6	0.1
3	0.098–98.1	0.997	78.3	–0.04 (–0.27, 0.18)	5	7	3.0	103	106	107	25	18	7	0.6
4	0.104–104	0.997	149	–0.1 (–0.6, 0.4)	11	5	2.0	112	104	104	17	12	8	0.6
5	0.096–95.6	0.999	99.5	–0.01 (–0.14, 0.11)	7	8	4.1	92	108	105	15	13	8	0.3
6	0.096–96.5	0.998	186	0 (–0.5, 0.4)	20	6	2.1	108	107	104	14	12	8	0.4
8	0.091–90.7	0.990	133	0 (–0.7, 0.7)	10	4	3.3	103	106	102	19	13	9	0.8
9	0.083–83.5	0.993	149	–0.1 (–0.7, 0.5)	10	11	3.2	103	96	108	19	17	7	0.2
10	0.102–102	0.999	107	–0.03 (–0.26, 0.19)	13	9	7	103	104	100	19	16	9	0.6
11	0.085–85	0.996	136	–0.1 (–0.5, 0.4)	12	5	3.1	115	106	105	18	14	10	0.4
12	0.081–80.9	0.995	147	–0.1 (–0.5, 0.4)	15	6	2.3	111	105	105	21	11	8	0.5

[a] Urine samples fortified at three different levels of 1.04 (l), 10.5 (m), and 313 μM (h) were measured six times to determine the relative standard deviation (RSD) of repeatability and the recovery. These samples were measured on six different days by three different technicians to determine the RSD of reproducibility. Blank urine was measured six times to determine the limit of quantification (LOQ). [b] Compound nr refers to isothiocyanate mercapturic acids depicted in **Figure 2**. Compound nr **7** is not included because this compound is used as the internal standard. [c] Linearity is defined as $Y = aX + b$ where Y = area ratio and X = concentration in mM. [d] The LOQ is quantified as 10/3 × (the average value of the noise + (3 × SD)).

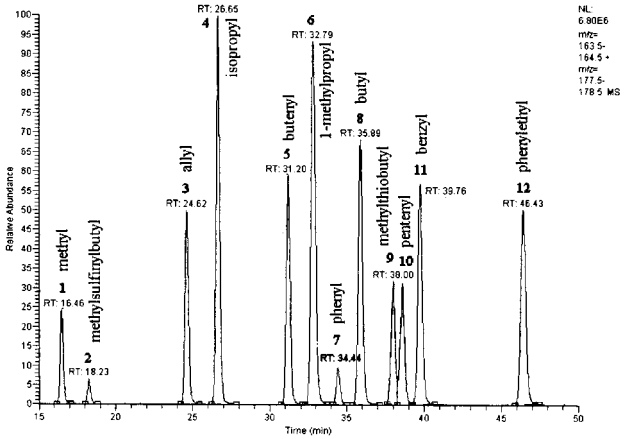

Figure 3. HPLC-MS/MS chromatogram of 12 available isothiocyanate mercapturic acids. Each peak is labeled with the side chain (R) mentioned in **Figure 2**. Concentrations are (μM) as follows: **1** (77), **2** (48), **3** (66), **4** (70), **5** (65), **6** (65), **7** (14), **8** (61), **9** (56), **10** (69), **11** (57), **12** (55).

methanol and water (data not shown). ESI proved more suitable than atmospheric pressure chemical ionization, because of its better performance at low HPLC eluent flow rates (0.25 mL/min) and negligible in-source fragmentation. In time segments of approximately 1 min surrounding the retention times of all 12 isothiocyanate mercapturic acids, the masses $[M + H]^+$ of each individual component were isolated and fragmented (**Figure 3**).

Mass Spectra. Fragmentation spectra generally contain fragments with relative abundances as shown in **Figure 4B** for allyl isothiocyanate mercapturic acid where *m*/*z* 122 is cysteine, 130 is 2-*N*-acetyl-propanoic acid, 164 is *N*-acetyl-L-cysteine, and 134 is the variable side chain allyl−N(H)−C(=S)−S. The most abundant fragment ion has a *m*/*z* of 164 except for compounds **2** and **7**. Phenyl isothiocyanate mercapturic acid (**7**) fragmented into ions with a base peak at *m*/*z* 170 (phenyl−N(H)−C(=S)−S), fragment *m*/*z* 164 (70%), and some minor fragments. 4-Methylsulfinylbutyl isothiocyanate mercapturic acid (**2**) fragmented into ions with a base peak at *m*/*z* 178 ($H_3C-S(=O)-(CH_2)_4-N-C=S$) and fragments at *m*/*z* 212 ($H_3C-S(=O)-(CH_2)_4-N(H)-C(=S)-S$, 45%) and 164 (1%). Quantitation was performed using *m*/*z* 164, except for compound **2** where *m*/*z* 178 was used. Clearly, this MS/MS detection method provides high specificity.

Method Validation. The ruggedness of the method was tested by changing six parameters slightly and analyzing urine spiked at the medium level. The tested parameters were the sample volume of urine applied to the SPE column, the volume of water for washing, the pH of the hydrochloric acid buffer, the composition of buffer for elution of the components from the SPE column, the injection volume for HPLC analysis, and the collision energy used for fragmentation of the compounds in the ion trap. Using the saturated design of Plackett and Burman (*31*), the method appears to be rugged for all but two parameters. These two parameters, applied sample volume and collision energy, were only found not to be rugged for one or two of the 11 isothiocyanate mercapturic acids. Concluding, no more than 200 μL of urine should be extracted and the collision energy used, to fragment trapped ions, should be no less than 30%.

From 11 solutions of mercapturic acids, two calibration standards were prepared. These standards were subsequently diluted to give the mentioned (**Table 1**) calibration range with a good correlation coefficient (0.990 or more), no intercept ($b = 0$), and with similar slopes (a). Calibration standards were added to blank urine at three levels: low, medium, and high. Results of repeated measurements of these samples are given in **Table 1**. Repeatability gave satisfactory results. Recoveries are all between 90 and 115%. High recoveries indirectly prove that there is no ion suppression in the MS source when analyzing urine. The reproducibility is for most compounds close to expected relative standard deviations (SDs) using the Horwitz equation (*32*). For methyl isothiocyanate mercapturic acid, relative SDs (RSDs) for reproducibility are high, which could be due to poor adsorption on the C18 SPE column leading to loss while extracting the compounds from urine. The limit of quantification is on average 0.5 μM, and as specifications of the method were tested for the concentration range of 1.0−313 μM, the latter is considered to be the range shown to be reliable for analysis. This range roughly corresponds to the amounts of allyl isothiocyanate mercapturic acid found in 24 h urine after the consumption of allyl glucosinolate (sinigrin) present in, respectively, one leaf of one Brussels sprout and 200 g of Brussels sprouts. The stability of compounds **1**−**12** (except **7**, which was used as the internal standard) added to blank urine at medium level (on average 10.5 μM) is at room temperature 1 day, in the refrigerator several days, and in the freezer over 1 year.

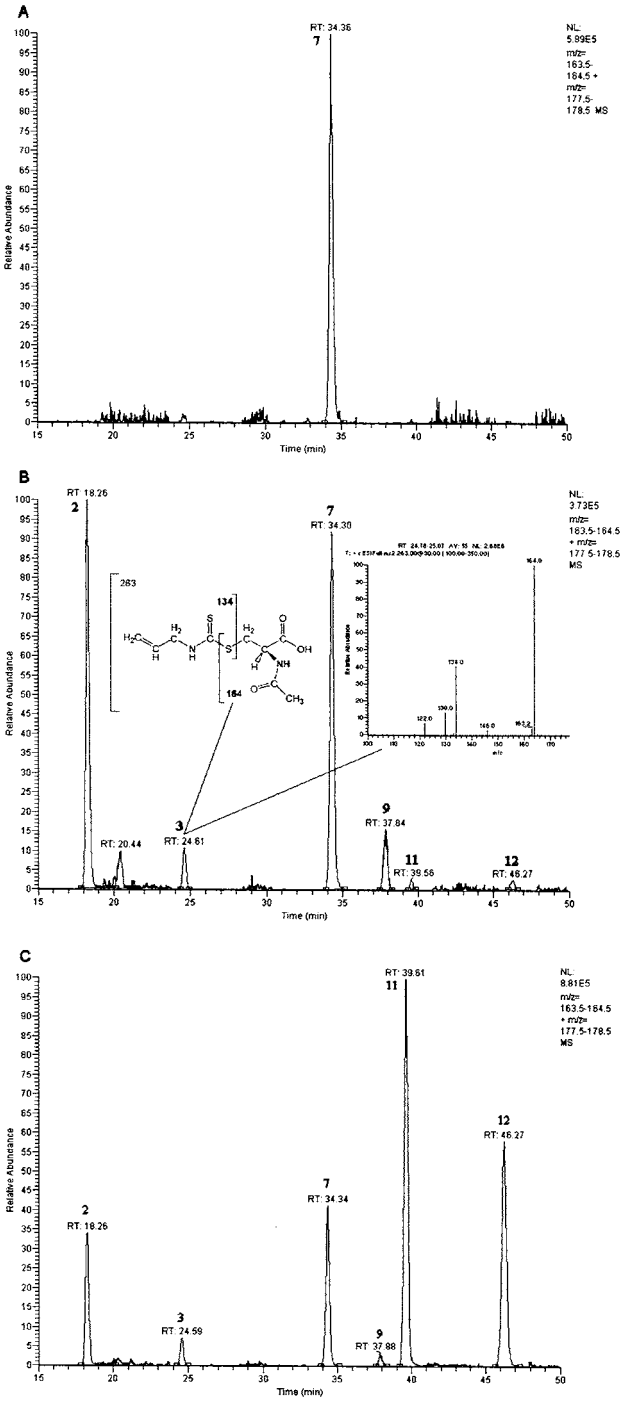

Figure 4. HPLC-MS/MS chromatograms of isothiocyanate mercapturic acids in urine. Each peak is labeled with the side chain (R) mentioned in **Figure 2**. The absence (<0.5 μM) of isothiocyanate mercapturic acids in blank urine is shown in chromatogram **A**. Peak 7 is the internal standard, 13.7 μM phenyl isothiocyanate mercapturic acid. After cauliflower and broccoli were consumed, chromatogram **B** was obtained. The fragmentation structures and spectrum of allyl isothiocyanate mercapturic acid are inserted. After mustard, garden cress, water cress, rocket, winter radish, and radish were consumed, chromatogram **C** was obtained. Method settings are as described in the Materials and Methods.

The described SPE-HPLC-MS/MS method proved to be a valid method for the analysis of isothiocyanate mercapturic acids in urine. In blank urine from six volunteers, collected after refraining from eating cruciferous vegetables and condiments during 36 h, no isothiocyanate mercapturic acids could be found (<0.5 μM, **Figure 4A**). Phenyl isothiocyanate mercapturic acid was absent from all urines tested (data not shown).

Mercapturic Acids in Urine After Brassica Consumption. After eating a meal with 200 g of both broccoli (*Brassica oleracea* L. *italica*) and cauliflower (*Brassica oleracea* L. *botrytis* subvar. *cauliflora*), the isothiocyanate mercapturic acids **2** (133 μmol), **3** (4.7 μmol), **9** (11.0 μmol), **11** (0.9 μmol), and **12** (1.0 μmol) were found in 24 h urine (**Figure 4B**). The content of 4-methylsulfinylbutyl glucosinolate is on average 80.7 μmol in broccoli and 63.8 μmol/100 g of fresh weight in cauliflower. Furthermore, cauliflower contains on average 27.9 μmol allyl glucosinolate/100 g of fresh weight (*33*). From the expected dose of 4-methylsulfinylbutyl glucosinolate (289 μmol), a large amount is recovered in the urine as its isothiocyanate mercapturic acid where only a minor amount of the expected dose of allyl glucosinolate (55.8 μmol) is recovered in the urine. The difference in relative excretion between the two compounds can be explained from the large variation in glucosinolate content in vegetables. A meal of mustard (*Brassica juncea* Coss.), garden cress (*Lepidium sativum* L.), water cress (*Nasturtium officinale* R.Br.), rocket (*Eruca sativa* Mill.) and winter radish (rettich, *Raphanus sativus var. alba* L.) (10 g each), and radish (*Raphanus sativus* L.) (90 g) resulted in the excretion in 24 h urine of compounds **2** (116 μmol), **3** (6.7 μmol), **9** (4.8 μmol), **11** (71.9 μmol), and **12** (51.7 μmol) (**Figure 4C**). The major glucosinolates (side chain mentioned) present in these condiments and salad crops are allyl for mustard, benzyl for garden cress, phenylethyl for water cress, 4-methylsulfinylbutyl and 4-methylthiobutyl for rocket, and 4-methylsulfinylbutenyl glucosinolate for both winter radish and radish, which is reflected in the excreted isothiocyanate mercapturic acids. Breakdown products of the principal glucosinolate of winter radish and radish, 4-methylsulfinylbutenyl glucosinolate, were not measured with the method described here.

Biomarker of Isothiocyanate Intake. Proof of the health benefit potential of fruits and vegetables and cruciferous vegetables in particular, is culminating, but the question remains, what are the responsible chemicals that make vegetables healthy? Because cruciferous vegetables differ from other vegetables in that they contain glucosinolates, these compounds might be the responsible bioactive phytochemicals. Glucosinolates themselves are not thought to diminish cancer risks. However, their breakdown products, isothiocyanates, have been proven (in in vitro and in vivo experiments) to modulate biotransformation enzyme activities resulting in reduced cancer risk (*7*−*9*). Glucosinolate intake is generally estimated using dietary questionnaires, which are not only subjective measures, but are not accurate because amounts of (cruciferous) vegetables in a whole meal are not easily estimated. Glucosinolates are thought to not be absorbed from the gut but partly converted into nitriles and isothiocyanates in the stomach and to isothiocyanates in the colon. Isothiocyanates are lipophilic and are readily absorbed from the gut. Spot urine cannot be used to quantify the uptake and excretion of isothiocyanates since the peak in excretion is between 3 and 6 h. Twelve hour urine collection contains the majority in isothiocyanate excretion, and most isothiocyanates, eaten with common meals, are excreted within 24 h (data not shown).

Glucosinolate metabolites can be analytically measured in vegetables, and intake of isothiocyanates can thus be estimated. Because glucosinolates are only partly converted into isothiocyanates and interindividual kinetics differ, it is not feasible to estimate the exact intake of isothiocyanates through analysis of glucosinolates and isothiocyanates in vegetables. Myrosinase,

which is still active in raw broccoli, is responsible for the breakdown of glucosinolates after chewing, resulting in an elevated isothiocyanate intake. Isothiocyanate conjugates, mostly mercapturic acids, quantitatively reflect isothiocyanate uptake but reflect glucosinolate intake only qualitatively. The amount of isothiocyanate conjugates excreted after eating raw broccoli is approximately three times greater than that after eating the same amount of cooked broccoli (*34*).

Indole glucosinolates do not yield isothiocyanates upon hydrolysis but are converted into indoles and thiocyanate ion (SCN^-). Measuring thiocyanate ion in urine can thus be a marker of indole glucosinolate intake and thus of cruciferous vegetable intake. High baseline concentrations in urine can be expected as thiocyanate ion is a metabolite from cyanide provided by cigarette smoke. Thiocyanate ion can be measured spectroscopically after ion-exchange cleanup (*35*) or can be measured using ion-exchange chromatography and suppressed conductivity detection. Isothiocyanate conjugate excretion can be measured using an assay, which gives the sum parameter of isothiocyanates after a cyclocondensation reaction (*28*, *29*). This is a fast assay, but chemical specificity is not restricted to isothiocyanates (*28*), and it returns a sum parameter of isothiocyanates and conjugates. The limitations of these two assays are not encountered when analyzing individual isothiocyanate mercapturic acids.

In conclusion, SPE-HPLC-MS/MS isothiocyanate mercapturic acid analysis in urine is a specific, rugged, and validated method, which can be applied to measure isothiocyanate exposure. This biomarker reflects the dose of isothiocyanates absorbed after intake of isothiocyanates or after breakdown in the human gut of glucosinolates and can thus be used to determine the health-promoting potential of cruciferous species in animal studies and clinical trials.

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LITERATURE CITED

(1) Steinmetz, K. A.; Potter, J. D. Vegetables, fruit, and cancer. I. Epidemiology. *Cancer Causes Control* **1991**, *2* (5), 325−357.

(2) Block, G.; Patterson, B.; Subar, A. Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. *Nutr. Cancer* **1992**, *18* (1), 1−29.

(3) Verhoeven, D. T.; Goldbohm, R. A.; van Poppel, G.; Verhagen, H.; van den Brandt, P. A. Epidemiological studies on brassica vegetables and cancer risk. *Cancer Epidemiol., Biomarkers Prev.* **1996**, *5* (9), 733−748.

(4) van Poppel, G.; Verhoeven, D. T.; Verhagen, H.; Goldbohm, R. A. Brassica vegetables and cancer prevention. Epidemiology and mechanisms. *Adv. Exp. Med. Biol.* **1999**, *472*, 159−168.

(5) Voorrips-L, E.; Goldbohm-R. A.; van Poppel, G.; Sturmans, F.; Hermus, R. J.; van den Brandt, P. A. Vegetable and fruit consumption and risks of colon and rectal cancer in a prospective cohort study: The Netherlands Cohort Study on Diet and Cancer. *Am. J. Epidemiol.* **2000**, *152* (11), 1081−1092.

(6) Verhoeven, D. T.; Verhagen, H.; Goldbohm, R. A.; van den Brandt, P. A.; van Poppel, G. A review of mechanisms underlying anticarcinogenicity by brassica vegetables. *Chem.-Biol. Interact.* **1997**, *103* (2), 79−129.

(7) Guo, Z.; Smith, T. J.; Wang, E.; Sadrieh, N.; Ma, Q.; Thomas, P. E.; Yang, C. S. Effects of phenethyl isothiocyanate, a carcinogenesis inhibitor, on xenobiotic-metabolizing enzymes and nitrosamine metabolism in rats. *Carcinogenesis* **1992**, *13* (12), 2205−2210.

(8) Sparnins, V. L.; Venegas, P. L.; Wattenberg, L. W. Glutathione S-transferase activity: enhancement by compounds inhibiting chemical carcinogenesis and by dietary constituents. *J. Natl. Cancer Inst.* **1982**, *68* (3), 493−496.

(9) Zhang, Y.; Talalay, P. Mechanism of differential potencies of isothiocyanates as inducers of anticarcinogenic Phase 2 enzymes. *Cancer Res.* **1998**, *58* (20), 4632−4639.

(10) Bogaards, J. J.; Verhagen, H.; Willems, M. I.; van Poppel, G.; van Bladeren, P. J. Consumption of Brussels sprouts results in elevated alpha-class glutathione-S-transferase levels in human blood plasma. *Carcinogenesis* **1994**, *15* (5), 1073−1075.

(11) Hecht, S. S. Chemoprevention by isothiocyanates. *J. Cell. Biochem.* **1995**, *22* (Suppl.), 195−209.

(12) Nijhoff, W. A.; Mulder, T. P.; Verhagen, H.; van Poppel, G.; Peters, W. H. Effects of consumption of brussels sprouts on plasma and urinary glutathione S-transferase class-alpha and -pi in humans. *Carcinogenesis* **1995**, *16* (4), 955−957.

(13) Nijhoff, W. A.; Grubben, M. J.; Nagengast, F. M.; Jansen, J. B.; Verhagen, H.; van Poppel, G.; Peters, W. H. Effects of consumption of Brussels sprouts on intestinal and lymphocytic glutathione S-transferases in humans. *Carcinogenesis* **1995**, *16* (9), 2125−2128.

(14) Kjær, A. Naturally derived isothiocyanates (mustard oils) and their parent glucosides. *Fortschr. Chem. Org. Naturstoffe*. **1960**, *18*, 122−176.

(15) Fenwick, G. R.; Heaney, R. K.; Mullin, W. J. Glucosinolates and their breakdown products in food and food plants. *Crit. Rev. Food Sci. Nutr.* **1983**, *18* (2), 123−201.

(16) Nugon-Baudon, L.; Szylit, O.; Raibaud, P. Production of toxic glucosinolate derivatives from rapeseed meal by intestinal microflora of rat and chicken. *J. Sci. Food Agric.* **1988**, *43* (4), 299−308.

(17) Nugon-Baudon, L.; Rabot, S.; Wal, J. M.; Szylit, O. Interactions of the intestinal microflora with glucosinolates in rapeseed meal toxicity: first evidence of an intestinal lactobacillus possessing a myrosinase-like activity in vivo. *J. Sci. Food Agric.* **1990**, *52* (4), 547−559.

(18) Krul, C. A. M.; Humblot, C.; Philippe, C.; Vermeulen, M.; van Nuenen, M.; Havenaar, R.; Rabot, S. Metabolism of sinigrin (2-propenyl glucosinolate) by the human colonic microflora in a dynamic in vitro large-intestinal model. *Carcinogenesis* **2002**, *23* (6), 101−108.

(19) Hulshof, K. F. A. M. *Dutch National Food Consumption Survey (DNFCS-2 1992)*; TNO Nutrition and Food Research: Zeist, The Netherlands, 1993.

(20) Brüsewitz, G.; Cameron, B. D.; Chasseaud, L. F.; Görler, K.; Hawkins, D. R.; Koch, H.; Mennicke, W. H. The metabolism of benzyl isothiocyanate and its cysteine conjugate. *Biochem. J.* **1977**, *162* (1), 99−107.

(21) Görler, K.; Krumbiegel, G.; Mennicke, W. H.; Siehl, H. U. The metabolism of benzyl isothiocyanate and its cysteine conjugate in guinea-pigs and rabbits. *Xenobiotica* **1982**, *12* (9), 535−542.

(22) Chung, F. L.; Morse, M. A.; Eklind, K. I. New potential chemopreventive agents for lung carcinogenesis of tobacco-specific nitrosamine. *Cancer Res.* **1992**, *52* (9 Suppl.), 2719−2722.

(23) Mennicke, W. H.; Görler, K.; Krumbiegel, G.; Lorenz, D.; Rittmann, N. Studies on the metabolism and excretion of benzyl isothiocyanate in man. *Xenobiotica* **1988**, *18* (4), 441−447.

(24) Chung, F. L.; Morse, M. A.; Eklind, K. I.; Lewis, J. Quantitation of human uptake of the anticarcinogen phenethyl isothiocyanate after a watercress meal. *Cancer Epidemiol. Biomarkers Prev.* **1992**, *1* (5), 383−388.

(25) Duncan, A. J.; Rabot, S.; Nugon-Baudon, L. Urinary mercapturic acids as markers for the determination of isothiocyanate release from glucosinolates in rats fed a cauliflower diet. *J. Sci. Food. Agric*. **1997**, *73* (2), 214−220.
(26) Jiao, D.; Ho, C. T.; Foiles, P.; Chung, F. L. Identification and quantification of the *N*-acetylcysteine conjugate of allyl isothiocyanate in human urine after ingestion of mustard. *Cancer Epidemiol. Biomarkers Prev.* **1994**, *3* (6), 487−492.
(27) Tiedink, H. G.; Davies, J. A.; van Broekhoven, L. W.; van der Kamp, H. J.; Jongen, W. M. Formation of mutagenic *N*-nitroso compounds in vegetable extracts upon nitrite treatment: a comparison with the glucosinolate content. *Food. Chem. Toxicol.* **1988**, *26* (11−12), 947−954.
(28) Zhang, Y.; Wade, K. L.; Prestera, T.; Talalay, P. Quantitative determination of isothiocyanates, dithiocarbamates, carbon disulfide, and related thiocarbonyl compounds by cyclocondensation with 1,2-benzenedithiol. *Anal. Biochem.* **1996**, *239* (2), 160−167.
(29) Chung, F. L.; Jiao, D.; Getahun, S. M.; Yu, M. C. A urinary biomarker for uptake of dietary isothiocyanates in humans. *Cancer Epidemiol. Biomarkers Prev.* **1998**, *7* (2), 103−108.
(30) Vermeulen, M.; Zwanenburg, B.; Chittenden, G. J. F.; Verhagen, H. Synthesis of isothiocyanate derived mercapturic acids. Submitted for publication.
(31) Placket, R. L.; Burman, J. P. The design of optimum multifactorial experiments. *Biometrika* **1946**, *33*, 305.
(32) Horwitz, W. International coordination and validation of analytical methods. *Food Addit. Contam.* **1993**, *10* (1), 61−69.
(33) Rosa, E. A. S.; Heaney, R. K.; Fenwick, G. R.; Portas, C. A. M. Glucosinolates in crop plants. *Hortic. Rev.* **1997**, 99−215.
(34) Conaway, C. C.; Getahun, S. M.; Liebes, L. L.; Pusateri, D. J.; Topham, D. K. W.; Botero-Omary, M.; Chung, F. L. Disposition of glucosinolates and sulforaphane in humans after ingestion of steamed and fresh broccoli. *Nutr. Cancer* **2000**, *38* (2), 168−178.
(35) Lundquist, P.; Kagedal, B.; Nilsson, L. An improved method for determination of thiocyanate in plasma and urine. *Eur. J. Clin. Chem. Clin. Biochem.* **1995**, *33* (6), 343−349.

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