Supplementation of the diet by exogenous myrosinase via mustard seeds to increase the bioavailability of sulforaphane in healthy human subjects after the consumption of cooked broccoli

Article

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Supplementation of the diet by exogenous myrosinase via mustard seeds to increase the bioavailability of sulforaphane in healthy human subjects after the consumption of cooked broccoli

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Shortened title: Increasing sulforaphane bioavailability from broccoli through exogenous myrosinase.

Abstract

Scope

Broccoli contains the glucosinolate glucoraphanin which, in the presence of myrosinase, can hydrolyse to the isothiocyanate sulforaphane, reported to have anti-carcinogenic activity. However, the myrosinase enzyme is denatured on cooking. Addition of an active source of myrosinase, such as from powdered mustard seed, to cooked *brassica* vegetables can increase the release of health beneficial isothiocyanates, however this has not previously been proven *in-vivo*.

Methods and results

The concentration of sulforaphane metabolite (sulforaphane N-acetyl-L-cysteine (SF-NAC) in 12 healthy adults after the consumption of 200g cooked broccoli, with and without 1 g powdered brown mustard, was studied in a randomized crossover design. During the 24 hour period following consumption of the study sample all urine was collected. SF-NAC content was assayed by HPLC. When study subjects ingested cooked broccoli alone, mean urinary SF-NAC excreted was 9.8 ± 5.1 μmol per g creatinine, whilst when cooked broccoli was consumed with mustard powder this increased significantly to 44.7 ± 33.9 μmol SF-NAC per g creatinine.

Conclusion

These results conclude that when powdered brown mustard is added to cooked broccoli the bioavailability of sulforaphane is over four times greater than that from cooked broccoli ingested alone.

Keywords: Mustard, broccoli, isothiocyanate, urine, myrosinase, bioavailability

1. Introduction

Globally, there is an increasing awareness of diet in maintaining good health and the prevention of diseases such as diabetes, cancer and cardio-vascular diseases. Increased consumption of vegetables is known to provide a variety of micro- and phytonutrients, which might have beneficial effects on health [1]. *Brassica* vegetables provide micronutrients such as folate, and phytochemicals such as glucosinolates. The later have been reported to possess potential anti-carcinogenic effects, as well as improving risk factors for cardiovascular diseases [2-4].

Broccoli (*Brassica oleracea* var. *Italica*) contains glucoraphanin as its predominant glucosinolate. Under favourable conditions, glucoraphanin is hydrolyzed either by plant myrosinases (co-existing in different segregated parts of the same plant alongside the glucosinolates) or by bacterial thioglucosidases (in human gut microflora) into the corresponding isothiocyanate, sulforaphane (SF) (4-methylsulfinyl butyl isothiocyanate) and a variety of other compounds depending on factors such as pH, metal ions and other protein elements [5].

Brassica are subjected to various methods of processing, including cooking, prior to consumption. Irrespective of the cooking methods, myrosinase will be denatured to varying extents, however, the glucosinolates are rarely affected by cooking if leaching out can be prevented [6-9]. Dekker et al., [9] pointed out that cooking *Brassica* vegetables alters the glucosinolate - myrosinase system due to a number of factors, amongst which are; partial or total inactivation of myrosinase, thermal/plant myrosinase mediated breakdown of glucosinolates, loss of enzymic cofactors, leaching of glucosinolates and their metabolites into the cooking medium, volatilization or thermal degradation of the metabolites. When myrosinases are inactivated during *Brassicaceae* processing, the production of beneficial health compounds is greatly diminished.

Most *Brassica* vegetables owe their chemo-preventive potentials to the presence of glucosinolates, the subsequent conversion of these glucosinolates to isothiocyanates, and bioavailability of the isothiocyanate metabolite [10]. SF is known to be an exceptionally active inducer of phase II enzymes [11]. SF is metabolized by the mercapturic acid pathway, predominantly appearing in urine as sulforaphane N-acetyl-L-cysteine (SF-NAC). SF is rapidly absorbed, metabolized, and excreted, with 80% appearing in the urine within 12-24 hours after

consumption [12], although its bioavailability and overall therapeutic benefit may be affected by pharmacokinetic properties, genetic variation, and food preparation [13]. SF-NAC is often used as a marker of SF bioavailability, although it is not the only metabolite present in urine [11, 12, 14-16].

Conaway et al., [17] investigated the metabolic fate of glucosinolates in humans after ingestion of steamed and raw broccoli, and reported that the excretion of urinary isothiocyanate metabolites was approximately 3 times greater in raw broccoli than from steamed broccoli. In broccoli sprouts, Shapiro et al., [16] concluded that the availability and excretion of glucosinolate was dictated by its conversion to isothiocyanates via myrosinase. Clarke et al., [13] concluded that the bioavailability of SF and erucin was lower in human subjects who consumed broccoli supplements compared to fresh broccoli sprouts. The same authors suggested that the food matrix, cooking, co-ingestion of other factors or the presence of proper enzymes for metabolism can affect the bioavailability of bioactive dietary constituents derived from *Brassica* vegetables [13, 18].

In some recent studies, the addition of an exogenous form of myrosinase isoenzymes to processed *Brassicaceae* has been shown *in vitro* as capable of re-initiating glucosinolate hydrolysis with the subsequent increase in beneficial hydrolysis products particularly SF [19, 20]. However, this increase in availability of SF had not yet been shown *in vivo* from such studies.

The hypothesis of this study was that the addition of brown mustard powder would improve the formation of SF when added to cooked broccoli because of the action of mustard myrosinase on the broccoli glucoraphanin *in situ* (i.e. either immediately before consumption, or during mastication and digestion). This study aims to investigate the bioavailability of the SF, by measuring SF-NAC in human urine, after the consumption of cooked broccoli with and without powdered brown mustard (*Brassica juncea*) by 12 healthy adults in a crossover design. This is not only important for the investigation of potential health effects, but also for the suitability of SF-NAC as a potential biomarker of intake.

2. Materials and methods

2.1 Study design

The study was given a favourable opinion for conduct by the University of Reading Research Ethics Committee (study number UREC 15/30). Twelve non-smoking apparently healthy adults, age 18-64 years, were recruited to attend the Hugh Sinclair Human Nutrition Unit, University of Reading. All adult subjects read and understood study information sheet,

completed a health and lifestyle questionnaire and signed an informed consent document prior to taking part in the study. Each subject attended a screening visit where physical examination, complete medical history and food preferences were taken. Volunteer exclusion criteria included: sufferers of chronic illnesses, individuals with food allergies and individuals who disliked *Brassica* vegetables. Two intervention visits were carried out in a randomized crossover study design, where participants were fed 200g cooked broccoli (*Brassica oleracea* var. *Italica*) with and without 1g powdered brown mustard seeds (*Brassica juncea* (L). *Czern*), with a 7-day washout period in-between the two visits. All 12 subjects screened completed both study intervention days.

2.1.1 Sample preparation

Freshly harvested matured broccoli (*Brassica oleracea* var. I*talica*) grown in the UK was purchased from Produce World Marshalls (Boston, UK). All broccoli used for the study was obtained 24 hours prior to sample preparation and refrigerated (4 °C). Brown mustard (*Brassica juncea* (L). *Czern*) seed, obtained from IPK Genebank (Gatersleben, Germany), was cultivated in a glasshouse at the University of Reading, and harvested in the pod after maturation. The harvested mustard seeds were allowed to dry in the pod under room temperature (18-23 °C) and then manually shelled. The dried brown mustard was ground using a coffee blender, sieved (30 μ mesh), stored in air-tight containers and refrigerated (4 °C). This was done 24 hours prior to consumption.

On study day, the broccoli was washed and allowed to drain. The broccoli heads were cut approximately 4 cm from the top, thoroughly mixed together and 200 g portions were weighed into low density polyethylene (LDPE) bags and vacuum sealed. Each weighed portion was then cooked *sous vide* in boiling water at 100 °C for 20 minutes. Cooking broccoli under this condition for 10 minutes is sufficient to completely inactivate myrosinase enzymes as established in our previous studies [18]. The cooked portion of broccoli was allowed to cool and then pureed using a Kenwood Multi Pro FDP 613 blender (Kenwood, UK) with the addition of 100 ml of potable water (25 °C). The pureed broccoli was then served to subjects (25 °C) with and without the addition of 1g brown powdered mustard seed. Where mustard powder was added this was done immediately prior to serving.

2.1.2 Study sample administration and urine collection

All study participants were asked to abstain from glucosinolate or isothiocyanates containing foods (a list of excluded foods was given to each participant at recruitment and stated on the study questionnaire), 48 hours prior to study visit day. Participants came in on the morning of

visit day fasted (no food and liquids other than water) from 8 pm of the evening preceding the visit day. Participants attended the Hugh Sinclair nutrition unit before 10 am. Each participant consumed 200 g of cooked broccoli, with or without 1 g powdered brown mustard powder (with supervision), according to the study design. Potable water was made available during sample consumption period, which was about 10-20 minutes. Participants were then directed to collect all urine for 24 hours after ingestion of study sample, in urine pots containing 0.5 g ascorbic acid (as preservative). The urine pots were placed in bags containing ice packs. The urine samples were returned on the morning of the following day. The urine volume was recorded, three 15 ml aliquot were centrifuged, and then stored at -20 °C until analysis. During study days participants avoided excluded foods. To enable control of food intake for the first meal after the broccoli intervention, a calorie controlled lunch was provided. This contained sandwiches, crisps and fruit juice. Participants were encouraged to eat these two hours after the broccoli intervention and they were encouraged to drink water during the 24 hour experimental period. Food consumed later in the study day was not controlled, other than that exclusion of the prohibited glucosinolate-containing foods.

2.2 Determination of sulforaphane in urine

SF-NAC quantification in urine was evaluated as described by Conaway et al., [17] with some modifications. 1 ml Urine was acidified with 10 µl 2 M HCL and frozen using dry ice. The frozen samples were allowed to thaw to room temperature and then centrifuged (1000 g) for 10 minutes. The supernatant was filtered (0.45 µm), 10 µl was used for HPLC analysis by Agilent HPLC-UV system (Agilent 1200, Manchester, UK) using a Nova-Pak C18 (4 µm) reverse phase column (4.6 mm x 250 mm) (Waters, Elstree, UK) with a flow rate of 1 ml/min and a wavelength of 365 nm. The mobile phase was 0.1% Trifluoro acetic acid in 9:1 water-acetonitrile with flow rate 1 ml/min. A standard NAC (Sigma Aldrich, UK) conjugate of SFN (Sigma Aldrich, UK) was synthesized [14]. Briefly, 123 mg of 0.4 mmol NAC was dissolved in 6 ml of 50 % aqueous ethanol, the pH of the solution was then adjusted to 7.8 with 1 N NaOH. 36 mg 0.2 mmol SF dissolved in 3 ml ethanol was added to the NAC solution and the mixture was stirred at ambient temperature on dry ice. The solvent was evaporated and a standard curve for urinary SFN-NAC was constructed by using the synthetic SFN-NAC dissolved in urine (0.5–2.5 mM).

2.3 Sulforaphane determination in broccoli samples

SF in broccoli (raw, cooked and cooked with brown mustard powder) was analyzed as described by Ghawi *et al*., [19] with some modifications. 150 mg Lyophilized broccoli powder

was mixed with 1.75 ml de-ionized water in 2 ml eppendorf tubes and incubated at 30 °C for 5hrs. The mixture was then centrifuged (13,000 g, 10 min) and the supernatant collected. A further 1 ml de-ionized water was then added to the mix and centrifuged (13,000 g, 10 min), following which the supernatant was removed and the procedure repeated. Supernatants were combined and filtered. 10 ml Dichloromethane was added to the combined supernatant, vortexed for 1 min and then centrifuged (13,000 g, 10 min). The organic phase was collected and this was repeated twice. The supernatants were combined and salted out using 10 g sodium sulfate. This was then dried using a rotary evaporator at 37 °C. The dried sample was re-dissolved in 0.7 ml acetonitrile and filtered (0.22 µm) before final injection onto HPLC for SF quantification. HPLC-UV system (Agilent 1200, UK) with Nova Pak C18 (4 µm) reverse phase column (4.6 mm x 250mm) was used. Flow rate was 1 ml/min at wavelength of 254 µm. The solvent system consisted of 10 % acetonitrile in water increased linearly to 60% acetonitrile over 22 min, then raised to 100 % acetonitrile in 4 min, finally, this was run isocratically using 100% acetonitrile for 4 min to purge the column. Column temperature was 30 °C with injection volume at 10 µl. SF was quantified by standard calibration curves, using standard SF (Sigma Aldrich, UK) in acetonitrile (10-1800 µg/ml). The linearity of the standard curves was expressed in terms of the determination coefficients from plot of the integrated peak area versus concentration of the standards.

2.4 Determination of effect of cooking time on myrosinase inactivation in broccoli

10 g portion of broccoli in vacuum sealed LDPE bags were cooked *sous vide* for different preset times (2-14 minutes). The cooked portions were rapidly cooled on ice. Myrosinase activity in cooked broccoli portions was then assayed as described and adapted by Okunade et al, [21]. Briefly, the ground material is extracted on ice with buffer (Tris-HCl 0.2 M, pH 7.5 containing EDTA 0.5 mM, dithiothreitol 1.5 mM and 0.4 g polyvinylpolypyrrolidone) and the protein in the filtered supernatant is precipitated with ammonium sulfate. The centrifuged pellet is re-suspended in Tris-HCL buffer and extensively dialysed to remove excess ammonium and sulfate ions. Following centrifugation, the supernatant was frozen (-80 °C), lyophilised, and the resulting powder was stored at -20 °C. Myrosinase activity was measured according to the coupled enzymatic procedure where the sample was added to a reaction mixture containing NADP/ATP, hexokinase/glucose-6-phosphate dehydrogenase, and ascorbic acid (cofactor). Following equilibration, sinigrin solution (0.6 M) was added. The change in absorbance due to the formation of NADP was measured at 340 nm using a D-glucose determination kit was used (R-Biopharm Rhone, Heidelberg, Germany). Myrosinase activity was determined from the

initial linear rate of increase in the curve of absorbance against reaction time. A standard myrosinase enzyme (Sigma Aldrich, UK) was used to establish the calibration curve of absorbance against concentration. One unit (un) of myrosinase was defined as the amount of enzyme that produces 1 μmol of glucose per minute when sinigrin is used as a substrate at 25 °C and pH 7.5.

2.5 Determination of creatinine in urine

Creatinine was determined using ILAB 600 clinical chemistry analyzer (Instrumentation Laboratories Werfen, Warrington, UK) and creatinine standard. Urine samples were defrosted at room temperature, vortexed (Whirl mixer, Fisons, UK) and centrifuged at 1,500 g for 5 min. 150 μl of centrifuged sample was then pipetted into a labelled 3 ml sample cup, ensuring that there were no air bubbles present in the sample. The standard creatinine was loaded in the test reagent compartment while urine samples were loaded into the sample compartment of the equipment. The samples were then analyzed after replacing the lid on the reagent and sample compartments, ensuring that the lid was in the correct position.

2.6 Data analysis and statistics

For both urine and broccoli samples, the mean (± SD) SF concentrations of triplicate analysis are presented. Significant differences between the two broccoli interventions (with and without mustard seed) were determined using two-way ANOVA where the intervention was a fixed effect and the participant a random effect. Analysis was carried out using PASW Statistics 21, IBM, UK.

3. Results and discussion

3.1 Effect of cooking time on broccoli myrosinase inactivation

The effect of cooking time on myrosinase inactivation in broccoli was studied (Figure 1). When broccoli was cooked *sous vide*, it was observed that myrosinase activity decreased with exposure time. This trend is similar to that observed in previous studies where gradual increase in temperature led to marked decrease in myrosinase activity [19, 21-23]. Cooking broccoli for 2 minutes led to 40% loss in myrosinase activity. Further increase in cooking time to 6 minutes increased activity loss (90%) and after 8 minutes of cooking, there was no measurable myrosinase activity recorded. Broccoli myrosinase is temperature sensitive and its temperature stability has been reported in the literature [24-27].

3.2 Sulforaphane in raw broccoli, cooked broccoli and cooked broccoli with mustard powder

Table 1 shows the SF content in raw broccoli, cooked broccoli and cooked broccoli with 1g mustard powder used for this study. The SF content of unprocessed broccoli was 2.05 ± 0.25 µmol/g dry weight while that of the cooked (*sous vide* cooking) sample was significantly lower 1.06 ± 0.18 µmol/g dry weight. This was expected as broccoli myrosinase is sensitive to thermal treatment and is inactivated on cooking which inhibits the formation of SF. However, when broccoli is eaten raw, sulforaphane nitrile is the main hydrolysis product [28] and processing broccoli at low temperatures (under 50 °C) encourages the formation of sulforaphane nitrile [25] as epithiospecifier protein activity is prominent at low temperatures [29]. However the same authors suggested that heating broccoli florets up to 60 °C favours the formation of SF.

The low concentration of SF in the raw and cooked broccoli samples was expected. However, when 1 g brown mustard was added to the *sous vide* cooked broccoli sample (*in vitro*), there was a significant 8 fold increase in SF content (to 8.58 ± 0.13 µmol/g dry weight). This indicates that the addition of brown mustard to cooked broccoli significantly improved the conversion of intact glucosinolate in the cooked broccoli (in which the myrosinase had been inactivated). Brown mustard myrosinase hydrolysed the intact glucosinolates in the cooked broccoli thereby improving the formation of more SF. Mustard myrosinase is robust and thermally stable compared to broccoli myrosinase [21, 23]. Recent research suggests alternative cooking modifications can also lead to increased SF formation from cooked broccoli. Soaking broccoli florets in water at 37°C for 90 minutes prior to stir frying was shown to promote hydrolysis before myrosinase was denatured on stir frying, leading to a 2.8 fold increase in SF [30].

3.3 Sulforaphane-N-acetyl cysteine in human subject urine

The amount of SF-NAC excreted in subject urine over 24 hours after the consumption of the study sample is shown in Figure 2. Shapiro et al., [16] had earlier pointed out that the extent of chewing before swallowing might influence the bioavailability of fresh unhydrolyzed broccoli sprouts. This was taken into consideration for this study, hence, the cooked broccoli was made into puree form with addition of 100 ml water. After the consumption of cooked broccoli alone, subjects excreted a mean 9.8 ± 5.1 µmol SF-NAC per g creatinine within 24 hours whereas following ingestion of the cooked broccoli with 1g powdered brown mustard, they excreted 44.7 ± 33.9 µmol SF-NAC per g creatinine within 24 hours. Conaway et al., [17] described the bioavailability of SF in raw and steamed broccoli in human subjects to be in the ratio 3:1. In the present study, the concentration of SF-NAC in the urine following a meal where mustard was added to cooked broccoli, to that of cooked broccoli alone, was on average in the ratio of

294 4.7:1. The relative increase in SF-NAC from the cooked broccoli alone meal to the cooked
295 broccoli with mustard meal varied between the individual subjects from 1.7 fold to 10 fold.
296 This indicates that adding brown mustard powder to cooked broccoli had a beneficial effect of
297 substantially increasing the formation of SF in all subjects (where SF-NAC was quantified as
298 the metabolite of SF). Due to the nature of the study, it cannot be proven whether the increase
299 in SF from hydrolysis of the broccoli glucoraphanin occurred before broccoli intake, during
300 mastication, or during digestion. However, it is clear is that the addition of mustard powder to
301 the cooked broccoli immediately prior to consumption enabled more effective conversion of
302 glucoraphanin to SF.
303 In the cooked broccoli, the myrosinase would have been largely inactivated [24], hence
304 glucosinolate would have remained predominantly intact and, therefore, the SF in the urine
305 post consumption of the standard cooked broccoli (9.8 ± 5.1 μmol/g creatinine) would have
306 resulted primarily from gut microflora conversion in the digestive tract. The action of human
307 gut microbiota can hydrolyse glucosinolates, however, the isothiocyanate yield is known to be
308 much considerably lower than that obtainable from plant myrosinase [17, 31].
309 In addition, the isothiocyanate produced by the action of the gut microflora might have
310 undergone further degradation leading to the formation of other compounds which reduces the
311 amount of isothiocyanate available for absorption [32, 33].
312 Broccoli myrosinase compared to myrosinase from mustard (*Brassica juncea, B. nigra* and
313 *Sinapis alba*) is known to be more temperature sensitive [24]. The powdered mustard seed
314 added to the cooked broccoli provided an active myrosinase source and, therefore rapidly
315 increased the formation of isothiocyanates. Brown mustard myrosinase is known to be more
316 robust with good thermal stability compared to broccoli myrosinase [21, 22]. On average, the
317 resulting amount of SF excreted in the urine (measured as SF-NAC) after consumption of
318 broccoli with mustard was 4.7 fold higher than that excreted from consumption of cooked
319 broccoli alone. The addition of 1g mustard to *sous vide* cooked broccoli (*in vitro*) (Table 1) led
320 to an increased SF content from 1.06 ± 0.18 to 8.58 ± 0.33 μmol/g dry weight, an 8 fold increase
321 in SF content. The rate of increase when mustard was added to cooked broccoli *in vitro* was
322 higher than that observed from the *in vivo* study (8:1 for *in vitro* study and 4.7:1 for the human
323 study). This slightly lower conversion rate is perhaps expected, and may be associated with
324 conversion in the gut. According to Lampe [34], the major metabolic pathway of
325 isothiocyanates in humans is by conjugation with glutathione and these conjugates are
326 subjected to further degradation by enzymes to yield the final metabolites. In the case of the

human study (*in vivo*), unhydrolysed glucosinolate may have been degraded by human gut microflora which could partly explain the difference in the relative results.

4 Conclusion

Broccoli myrosinase is relatively temperature sensitive and is inactivated when core temperature exceeds 60 °C. From the results of the current study, it can be concluded that the addition of powdered brown mustard to cooked broccoli greatly enhances the formation of SF compared to the cooked broccoli in which the myrosinase isoenzymes has been inactivated, confirming that the presence of plant myrosinase is important for SF bioavailability. When brown mustard powder was added to cooked broccoli, SF formation was promoted (8-fold), hence improving its bioavailability, and resulting in an increase in the metabolite sulforaphane N-acetyl-L-cysteine (SF-NAC) of over 4-fold. However, this conclusion must be considered within the context of both plant and human variability. Bioavailability of glucosinolates and isothiocyanates can be influenced by plant storage, processing, genetic variation of the plant and the food matrix. In addition, human genetic variation influences the bioavailability of SF. Glutathione S-transferase (GST) metabolises isothiocyanates and lack of this enzyme is associated with more rapid excretion of SF; hence individuals with this genetic variation may derive less benefit from consumption of *Brassica* vegetables.

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Figures and tables

Figure 1: Effect of cooking time on myrosinase inactivation in *sous vide* cooked broccoli (water temperature 100 °C). Error bars represent standard error of the mean.

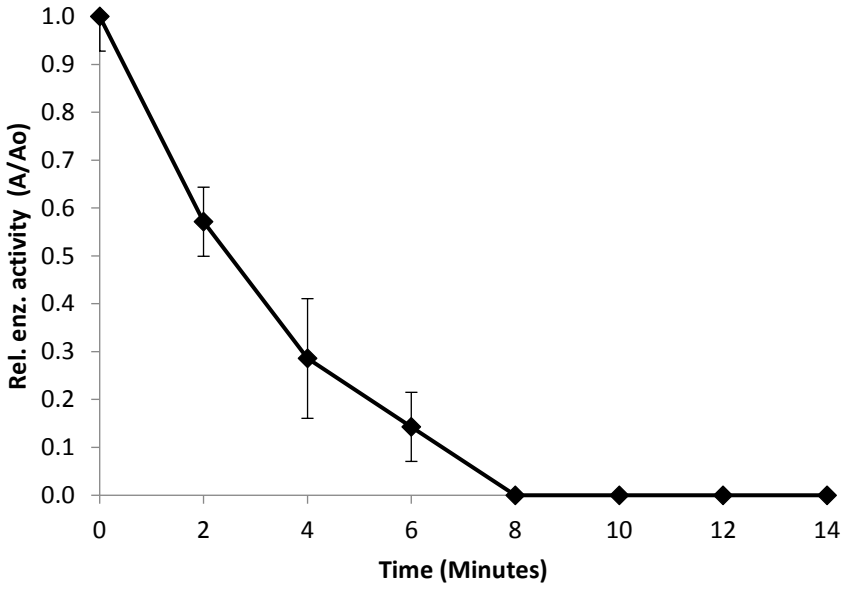

Figure 2: Sulforaphane N-acetyl-L-cysteine (SF-NAC) /g creatinine excreted in 24 hours by 12 healthy adults (mean data). Error bars represent standard error of the mean.

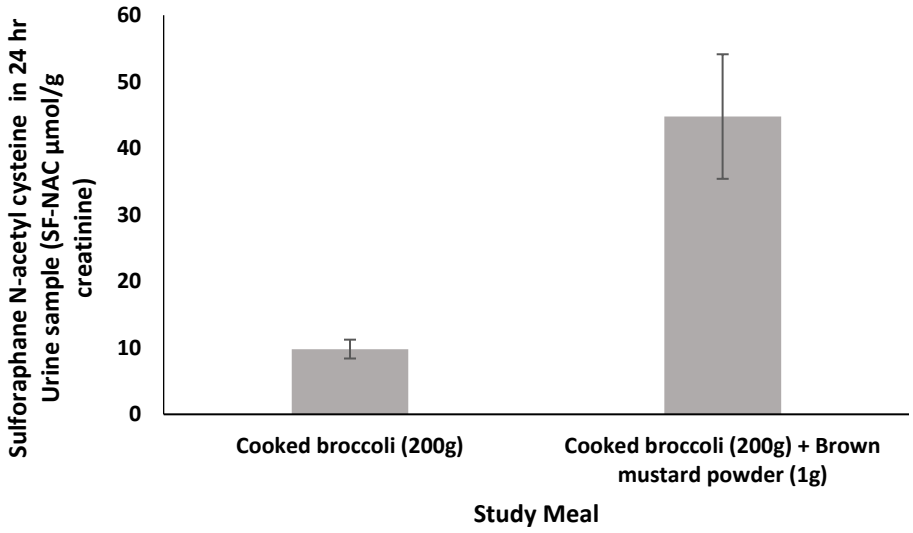

Table 1: Sulforaphane content of raw broccoli, cooked broccoli and cooked broccoli with 1g brown mustard powder added post cooking. Broccoli was cooked *sous vide* in boiling water at 100 °C for 20 minutes

Sample	Sulforaphane (µmol/g dry weight)*
Raw broccoli	2.05 ±0.25[b]
Cooked broccoli	1.06 ± 0.18[a]
Cooked broccoli + 1g brown mustard powder	8.58 ±0.33[c]

*Values not sharing a common letter are significantly different at $P < 0.05$.

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