

## Effect of meal composition and cooking duration on the fate of sulforaphane following consumption of broccoli by healthy human subjects

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(Received 26 June 2006 – Revised 18 October 2006 – Accepted 20 October 2006)

The isothiocyanate, sulforaphane, has been implicated in the cancer-protective effects of brassica vegetables. When broccoli is consumed, sulforaphane is released from hydrolysis of glucoraphanin by plant myrosinase and/or colonic microbiota. The influence of meal composition and broccoli-cooking duration on isothiocyanate uptake was investigated in a designed experiment. Volunteers ( $n$  12) were each offered a meal, with or without beef, together with 150 g lightly cooked broccoli (microwaved 2.0 min) or fully cooked broccoli (microwaved 5.5 min), or a broccoli seed extract. They received 3 g mustard containing pre-formed allyl isothiocyanate (AITC) with each meal. Urinary output of allyl (AMA) and sulforaphane (SFMA) mercapturic acids, the biomarkers of production of AITC and sulforaphane respectively, were measured for 24 h after meal consumption. The estimated yield of sulforaphane *in vivo* was about 3-fold higher after consumption of lightly cooked broccoli than fully cooked broccoli. Absorption of AITC from mustard was about 1.3-fold higher following consumption of the meat-containing meal compared with the non meat-containing alternative. The meal matrix did not significantly influence the hydrolysis of glucoraphanin and its excretion as SFMA from broccoli. Isothiocyanates may interact with the meal matrix to a greater extent if they are ingested pre-formed rather than after their production from hydrolysis of glucosinolates *in vivo*. The main influence on the production of isothiocyanates *in vivo* is the way in which brassica vegetables are cooked, rather than the effect of the meal matrix.

### Allyl isothiocyanate: Sulforaphane: Mercapturic acid: Meal composition

Epidemiological studies suggest that the cancer-protective effects of brassica vegetables may be partly related to their content of glucosinolates (van Poppel *et al.* 1999). During the preparation and cooking of brassica, followed by their ingestion and digestion in the upper alimentary tract, glucosinolates are hydrolysed to thiohydroxamate-*O*-sulfonate by a plant thioglucosidase, myrosinase (EC 3.2.1.147), which is released from ruptured plant cells. Thiohydroxamate-*O*-sulfonate is then immediately converted to different classes of metabolites depending on the properties of the hydrolysis medium (Fenwick & Heaney, 1983). A further stage of hydrolysis of glucosinolates occurs in the colon under the action of colonic microbiota (Elfoul *et al.* 2001; Krul *et al.* 2002). Isothiocyanates, produced from hydrolysis of glucosinolates at a neutral pH, are one of the main metabolites implicated in the cancer-protective effects of brassica (Zhang *et al.* 2006).

An understanding of the digestive and absorptive fate of dietary glucosinolates and their metabolites has emerged mostly from mechanistic studies in animal models (Brüsewitz *et al.* 1977; Mennicke *et al.* 1983; Duncan *et al.* 1997; Elfoul *et al.* 2001). A few studies, involving human subjects, have used urinary biomarkers to demonstrate the absorption of isothiocyanates *in vivo* after the intake of glucosinolates from brassica vegetables (Getahun & Chung, 1999; Conaway *et al.* 2000; Shapiro *et al.* 2001; Rouzaud *et al.* 2004). After their

absorption, isothiocyanates are metabolised by the mercapturic acid pathway *in vivo*. Isothiocyanates initially form conjugates with glutathione, then undergo enzymatic modifications, and are excreted in urine as their corresponding *N*-acetylcysteine conjugates or mercapturic acids (MA) (Brüsewitz *et al.* 1977). Urinary excretion of MA of isothiocyanates therefore reflects their production and uptake *in vivo*.

The production of isothiocyanates following the ingestion of brassica is influenced by residual glucosinolate concentration and plant myrosinase activity in the ingested vegetable. However, brassica vegetables are normally consumed as part of a meal and the digestive fate of glucosinolates may be further influenced by the composition of the meal matrix.

Sulforaphane, the alkyl isothiocyanate in broccoli, is produced from hydrolysis of its alkyl glucosinolate precursor, glucoraphanin. The aim of the present study was to determine the effect of the meal matrix on the production of sulforaphane from lightly cooked or fully cooked broccoli in healthy human volunteers.

### Materials and methods

#### Human subjects

Twelve healthy, non-smoking, human volunteers (5 males and 7 females), with mean BMI 26.1 (SEM 1.52) kg/m<sup>2</sup> and mean

age 38.4 (SEM 3.11) years, were recruited for the study after giving their informed consent. None of the subjects was on any drug or dietary supplement just before the start of or during the experiment. Volunteers were low to moderate consumers of brassica vegetables. They were given information on the study and provided with a urine collection kit and instructions for the collection and storage of urine following their experimental meals. Volunteers were asked to avoid foods containing glucosinolates and isothiocyanates, and nutritional supplements for 48 h before their first experimental meal and throughout the study. They were also required to keep a simple food diary of their daily dietary intake during that period. Ethical approval for the study was obtained from the Grampian Research Ethics Committee within the Grampian Hospitals NHS Trust.

### Experimental design

The experiment consisted of a  $2 \times 3$  factorial design with factors involving a meal containing meat or a vegetarian equivalent, and three broccoli treatments comprising 150 g lightly cooked broccoli, 150 g fully cooked broccoli or an extract of broccoli seeds (70 ml). Amounts of cooked broccoli corresponded to a portion size slightly larger than generally consumed (MAFF, 1988), while the amount of seed extract was adapted from Rouzaud (2001) and aimed to contain substantial concentrations of sulforaphane. These amounts were chosen to

enhance the estimation of recovery of MA in urine. The vegetarian meal consisted of rice and a tomato and basil sauce, while the meat-containing alternative also contained lean, minced beef. The broccoli seed extract was a source of pre-formed metabolites of glucosinolates, generated from hydrolysis of glucosinolates during its preparation. It was designed to determine the recovery of metabolites of glucosinolates after their ingestion and absorption *in vivo*. Six experimental meals were offered to each volunteer. Meals were served on Mondays and Thursdays over 3 weeks, and were thus separated by a wash-out period of at least 48 h to avoid potential carry-over effects. Meals were allocated to volunteers by randomising type of meal (vegetarian or meat-containing meal) and broccoli treatment (lightly cooked, fully cooked and seed extract) within two combined  $6 \times 6$  complete Latin squares. The order of allocation of each combination of type of meal and cooking treatment of broccoli was balanced to resolve possible carry-over effects of dietary treatment. Since each individual received all combinations of type of meal and broccoli-cooking treatment, each subject was treated as a block in ANOVA to account for inter-individual variation in urinary excretion of MA.

Volunteers were offered 3 g English mustard with each experimental meal. Mustard is an abundant and stable source of pre-formed allyl isothiocyanate (AITC; Rouzaud *et al.* 2004). It was used to control for intra- and inter-individual variation in the absorption and excretion of isothiocyanates. Fig. 1 illustrates

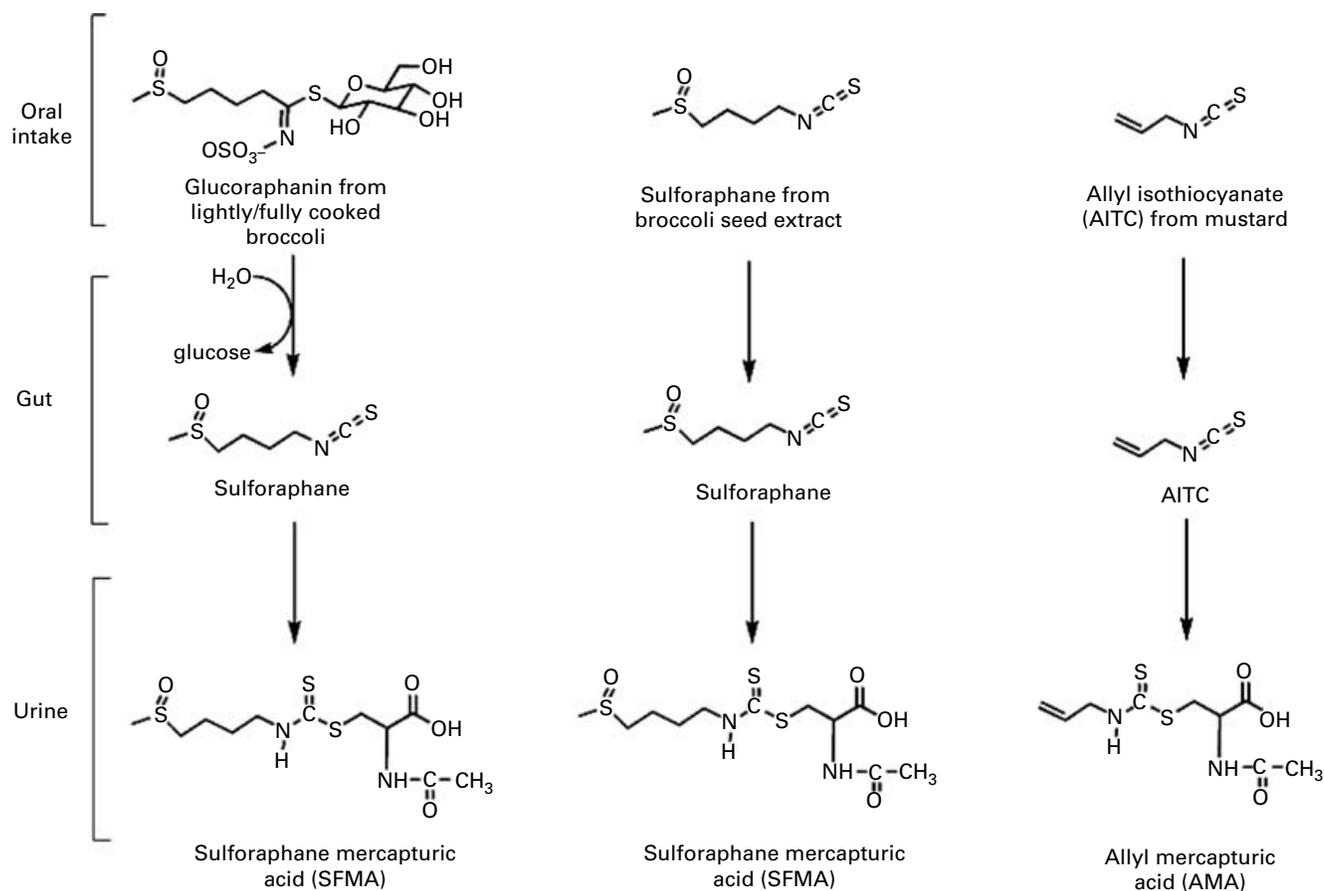


Fig. 1. Metabolic fate of glucosinolate precursors and isothiocyanates following consumption of brassica within experimental meals by volunteers.

the expected metabolic fate of glucosinolate precursors and isothiocyanates following consumption of brassica within the experimental meals by human volunteers.

### Experimental meals

Broccoli heads (Var. Monaco), each weighing about 300 g and pre-packed with PVC film, were purchased from a local supermarket (Sainsbury's, Aberdeen, UK) on the evening before each experimental day and held at 4°C until they were cooked. The variety of broccoli and its wholesale source were kept constant throughout the experimental period. Broccoli florets were cut and maintained at 4°C, 30 min before being cooked and served to volunteers. Florets were divided into portions, weighing 150 g each, from seven broccoli heads for each group of six individuals to obtain a uniform product within groups. Lightly cooked or fully cooked broccoli was prepared by cooking a portion of florets with 16 ml tap water in a heat-resistant dish, covered with pierced PVC film, in a microwave oven at 750 W for 2.0 or 5.5 min respectively. Test samples of lightly cooked or fully cooked broccoli were prepared at the same time as broccoli that was offered to volunteers within each group. They were maintained at room temperature while volunteers consumed their meals, and frozen in liquid N<sub>2</sub> to be stored at -80°C for subsequent analysis of residual glucoraphanin concentration and plant myrosinase activity.

Forty portions of broccoli seed extract were prepared 2 d before the start of the experiment and were frozen at -20°C for use throughout the experiment. GEO organic broccoli seeds (UK Juicers Ltd, York, UK) were milled in a coffee grinder. For each extract, 9 g ground broccoli seed was mixed with 50 ml tap water and incubated for 1 h at room temperature to allow hydrolysis of glucoraphanin to sulforaphane and sulforaphane nitrile. The suspension was sieved and decanted into a polypropylene bottle. All bottles containing the extract were sealed and placed in a water bath at 80°C for 10 min to inactivate plant myrosinase (Rouzaud *et al.* 2004). Extracts were cooled on ice for 15 min, followed by the addition of 20 ml pure orange juice (Del Monte Foods International, Staines, Middx, UK) and 10 g Canderel (aspartame) artificial sweetener (Merisant UK Ltd, Bucks, UK) to improve palatability. The seed extracts were stored at -20°C and defrosted at 4°C on the evening before each experimental meal.

The meat-containing and vegetarian meals were prepared and blast-frozen 2 weeks before the start of the experiment. For the meat-containing component, 5000 g fresh, lean minced beef topside was purchased from a local supermarket (Tesco, Aberdeen, UK). It was cooked in two equal batches, each in 10 ml olive oil followed by the addition of 3000 g tomato and basil sauce (Bertolli UK, West Sussex, UK) and further cooking for 50 min. For the vegetarian component, two batches of 3000 g tomato and basil sauce were each heated for 30 min. Following cooking, the meat-containing and vegetarian components were portioned into 175 g and 100 g respectively, each type of meal thus containing equal amounts of sauce, and were blast frozen in heat-proof, disposable containers. They were stored at -20°C and heated in an oven at 200°C for 20 min before being served to volunteers.

Basmati rice was cooked and held in a rice cooker 30 min before the experimental meals were served.

Portions of smooth English mustard (Colman's, Norwich, Norfolk, UK), weighing 30 g each, were incubated in a water bath at 80°C for 10 min to inactivate plant myrosinase. They were cooled on ice for 40 min and frozen at -20°C. Mustard was defrosted at 4°C on the evening before each experimental meal and volunteers received 3 g (actual amount, 3.011 (SEM 0.002) g at each meal.

### Urine collection

Volunteers were asked to collect the total volume of one micturition just before consuming each experimental meal and then all urine excreted for 24 h after the meals. The latter was split into four batches, from 0–4 h, 4–10 h, 10–19 h and 19–24 h after the experimental meals. Each batch was collected into separate containers which were sealed and kept in cool bags containing cool blocks. Total volume of urine excreted within each batch was measured on receipt of samples from volunteers and 20 ml-aliquots of each sample were stored at -20°C for analysis of MA.

### Analysis of test meals

*Glucoraphanin in broccoli.* Glucoraphanin was extracted from freeze-dried broccoli, desulfated and analysed by HPLC, as modified from Minchinton *et al.* (1982) and European Union (1997), and described by Rungapamestry *et al.* (2006). Glucoraphanin concentration was quantified using published response factors that have been experimentally determined (European Union, 1997). A standard curve was also constructed for glucoraphanin (KVL, Frederiksberg, Denmark) at concentrations from 0 to 20 µmol/g DM within a freeze-dried watercress matrix ( $r^2$  0.9905) to verify linearity over the analytical range. The limit of quantification for desulfoglucoraphanin was 0.044 µmol/g DM.

*Metabolites of glucosinolates in mustard and broccoli seed extract.* AITC from mustard, and sulforaphane and sulforaphane nitrile from broccoli seed extract were analysed using the methods of Rouzaud *et al.* (2004), modified as follows. AITC was extracted twice in 10 ml dichloromethane from 1 g mustard diluted in 10 ml deionised water, which contained 100 µl 67 mM butyl isothiocyanate (Aldrich Chemical Company, Milwaukee, WI, USA) as internal standard. Sulforaphane and sulforaphane nitrile were extracted from 5 ml broccoli seed extract, which contained 100 µl 250 mM benzyl isothiocyanate (Aldrich Chemical Company) as internal standard. In each case, the pooled dichloromethane extracts were concentrated to 100 µl. Metabolites of glucosinolates were analysed by GC with a flame ionisation detector, using an Equity-1 fused silica capillary column with a non-polar poly(dimethylsiloxane) bonded phase 30 m × 0.25 mm i.d. × 0.25 µm film thickness (Supelco, Bellefonte, PA, USA). AITC was analysed at an isothermal column temperature of 70°C for 20 min, as adapted from Rouzaud *et al.* (2004). He gas was used as a carrier at a constant flow rate of 1 ml/min and a split flow rate of 30 ml/min. The injector and detector were held at 200°C. For sulforaphane and sulforaphane nitrile, the initial temperature of the column was maintained at 150°C for 2 min, and then increased linearly at

10°C/min to 270°C, and was maintained for 10 min at 270°C (Matusheski *et al.* 2004).

An authentic standard of AITC (Sigma-Aldrich, St Louis, MO, USA) was extracted at nominal concentrations from 3 to 30 µmol/ml within an aqueous matrix ( $r^2$  value for calibration curve was 0.9999). Sulforaphane and sulforaphane nitrile (synthesised by N. P. Botting, Department of Chemistry, University of St Andrews, UK) were added, at nominal concentrations from 0 to 0.68 and 0 to 3.14 µmol/ml respectively, to an extract of ground mustard seed, prepared using the same protocol as for the broccoli seed extract. They were then extracted from the mustard seed extract to construct a standard curve within a matrix similar to the broccoli seed extract ( $r^2$  values for calibration curves were 0.9961 and 0.9971 respectively). Metabolites of glucosinolates had an extraction efficiency of about 98% from a freeze-dried vegetable matrix and their concentrations were not corrected for this efficiency since the internal standard was recovered to a similar extent.

**Myrosinase activity.** The determination of myrosinase activity in broccoli was adapted from Shapiro *et al.* (2001), as described by Rungapamestry *et al.* (2006). A standard curve was prepared using myrosinase from a commercial source (Sigma-Aldrich) ( $r^2$  0.9930). Myrosinase activity was expressed as units/portion of cooked broccoli (1 unit myrosinase will produce 1 µmol glucose from hydrolysis of 1 µmol 2-propenyl glucosinolate at 25°C and pH 6.0).

**Macronutrient analysis in meat-containing and vegetarian meals.** N content in freeze-dried test meals of the mince and tomato and basil sauce component of the meat-containing meal and the tomato and basil sauce component of the vegetarian meal was determined by the Dumas Combustion method (Pella & Colombo, 1973). Crude protein content was calculated by multiplying the N content by a factor of 6.25. Total fat content in freeze-dried test meals was determined by gravimetric extraction with iso-hexane for 6 h (British Standards, 1996).

**Analysis of urinary mercapturic acids.** *N*-acetyl-*S*-(*N*-4-methylsulfinylbutylthiocarbonyl)-*L*-cysteine (SFMA) and *N*-Acetyl-*S*-(*N*-allylthiocarbonyl)-*L*-cysteine (AMA), the mercapturic acids of sulforaphane and AITC respectively, were extracted from human urine and analysed by HPLC according to Rouzaud *et al.* (2004), as adapted from analysis of MA in rat urine (Mennicke *et al.* 1987). The dicyclohexylamine salt of *N*-Acetyl-*S*-(*N*-propylthiocarbonyl)-*L*-cysteine or propyl mercapturic acid (PMA) (synthesised according to Mennicke *et al.* (1983)) was used as the internal standard. Urinary SFMA and AMA concentrations were determined from calibration curves of SFMA (synthesised by N. P. Botting, Department of Chemistry, University of St Andrews, UK) and the dicyclohexylamine salt of AMA (synthesised according to Mennicke *et al.* (1983)) ( $r^2$  values of 0.9976 to 1.000 for AMA and 0.9865 to 1.000 for SFMA). The purity of SFMA standard was unknown and absolute concentrations of SFMA were calculated, assuming SFMA had a similar response to AMA and PMA in the UV detector (Rouzaud, 2001).

#### Calculations and statistical analysis

Samples were analysed in duplicate and data were presented as means with their standard errors for a minimum of six

observations for analysis of test meals and twelve observations for MA analysis. Statistical analyses were performed using the software GenStat Release 8.1 (Genstat 5 Committee, 2005). The data for excretion of AMA and SFMA were analysed and interpreted using two approaches. Firstly, total molar output of AMA or SFMA, for 24 h following consumption of meals, was expressed as a proportion of the molar intake of their ingested precursors. AMA output was calculated as a proportion of AITC intake and, SFMA output, as a proportion of glucoraphanin or sulforaphane intake. The effect of broccoli-cooking treatment and meal composition on total AMA or SFMA output as a proportion of ingested precursors was tested by ANOVA. Since each volunteer received all combinations of the dietary treatments, the significance of cooking treatment and meal matrix was assessed within volunteers. The inter-individual variability in MA excretion as a proportion of precursor ingested was tested by ANOVA.

Secondly, the amount of AMA or SFMA excreted in urine collected during each of the four periods, following consumption of the meals, was expressed as a proportion of the amount of precursors consumed. The effect of dietary treatment and time of urine collection on MA excretion after meal consumption was tested by ANOVA. The difference in mean MA output between the four periods of their excretion after meal consumption was tested using orthogonal contrasts within ANOVA at  $P < 0.001$ .

## Results

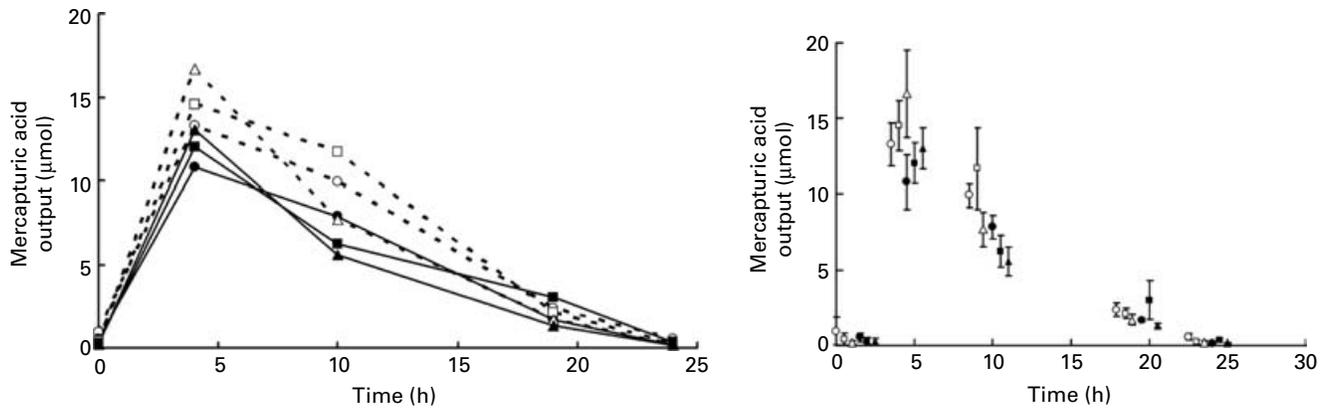
### Composition of experimental meals

**Intake of glucosinolates, isothiocyanates and myrosinase.** Glucoraphanin concentration comprised approximately 20% (µmol/µmol) total glucosinolate concentration in cooked broccoli. Mean glucoraphanin intake by volunteers was not significantly different between lightly cooked broccoli (62.0 (SEM 6.17) µmol/portion) and fully cooked broccoli (71.7 (SEM 5.73) µmol/portion) ( $P = 0.301$ ). The extract of broccoli seeds contained 2.7 (SEM 0.63) µmol sulforaphane and 121.6 (SEM 4.72) µmol sulforaphane nitrile/portion, while mustard contained 51.9 (SEM 0.08) µmol AITC/portion. Intake of plant myrosinase by volunteers was significantly higher after lightly cooked broccoli (55.7 (SEM 4.69) units/portion) than fully cooked broccoli (15.6 (SEM 1.17) units/portion) ( $P < 0.001$ ).

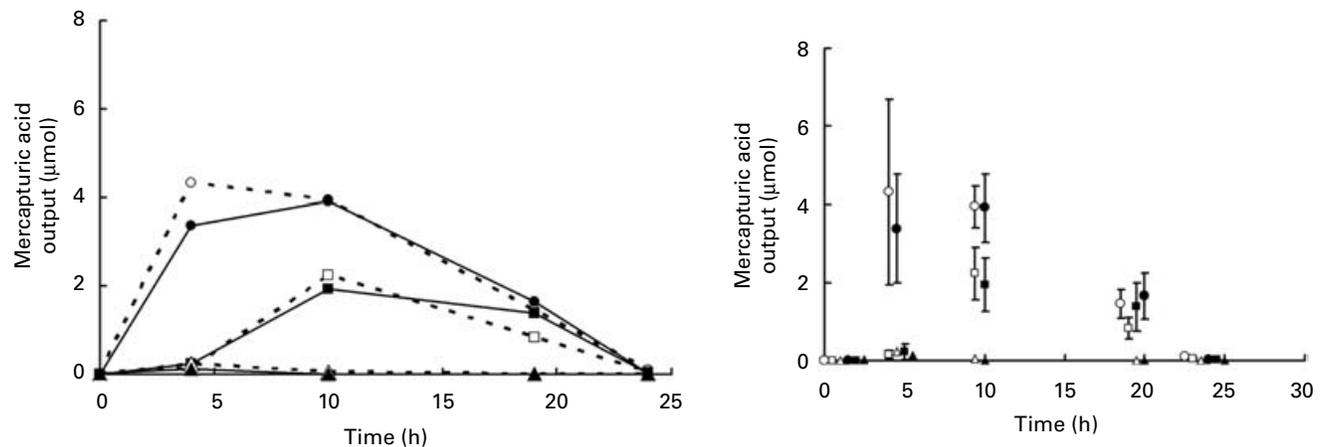
**Macronutrient composition of meat-containing and vegetarian meals.** The two types of meals were significantly different in their crude protein ( $P < 0.001$ ) and total fat content ( $P < 0.001$ ). The meat-containing and vegetarian components of the experimental meals contained 26.7 (SEM 0.41) and 2.2 (SEM 0.01) g protein/portion respectively. Total fat content was 7.4 (SEM 0.03) and 1.5 (SEM 0.02) g/portion respectively.

### Excretion of mercapturic acids

**Kinetics of allyl mercapturic acid and sulforaphane mercapturic acid output.** The kinetics of AMA and SFMA excretion in urine collected following the experimental meals are shown in Fig. 2 and Fig. 3 respectively. Excretion of AMA and SFMA by volunteers was complete 24 h after consumption



**Fig. 2.** Excretion of allyl mercapturic acid (AMA;  $\mu\text{mol}$ ), in baseline urine at time 0 before the experimental meals and from 0–4, 4–10, 10–19 and 19–24 h after each meal. Experimental meals consisted of lightly cooked broccoli with a meat-containing ( $\circ$ ) or vegetarian ( $\bullet$ ) meal, fully cooked broccoli with a meat-containing ( $\square$ ) or vegetarian ( $\blacksquare$ ) meal, and broccoli seed extract with a meat-containing ( $\triangle$ ) or vegetarian ( $\blacktriangle$ ) meal. Each point represents the mean of twelve observations. Mean values with their standard errors depicted by vertical bars are presented separately for clarity.



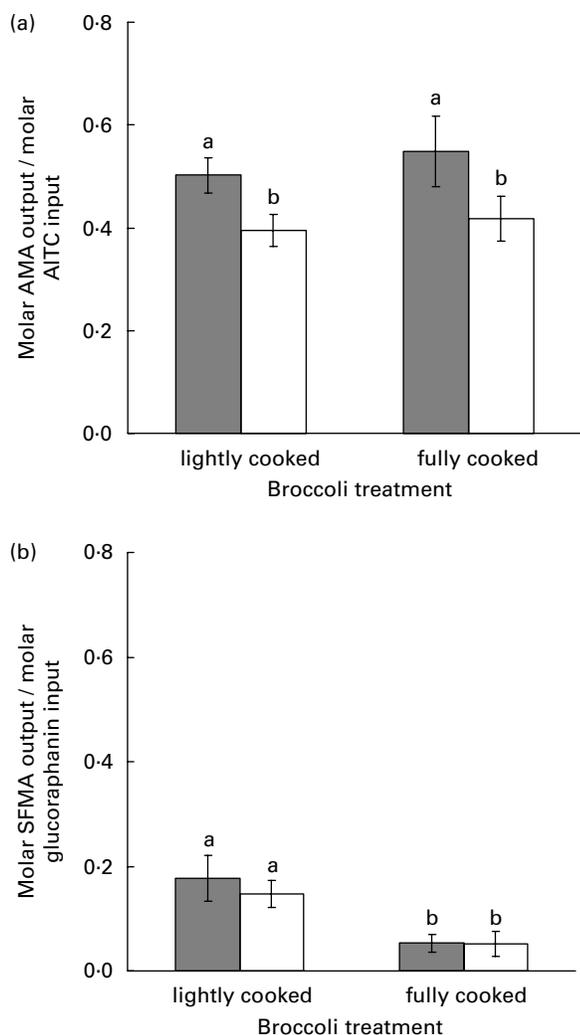
**Fig. 3.** Excretion of sulforaphane mercapturic acid (SFMA;  $\mu\text{mol}$ ), in baseline urine at time 0 before the experimental meals and from 0–4, 4–10, 10–19 and 19–24 h after each meal. Experimental meals consisted of lightly cooked broccoli with a meat-containing ( $\circ$ ) or vegetarian ( $\bullet$ ) meal, fully cooked broccoli with a meat-containing ( $\square$ ) or vegetarian ( $\blacksquare$ ) meal, and broccoli seed extract with a meat-containing ( $\triangle$ ) or vegetarian ( $\blacktriangle$ ) meal. Each point represents the mean of twelve observations. Mean values with their standard errors depicted by vertical bars are presented separately for clarity.

of the experimental meals. Trace concentrations of SFMA were observed after consumption of the broccoli seed extract.

*Total allyl mercapturic acid and sulforaphane mercapturic acid output as a proportion of ingested precursors.* Total AMA output over 24 h, as a proportion of AITC intake, was significantly higher after consumption of the meat-containing meal compared with the vegetarian meal by volunteers ( $P < 0.001$ ), but was not influenced by broccoli treatment ( $P = 0.563$ ) (Fig. 4(a)). Total SFMA output over 24 h, as a proportion of glucoraphanin intake, was significantly higher after consumption of lightly cooked than fully cooked broccoli by volunteers ( $P < 0.001$ ) (Fig. 4(b)). SFMA output was not affected by meal composition ( $P = 0.459$ ). The average proportion of AITC in mustard absorbed and excreted as AMA (Fig. 4(a)) was higher than the mean fraction of glucoraphanin hydrolysed and excreted as SFMA after the consumption of cooked broccoli (Fig. 4(b)). Both total AMA and SFMA output, expressed as a proportion of precursor ingested, varied significantly among volunteers ( $P < 0.001$ ). As determined from the CV among individuals, SFMA output was

about 2- and 4-fold more variable than AMA output after consumption of lightly cooked broccoli with the meat-containing and vegetarian meals respectively ( $P < 0.001$ ). After consumption of fully cooked broccoli with the meat-containing or vegetarian meals, SFMA output was about 3- or 4-fold more variable than AMA output ( $P < 0.001$ ) (Fig. 5a,b).

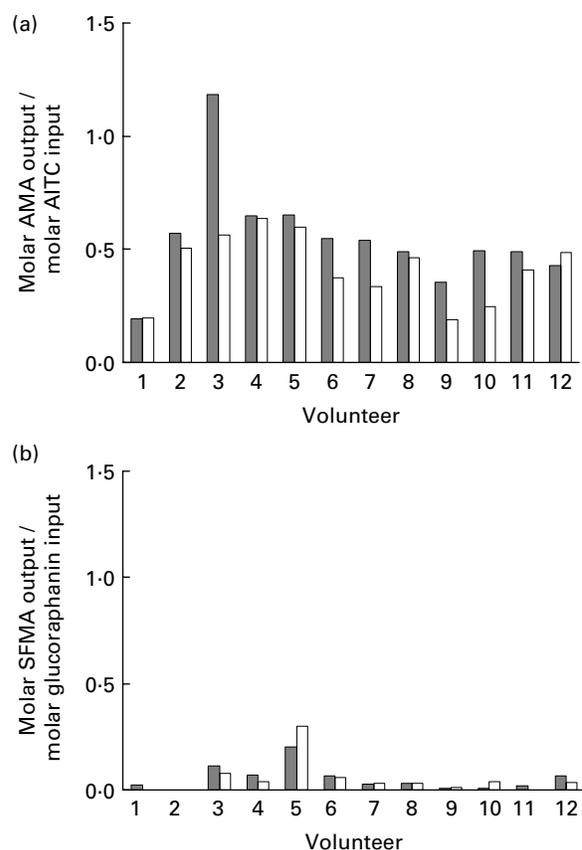
*Rate of allyl mercapturic acid and sulforaphane mercapturic acid output as a proportion of intake of precursor.* Approximately 50% AITC ingested was recovered as AMA 24 h after consumption of the meat-containing meal compared with about 40% after the vegetarian meal ( $P = 0.001$ ). AMA output was highest 4 h after consumption of the meals ( $P < 0.001$ ), with about one-third of AITC ingested being excreted in that time. The proportion of AITC absorbed and excreted as AMA after dietary intake was then significantly reduced over time ( $P < 0.001$ ). The proportion of AITC excreted was 2-fold lower in the second than in the first urine collection when mustard was ingested along with fully cooked broccoli and the vegetarian meal ( $P < 0.001$ ), but was similar in the first two urine collections after its consumption with fully cooked



**Fig. 4.** Total excretion of (a) allyl mercapturic acid (AMA;  $\mu\text{mol}$ ) over 24 h as a proportion of allyl isothiocyanate (AITC) intake ( $\mu\text{mol}$ ), and (b) sulforaphane mercapturic acid (SFMA;  $\mu\text{mol}$ ) over 24 h as a proportion of glucoraphanin intake ( $\mu\text{mol}$ ), after consumption of lightly cooked or fully cooked broccoli along with a meat-containing (■) or vegetarian (□) meal. Values are means for twelve replicates, with their standard errors represented by vertical bars. <sup>a,b</sup>Mean values with different letters were significantly different at  $P < 0.001$ . Allyl mercapturic acid (AMA) output was significantly affected by type of meal ( $P < 0.001$ ). Sulforaphane mercapturic acid (SFMA;  $\mu\text{mol}$ ) output was significantly influenced by broccoli treatment ( $P < 0.001$ ).

broccoli and the meat-containing meal ( $P = 0.181$ ). This led to an interaction between the effect of type of meal and time of urinary excretion on AMA output ( $P = 0.020$ ).

The proportion of glucoraphanin hydrolysed to sulforaphane and excreted as SFMA 24 h after the meals was approximately 20% from lightly cooked broccoli and 5% from fully cooked broccoli. SFMA was not recovered after consumption of the broccoli seed extract. Excretion of SFMA, as a proportion of glucoraphanin intake, was highest 4–10 h after intake of lightly cooked or fully cooked broccoli and was subsequently reduced over time ( $P < 0.001$ ). Most of the hydrolysed glucoraphanin was excreted as SFMA in the first and second urine collections following consumption of lightly cooked broccoli but in the second and third urine collections after intake of fully cooked broccoli. After the intake of lightly cooked



**Fig. 5.** Inter-individual variation ( $P < 0.001$ ) in total excretion of (a) allyl mercapturic acid (AMA;  $\mu\text{mol}$ ) over 24 h as a proportion of allyl isothiocyanate (AITC) intake ( $\mu\text{mol}$ ), and (b) sulforaphane mercapturic acid (SFMA;  $\mu\text{mol}$ ) over 24 h as a proportion of glucoraphanin intake ( $\mu\text{mol}$ ), after consumption of fully cooked broccoli along with a meat-containing (■) or vegetarian (□) meal.

broccoli, the proportion of glucoraphanin excreted as SFMA in the first urine collection was similar to that in the second collection ( $P = 0.372$ ), but higher than in the fourth urine collection ( $P < 0.001$ ). Following the consumption of fully cooked broccoli, it was higher in the second urine collection ( $P < 0.001$ ), but was not different in the last urine collection ( $P = 0.821$ ). This trend caused a significant interaction between the effect of broccoli treatment and time of urine excretion on SFMA output ( $P = 0.002$ ), irrespective of type of meal.

## Discussion

Previous feeding trials with brassica, involving human subjects, have offered raw or cooked vegetables or both, and measured urinary excretion of MA of isothiocyanates (Gethahun & Chung, 1999; Conaway *et al.* 2000; Rouzaud *et al.* 2004). Brassica are usually consumed as part of a complex meal. An understanding of the influence of the meal matrix on the absorption of isothiocyanates is important to complement research on the metabolic fate of glucosinolates from processed brassica vegetables and cancer-prevention. In brassica, sulforaphane may be one of the most potent chemoprotective isothiocyanates (Zhang *et al.* 1992; Myzak & Dashwood, 2006).

The present study investigated the effect of cooking broccoli together with the influence of the meal matrix on the time-course of production of sulforaphane in healthy volunteers. The durations for cooking broccoli were based on results from a previous experiment in which the highest production of isothiocyanates following hydrolysis, *in vitro*, was in lightly cooked brassica compared with raw or fully cooked brassica (Rungapamestry *et al.* 2006). Production of sulforaphane was quantified using its urinary MA biomarker, SFMA, a specific means of measuring excretion of individual isothiocyanates compared with measurement of the output of total isothiocyanates by their cyclocondensation products, used in previous studies (Getahun & Chung, 1999; Conaway *et al.* 2000; Shapiro *et al.* 2001). The current experiment is the first to show that the meal matrix can influence the production of pre-formed isothiocyanates *in vivo*.

Sulforaphane and AITC absorbed from cooked broccoli and mustard respectively were completely excreted 24 h after consumption of brassica by volunteers. This observation is in agreement with previous experiments on the time-course of excretion of MA after ingestion of glucosinolate precursors or isothiocyanates by human subjects (Chung *et al.* 1992; Conaway *et al.* 2000; Rouzaud *et al.* 2004). The peak in AMA excretion within 4 h of AITC intake and the proportion of ingested AITC converted to AMA after complete excretion of the metabolite are comparable to previous reports (Jiao *et al.* 1994).

Total AMA output, expressed as the molar proportion of AITC intake, was significantly influenced by the meal matrix but not by the cooking treatment of broccoli. The effect of meal composition on the postprandial absorption of isothiocyanates was expected to be partly dictated by the protein or fat content of the meal. Isothiocyanates have been shown to interact with proteins *in vitro* (Björkman, 1973). Contrary to our hypothesis that protein would delay or lower the absorption of isothiocyanates in the present experiment, excretion of AMA was higher after ingestion of AITC together with the meat-containing meal, which was higher in protein than the vegetarian meal. The fat content of the meat-containing meal was also 5-fold higher than that of the vegetarian meal. Isothiocyanates are relatively non-polar and the fat content of the meals may have been responsible for the subtle but significant differences in AITC uptake from the two types of meal. The improved bioavailability of many lipophilic drugs taken with food is often due to the lipid content of food, while lipid-based formulations can assist in the formation of solubilised phases for absorption of lipophilic drugs (Humberstone & Charman, 1997). The higher lipophilicity of AITC, compared with sulforaphane, may partly explain the influence of meal composition on absorption of AITC but not on sulforaphane. Isothiocyanates may also be better incorporated into lipids during the chewing phase, if they are ingested pre-formed, rather than being formed during the later stages of digestion within the gastrointestinal chyme, after hydrolysis of their glucosinolate precursors.

Total SFMA output was approximately 3 and 10  $\mu\text{mol}$ , corresponding to 5 and 20 % of glucoraphanin intake, after consumption of fully cooked and lightly cooked broccoli respectively. Excretion of SFMA was therefore expected to be higher following the intake of the broccoli seed extract due to its expected high concentrations of pre-formed

sulforaphane, as opposed to the comparably lower glucoraphanin concentrations in mature broccoli. However, trace concentrations of SFMA were observed due to the predominant yield of sulforaphane nitrile, which was 45 times higher than sulforaphane during preparation of the extract. The low concentration of sulforaphane in the seed extract was below the sensitivity of the MA analysis, compared with its yield from cooked broccoli.

Although mean intake of glucoraphanin was similar in lightly cooked or fully cooked broccoli, consumption of lightly cooked broccoli produced a higher yield of sulforaphane, expressed as the molar proportion of glucoraphanin ingested, *in vivo*. This observation is in agreement with previous experiments in human subjects, showing that production of isothiocyanates is higher following ingestion of brassica with active plant myrosinase, compared with those in which myrosinase has been denatured (Getahun & Chung, 1999; Conaway *et al.* 2000; Shapiro *et al.* 2001; Rouzaud *et al.* 2004). In the current study, lightly cooked broccoli had a significantly higher plant myrosinase activity than fully cooked broccoli. No direct evidence for the release of isothiocyanates within the colon is available *in vivo*. Colonic microbiota has been estimated to account for about 8 % of isothiocyanates recovered as MA in gnotobiotic rats harbouring a human faecal microbiota, compared with their germ-free counterparts, when given a diet with or without myrosinase (Rouzaud *et al.* 2003). This is comparable to the recovery of sulforaphane of 5 % as SFMA after fully cooked broccoli in our experiment. It has been suggested that the lower recovery of isothiocyanates in the colon may be due to the predominant formation of nitriles (Cheng *et al.* 2004) or amines (Combourieu *et al.* 2001), as shown after glucosinolate hydrolysis by human faecal microbiota *in vitro*.

The output of AITC and sulforaphane, as a proportion of ingested precursors, was highly variable among volunteers. Inter-individual variability in SFMA output was more pronounced after consumption of fully cooked compared with lightly cooked broccoli, suggesting significant inter-individual variation in colonic hydrolysis of glucoraphanin. Large inter-individual differences in faecal microbiota populations of genera such as *Bacteroides*, *Bifidobacteria* and *Lactobacilli*, implicated in glucosinolate hydrolysis (NugonBaudon *et al.* 1990; Elfoul *et al.* 2001; Cheng *et al.* 2004), have been reported among human subjects (Nagashima *et al.* 2003). Furthermore, isothiocyanate metabolism and excretion can vary markedly among individuals (Zhang *et al.* 1995). Inter-individual variability associated with the digestive fate of glucoraphanin was higher than that associated with the output of AITC. The AITC was delivered directly to the stomach and small intestine, where it was presumably more readily available for absorption from the uniform matrix of mustard. Conversely, sulforaphane was produced in the alimentary tract following hydrolysis of glucoraphanin *in situ* within the non-homogeneous matrix of cooked broccoli. The higher variability in SFMA compared with AMA excretion could therefore be a function of the combined effect of plant and microbial myrosinase in the former. It could be further explained by temporal differences in the degree of chewing and gastro-intestinal transit time among individuals. This larger inter-individual variation in SFMA may have masked any influence of meal composition on SFMA excretion.

The output of AMA was considerably higher than that of SFMA, when expressed as a proportion of ingested precursors. Pre-formed AITC was readily available for absorption from the stomach and small intestine, while only a relatively small fraction of ingested glucoraphanin was hydrolysed to and absorbed as sulforaphane *in vivo*. Excretion of MA can be considerably higher after ingestion of pre-formed isothiocyanates than after consumption of their glucosinolate precursors (Shapiro *et al.* 2001). Furthermore, sulforaphane has been shown to have a slower metabolic disposal via its conjugation with glutathione at physiological pH, compared with AITC (Zhang *et al.* 1995).

The findings from the present experiment suggest that the uptake of cancer-protective isothiocyanates following consumption of lightly cooked brassica vegetables is more extensive than after intake of the fully cooked vegetables. We have also shown that individuals vary extensively in the way in which they metabolise glucosinolates to isothiocyanates. Moreover, the nature of the meal matrix can considerably influence the digestive fate of pre-formed isothiocyanates. The main determinant in the production of isothiocyanates from their glucosinolate precursors was the way in which the brassica vegetable was cooked.

### Acknowledgements

We thank the volunteers for their participation and compliance in this study. We acknowledge The Rowett Research Institute who provided the facilities for blast freezing the meals, Mrs Heather Scott (The Robert Gordon University) who helped in the preparation and serving of the meals, and Dr Jason Owen and Mrs Gillian Telfer (Macaulay Institute) for the analysis of nitrogen and fat content of the meals. We also thank Dr Mark Brewer (Biomathematics & Statistics Scotland) for statistical advice. This study was supported by the Food Standards Agency (contract No. T01027, Influence of cooking and processing of brassica vegetables on the release of beneficial and harmful metabolites of glucosinolates in the digestive tract).

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