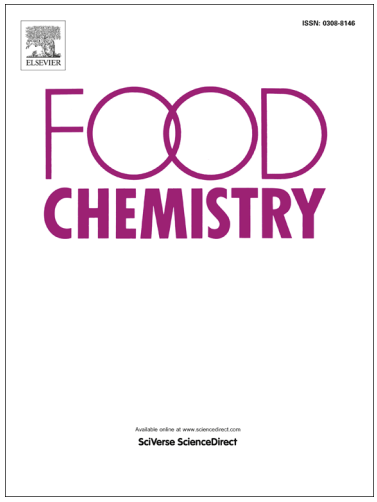

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PII: S0308-8146(18)31062-8
DOI: https://doi.org/10.1016/j.foodchem.2018.06.089
Reference: FOCH 23050

To appear in: *Food Chemistry*

Received Date: 12 February 2018
Revised Date: 13 June 2018
Accepted Date: 18 June 2018

Please cite this article as: Bello, C., Maldini, M., Baima, S., Scaccini, C., Natella, F., Glucoraphanin and sulforaphane evolution during juice preparation from broccoli sprouts, *Food Chemistry* (2018), doi: https://doi.org/10.1016/j.foodchem.2018.06.089

Glucoraphanin and sulforaphane evolution during juice preparation from broccoli sprouts

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Declarations of interest: none

Abbreviations: Cys, Cysteine; ESP, epithiospecifier protein; ESI-MS, Electrospray Ionization Source-Mass Spectrometry; FW, Fresh Weight; GL, Glucosinolate; GR, glucoraphanin; GSH, glutathione; GR, glucoraphanin; HPLC, High Performance Liquid Chromatography; ITC, isothiocyanates; Lys, Lysine; PEITC, phenethyl isothiocyanate; SFN, sulforaphane;

Abstract

Broccoli sprouts are considered functional food as they are naturally enriched in glucoraphanin (GR) that is the biological precursor of the anticancer compound sulforaphane (SFN). Due to its health promoting value, also broccoli sprout juice is becoming very popular.

The present study aimed to quantitatively assess the conversion of GR to its hydrolysis products, SFN and SFN-nitrile, during the juice preparation process. We demonstrated that SFN plus SFN-nitrile yield from glucoraphanin is quite low (≈25%) and that some SFN is lost during the juice preparation partially due to the spontaneous conversion to sulforaphane-amine or conjugation to GSH and proteins naturally present in the juice. Our results demonstrate that the detection of the sole SFN free form does not provide reliable information about the real concentration of this functional compound in the juice.

Key words: broccoli sprouts, bioactive molecules, glucosinolate, isothiocyanates, sulforaphane

Chemical compounds studied in this article: Glucoraphanin (PubChem CID: 9548634); Sulforaphane (PubChem CID: 5350); Sulforaphane nitrile (PubChem CID: 543743)

1. Introduction

Several epidemiological studies demonstrate that cruciferous vegetable consumption is associated with a reduction of cancer risk (Tse & Eslick, 2014) and of other chronic diseases (Dinkova-Kostova & Kostov, 2012). Currently, the role of these vegetables on cancer chemoprevention has been attributed to the hydrolysis products of glucosinolates (GLs) that are secondary plant metabolites typical of *Brassica* vegetables (Possenti et al., 2016), where they are particularly abundant. GLs are *beta*-D-thioglucoside-*N*-hydroxy sulphates with a variable side chain characterizing the molecule. GLs as such are biologically inactive, but when plant tissues are crushed or chewed, GLs are hydrolyzed by myrosinase releasing isothiocyanates (ITCs), a class of very reactive compounds. Several evidence, demonstrate that ITCs are inducer of phase 2 detoxification enzymes (Cornblatt, et al., 2007) and of the Nrf2 signaling pathway (Bauman et al., 2016), suggesting that they can affect cancer development. Beside anti-cancerogenic activity, ITCs possess protective effects against cardiovascular disease, neurodegeneration, diabetes and several inflammatory disorders (Bahadoran et al., 2011; Mirmiran, Bahadoran, Hosseinpanah, Keyzad, & Azizi, 2012).

Because of the potential health benefits of GL/ITC intake, several attempts have been made to increase *Brassica* vegetable consumption. Unfortunately, due to their sensory characteristics these vegetables tend to be disliked (Cox & Poelman, 2015), prompting food researchers and food industry to develop new food products rich in cruciferous bioactive molecules (Amofa-Diatuo, Anang, Barba, & Tiwari, 2017).

Among ITCs, sulforaphane (SFN) is the most studied compound and is the one that has the strongest evidence of beneficial effects (recently it has also been utilized to improve some symptoms of autism spectrum disorders (Singh et al., 2014)). SFN is produced by the hydrolysis of glucoraphanin (GR), in a myrosinase-catalysed reaction yielding glucose and an unstable aglycone. This unstable product undergoes spontaneous rearrangement into two different products, SFN and

SFN-nitrile, whose relative formation is related to the differences in the hydrolysis conditions (e.g. the presence and activity of the epithiospecifier protein (ESP) and Fe^{2+}, that direct the hydrolysis reaction favouring the formation of SFN nitrile in place of SFN (Matusheski et al., 2006); high temperature that can differently inhibit myrosinase and ESP activities (70°C and 50°C, respectively) (Matusheski, Juvik, & Jeffery, 2004).

GR is particularly abundant in broccoli (where it represents about 50% of total GL) with a content ranging from 25 to 650 mg/100 g FW, and is even higher in broccoli sprouts (up to 1400 mg/100 g FW) (Possenti et al., 2016).

As domestic processing and cooking decrease the content of GR, SFN and other bioactive molecules (Nugrahedi, Verkerk, Widianarko, & Dekker, 2015), the market and consumers are driven towards uncooked, healthy and easy to eat products, such as broccoli and broccoli sprout juice. Indeed, several *Brassica* vegetable beverages and juices have been put onto the market and tested for their functional capacity in *in vitro* (Lee et al., 2013) and *in vivo* experiments (Egner et al., 2014; Kensler et al., 2012).

In most paper studying the biological activity of these products, only the concentration of GR present in the starting raw material is given and the assumption is made that all the GR present in the raw vegetable is converted into SFN. However, matching the results of our previous studies (Ferruzza et al., 2016; Natella et al., 2016) we observed that during broccoli sprout juice preparation the conversion of GR to SFN and SFN-nitrile seems to be not quantitative. The aim of the present study was to monitor the conversion of GR to SFN and SFN-nitrile during the juice preparation process.

2. Materials and Methods

2.1. Materials

Solvents and HPLC grade methanol used for extraction were of high purity (Carlo Erba, Milano, Italy). Acetonitrile (LC-MS grade) and formic acid (MS grade) were from Sigma-Aldrich Chemical Company (St Louis, MO). HPLC grade water (18 mΩ) was prepared using a Millipore (Bedford, MA, USA) Milli-Q purification system. Glucoraphanin potassium salt (purity 93.4%) was purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). Sulforaphane (purity >95%), Pronase E, Glutathione (reduced form) and Lysine were from Sigma-Aldrich (St. Louis, MO, USA). 4-(Methylsulfiny)-1-butylamine (Sulforapahne-amine) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

2.2. Growth condition of Broccoli Sprouts

Broccoli seeds (*Brassica oleracea* L. var. *botrytis* subvar. *cymosa*) were purchased from SUBA&UNICO (Longiano, FC, Italy). Seeds were surface sterilized by soaking for 15 min in 2% sodium hypochlorite under shaking, then drained and rinsed 10 times with distilled water. After soaking in distilled water for 16-18 h at 21 °C, seeds were rinsed in distilled water and transferred in the germination cylinder of Vitaseed sprouter (SUBA&UNICO) filled with distilled water. Sprouts were grown at 21°C and 70% humidity in a plant growth chamber (Weiss Gallenkamp, Loughborough, United Kingdom) equipped with PHILIPS Master TL-D 36W/840 cool-white fluorescent tubes providing a photosynthetic photon flux density of 110 µmol m^{-2} s^{-1}, with a 16 h light/8 h dark photoperiod. Five days old sprouts were rapidly but gently collected from the germination cylinder and weighted. For each germination cylinder, 100 g of sprouts were used for juice extraction

2.3 Juice extraction

Immediately after harvesting broccoli sprouts were squeezed with a mechanical press (Angel 8500S, Living Juice srl, Lecco, LC). The juice was collected in ice-cold tubes, cleared by centrifugation (30 min, 4000 rpm, 4 °C) and aliquots, immediately frozen in liquid nitrogen, were stored at –80 °C until further analysis. For some analyses, a microwaving treatment was applied before squeezing: 100 gr sprouts were immersed in 150 ml of double distilled water and exposed for 1 minute at 850 Watt in a domestic microwave oven (Samsung GE82P), to reach an internal temperature of 58 °C. Then, sprouts were drained, gently soaked up with tissue paper, re-weighted and squeezed as described above.

The volumes of crude and cleared juice and the weights of squeezed sprouts residue and insoluble debris pellet were recorded. Aliquots of all the intermediate fractions were immediately frozen in liquid nitrogen and stored at –80 °C until analysis.

The release of SFN from its protein-conjugates was studied using freshly prepared juice samples, diluted 1:10 with TRIS 20 mM pH 8 and incubated for 120 min at room temperature or at 65°C (Hu et al., 2001).

2.4 Proteins digestion for the detection of amino acid adducts

Proteins from broccoli sprout juice were precipitated using trichloroacetic acid (TCA)/acetone following the procedure described by Méchin (Méchin, Damerval, & Zivy, 2007). Briefly, 200 µL of juice were precipitated with 1.8 mL of cold acetone containing 10% TCA (w/v) and 0.07% 2-mercaptoethanol (v/v) and centrifuged at 13000 rpm for 30 min (3°C). Supernatant was discarded and the pellet was re-suspended in 1.8 mL cold acetone containing 0.07% of 2-mercaptoethanol (v/v). This step was repeated twice. Pellets were dried with nitrogen and incubated with 300 µL of Pronase E (5 mg/mL) in 50mM sodium bicarbonate pH 8.9 at 37°C for 20 hours (Kumar & Sabbioni, 2010). The digested samples were acidified (up to pH 4.0) with 2 M hydrochloric acid and diluted twice with methanol prior to the analysis by LC-MS/MS.

2.5 Synthesis of Sulforaphane–Glutathione adduct (SFN-GSH) and Sulforaphane–Lysine adduct (SFN-Lys)

SFN-GSH was synthesized according to the following procedure (Pernice et al., 2009): 2.2 µmol of SFN and 4.5 µmol of GSH were dissolved in 500µL of 0.5 M sodium hydrogen carbonate buffer pH 10 and stirred at 25°C for 1h. The reaction was stopped by adjusting the pH to 5 using 36% hydrochloric acid. For SFN-Lys preparation 2.2 µmol of SFN and 5.4 µmol of lysine were dissolved in 500 µL of 0.5 M sodium hydrogen carbonate buffer pH 10 and stirred at 25°C for 1h. Molecular weight and structures of the conjugates were confirmed by electrospray ionization MS and their retention time and mass spectra were utilized to identify the adduct in the samples.

2.7 Glucoraphanin, Sulforaphane, Sulforaphane-nitrile, Sulforaphane-amine and Sulforaphane adducts analysis

Glucoraphanin (GR), Sulforaphane (SFN), Sulforaphane-amine, SFN-GSH and SFN-Lys were identified by using an HPLC system (Perkin-Elmer, USA) interfaced to an Applied Biosystems (Foster City, CA, USA) API3200 Q-Trap spectrometer. Quantitative on-line HPLC-ESI-MS/MS analyses were performed using mass spectrometer in Multiple Reaction Monitoring (MRM) mode.

GR measurement was performed according Maldini et al. (2012); briefly, samples were extracted with methanol:water (70:30 v/v; sample to solvent ratio 1:25 w/v) at 70 °C for 30 min under vortex mixing. Samples were centrifuged (4000 rpm, 30 min, 4 °C), supernatants were collected and the solvent was completely removed using a rotary evaporator at 40 °C. The dried samples were dissolved in ultrapure water with the same volume of extraction and filtered through 0.20 µm syringe PVDF filters (Whatmann International Ltd., UK). Samples were injected (10 µL) into a Luna C_{18} column (Phenomenex, USA) (150x2.1 mm i.d., 5 µm) and eluted at flow rate of 0.3 mL

min^{-1}. Mobile phase A was H_2O containing 0.1% formic acid while mobile phase B was acetonitrile containing 0.1% formic acid. Elution gradient was: 94% A, 88:12 (A:B) in 15 min, from 88:12 (A:B) to 75:25 (A:B) in 6 min, from 75:25 (A:B) to 40:60 (A:B) in 9 min, and finally to 100% B in 1 min. The column was kept at 25 °C, using a Peltier Column Oven Series 200 (Perkin Elmer). MS detection was performed in ESI negative ion mode; source temperature was held at 450°C, and MS parameters were: ion spray voltage at -4500; dwell time was 60 ms; declustering potential -50 eV, entrance potential -5 eV, collision energy -27 eV; selected transition was 436/178.

For SFN, SFN-nitrile, Sulforaphane-amine, SFN-GSH, SFN-Cys and SFN-Lys identification and/or determination, juice or pronase digested juice samples were diluted with H_2O containing 0.1% formic acid. For SFN and SFN-nitrile determination sprout, waste and pellet were extracted with methanol:water (70:30 v/v; sample to solvent ratio 1:25 w/v) at 70 °C for 30 min under vortex mixing. Samples were centrifuged (4000 rpm, 30 min, 4 °C), supernatants were collected and the solvent was completely removed using a rotary evaporator at 40 °C. The dried samples were dissolved in ultrapure water with the same volume of extraction and filtered through 0.20 µm syringe PVDF filters (Whatmann International Ltd., UK).

Samples were filtered, injected into a Luna C_{18} column (Phenomenex, USA) (150 x 2.1 mm i.d., 5 µm d) and eluted at flow rate of 0.3 mL min^{-1}. Mobile phase A was H_2O containing 0.1% formic acid while mobile phase B was acetonitrile containing 0.1% formic acid. Elution gradient was: 100% A, 20:80 (A:B) in 20 min, from 20:80 (A:B) to 0:100 (A:B) in 1 min. The column was kept at 25 °C, using a Peltier Column Oven Series 200 (Perkin Elmer). MS detection was performed in ESI positive ion mode; Source temperature was held at 500°C, and MS parameters were: ion spray voltage at +5500; dwell time was 100 ms; declustering potential 46 eV, entrance potential 6 eV, cell exit potential 4 eV. For quantitative measure the following transitions were selected: SFN 178/114 and 178/55 (CE=18); SFN-nitrile 146/87 and 146/55 (CE=25); Sulforaphane-amine 136/72 (CE=18) and 136/55 (CE=30). SFN and Sulforaphane-amine concentrations were calculated over

an external standard curve of the respective standard, while SFN-nitrile, SFN-GSH and SFN-Lys concentrations were calculated over an external standard curve of SFN.

For the above analysis, data acquisition and processing were performed using Analyst software 1.5.1.

3. Results and Discussion

In previous studies we analysed the GL content in broccoli sprouts (Maldini et al., 2012), successively we used broccoli sprouts grown in the same condition to produce a juice whose biological effect was studied on a cellular model (Ferruzza et al., 2016). Matching the results obtained in these two different studies we observed that SFN content of the juice was notably lower than expected from the GR content of sprouts. In order to study this apparent inconsistency, the fate of GR and SFN in broccoli sprouts during juice preparation was studied here. Immediately after harvesting, broccoli sprouts were squeezed with a mechanical press (usually used for homemade juice preparation), then, the collected juice (cloudy juice) was centrifuged to remove particulate matter and cellular debris, yielding the final clear juice and pellet; the waste material remaining in the press after the squeezing was also collected (waste).

The total content in GR, SFN and SFN-nitrile during the whole juice preparation process (in sprouts, waste, cloudy juice, clear juice and pellet) is shown in Figure 1A.

The GR content in 100 g of the starting broccoli sprouts was 458±136 µmol, a value comparable to that observed in our previous work (Maldini, 2012) and similar to that reported by Baenas for broccoli sprouts grown on soil trays (Baenas, García-Viguera, & Moreno, 2014). As shown in the figure 1A, broccoli sprouts also contained small amounts of SFN and SFN-nitrile (41±49 and 33±18 µmol, respectively), whose presence is presumably due to slight mechanical injuries suffered by the sprouts during harvesting.

As expected, the clear juice extracted from fresh sprouts (about 50 mL of clear juice were collected from 100g of sprouts) did not contain GR, while the predominant product of GR hydrolysis was SFN-nitrile (0.90±0.46 µmol/mL juice, which corresponds to 51±28 µmol in the juice extracted from 100 g fresh sprouts).

These results are in agreement with previous data, that reported that when broccoli or broccoli sprouts are crushed at room temperature and endogenous plant pH, SFN-nitrile is the principal GR hydrolysis product (Jones, Frisina, Winkler, Imsic, & Tomkins, 2010; Matusheski et al., 2004).

The total amount of SFN extracted in the juice from 100 g fresh sprouts was about 7±3 µmol (0.12±0.04 µmol/mL juice), a value a bit higher than that previously reported in broccoli juice or broccoli sprouts juices (about 0.05-0.1 µmol/mL and 0.02 µmol/mL, respectively) (Houška et al., 2006; Kassie et al., 1996; Totušek et al., 2011).

A common assumption in the current literature concerning the health benefits of SFN is that the GR present in broccoli is almost entirely converted to SFN and SFN-nitrile during crushing or squeezing. However, unexpectedly, we observed that the sum of SFN and SFN-nitrile contained in the juice obtained from 100 g broccoli sprouts represented only 10% (57.8±30.9 µmol) of the total amount of GR plus the small amount of SFN and SFN-nitrile found in the sprouts (532±145 µmol) (Figure 1A). We thought to look for the missing SFN, SFN-nitrile and GR amounts in the waste produced during broccoli sprouts squeezing and in the pellet obtained after juice clearing by centrifugation (Figure 1A). The overall contents of GR, SFN and SFN-nitrile in these two by-products of juice preparation were 73.9±30.7 µmol and 10.2±5.8 µmol, respectively for all waste and pellet obtained from the starting 100 g broccoli of sprouts. This means that the total amount of GR breakdown products present in juice, pellet and waste produced from 100 g of sprouts is 141.9±65.0 µmol, which accounts for about a quarter of the amount present in the sprouts. As expected, similar results have been obtained measuring SFN and SFN-nitrile on the un-centrifuged cloudy juice (figure 1A).

In order to verify whether the ESP inactivation (that redirects GR hydrolysis toward SFN instead of SFN-nitrile) could somewhat explain the missing amounts of GR breakdown products, before squeezing the sprouts were heated at 58 °C using a microwave oven (100 g broccoli sprouts in 150 mL of water for 1 min at 750 W). Due to the higher sensitivity to heat of ESP respect to myrosinase, the temperature reached by broccoli sprouts after this microwave treatment allows the inactivation of ESP without affecting myrosinase activity (Matusheski et al., 2004), as demonstrated by the absence of GLs in the juice (figure 1B). As expected the thermal inactivation of ESP promote SFN production in place of SFN nitrile. Nevertheless, also in this case we did not obtain a higher recovery of GR hydrolysis products. In fact, the sum of SFN and SFN-nitrile present in juice, pellet and waste corresponds only to 23% of GR found in starting broccoli sprout GR (Figure 1B).

A non-quantitative yield of GR to SFN+SFN-nitrile has been previously reported in few different papers and samples (broccoli sprouts and broccoli seeds, respectively 50 and 20-70% recovery) (Guo, Yang, Wang, Guo, & Gu, 2014; Matusheski et al., 2001; Shen, Su, Wang, Du, & Wang, 2010), but unfortunately it was not further investigated.

SFN and SFN-nitrile could be further enzymatically or chemically degraded and the possible catabolic pathways are shown in figure 2.

In *in vitro* experiments it has been demonstrated that ITC can decompose to their corresponding amine (Hanschen et al., 2012), but clearly no ITC derived amines have been detected in intact *Brassica* vegetables (Song, Morrison, Botting, & Thornalley, 2005).

In order to investigate whether SFN or SFN-nitrile catabolism could explain the apparent non–quantitative conversion of GR during juice preparation, we searched for the presence of these breakdown compounds by HPLC MS/MS. Sulforaphane-amine was identified in broccoli sprout juice and quantified using authentic standard; the amount found in broccoli sprout juice was 0.46±0.19 µmol/mL (data not shown). Although indicative of the reactivity of SFN, this small amount cannot explain the GR-derivatives loss observed in our study; in fact, the amount of

sulforaphane-amine originating from 100 g of broccoli sprouts is about only 3% of the starting GR. Unfortunately, we did not investigate further possible reactions of this amine; in fact, sulforaphane-amine could react with SFN (or other ITCs occurring in the juice) and form disulforaphanylthiourea (or other thiourea derivatives) (figure 2). The formation of this thiourea derivatives has been suggested for phenethyl isothiocyanate (PEITC), whose *in vitro* hydrolysis produces both phenethylamine and N,N-diphenethylthiourea (Xu & Thornalley, 2000).

ITCs are strong electrophiles able to react with nucleophilic compounds, such as thiol, hydroxyl and amino groups of proteins and/or amino acids (Hanschen et al., 2012). Few and old studies describe the interaction between ITCs and food proteins (Björkman, 1973; Hernaindez-triana & Petzke, n.d.; Kishore Kumar Murthy & Narasinga Rao, 1986), and demonstrate that ITCs binding takes place especially on cysteine and lysine residues (Kawakishi & Kaneko, 1987), leading to the formation of thiocarbamoylated protein and/or thiourea-like derivatives. However, these studies are focused on the alteration and/or loss of nutritional quality of proteins rather than on the possible effect on ITC availability (Hanschen, Lamy, Schreiner, & Rohn, 2014; Kawakishi & Kaneko, 1987). Therefore, we further investigated the possible reactions between SFN and some nucleophilic compounds, specially GSH and protein.

As the ITC binding to cysteine sulfhydryl group is reversible under slightly alkaline pH (Conaway, Krzeminski, Amin, & Chung, 2001), broccoli sprout juice was diluted in an alkaline buffer (TRIS at pH 8.5) and incubated both at room temperature and at 65°C to improve the dissociation. Figure 3 shows that the alkalinization of the juice induces an increase in the measurable SFN (+16% and +45%, respectively for RT and 65°C), suggesting its release from protein and other easily hydrolysable adducts, such as SFN-GSH.

On the basis of these results, the binding of SFN to the free cysteine sulfydryl group of plant GSH was investigated. The presence of SFN-GSH in broccoli sprout juice was studied by HPLC MS/MS, that allowed to demonstrate the occurrence of this adduct in the juice. The identity of SFN-GSH adduct was initially verified by comparing the MS^2 spectrum with that reported in literature

(Kassahun, Davis, Hu, Martin, & Baillie, 1997) and then confirmed by comparing both the retention time and the MS^2 spectrum with those of synthetized SFN-GSH. The chromatogram and mass spectral data of the adduct are shown in Figure 4. Quantifying the SFN-GSH adduct on SFN standard curve, we obtained a value of 9±2 nmol/mL juice, that corresponds to 0.3±0.1 µmol produced from 100 g broccoli sprouts. Then also the amount of SFN "lost" in the form of GSH adduct is quite low and cannot explain the poor recovery.

Interestingly, an ITC-GSH conjugate was already observed (unfortunately only once) in a plant extract. About 12.5 pmol/mL of PEITC-GSH conjugate (1% of the parent GLS) has been, in fact, reported in a crude watercress extract (Rose, 2000).

The studies on ITC-food protein interactions were carried out in model system using isolated protein or protein fractions (Kawakishi & Kaneko, 1987; Keppler et al., 2017; Kishore Kumar Murthy & Narasinga Rao, 1986). In these studies, the interaction between ITC (such as benzyl-ITC, allyl-ITC, etc.) and aminoacids of food proteins has been indirectly demonstrated through the determination of the available aminoacids before and after ITC treatment (Hernaindez-Triana & Petzke, 1996; Kishore Kumar Murthy & Narasinga Rao, 1986). Currently, there is only one investigation about the interaction of SFN with aminoacids (namely cysteine) directly in food, specifically in broccoli sprouts (Hanschen et al., 2012). Hanschen and collaborators found that one fifth of the SFN formed after the addition of exogenous myrosinase reacted with a cysteine derivative (Nα-(tert-butoxycarbonyl)-L-cysteine methyl ester) that was specifically added to the broccoli sprout extract. They also hypothesised that part of the produced SFN could react with food proteins, as only 63% of breakdown products were recovered after GR hydrolysis.

Differently from this paper, we specifically studied the possibility that naturally produced SFN reacted with amino group of proteins naturally present in the sprout juice. For such purpose, the proteins precipitated from sprout juice were enzymatically digested by Pronase E and then analyzed for the presence of SFN-Lys adducts by LC-MS/MS following the method of Kumar (Kumar & Sabbioni, 2010).

We were able to identify the SFN–lysine adduct, whose identity was verified by comparison of both retention time and MS^2 spectrum with that of synthetized SFN-Lys. The mass spectrum (ESI positive mode) shows the characteristic fragment ions (324.2: $[M + H]^+$, 136.0 $[M - C_7H_{12}N_2O_2S]^+$, 147.2 $[M\ C_6H_{11}NOS_2]^+$, 260.2 $[M - CH_3OS]^+$) as reported by Kumar (Kumar & Sabbioni, 2010) (Figure 5). SFN-Lys adducts, quantified using a SFN standard curve, were estimated at about 20 pmol/mg proteins (data not shown), an amount too low to explain the poor recovery.

The presence of SFN-Cys was also monitored, but as expected no SFN–cysteine adducts were found in digested proteins. In fact, as already demonstrated by Kumar (Kumar & Sabbioni, 2010) and Conaway (Conaway et al., 2001), the ITC binding to thiol group are too labile to survive the experimental procedure needed for protein precipitation and sample preparation.

In summary, we demonstrated that the juice contains sulforaphane-amine that can be formed from SFN idrolysis ~~or can be released from the cleavage of dithiocarbamate and thiourea-like derivatives coming from the reaction among SFN and amino group of GSH and protein~~ (Figure 2). We also suggest that other thiourea derivatives can be formed from this compound. In addition, we can state that part of the SFN is "sequestered" as conjugate with GSH and proteins, but we cannot demonstrate that all the "missing" SFN is present in its conjugated forms. At the best of our knowledge this is the first time that the interaction of an ITC with food proteins/GSH has been directly demonstrated in food.

An important limitation of our study is that the quantification of SFN-nitrile and bound SFN were done using the calibration curve of SFN, and this does not allow to exactly quantify the presence of these molecules. Actually, SFN-nitrile could have been underestimated, then the real recovery could be higher than that we estimated. A second limitation is that we did not determine the presence of sulforaphane-amine and SFN-adducts in waste.

Then, further studies are needed to answer these points and to understand if broccoli sprout juice could be a real alternative, in terms of bioactive molecules, to its fresh counterpart (as seems to be demonstrated by broccoli juice supplementation studies (Smith, Mithen & Johnson, 2003), or if the application of different processing technologies could help in the preservation of SFN and/or GR. Even if, it should be also considered that the concentrations of SFN and/or GR are not the sole determinants of their *in vivo* bioavailability and activity (Fahey et al., 2015) and that there is still need to perform in depth studies evaluating the impact of processing on the nutritional properties of the GLS containing vegetables (Barba & Orliena, 2017).

4. Conclusions

Studying the fate of GR and SFN during the process of juice preparation we observed that, despite all GR present in the starting broccoli sprouts was hydrolyzed during the process, the resulting juice did not contain an equivalent amount of SFN and SFN-nitrile. Even considering their loss in the waste discarded during juice preparation, the apparent yield of these two compounds was about 25% percent of the starting GR. In our study we were not able to utterly explain the reason of this low recovery, however, analyzing the possible "escape route" of SFN, we found that SFN can 1) be degraded to sulforaphane-amine, 2) form an adduct with GSH and 3) bind to the plant proteins present in the juice.

It is widely accepted that, at least in human cellular model, the ITC bound forms can release the biological active free form, then these conjugates exhibit biological activities similar to those of free ITCs (Rose, 2000). However, it is also known that the binding to proteins can cause an interference with ITC action. For example, it has been demonstrated that the antimicrobial activity of AIT in meat can be neutralized by the presence of GSH and Cys (Luciano, Hosseinian, Beta, & Holley, 2008), while the antimicrobial activity of cauliflower juice is reversed by addiction of cysteine (Brandi, Amagliani, Schiavano, De Santi, & Sisti, 2006).

Therefore, as an amount of SFN is bound to juice proteins, the detection of the sole free form does not provide information about the effective availability of this functional compound in foods.

Acknowledgements

This work was supported by Italian Ministry of Agriculture, Food & Forestry (MiPAAF) grant "FoodBall" (DM 28331/7303/14 del 9/12/2014).

Kariklia Pascucci is acknowledged for her kind support in the daily lab work.

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Figure Captions

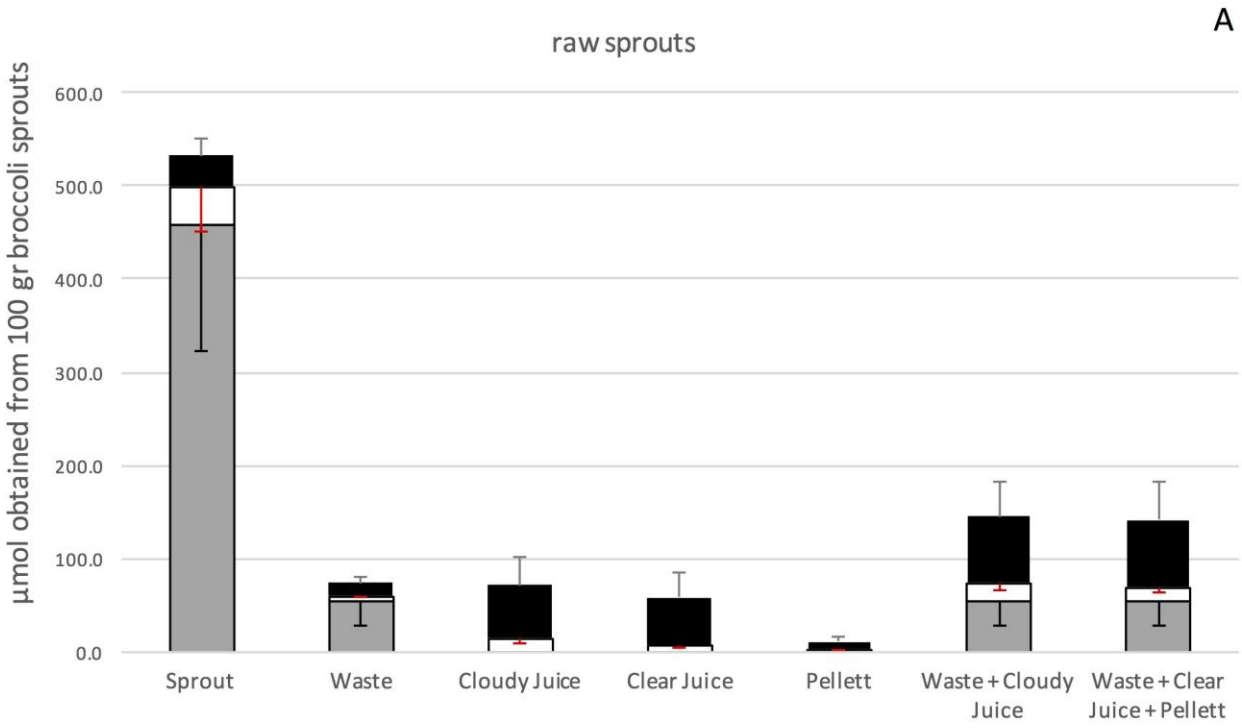
Figure 1: Amount of GR, SFN and SFN-nitrile (µmol) present in 100 gr of broccoli sprouts and in the different fractions obtained from 100 gr broccoli sprouts raw (A) and after microwaving treatment (B)

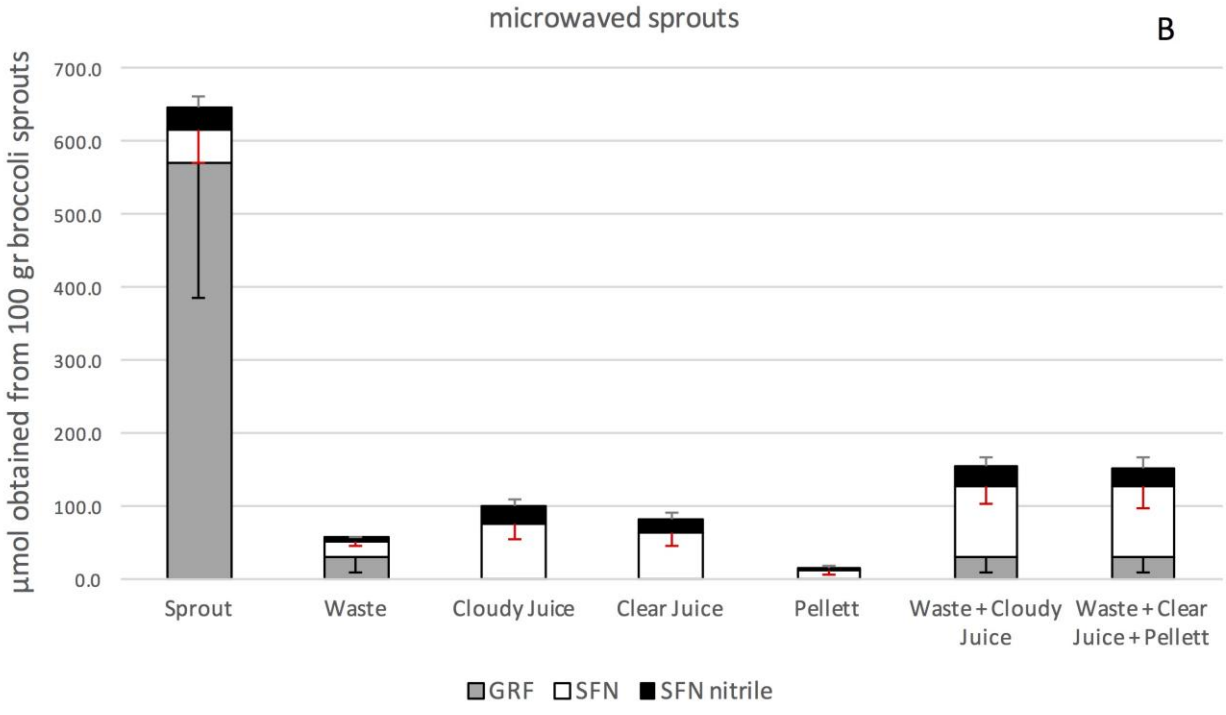
Figure 2: Scheme of the catabolic pathways of GR, SFN and SFN-nitrile. Reaction 1: SFN-nitrile hydrolysis to carboxylic acids by nitrilases (our hypothesis); Reaction 2 reversible SFN binding to SH group of protein (Kawakishi & Kaneko, 1987; Conaway et al., 2001; Song et al., 2006; Hu et al., 2011; Hanschen et al., 2012); Reaction 3: SFN binding to protein NH_2 group (Kawakishi & Kaneko, 1987; Hernaindez-Triana & Petzke, 1996; Kumar & Sabbioni, 2010; Hanschen et al., 2012); Reaction 4: SFN hydrolysis to sulforaphane-amine (Pecháček et al, 1997; Xu et al., 2000; Song et al., 2005; Hanschen et al., 2012); Reaction 5: SFN binding to GSH (Rose et al., 2000; Song et al., 2005); Reaction 6: sulforaphane-amine reaction with SFN to form disulforaphanylthiourea (or other thiourea derivatives) (Pecháček et al, 1997; Xu et al., 2000).

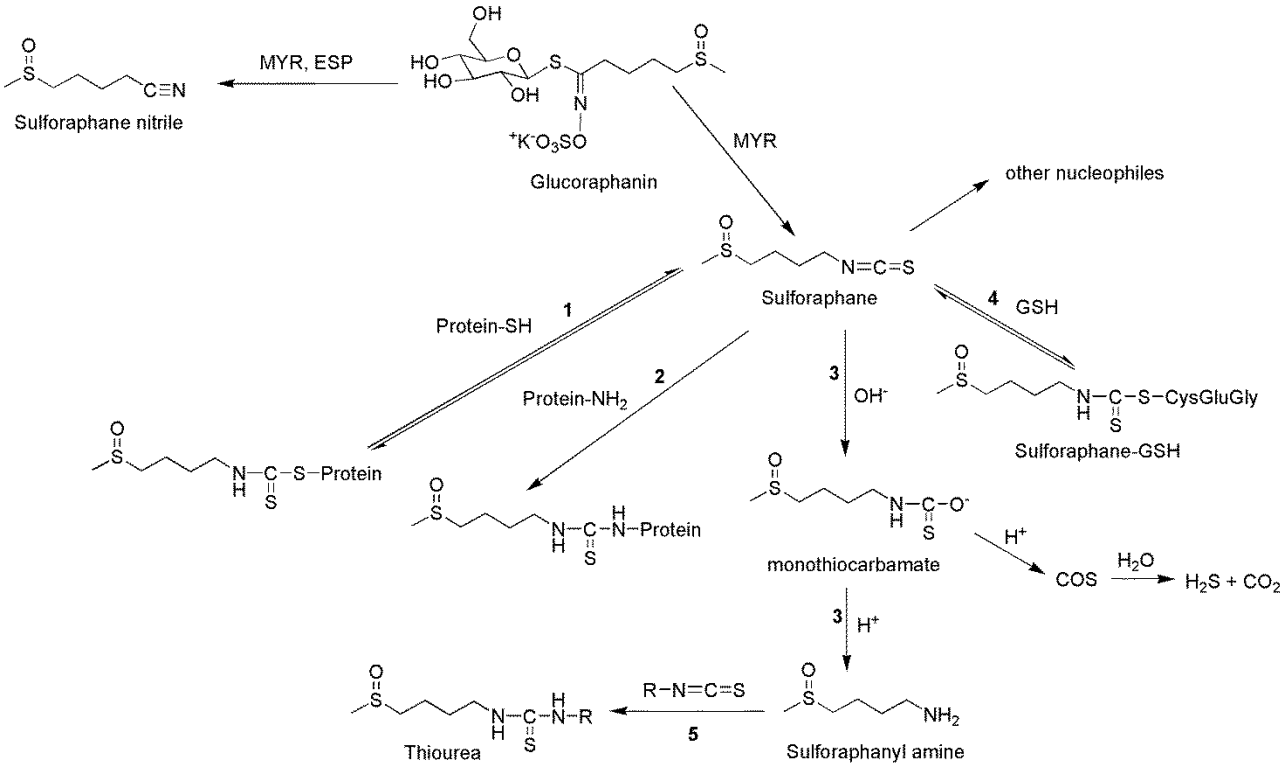
Figure 3: SFN concentration in broccoli sprout juice diluted in an alkaline buffer (TRIS at pH 8.5) and incubated at room temperature or at 65°C

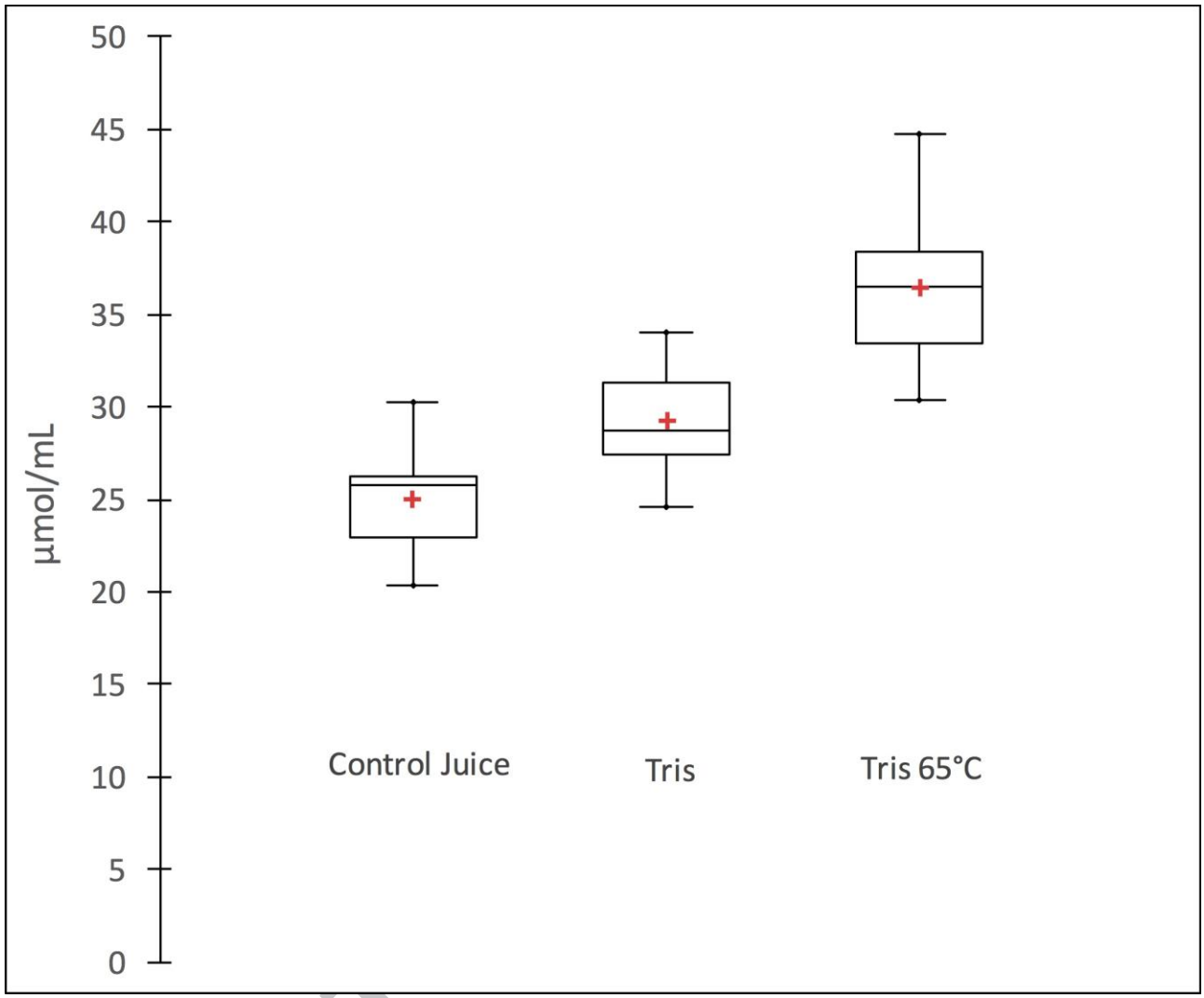
Figure 4: LC-MS/MS analysis of SFN-GSH in broccoli sprout juice; (A) SFN-GSH spectrum (B) chromatogram of SFN-GSH detected in the juice using three different MRM transitions (485/136, 485/356 and 485/178 m/z)

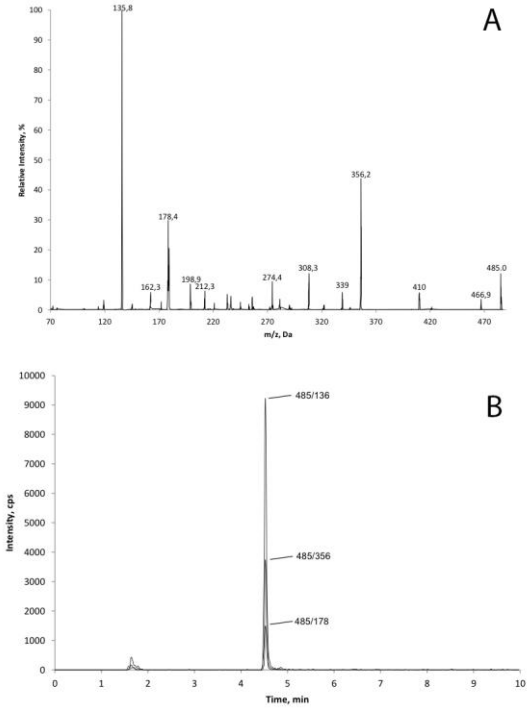
Figure 5: SFN-Lys mass spectrum

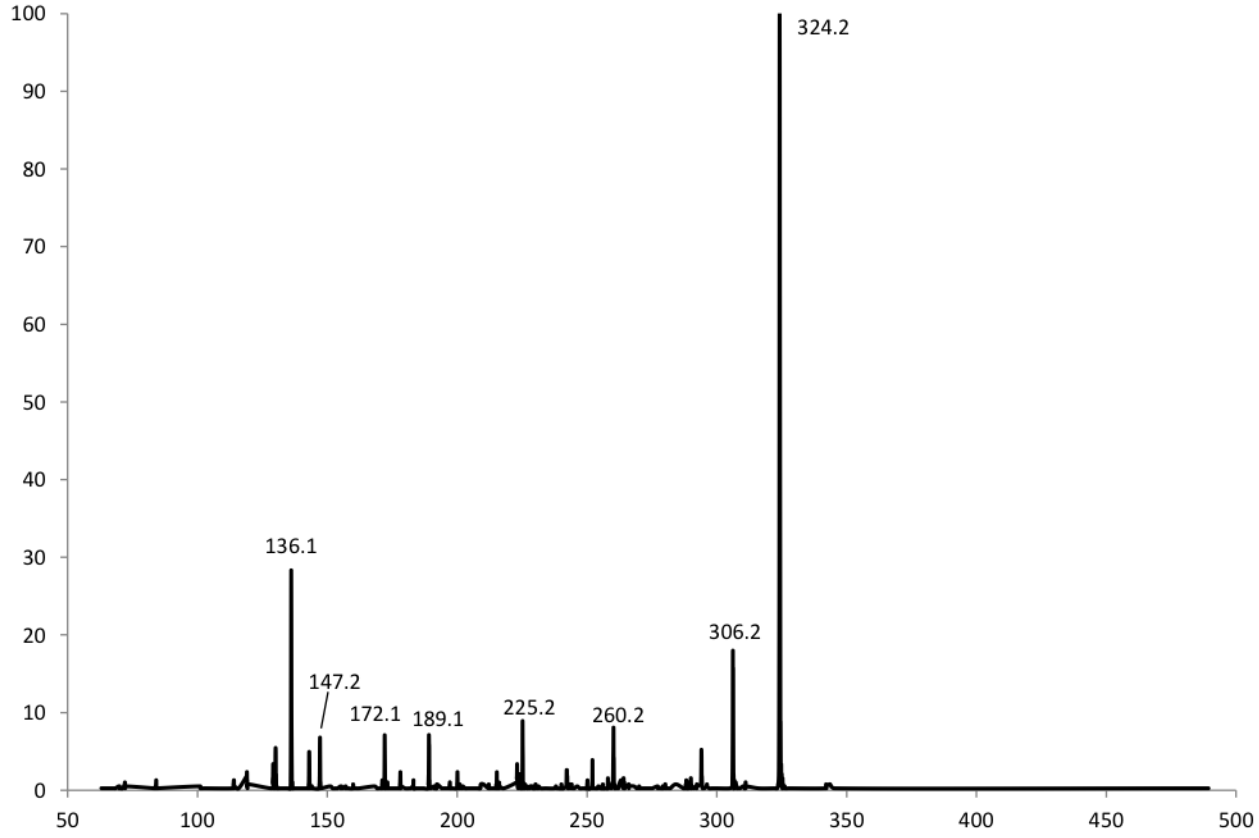

A
raw sprouts
µmol obtained from 100 gr broccoli sprouts
600.0
500.0
400.0
300.0
200.0
100.0
0.0
Sprout
Waste
Cloudy Juice
Clear Juice
Pellett
Waste + Cloudy Juice
Waste + Clear Juice + Pellett


B
microwaved sprouts
µmol obtained from 100 gr broccoli sprouts
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
Sprout
Waste
Cloudy Juice
Clear Juice
Pellett
Waste + Cloudy Juice
Waste + Clear Juice + Pellett
GRF
SFN
SFN nitrile









Highlights

- The sulforaphane yield from glucoraphanin is non-quantitative;
- Broccoli sprout juice contains sulforaphanyl-amine;
- Some sulforaphane is present in the juice as conjugate with glutathione and proteins.