Bioavalability and new biomarkers of cruciferous sprouts consumption

Nieves Baenas[1†*], Clara Suárez-Martínez[2†], Cristina García-Viguera[1], Diego A. Moreno[1]

[1]Phytochemistry Lab., Dept. of Food Science and Technology, CEBAS-CSIC, Espinardo, 30100, Murcia, Spain. [2] Dept. of Food Science and Nutrition, Veterinary Faculty, University of Murcia, Espinardo, 30100, Murcia, Spain.

[†] These authors contributed equally to this work.

[*]Corresponding autor: Nieves Baenas Navarro. Tel.: +34 968 396200 Ext. 6253. fax: +34 968 396213. E-mail address: nbaenas@cebas.csic.es. C.S-M. (clara.suarez@um.es), D.A.M. (dmoreno@cebas.csic.es), C. G-V. (cgviguera@cebas.csic.es).

ABSTRACT

The evaluation of the bioavailability of cruciferous compounds is one of several challenges in the design of clinical trials. A 7-days-cross-over study with fourteen women was undertaken to compare the bioavailability of glucosinolates from broccoli and radish sprouts. The urinary excretion of isothiocyanates, indoles and metabolites was analysed by UHPLC-QqQ-MS/MS. Even though the bioavailability of broccoli compounds has been studied, as far as we are aware, there are not any biomarkers established for radish sprouts intake. For the first time, sulforaphene, sulforaphane-N-acetyl-L-cysteine (SFN-NAC) and 3,3'diindolylmethane (DIM), were studied as biomarkers of dietary exposure to radish. The SFN-NAC and DIM were already considered biomarkers of broccoli consumption. Higher excretion of conjugated isothiocyanates and homogeneous excretion of indoles were found during the first 12 h after ingestion. Metabolites were excreted homogeneously during the study, suggesting no accumulation. These results provide valuable information to better understand the bioavailability of cruciferous bioactives.

Keywords: *Glucosinolates, sulforaphane, sulforaphene, indole-3-carbinol, 3,3'diindolylmethane.*

1. Introduction

There are epidemiological evidences of the benefit of consuming cruciferous food on the reduction of cancer risk (Jeffery & Keck, 2008), degenerative diseases (Tarozzi, et al., 2013) and the modulation of obesity-related metabolic disorders (Zhang, et al., 2016) after cruciferous intake. Cruciferous sprouts are especially rich in bioactive compounds compared to the adult plants, being further healthier than other vegetables (Pérez-Balibrea, Moreno, & García-Viguera, 2011; Vale, Santos, Brito, Fernandes, Rosa, & Oliveira, 2015). The highest benefit of cruciferous foods occurs when are consumed fresh, as young sprouts, avoiding degradation of the enzyme myrosinase by cooking, which is necessary to hydrolyse their characteristic sulphur and nitrogen compounds, the glucosinolates (GLS), to the bioactive isothiocyanates (ITC) and indoles. In case of sprouts, the degradation of GLS after consumption occurs during chewing, in presence of the plant's enzyme myrosinase, and also is mediated by β-thioglucosidases in the gut microbiota (Angelino & Jeffery, 2014).

There is growing evidences that ITC, such as sulforaphane (SFN) and sulforaphene (SFE), as well as the indole-3-carbinol (I3C), play antioxidant, anti-inflammatory and multi-faceted anticancerogenic activities in cells (Stefanson & Bakovic, 2014), through the inhibition of the activation of the central factor of inflammation NF-*κ*B (Egner, et al., 2011), and the induction of the Keap1/Nrf2/ARE pathway related with antioxidant genes and detoxifying enzymes, such as glutathione *S*-transferases (GST) (Baenas, Silván, Medina, de Pascual-Teresa, García-Viguera, & Moreno, 2015; Myzak, Tong, Dashwood, Dashwood, & Ho, 2007), and also blocking carcinogenic stages by induction of apoptosis, cell cycle arrest and inhibition of histone deacetylases, among others (Myzak, et al., 2007).

The majority of studies about the bioavailability of bioactive compounds from crucifers are focused in glucoraphanin (GRA), the main GLS of broccoli sprouts, and its bioactive isothiocyanate SFN, which is metabolised by the mercapturic acid pathway, first with a conjugation with glutathione (-GSH), following modifications with cysteine (-CYS), and finally, metabolised in the liver with N-acetyl-L-cysteine (-NAC). During the last years, some conjugated ITC, such as SFN-NAC, and other secondary compounds, such as 3,3'diindolylmethane (DIM),

which is released by I3C in acid medium (i.e. the stomach), have been used as biomarkers of cruciferous intake (Angelino & Jeffery, 2014; Fujioka, et al., 2016a). However, the bioavailability of the GLS glucoraphenin (GRE) and its isothiocyanate SFE, from radish sprouts, which only differ from SFN in a double bond between the third and fourth carbon, have not been yet investigated.

To our concern, there are no publications studying the bioavailability of radish sprouts compounds, and it is unknown if SFE is metabolised also by the mercapturic acid pathway. Furthermore, there are no commercially available conjugated metabolites of SFE, which would be needed for study its bioavailability by a rapid and sensitive UHPLC-QqQ-MS/MS method, stablishing their appropriate ionization conditions and MRM transitions.

On the other hand, the presence of SFN has been described in radish (Pocasap, Weerapreeyakul, & Barusrux, 2013), maybe by the hydrolysis of GRE, or through a modification of SFE once formed to SFN, suggesting its possible transformation by the mercapturic acid pathway.

Therefore, the aim of this study was to evaluate and compare the bioavailability and metabolism of GRA and GRE, from broccoli and radish sprouts, respectively, and the study of possible biomarkers of radish consumption. Also the excretion kinetics of ITC, indoles and conjugated metabolites, after consumption of both broccoli and radish sprouts, were evaluated in a 7 days-cross-over trial with healthy adult women.

2. Material and methods

2.1. Plant material

Broccoli (*Brassica oleracea* var. *italica*) and radish (*Raphanus sativus* cv. Rambo) 8-day-old sprouts were supplied by Aquaporins & Ingredients, S.L. (Murcia, Spain). These sprouts were bioestimulated during production (4 days previous to delivery) with the natural compound methyl jasmonate 250 µM (Baenas, Villaño, García-Viguera, & Moreno, 2016), in order to obtain cruciferous sprouts up to 2-fold richer in bioactive compounds. Three trays of sprouts (n=3) were collected once a week during the study, then, samples were flash frozen and lyophilised prior analysis of GLS and ITC (see 2.3. section), through an hydro-methanolic (Baenas, Garcia-

Viguera, & Moreno, 2014) and aqueous extraction (Cramer & Jeffery, 2011), respectively, as previously described.

2.2. Human subjects and study design.

A total of fourteen women, aged 27-36 years, non-smokers with stable food habits and not receiving medication, during the experimental procedure, were selected to participate in the study. Written informed consent was obtained from all subjects. The present study was conducted according to guidelines and procedures approved by the CSIC Committee of Bioethics for the AGL-2013-46247-P project. Subjects were randomly assigned to a seven-by-seven cross-over design (Figure 1), one group receiving broccoli sprouts and the second receiving radish sprouts. Nobody dropped out of the study. A list of foods containing glucosinolates was given to all the participants in order to avoid consumption during the study.

Experimental doses (7 trays of broccoli or radish sprouts of 20 grams each) were given at once, on Friday. Subjects were instructed to ingest 1 tray per day, at 10 a.m., according to the cross-over design and to keep trays refrigerated (4 °C) at home. The first day of the study urine samples were collected from 0 to 12 h, and from 12 to 24 h after ingestion. From day 2 to 7 the urine samples were collected for 24 h periods. All urine samples were kept refrigerated during collection and frozen once in the laboratory.

2.3. Metabolites analysis

The quantitative analysis of GLS in freeze-dried sprouts was carried out by HPLC-DAD (Agilent Technologies 1260 Infinity), according to UV spectra and order of elution already described for similar acquisition conditions (Baenas, et al., 2014). Measurement of metabolites in sprouts and urine (GRA, SFN, SFN-GSH, SFN-CYS, SFN-NAC) was performed following their MRM transition by a rapid, sensitive and high throughput UHPLC-QqQ-MS/MS (Agilent Technologies, Waldbron, Germany) method, with modifications of the protocol of Dominguez-Perles *et al*. (2014), for the optimization of new compounds: GRE, SFE, glucobrassicin (GB), I3C, DIM; assigning their retention times, MS fragmentation energy parameters and preferential transitions

(Supplemental File 1). GRA and GRE were obtained from Phytoplan (Diehm & Neuberger GmbH, Heidelberg, Germany) and ITC and indoles were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). No commercially available conjugated metabolites of SFE from radish were found.

2.4. Statistical analysis

All assays were conducted in triplicate. Data were processed using SPSS 15.0 software package (LEAD Technologies Inc., Chicago, USA). First of all, data were tested by Shapiro-wilk normality test, as these values do not follow a normal distribution (non-parametric data), statistical differences were determined by the Wilcoxon signed-rank test when comparing two samples and by the Friedman test when comparing multiple samples. Values of $P<0.05$ were considered significant.

3. Results and discussion

3.1. Bioactive compounds present in cruciferous sprouts

Broccoli and radish sprouts from each week of the study were characterised in GLS and ITC (Table 1). Results are presented as serving dose (20 grams of fresh weight (F.W.)). The amount of cruciferous sprouts consumed daily by the participants was considered a normal serving.

In broccoli sprouts, glucoraphanin (GRA), in the hydromethanolic extract, and its hydrolysis compound sulforaphane (SFN), in the aqueous extract were the predominant compounds, according to previous findings (Angelino & Jeffery, 2014; Cramer & Jeffery, 2011).

Results showed that radish sprouts presented glucoraphenin (GRE) and glucoraphasatin (GPH) as predominant GLS (Table 1), which were hydrolysed to sulforaphene (SFE) and raphasatin (RPS), respectively. Only SFE was detected in the aqueous extract, as RPS is very unstable and rapidly degraded to less bioactive compounds in aqueous media (Kim, Kim, & Lim, 2015).

GRA was not detected in radish sprouts, but the hydrolysis product SFN was present in the aqueous extract (Pocasap, et al., 2013). This could be due to the formation of SFN derived from SFE, losing its double bond, or directly hydrolysed from GRE (Figure 2). Forthcoming

146 evaluations of GRE hydrolysis under different conditions would provide more information about
147 its possible transformations.
148
149 **3.2. Bioavailability and metabolism of GLS/ITC**
150 After ingestion of a serving portion of broccoli sprouts (20 g F.W.), GRA (64 μmol) was
151 hydrolysed, absorbed and metabolised, through the mercapturic acid pathway, by a 12 % on
152 average. SFN and its conjugated metabolites, with glutathione (-GSH), cysteine (-CYS) and N-
153 acetyl-L-cysteine (-NAC) (Angelino & Jeffery, 2014), were found in urine (~7.6 μmol/ 24 h as
154 the sum of SFN, SFN-GSH, SFN-CYS and SFN-NAC) (Figure 3A), considered markers of
155 bioavailability.
156 In the case of radish sprouts, the metabolism of SFE, the predominant ITC, has not been described
157 to the present date, and there are not any conjugated SFE metabolites commercially available to
158 evaluate its bioavailability, by optimization of MRM-transitions in UHPLC-QqQ-MS/MS
159 system. Therefore, it was hypothesised that conjugated SFN metabolites could be found also in
160 urine after radish sprouts consumption, being also possible biomarkers of intake.
161 Results showed that GRE (61 μmol in 20 g F.W.) was metabolised in SFE, SFN and SFN
162 metabolites (SFN-NAC, SFN-CYS, SFN-GSH), corresponding to 8 % on average (~4.9
163 μmol/24 h) of the GRE consumed (Figure 3B). Therefore, SFN metabolites (SFN-NAC, SFN-
164 CYS, SFN-GSH) could act also as biomarkers of radish consumption.
165 The values of bioavailability ranged from 9 to 100 % according to different GLS/ITC profiles of
166 the cruciferous vegetables administered, and the consumption as raw or cooked foods and the
167 influence of the microbiota (Shapiro, Fahey, Wade, Stephenson, & Talalay, 1998; Vermeulen,
168 Klopping-Ketelaars, van den Berg, & Vaes, 2008). In our case, after broccoli sprouts
169 consumption, the SFN-NAC was the predominant metabolite found in urine (~80 %), followed
170 by SFN-CYS (~11 %), SFN (7.5 %), and SFN-GSH (~0.9 %) (Figure 3A), as previously found
171 (Clarke, et al., 2011; Dominguez-Perles, et al., 2014). In the case of radish sprouts, the SFE was
172 excreted in higher amounts (~65 %) (Figure 3B), followed by the conjugated metabolites: SFN-
173 NAC (~19 %), SFN-CYS (~4%), SFN (~1.1%) and SFN-GSH (~0.7%).

174 SFN and SFE present the isothiocyanate group (−N=C=S), which central carbon is highly
175 electrophilic and actively interacts with cellular nucleophilic targets; such as, the GSH and/or
176 cysteine residues (Kim, Kim, & Lim, 2010). Little information is available about SFE bioactivity
177 and, only recently, Byun, *et al*., (2016) showed that SFE reduced the cellular GSH levels *in vitro*,
178 which could indicate its conjugation with GSH. Thus, the synthesis of SFE conjugates with GSH,
179 CYS or NAC could help to generate knowledge on the metabolism of this compound.
180 On the other hand, the higher excretion of pure SFE after radish consumption compared to pure
181 SFN after broccoli ingestion, may suggest that this compound could follow a different
182 transformation pathway. Contrary to SFN, SFE contains a double bond between the third and
183 fourth carbon, which could result in a decrease in the electrophilicity of the –N=C=S group (Kim,
184 et al., 2010), and different excretion kinetics and transformation.
185 According to Holst & Williamson (2004), human studies about Phase I metabolism may
186 contribute considerably to understand the biotransformation of ITC and, consequently, the limit
187 of their bioavailability and health-promoting effects. Therefore, further studies about SFE
188 metabolism and bioactivity are needed to support the health-promoting activities of SFE, now
189 insufficiently studied. For instance, Myzak, *et al*, (2006) showed differences in the bioactivity of
190 SFN conjugated metabolites, as being SFN-CYS and SFN-NAC, significantly active as HDAC
191 inhibitors, with cancer therapeutic potential, but not for SFN and SFN-GSH (Myzak, Ho, &
192 Dashwood, 2006). On the other hand, pure SFN and SFE have shown bioactivities, inhibiting
193 growth of several colon cancer cells (Byun, et al., 2016). Therefore, whether SFE is metabolised
194 by the mercapturic acid pathway or acting in the cell without transformation, this ITC may provide
195 health-promoting effects through induction of detoxification enzymes and antioxidant activity,
196 which did not appear to be affected by their hydrophilicity or other structural factors (La Marca,
197 et al., 2012).
198
199 **3.3. Excretion kinetics of SFN, SFE and its metabolites.**
200 The values of individual metabolites excreted, analysed in urine samples, were represented
201 according to two different criteria: 1) the levels of excretion during 24 h were normalised, first

from 0 to 12 h after consumption and then from 12 to 24 h, using creatinine as an index to which refers the results, since the creatinine excretion is a relatively constant value between subjects; 2) when comparing the daily excretion during 7 days, the data were represented in volumes collected every 24 h h.

The excretion of SFN and its conjugated metabolites, as well as SFE after radish sprouts ingestion was higher during the first 12 h after consumption of sprouts (Figure 4). It has been described that urinary excretion of SFN metabolites after consumption of fresh broccoli reaches peak concentration 3-6 h after consumption, but it could be delayed until 6-12 h (Vermeulen, et al., 2008). After broccoli sprouts ingestion, non-significant differences were found (Figure 4A) between the two periods. In contrast, significant differences were shown in the values of excretion after radish sprouts consumption in all metabolites except for SFN-GSH and SFN-CYS (Figure 4B). The delayed excretion of metabolites, after broccoli sprouts consumption, could be related to a saturation of the membrane transporters (such as P-glycoprotein) in the cells, as SFN is conjugated with GSH and cysteinylglycine in the cells and exported after protein binding, being available for metabolism and excretion (Hanlon, Coldham, Gielbert, Sauer, & Ioannides, 2009). SFE is mostly excreted during the first 12 h after ingestion of radish sprouts (Figure 4B), suggesting that it might be not subjected to the mercapturic acid metabolism. Nevertheless, the biological activity of the pure ITC has shown to be similar than the N -acetylcysteine conjugates (Tang, Li, Song, & Zhang, 2006), being of great interest either if are metabolised or not.

The levels of excretion after 7 days of ingestion (Supplemental files 2 and 3) were also studied and no differences were found in the median daily excretion of SFE, SFN and its metabolites in both broccoli and radish studies. This suggests that repetitive dosing of sprouts should not produce accumulation of any metabolite in the body, as any factor that increases the metabolites amount in the body will cause a decrease in its excretion (Hanlon, et al., 2009). Also, there is a high interindividual variation in excretion values related to human assays, which could be explained by different factors, such as the intensity of chewing, where myrosinase enzymes come into contact with intact GLS, gastric pH and the activity of the microbiota, where one subject could metabolize three times more GLS into ITC than another, and also the polymorphisms of GST

230 enzymes may affect ITC metabolism (Clarke, et al., 2011; Egner, et al., 2011; Fujioka, Fritz,
231 Upadhyaya, Kassie, & Hecht, 2016b). Low amounts of intact GRA and GRE, on average 0.011
232 and 0.04 μmol/24 h, respectively, were also recovered in the urine.
233 One of several challenges in the design of clinical trials is the selection of the appropriate dosage.
234 In this work, commercial trays of sprouts were used, facilitated and quality certified by the
235 company. The average amount of sprouts (~ 20 g per tray) was chosen as one serving, representing
236 a realistic dietary supply. The consumption of 3-5 servings per week of cruciferous sprouts may
237 produce a potential effect decreasing the risk for cancer, according to Jeffery and Araya (2009).
238
239 **3.4. Bioavailability, metabolism and excretion kinetics of GLS/indoles**
240 Glucobrassicin (GB) present in broccoli and radish sprouts is an indole GLS derived from
241 tryptophan and releases bioactive indole-3-carbinol (I3C) upon hydrolysis. This bioactive
242 compound requires acid modification in the stomach to form 3,3'-diindolylmethane (DIM) and
243 other condensates to optimize activity, increasing levels of Phase II enzymes, related to
244 detoxification against lung, colon and prostate cancers (Egner, et al., 2011), and the
245 antiproliferative effects on estrogenic-sensitive tumours (Fujioka, et al., 2016a). In particular,
246 DIM has been associated with the suppression of epigenetic alterations related to carcinogenesis,
247 by suppression of DNA methylation and aberrant histone modifications (Fujioka, et al., 2016b).
248 Additionally, the induction of Phase I enzymes, including the CYP 1 family, catalysing the
249 oxidation of xenobiotics may also be responsible of the action (Ebert, Seidel, & Lampen, 2005;
250 Watson, Beaver, Williams, Dashwood, & Emily, 2013).
251 Because of the rapid hydrolysis of I3C to DIM *in vivo*, high stability of DIM, and the great
252 correlation between GB intake and the amount of DIM excreted, this compound has been
253 described as a biomarker of cruciferous vegetables consumption (Fujioka, et al., 2014). Other
254 condensated compounds such as indol-[3,2-b]-carbazole and related oligomers, were in non-
255 quantifiable concentrations in other studies (Reed, et al., 2006).
256 Little is known about the bioavailability of other indole GLS present in broccoli and radish
257 sprouts, such as hydroxyglucobrassicin (HGB), methoxyglucobrassicin (METGB) and

neoglucobrassicin (NEOGB), which might be also hydrolysed leading indolyl-3-methyl isothiocyanates, unstable and hydrolysed to their corresponding carbinols (Agerbirk, De Vos, Kim, & Jander, 2008; Hanley & Parsley, 1990).

Additional studies are required to confirm if these indole GLS could be hydrolysed also in I3C and DIM, as well as to evaluate the possible health-promoting effects of their hydrolysis and condensate metabolites.

Results demonstrated that broccoli and radish sprouts content in GB were ~11.4 and ~7.7 μmol/20 g F.W, respectively. After ingestion of broccoli sprouts, 49 % of GB was suitably metabolised and excreted as hydrolysis metabolites, calculated as the sum of I3C and DIM (~5.57 μmol /24 h). Following radish ingestion, the percentage of GB hydrolysed and absorbed was 38 % (~2.92 μmol /24 h). It is remarkable that the DIM excreted correspond to over 99 % of these total metabolites. These results of bioavailability contrast with the extremely low percentage (< 1 %) of GB excreted as DIM after consumption of Brussels sprouts and cabbage in a previous study (Fujioka, et al., 2014). Nevertheless, results show relevant bioavailability of GB and the successful use of DIM as biomarker of cruciferous intake. Further studies about conversion of other indole GLS to I3C and DIM are needed to know more about bioavailability of these compounds, as there is no information in literature.

When urine samples were collected in two periods after the ingestion of the sprouts, higher values of excretion of I3C from 12 to 24 h than from the first period were detected (Figure 5), although non-statistically different. Regarding excretion values of DIM, no differences among results were found from 0 to 12 h and from 12 to 24 h (Figure 5). Even if a previous study in humans has shown that the majority of DIM was excreted in the first 12 h (Fujioka, et al., 2016b), other authors have detected DIM in plasma at 12 and 24 h post ingestion (Reed, et al., 2006). Therefore, the excretion of this compound might be longer than for the ITC, which were almost totally excreted during the first 12 h. According to these results, *in vivo* evidences show that I3C condensation products were absorbed, preferentially targeting the liver, and were detected within the first hour in urine. However, the amount increased significantly between 12 and 72 h, implying effects on the xenobiotic metabolism (WHO, 2004).

No statistical differences were found in the 24 h urine excretion values of I3C and DIM after 7-days of consumption of sprouts (Supplemental File 3). The high variability between subjects has been described before within a low dose level of GB consumed (50 μmol), which was considerably higher than in this study (Fujioka, et al., 2016a).

Furthermore, the results showed no accumulation of metabolites after 7 days of intervention, an important result for safe consumption, also proven with hyper-doses of GB (400-500 μmol) in humans (Fujioka, et al., 2016a).

Low amounts of intact GB (~0.011 and ~0.04 μmol/24 h, after broccoli and radish ingestion, respectively) were also recovered in the urine, but the biological activities of I3C, administered orally to humans, cannot be attributed to the parental compound but rather to DIM and other oligomeric derivatives (Reed, et al., 2006). Therefore, the evaluation of DIM in urine after broccoli and radish consumption provided a susceptible tool to design future clinical trials.

4. Conclusions

The measurement of ITC, indoles and conjugated metabolites are useful biomarkers of dietary exposure to cruciferous foods. The SFN-NAC is not the only metabolite present in urine and, as along with DIM, could be used as biomarker of the consumption of cruciferous vegetables. After ingestion of radish sprouts, SFE, together with SFN-NAC and DIM, could be considered as biomarkers, however, metabolites of SFE are not commercially available yet and, consequently, understudied. Repeated dosing of sprouts does not lead to accumulation or higher urine levels of metabolites over time; nevertheless, more research is needed before definitive statements and larger interventions and clinical trials.

Acknowledgements

This work was supported by the Spanish Ministry of Economy and Competitiveness of the Spanish Government (Research Project AGL2013-46247-P) and the Grant for Research Groups of Excellence from the Murcia Regional Agency for Science and Technology (Fundación Séneca

- Project 19900/GERM/15). N. Baenas was funded by a FPU (Formación Profesorado Universitario) grant of the Fellowships Programme from the Spanish Ministry of Education.

7. References

Agerbirk, N., De Vos, M., Kim, J. H., & Jander, G. (2008). Indole glucosinolate breakdown and its biological effects. *Phytochemistry Reviews, 8*, 101.

Angelino, D., & Jeffery, E. (2014). Glucosinolate hydrolysis and bioavailability of resulting isothiocyanates: Focus on glucoraphanin. *Journal of Functional Foods, 7*, 67-76.

Baenas, N., Garcia-Viguera, C., & Moreno, D. A. (2014). Biotic elicitors effectively increase the glucosinolates content in *Brassicaceae* sprouts. *Journal of Agricultural and Food Chemistry, 62*, 1881-1889.

Baenas, N., Silván, J. M., Medina, S., de Pascual-Teresa, S., García-Viguera, C., & Moreno, D. A. (2015). Metabolism and antiproliferative effects of sulforaphane and broccoli sprouts in human intestinal (Caco-2) and hepatic (HepG2) cells. *Phytochemistry Reviews, 14*, 1035-1044.

Baenas, N., Villaño, D., García-Viguera, C., & Moreno, D. A. (2016). Optimizing elicitation and seed priming to enrich broccoli and radish sprouts in glucosinolates. *Food Chemistry, 204*, 314-319.

Byun, S., Shin, S. H., Park, J., Lim, S., Lee, E., Lee, C., Sung, D., Farrand, L., Lee, S. R., Kim, K. H., Dong, Z., Lee, S. W., & Lee, K. W. (2016). Sulforaphene suppresses growth of colon cancer-derived tumors via induction of glutathione depletion and microtubule depolymerization. *Molecular Nutrition & Food Research*, 60(5), 1068-1078.

Clarke, J. D., Hsu, A., Riedl, K., Bella, D., Schwartz, S. J., Stevens, J. F., & Ho, E. (2011). Bioavailability and inter-conversion of sulforaphane and erucin in human subjects consuming broccoli sprouts or broccoli supplement in a cross-over study design. *Pharmacological Research, 64*, 456-463.

Cramer, J. M., & Jeffery, E. H. (2011). Sulforaphane absorption and excretion following ingestion of a semi-purified broccoli powder rich in glucoraphanin and broccoli sprouts in healthy men. *Nutrition and Cancer, 63*, 196-201.

Dominguez-Perles, R., Medina, S., Moreno, D. A., Garcia-Viguera, C., Ferreres, F., & Gil-Izquierdo, A. (2014). A new ultra-rapid UHPLC/MS/MS method for assessing glucoraphanin and sulforaphane bioavailability in human urine. *Food Chemistry, 143*, 132-138.

Ebert, B., Seidel, A., & Lampen, A. (2005). Induction of phase-1 metabolizing enzymes by oltipraz, flavone and indole-3-carbinol enhance the formation and transport of

benzo[a]pyrene sulfate conjugates in intestinal Caco-2 cells. *Toxicology Letters, 158*, 140-151.

Egner, P. A., Chen, J. G., Wang, J. B., Wu, Y., Sun, Y., Lu, J. H., Zhu, J., Zhang, Y. H., Chen, Y. S., Friesen, M. D., Jacobson, L. P., Muñoz, A., Ng, D., Qian, G. S., Zhu, Y. R., Chen, T. Y., Botting, N. P., Zhang, Q., Fahey, J. W., Talalay, P., Groopman, J. D., & Kensler, T. W. (2011). Bioavailability of sulforaphane from two broccoli sprout beverages: Results of a short term, cross-over clinical trial in Qidong, China. *Cancer prevention research (Philadelphia, Pa.), 4*, 384-395.

Fujioka, N., Ainslie-Waldman, C. E., Upadhyaya, P., Carmella, S. G., Fritz, V. A., Rohwer, C., Fan, Y., Rauch, D., Le, C., Hatsukami, D. K., & Hecht, S. S. (2014). Urinary 3,3'-diindolylmethane: a biomarker of glucobrassicin exposure and indole-3-carbinol uptake in humans. *Cancer Epidemiol Biomarkers Prev, 23*, 282-287.

Fujioka, N., Ransom, B. W., Carmella, S. G., Upadhyaya, P., Lindgren, B. R., Roper-Batker, A., Hatsukami, D. K., Fritz, V. A., Rohwer, C., & Hecht, S. S. (2016a). Harnessing the Power of Cruciferous Vegetables: Developing a Biomarker for *Brassica* Vegetable Consumption Using Urinary 3,3′-Diindolylmethane. *Cancer Prevention Research*. 9(10):788-793

Fujioka, N., Fritz, V., Upadhyaya, P., Kassie, F., & Hecht, S. S. (2016b). Research on cruciferous vegetables, indole-3-carbinol, and cancer prevention: A tribute to Lee W. Wattenberg. *Molecular Nutrition & Food Research, 60*, 1228-1238.

Hanley, A., & Parsley, K. R. (1990). Identification of 1-methoxyindolyl-3-methyl isothiocyanate as an indole glucosinolate breakdown product. *Phytochemistry, 29*, 769-771.

Hanlon, N., Coldham, N., Gielbert, A., Sauer, M. J., & Ioannides, C. (2009). Repeated intake of broccoli does not lead to higher plasma levels of sulforaphane in human volunteers. *Cancer Letters, 284*, 15-20.

Holst, B., & Williamson, G. (2004). A critical review of the bioavailability of glucosinolates and related compounds. *Natural Product Reports, 21*, 425-447.

Jeffery, E. H., & Araya, M. (2009). Physiological effects of broccoli consumption. *Phytochemistry Reviews, 8*, 283-298.

Jeffery, E. H., & Keck, A. S. (2008). Translating knowledge generated by epidemiological and in vitro studies into dietary cancer prevention. *Molecular Nutrition & Food Research, 52 Suppl 1*, S7-17.

Kim, M. J., Kim, S. H., & Lim, S. J. (2010). Comparison of the apoptosis-inducing capability of sulforaphane analogues in human colon cancer cells. *Anticancer Research, 30*, 3611-3619.

Kim, J.-W., Kim, M.-B., & Lim, S.-B. (2015). Formation and Stabilization of Raphasatin and Sulforaphene from Radish Roots by Endogenous Enzymolysis. *Preventive Nutrition and Food Science, 20*, 119-125.

La Marca, M., Beffy, P., Della Croce, C., Gervasi, P. G., Iori, R., Puccinelli, E., & Longo, V. (2012). Structural influence of isothiocyanates on expression of cytochrome P450, phase II enzymes, and activation of Nrf2 in primary rat hepatocytes. *Food and Chemical Toxicology, 50*, 2822-2830.

Myzak, M. C., Ho, E., & Dashwood, R. H. (2006). Dietary agents as histone deacetylase inhibitors. *Molecular Carcinogenesis, 45*, 443-446.

Myzak, M. C., Tong, P., Dashwood, W. M., Dashwood, R. H., & Ho, E. (2007). Sulforaphane retards the growth of human PC-3 xenografts and inhibits HDAC activity in human subjects. *Experimental Biology and Medicine (Maywood), 232*, 227-234.

Pérez-Balibrea, S., Moreno, D. A., & García-Viguera, C. (2011). Genotypic effects on the phytochemical quality of seeds and sprouts from commercial broccoli cultivars. *Food Chemistry, 125*, 348-354.

Pocasap, P., Weerapreeyakul, N., & Barusrux, S. (2013). Cancer preventive effect of Thai rat-tailed radish (Raphanus sativus L. var. caudatus Alef). *Journal of Functional Foods, 5*, 1372-1381.

Reed, G. A., Arneson, D. W., Putnam, W. C., Smith, H. J., Gray, J. C., Sullivan, D. K., Mayo, M. S., Crowell, J. A., & Hurwitz, A. (2006). Single-dose and multiple-dose administration of indole-3-carbinol to women: pharmacokinetics based on 3,3'-diindolylmethane. *Cancer Epidemiology Biomarkers & Prevention, 15*, 2477-2481.

Shapiro, T. A., Fahey, J. W., Wade, K. L., Stephenson, K. K., & Talalay, P. (1998). Human metabolism and excretion of cancer chemoprotective glucosinolates and isothiocyanates of cruciferous vegetables. *Cancer Epidemiology Biomarkers & Prevention, 7*, 1091-1100.

Stefanson, A. L., & Bakovic, M. (2014). Dietary regulation of Keap1/Nrf2/ARE pathway: focus on plant-derived compounds and trace minerals. *Nutrients, 6*, 3777-3801.

Tang, L., Li, G., Song, L., & Zhang, Y. (2006). The principal urinary metabolites of dietary isothiocyanates, N-acetylcysteine conjugates, elicit the same anti-proliferative response as their parent compounds in human bladder cancer cells. *Anti-Cancer Drugs, 17*, 297-305.

Tarozzi, A., Angeloni, C., Malaguti, M., Morroni, F., Hrelia, S., & Hrelia, P. (2013). Sulforaphane as a Potential Protective Phytochemical against Neurodegenerative Diseases. *Oxidative Medicine and Cellular Longevity, 2013*, 10.

418 Vale, A. P., Santos, J., Brito, N. V., Fernandes, D., Rosa, E., & Oliveira, M. B. (2015). Evaluating
419 the impact of sprouting conditions on the glucosinolate content of *Brassica oleracea*
420 sprouts. *Phytochemistry, 115*, 252-260.
421 Vermeulen, M., Klopping-Ketelaars, I. W., van den Berg, R., & Vaes, W. H. (2008).
422 Bioavailability and kinetics of sulforaphane in humans after consumption of cooked
423 versus raw broccoli. *Journal of Agricultural and Food Chemistry, 56*, 10505-10509.
424 Watson, G. W., Beaver, L. M., Williams, D. E., Dashwood, R. H., & Emily, H. (2013).
425 Phytochemicals from Cruciferous Vegetables, Epigenetics, and Prostate Cancer
426 Prevention. *The AAPS Journal, 15*, 951-961.
427 Zhang, H. Q., Chen, S. Y., Wang, A. S., Yao, A. J., Fu, J. F., Zhao, J. S., Chen, F., Zou, Z. Q.,
428 Zhang, X. H., Shan, Y. J., & Bao, Y. P. (2016). Sulforaphane induces adipocyte browning
429 and promotes glucose and lipid utilization. *Molecular Nutrition & Food Research, 60*,
430 2185-2197.

FIGURE CAPTIONS

Figure 1. Outline of the cross-over study design. Young adult women were randomised to receive either broccoli and radish sprouts (20 g fresh weight) once a day during 7 days, with a 7-day washout period between the 7-day test phases. Urine was collected through 0-12 h and 12-24 h after dosing in the first day and for 24 h after ingestion from day 2 to 7.

Figure 2. Scheme of possible hydrolysis pathway and transformation of glucoraphenin from radish sprouts to sulforaphene and sulforaphane.

Figure 3. Levels of **s**ulforaphane, sulforaphene, metabolites derived from the mercapturic acid pathway (SFN-glutathione, SFN-cysteine, SFN-N-acetyl-L-cysteine) and the sum of those compounds in urine after consumption of broccoli (Figure 3A) or radish sprouts (Figure 3B) during 7 days of the study. Results are presented in μmol/24 h (Mean ± SEM). Not statistically significant differences ($p<0.05$) were found among days.

Figure 4. Levels of **s**ulforaphane, sulforaphene and metabolites derived from the mercapturic acid pathway (SFN-glutathione, SFN-cysteine, SFN-N-acetyl-L-cysteine) in urine after consumption of broccoli (Figure 4A) or radish sprouts (Figure 4B) separated in two periods: 0-12 h and 12-24 h in the first day. Results are presented in nmol/mg creatinine (Mean ± SEM).

* Indicates statistically significant differences between urine collection phases $p<0.05$.

Figure 5. Levels of indole-3-carbinol (I3C) and 3,3'diindolylmethane (DIM) in urine after consumption of broccoli (Figure 5A) or radish sprouts (Figure 5B) separated in two periods: 0-12 h and 12-24 h in the first day. Results are presented in nmol/mg creatinine (Mean ± SEM). Not statistically significant differences ($p<0.05$) were found among two periods.

456 **Table 1.** Glucosinolates and isothiocyanates (μmol/20 g F.W.) in broccoli and radish sprouts.

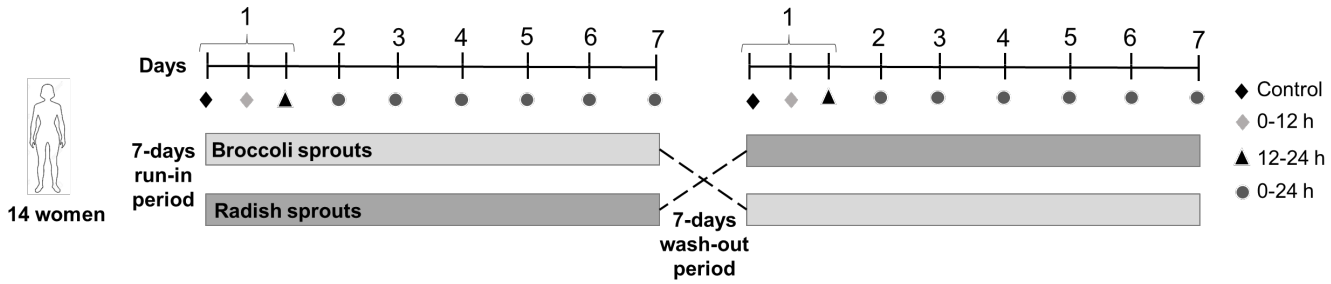
	Broccoli sprouts		Radish sprouts	
	Week 1	**Week 2**	**Week 1**	**Week 2**
Glucosinolates				
Glucoiberin	30.91± 6.21[a]	26.21 ± 0.86	-	-
Glucoraphanin (GRA)	63.91 ± 3.36	64.98 ± 0.33		
Glucoraphenin (GRE)	-	-	60.81 ± 2.13	61.25 ± 2.23
4-Hydroxyglucobrassicin	5.43 ± 0.39	4.17 ± 0.57	6.93 ± 0.65	6.23 ± 0.18
Glucoerucin	13.42 ± 1.03	12.77 ± 1.85	-	-
Glucoraphasatin (GPH)	-	-	78.52 ± 4.33	78.22 ± 14.11
Glucobrassicin	13.30 ± 0.76	9.39 ± 0.50	8.00 ± 0.18	7.42 ± 0.23
4-Methoxyglucobrassicin	6.23 ± 0.30	7.28 ± 0.75	3.25 ± 0.02	4.27 ± 0.45
Neoglucobrassicin	35.51 ± 0.14	42.78 ± 2.88	-	-
Isothiocyanates				
Sulforaphane (SFN)	11.39 ± 0.04	11.58 ± 0.31	0.93 ± 0.01	0.91 ± 0.03
Sulforaphene (SFE)	-	-	11.49 ± 0.25	11.30 ± 0.03
Indole-3-carbinol (I3C)	0.24 ± 0.05	0.26 ± 0.03	1.22 ± 0.11	1.24 ± 0.14

[a] Mean values (n=3) ± SD

457

458

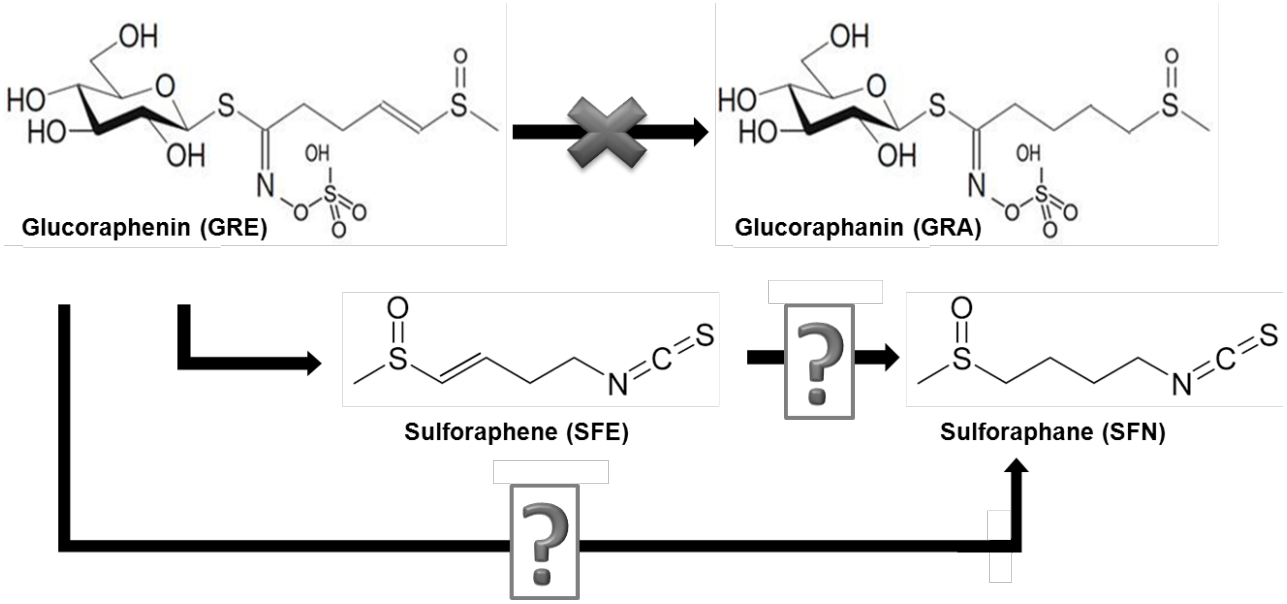
459 **FIGURE 1**



460

461

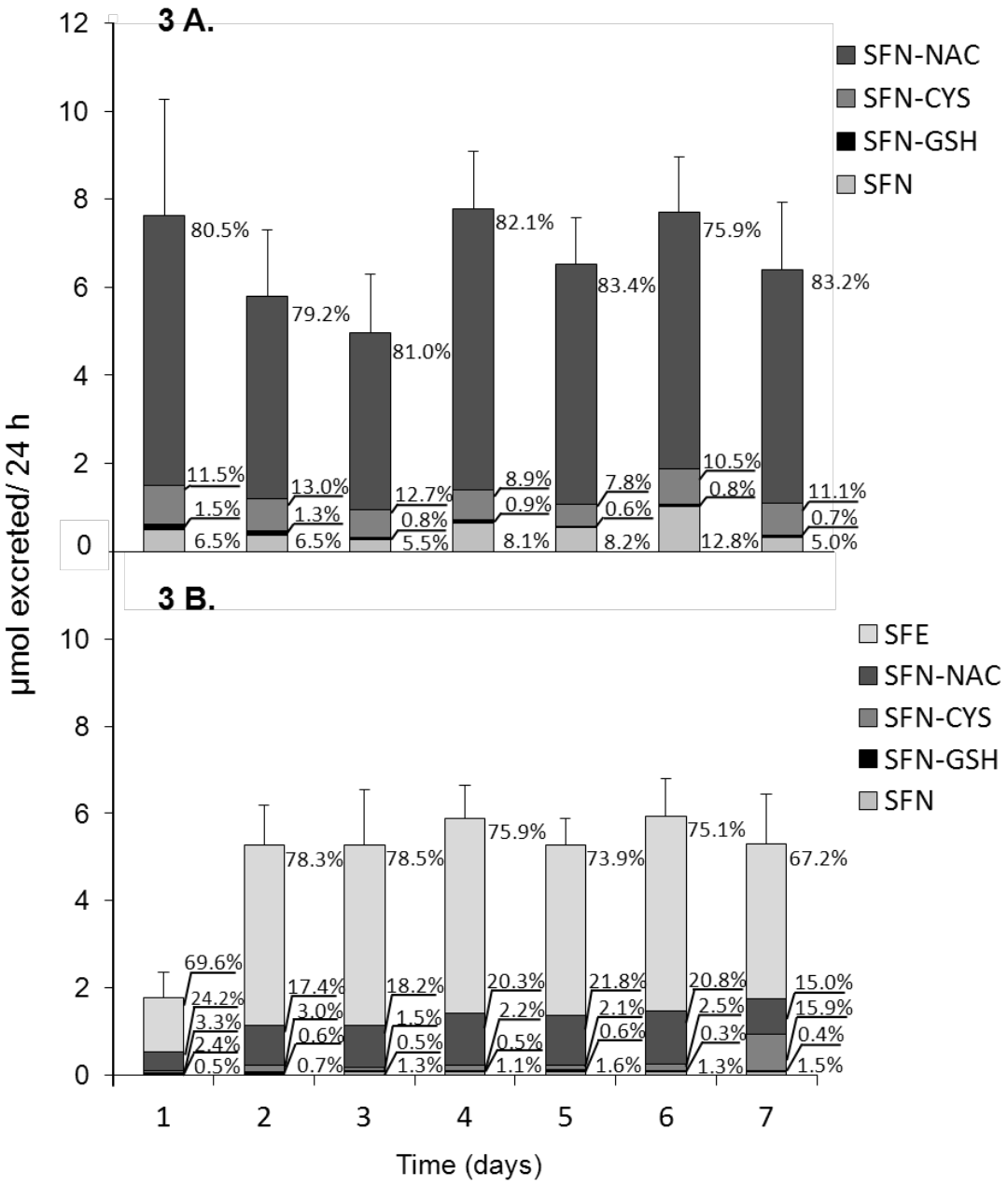
462 **FIGURE 2**



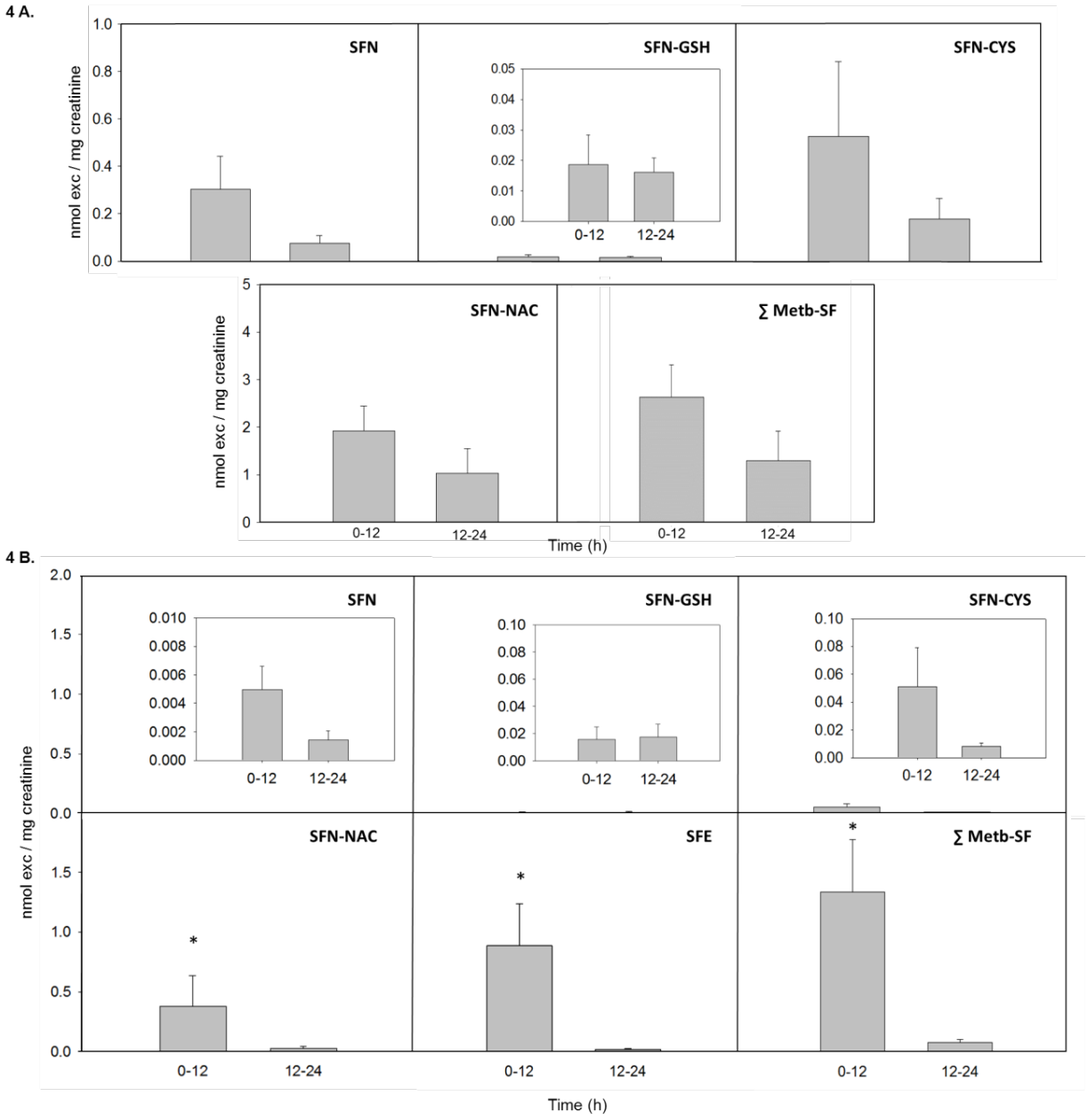
463

464

FIGURE 3

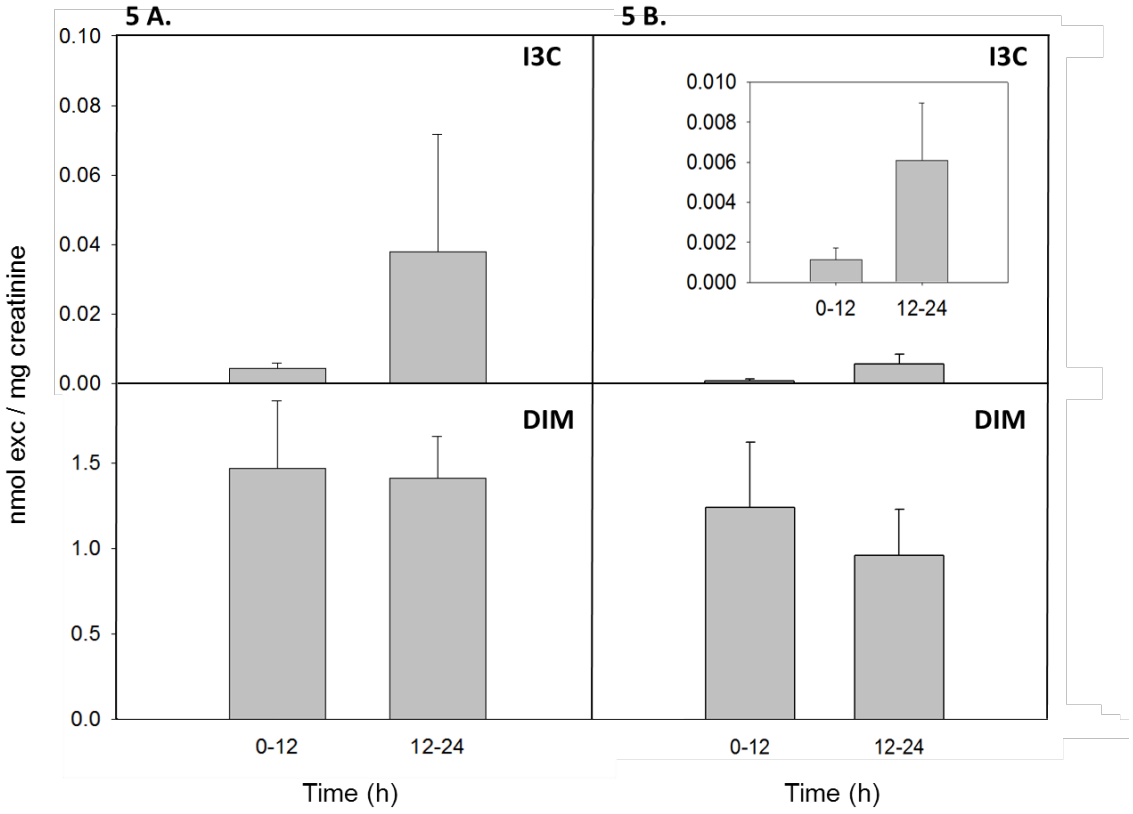

3 A.
SFN-NAC
SFN-CYS
SFN-GSH
SFN
80.5%
11.5%
1.5%
6.5%
79.2%
13.0%
1.3%
6.5%
81.0%
12.7%
0.8%
5.5%
82.1%
8.9%
0.9%
8.1%
83.4%
7.8%
0.6%
8.2%
75.9%
10.5%
0.8%
12.8%
83.2%
11.1%
0.7%
5.0%
3 B.
SFE
SFN-NAC
SFN-CYS
SFN-GSH
SFN
69.6%
24.2%
3.3%
2.4%
0.5%
78.3%
17.4%
3.0%
0.6%
0.7%
78.5%
18.2%
1.5%
0.5%
1.3%
75.9%
20.3%
2.2%
0.5%
1.1%
73.9%
21.8%
2.1%
0.6%
1.6%
75.1%
20.8%
2.5%
0.3%
1.3%
67.2%
15.0%
15.9%
0.4%
1.5%
µmol excreted/ 24 h
Time (days)
0
2
4
6
8
10
12
1
2
3
4
5
6
7

467 **FIGURE 4.**



468

469 **FIGURE 5**



470