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Improved synthesis methods of standards used for quantitative determination of total isothiocyanates from broccoli in human urine

Mette Kristensen [a,*], Kirstine S. Krogholm [a], Hanne Frederiksen [a], Fritz Duus [c], Claus Cornett [d], Susanne H. Bügel [b], Salka E. Rasmussen [a]

[a] *Department of Toxicology and Risk Assessment, Danish Institute for Food and Veterinary Research, 2860 Søborg, Denmark*
[b] *Department of Human Nutrition and Centre for Advanced Food Studies, The Royal Veterinary and Agricultural University, 1958 Frederiksberg, Denmark*
[c] *Department of Life Sciences and Chemistry, Roskilde University, 4000 Roskilde, Denmark*
[d] *Department of Analytical Chemistry, The Danish University of Pharmaceutical Sciences, 2100 Copenhagen, Denmark*

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Abstract

A well-known method for quantification of isothiocyanates (ITCs) and their metabolites is the condensation reaction with 1,2-benzenedithiole to produce 1,3-benzodithiole-2-thione, which can be quantified by high-performance liquid chromatography. Standards of an ITC metabolite and 1,3-benzodithiole-2-thione are required for this assay but are not commercially available. In the present study, we report on an improved synthesis of the ITC metabolite *N*-acetyl-*S*-(*N*-4-methylsulfinylbutylthiocarbamoyl)-L-cysteine and 1,3-benzodithiole-2-thione. The standards were used to quantify the urinary excretion of ITCs from 10 healthy subjects who consumed 350 g broccoli. The excretion was investigated throughout 48 h showing a cumulative urinary ITC excretion of 49.1 ± 25.2% of the dose.

Keywords: Isothiocyantes; Biomarker; Synthesis; 1,3-Benzodithiole-2-thione; *N*-acetyl-*S*-(*N*-4-methylsulfinylbutylthiocarbamoyl)-L-cysteine

1. Introduction

Consumption of cruciferous vegetables is known to be associated with a lower risk of developing cancer [1]. Cruciferous vegetables differ from other vegetables by the presence of glucosinolates, and the preventive effect is assumed to be related to these compounds. Glucosinolates become bioactive when they are broken down to a range of different degradation products by the enzyme myrosinase (E.C. 3.2.3.1), which is present in cruciferous vegetables and in human gut microbes [2]. The individual breakdown product depends on the parent glucosinolate and on the conditions under which the glucosinolate hydrolysis occurs. Isothiocyanates (ITCs) are an important group of enzymatic breakdown products from glucosinolates. ITCs are thought to be strong cancer chemopreventors through their ability to induce phase II enzymes [3,4] and potentially also to inhibit phase I enzymes [5]. Furthermore, ITCs have also been reported to induce growth arrest and apoptosis in human cancer cells [6]. Despite extensive knowledge regarding the anticarcinogenic role of ITCs, our understanding of the extent to which these compounds are released, absorbed and excreted after consumption of cruciferous vegetables is less well established.

In humans ITCs are primarily metabolised via the mercapturic acid pathway to the *N*-acetylcystein conjugate of ITC, and this metabolite is excreted in urine and could thereby serve as a marker for absorbed ITC [7,8]. In this present investigation, we have chosen to look at total ITC excretion as a biomarker of overall ITC exposure. Previous studies [8,9] have investigated how individual ITC metabolites can be reliably quantified and used as biomarker for exposure for individual glucosinolates. However, precaution must be taken when assuming that the excreted ITC conjugates are equivalent to the consumed amounts of ITCs, since ITC conjugates are unstable and rapidly dissociate back to ITCs [10]. We, therefore decided to look

* Corresponding author. Tel.: +45 72346000; fax: +45 72347698.
E-mail address: mehxy@dfvf.dk (M. Kristensen).

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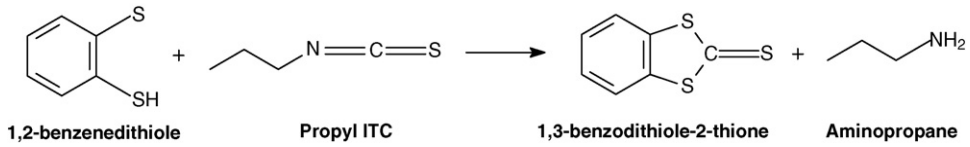

Fig. 1. Cyclocondensation of ITC with 1,2-benzenedithiole forms 1,3-benzodithiole-2-thione.

at the total excretion of ITCs instead of individual ITCs as a biomarker of total ITC exposure. Using a previously developed assay, where ITCs and their thiol conjugates react quantitatively with the vicinal mercapto groups of 1,2-benzenedithiole giving rise to the cyclic condensation product 1,3-benzodithiole-2-thione [11] (Fig. 1), it is possible to measure the total amount of ITC and their conjugates excreted in urine by reversed phase HPLC [7]. To quantify ITCs in human urine by the cyclocondensation reaction, and to assess the performance of the HPLC method, a standard of the cyclocondesations product is required. Moreover, since the ITC mercapturic acid, *N*-acetyl-*S*-(*N*-4-methylsulfinylthiocarbamyl)-L-cystein (SFN-NAC), is the main urinary ITC metabolite after intake of broccoli [7], a standard of this compound additionally has to be included to ensure that the conversion of all ITCs to 1,3-benzodithiole-2-thione in the cyclocondensation reaction is complete. Neither of these two standards is commercially available, but methods for synthesis of both compounds have previously been described by Zhang et al. [11] and Kassahun et al. [12]. However, difficulties with the final purification of the synthesis products prompted us to develop improved, easier and more cost-effective methods for synthesis of these compounds, as described in this paper. By utilizing these standards we investigated the relationship between consumption of broccoli and urinary excretion of ITCs in 10 humans.

2. Experimental

2.1. Materials

Propyl ITC, 1,2-benzenedithiole and *N*-acetylcysteine (NAC) were purchased from Aldrich (Milwaukee, WI, USA). D,L-Sulforaphane (4-methylsulfinylbutyl isothiocyanate) (SFN) was purchased from LKT Laboratories (St. Paul, MI, USA). Reversed phase C18 Mega Bond Elut cartridges were purchased from Varian (Harbor City, CA, USA). Myrosinase and all other chemicals and solvents used were of analytical grade and purchased from Sigma–Aldrich (St. Louis, MO, USA). The broccoli used was of Spanish origin and purchased from the local grocery store.

2.2. Equipment

HPLC analyses were performed on a HP 1090-system equipped with a DAD UV/vis-detector from Agilent Technologies (Waldbronn, Germany). A Zorbax SB-C18 column (4.6 mm × 150 mm, 3.5 μm) with a guard column (C18, 4.6 mm × 12.5 mm, 5 μm), both from Agilent Technologies, was used. The columns were kept at 40 °C during analyses. HPLC/MS-analyses were carried out on an 1100 HPLC with DAD UV/vis-detector connected to a mass spectrometer (G1946 MSD, B-model), both from Agilent Technologies (Waldbronn, Germany). The system was using the same chromatographic columns and conditions as for the 1090-HPLC analysis.

2.3. Synthesis of 1,3-benzodithiole-2-thione

The synthesis of 1,3-benzodithiole-2-thione (Fig. 1) was modified by Zhang et al. [11] to ease purification of the synthesis product. An amount of 5.6 mmol of 1,2-benzenedithiole was dissolved in a solution of 12 ml of methanol and 24 ml potassium phosphate buffer (pH 8.5). Propyl ITC (9.9 mmol) was mixed with 12 ml methanol and transferred to a closed funnel with pressure equalization, and dropwise added into the 1,2-benzenedithiole solution under constant stirring. The mixture was left to react overnight at room temperature under nitrogenous atmosphere and constantly stirred. The solution was extracted with chloroform three times (50, 25, 25 ml, respectively) to isolate the reaction product. Any residues of water in the chloroform phase were removed by drying with solid sodium sulphate (Na_2SO_4) and the product was isolated by subsequent evaporation of the chloroform on a rotary evaporator (RotaVapor R-114, Bie & Bertensen A/S, Copenhagen, Denmark). Purification of 1,3-benzodithiole-2-thione was conducted using thin layer chromatography (TLC) plates (Analtech-uniplate, preparative layer, UV 254, 20 cm × 20 cm, Inc. Nework, Germany). 1,3-Benzodithiole-2-thione was dissolved in chloroform and applicated on the plates. Dichloromethane was used as mobile phase. The yellow 1,3-benzodithiole-2-thione zone was identified by UV-light (254 nm) and scraped off the TCL plates, extracted in chloroform and the silica removed by filtration. The chloroform was evaporated by rotary evaporation and the molecular mass of the compound was confirmed by HPLC/MS using the same method as described for measurement of total ITC in urine and broccoli. The ^{1}H NMR spectrum of the product, as a ~0.2 M solution in chloroform-*d*, was recorded at 300 MHz on a Varian Mercury spectrometer (Varian, Palo Alto, CA, USA) using tetramethylsilane (TMS) as internal standard.

2.4. Synthesis of SFN-NAC

The synthesis of SFN-NAC was modified from Kassahun et al. [12] to facilitate the purification of the synthesis product (Fig. 2). One hundred and ten micromoles of NAC was dissolved in 2 ml aqueous ethanol (50%) and pH was adjusted to 7.8 using 1 M NaOH. Fifty six micromoles of SFN dissolved in 3 ml ethanol was added to the NAC-solution and the mixture was stirred at room temperature under nitrogenous atmosphere for

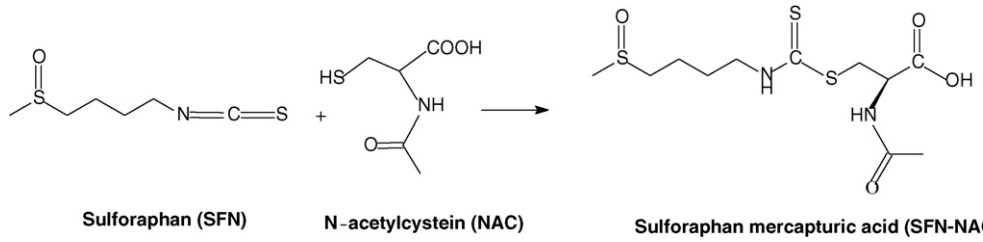

Fig. 2. Reaction of 4-methylsulfinylbutyl ITC (SFN) with *N*-acetylcystein (NAC) gives rise to formation of *N*-acetyl-*S*-(*N*-4-methylsulfinylbutylthiocabamoyl)-L-cysteine (SFN-NAC).

3 h. Subsequently, the solvent was evaporated by rotary evaporation. The crude product was dissolved in 40 μl MeOH and 4.8 ml 1% HCOOH and purified by solid phase extraction (SPE) (2 g Bond Elut, reversed phase C18). Prior to application of the dissolved product, the SPE column was flushed with 4.8 ml MeOH, followed by 4.8 ml 1% HCOOH. To separate impurities from SFN-NAC, six washing steps with 4.8 ml of 6% (×3), 10% (×1) and 20% (×2) MeOH in 1% HCOOH, respectively, was conducted. SFN-NAC was finally eluted by 2 ml × 4.8 ml 35% MeOH in 1% HCOOH. The molecular mass of the compound was confirmed by HPLC/MS using mobile phases A: 1% formic acid (v/v) and B: 100% MeOH (flow-rate 1 ml/min). The elution profile was: linear gradient from 0 to 11 min: 0–30% B (v/v), 11–19 min: 100% B, 19–20 min: 1% B (v/v). The UV/vis spectra were recorded simultaneously during analysis at 269 and 250 nm (reference at 450 ± 80 nm) with peak scanning between 230 and 400 nm.

The pooled SFN-NAC fractions, collected from the SPE, were evaporated to dryness with a refrigerated vaportrap (Savant and Heto UR2 rotor, RTV4104, Bie & Berntsen A/S, Copenhagen, Denmark). Finally, NMR spectroscopy confirmed structure and purity of the isolated product. For NMR the SFN-NAC was dissolved in DMSO-*d*, and ^{1}H NMR spectra were recorded on a Bruker Av 400 WB spectrometer (Bruker Biospin, Karlsruhe, Germany) at 400.13 MHz with TMS as internal standard.

2.5. Human intervention study

Ten non-smoking subjects, nine females and one man (19–29 years) with a normal BMI (19.6–24.6 kg/m^2) volunteered for the study. One week prior to and during the trial, subjects had to exclude glucosinolate-containing foods from their diet and were instructed to avoid all kinds of fruit and vegetables as well as coffee, tea and chocolate. A urine sample was collected after 12 h overnight fasting on the first day of the trial, and subsequently a standardized breakfast was consumed including 350 g broccoli, which had been steamed for 2 min. A strictly controlled glucosinolate-free diet based on bread, milk, cream cheese, eggs, meat, potatoes and biscuits (women 10 MJ and men 12 MJ, 48.6 E% from carbohydrate, 16.3 E% from protein and 35.1 E% from fat) was provided for the rest of the first trial day and the following day. Urine samples were collected in intervals between 0 and 2 h, 2 and 4 h, 4 and 6 h, 6 and 8 h, 8 and 12 h, 12 and 24 h, and 24 and 48 h after consumption of the broccoli. Urine was collected in 2500 or 500 ml plastic bottles containing 2.5 or 0.5 g l-ascorbic acid, respectively, as stabilizing agent. Urine samples were stored at −80 °C until analyses. The study was approved by the Ethics Committee of Copenhagen and Frederiksberg (J.nr. KF 01-161/01).

2.6. Measurement of total ITC in urine and broccoli

The total amount of ITC in urine was determined by UV detection as the cyclocondensation product as described previously [11,13,14] although with a few minor adjustments. All samples were blinded by technicians and analysed in randomised order. Five hundred microlitres of 500 mmol/l sodium borate buffer (pH 9.25), 1 ml of 20 mmol/l 1,2-benzenedithiole in acetonitrile and 500 μl of urine sample were mixed in a 4 ml glass vial with screw cap. Mixtures were incubated for 2 h at 65 °C to ensure that all ITCs were converted to 1,3-benzodithiole-2-thione. After cooling at room temperature and centrifugation at 3000 rpm, 50 μl of the supernatant was injected onto the HP 1090-system. The flow-rate of the system was 1 ml/min and mobile phases A: 1% formic acid (v/v) and B: 100% MeOH. The elution profile was: 0–7 min with 80% B (v/v), 7–8 min with 80–100% B (v/v), and 8–12 min with 100% B. Post time: 5 min with 1% B (v/v). The UV/vis spectra were recorded simultaneously during analysis at 365 and 290 nm, with peak scanning between 230 and 400 nm, using the 365 nm signal to quantify the amount of 1,3-benzodithiole-2-thione.

Measurement of ITCs in broccoli was conducted as described by Conaway et al. [14] although modified by excluding the MeOH extraction steps, since these were found not to affect the final measurable ITC level (data not shown). Twenty grams of the intervention broccoli was chopped and 1 g was mixed with 12.5 ml 0.35 U myrosinase in 0.1 mol/l potassium phosphate buffer (pH 6.6) and homogenised by using an Ultra-Turax DI25 Basic (IKA-Werke, Staufen, Germany). The homogenate was incubated in a water bath shaker for 2 h at 37 °C to ensure that all glucosinolates had been hydrolysed. The sample was centrifuged at 4000 rpm for 10 min and 500 μl of the supernatant was added to the cyclocondensation assay, as described for urine analysis, except the sodium borate buffer that was replaced with 500 mmol/l potassium phosphate buffer (pH 8.5).

2.7. Calibration and standards

Quantification of the produced cyclocondensations product in urine samples was based on calibration curves achieved by spiking blank urine samples in triplicate with different

concentrations of the synthesised 1,3-benzodithiole-2-thione standard (six levels from 0.1 to 50 μM). Reproducibility of the method was controlled by including spiked urine samples and a pure standard in each series of analyses; two levels of SFN-NAC in blank urine (1.25 and 2.5 μmol/l) were carried through the cyclocondensation and analysed along with a pure standard of 10 μg/ml 1,3-benzodithiole-2-thione. The limit of quantification of 1,3-benzodithiole-2-thione was determined to 5 pmol in urine based on the lowest level in the calibration curve (0.1 μM).

2.8. Statistical design

All data are expressed as the mean value of indicated numbers of measurements ± standard deviation (S.D.). Coefficient of variation (CV) was determined as (S.D./mean) × 100%.

3. Results and discussion

3.1. Synthesises

To reduce the cost and simplify the purification process, we modified the synthesis of 1,3-benzodithiole-2-thione reported by Zhang et al. [11]. By changing the molar ratio of the reactants, we avoided difficulties with separation of excess of 1,2-benzenedithiole from 1,3-benzodithiole-2-thione, since propyl ITCs are easily separated from the product 1,3-benzodithiole-2-thione. ^{1}H NMR and MS data of 1,3-benzodithiole-2-thione were in agreement with literature [11,12]. The yield of 1,3-benzodithiole-2-thione was 1.73 mmol (320 mg, 31%) as yellow crystals.

The purification of SFN-NAC with solid phase extraction facilitated the purification of larger amounts of the product compared to the reversed phase HPLC method described for purification in Kassahun et al. [12]. After the synthesis, the crude product of SFN-NAC appeared as a light brown oily product. When analysed by HPLC/MS, SFN-NAC and two impurities were detected. SFN-NAC was eluted from the SPE column in the fraction containing 35% MeOH in 1% HCOOH. The two impurities were eluted with 6 and 10–20% MeOH in 1% HCOOH, respectively. The most polar impurity was identified as NAC with $[M + H]^+ = m/z$ 164, and the other with mass $[M + H]^+ = m/z$ 325 was tentatively identified by ^{1}H NMR as the dimer of NAC. ^{1}H NMR and MS data for SFN-NAC were in agreement with literature [12,15]. After SPE purification, SFN-NAC appeared as a white powder and the yield was 19.4 μmol (6.6 mg, 35%). Neither Zhang et al. [11] nor Kassahun et al. [12] have reported the yield of their synthesis products, so conclusions on whether the yields of our modified methods are higher cannot be drawn.

3.2. Human intervention study

The cyclocondensation reaction was used to quantify ITCs in the urine from the broccoli intervention. Urinary content of 1,3-benzodithiole-2-thione was quantified by HPLC after the cyclocondensation reaction. Fig. 3 shows the HPLC chromatograms of a pure standard solution of 1,3-benzodithiole-2-thione (A), 1,3-benzodithiole-2-thione in urine after broccoli ingestion (B) and in blank urine (C). By spiking blank urine with a SFN-NAC standard, we have shown that the conversion of the SFN-NAC to the cyclocondensations product corresponded to 80.53 ± 8.63% and the resulting standard curve was linear with a correlation coefficient at 0.999. Reproducibility of the method, verified by the two levels of SFN-NAC and athe pure standard of 1,3-benzodithiole-2-thione, had a CV% at 12.3 and 3.3, respectively.

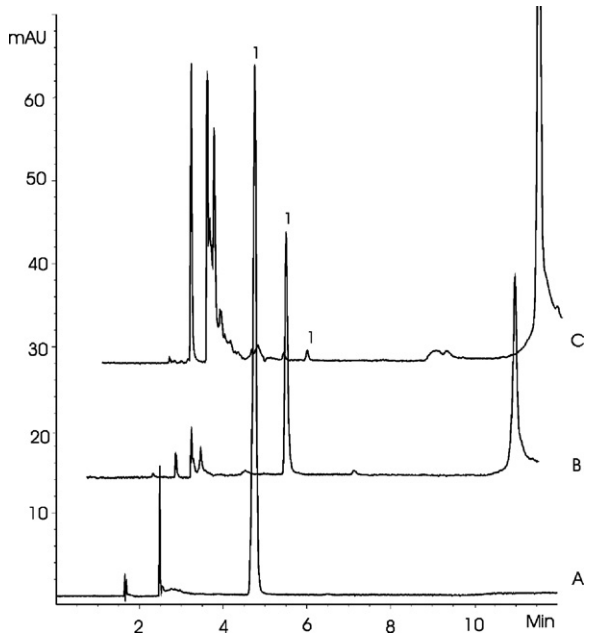

Fig. 3. High-performance liquid chromatography analysis of 1,3-benzodithiole-2-thione in a pure standard (10 μM) (A), in a urine sample after broccoli ingestion (B) and in blank urine (C). (1) Shows the peak of 1,3-benzodithiole-2-thione eluted after 4.8 min.

The total amount of ITC equivalents excreted in the urine, collected at different time intervals, 48 h after ingestion of broccoli are illustrated in Fig. 4 for all subjects. Baseline values ranged from non-detectable to 0.38 μmol with a mean of

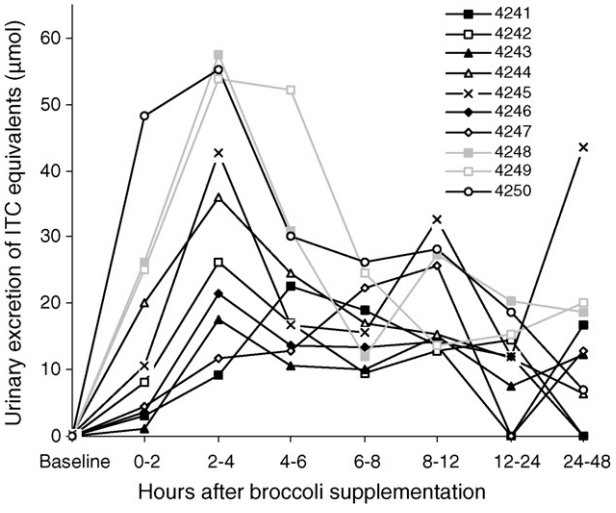

Fig. 4. Urinary ITC levels for individual subjects at a given time interval after ingestion of 350 g lightly steamed broccoli.

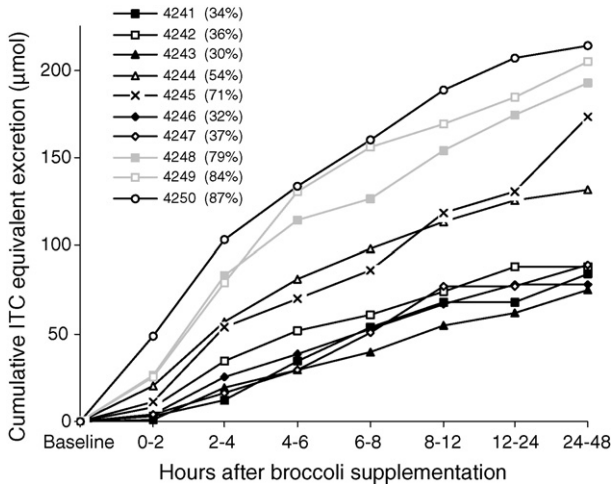

Fig. 5. Cumulative excretion of urinary ITC equivalents for individual subjects, through 48 h after broccoli supplementation. Percentage of ITC equivalent excreted of each subject compared to ITC amount analysed in the supplemented broccoli is given in the parenthesis after the subject code.

0.09 ± 0.14 μmol. Content of ITC equivalents in urine 24–48 h after broccoli supplementation was 13.68 ± 12.6 μmol indicating that ITC excretion continues after 24 h. Five subjects illustrated an increase in ITC equivalent excretion in the 24–48 h interval, assumable due to unintentional consumption of food elements containing glucosinolates. Most subjects had a maximum urinary ITC equivalent excretion (C_{max}) within the 2–4 h interval and a minor increase in excretion in the 8–12 h interval, but inter-individual variation was observed. The ITC equivalents excreted in urine up to 4 h after broccoli ingestion is most likely due to absorption of ITC in the small intestine. The second peak in excretion after 8–12 h is presumably due to ITC absorption in the colon. The broccoli used in this intervention study was steamed for 2 min before serving and this may have reduced the myrosinase activity level in the broccoli. A reduced myrosinase activity in the broccoli may result in that part of the glucosinolates is transferred unhydrolysed through the small intestine to the colon. Bacteria in the colon have been shown to exert myrosinase activity [2,16] and unhydrolysed glucosinolates may thus, be converted to ITCs in the colon. Furthermore, Petri et al. used an intestinal perfusions technique (Loc-I-Gut®) to study intestinal absorption of the ITC sulforaphane [17]. They found that sulforaphane was extensively absorbed by enterocytes, metabolised and partly effluxed back into the lumen as a glutatione (GSH) conjugate. This ITC–GSH conjugate is presumably absorbed later in the duodenum or colon and may also contribute to the biphased urinary excretion curve.

The cumulative excretion rate of ITC equivalents (Fig. 5) shows a large variation between individuals. Especially subjects 4248, 4249 and 4250 were high excreters compared to subjects 4241, 4242, 4243 and 4246. All excretion curves have a relatively smooth and gradual rise, except for subject 4245, who showed higher excretion from 8 to 48 h than the other subjects. The total content of glucosinolates that were converted to ITCs in the broccoli used for the intervention was found to be 0.7 ± 0.08 μmol ITC/g. This is equivalent to an ingestion of 245 μmol ITC per person, if all glucosinolates were converted to ITCs. The total cumulative excretion of ITC equivalents in the subjects ranged from 74.5 to 213.6 μmol, corresponding to an excretion of 30–87% of the total amount of ITCs ingested (Fig. 5). The coefficient of variation of 51.4% among the individuals illustrates a large inter-individual variation in ITC excretion. There may be a number of reasons for this wide variability in ITC excretion among humans. Differences in individual patterns through mastication [18] and processing in the small intestine, whereby myrosinase is released from the broccoli cells, may be one reason. Amount and variation in gut bacterial strains with myrosinase activity between individuals may be another factor [19]. Furthermore, ITCs are metabolised by an initial and rapid conjugation reaction with glutathione in the human enterocytes [20] catalysed by glutathione-*S*-transferases. These transferases are subject to considerable polymorphism in individuals and could possibly account for some differences observed in urine [21]. Mean excretion of total ITC equivalents among the 10 individuals was 49.1 ± 25.2%. Conaway et al. [14] found an average 24 h urinary excretion of ITC equivalents of only 10.2 ± 5.9% after ingestion of 200 g steamed broccoli. The broccoli in the study by Conaway et al. was steamed for 15 min and all myrosinase activity was assumed completely inactivated. Thereby only glucosinolates converted to ITCs in colon is excreted in urine. In our study the broccoli was steamed for only 2 min, whereby much higher myrosinase activity is maintained resulting in a higher conversion of glucosinolates to ITCs. Furthermore, Conaway et al. [14] only collected urine for 24 h after supplementation, whereas we collected urine for 48 h.

4. Conclusion

In the present study, we have demonstrated optimised synthesis methods of standards required to analyse dietary exposure to ITCs. This improved methodology facilitates the use of the cyclocondensation reaction for future studies of human exposure to ITCs and the potential cancer preventive effects of ITCs.

Acknowledgements

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