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Comparison of bioactive phytochemical content and release of isothiocyanates in selected brassica sprouts

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ABSTRACT

The consumption of brassica sprouts as raw vegetables provides a fair amount of glucosinolates (GLs) and active plant myrosinase, which enables the breakdown of GLs into health-promoting isothiocyanates (ITCs). This study reports the determination of the main constituents related to human health found in edible sprouts of two *Brassica oleracea* varieties, broccoli and Tuscan black kale, and two *Raphanus sativus* varieties, Daikon and Sango. Radish sprouts exhibited the highest ability to produce ITCs, with Daikon showing the greatest level of conversion of GLs into bioactive ITCs (96.5%), followed by Sango (90.0%). Tuscan black kale gave a value of 68.5%, whereas broccoli displayed the lowest with 18.7%. ITCs were not the exclusive GL breakdown products in the two *B. oleracea* varieties, since nitriles were also produced, thus accounting for the lower conversion observed. Measuring the release of plant ITCs is a valuable tool in predicting the potential level of exposure to these bioactive compounds after the consumption of raw brassica sprouts.

1. Introduction

Sprouts are natural functional foods that fully meet the demand of modern consumers for quality easy-to-eat food that can be tasty and provide health benefits beyond the basic function of supplying nutrients. Nowadays, the market offers a large variety of seeds and ready-to-eat sprouted seeds, among which the brassica family is well represented with broccoli (*Brassica oleracea* L. ssp. *italica*) and radish (*Raphanus sativus* L.) being the most popular.

Several studies have demonstrated that brassica sprouts are a good source of vitamin C, vitamin A, folic acid, dietary fibre and minerals, as well as of a high variety of phytochemicals, namely glucosinolates (GLs) and phenols (Pérez-Balibrea, Moreno & Garcia-Viguera, 2011; Zielinski, Frias, Piskula, Kozlowska, & Vidal-Valverde, 2005).

Brassica sprouts differ from others since they are a source of GLs, well known sulfur-containing secondary metabolites (ca. 130 molecules identified to date), which display a structural homogeneity based on a β-D-glucopyranosyl unit and an *O*-sulfated anomeric (*Z*)-thiohydroximate function connected to a variable side chain depending on the amino acid metabolism of the plant species (Agerbirk & Olsen, 2012).

The positive health effect of brassica vegetables against various pathologies and chronic diseases, recently reviewed by Dinkova-Kostova and Kostov (2012), is widely recognised to be due to GLs, which act as precursors of bioactive isothiocyanates (ITCs), the products of hydrolysis mediated by plant endogenous myrosinase (β-thioglucoside glucohydrolase; E.C. 3.2.1.147) or by the intestinal microflora (Fahey et al., 2012).

Most reported health effects are based on *R*-sulforaphane (4(*R*)-methylsulfinylbutyl ITC) derived from glucoraphanin (GRA, 4(*R*)-methylsulfinylbutyl GL) contained in broccoli sprouts. Sulforaphane is one of the most potent naturally occurring inducers of the Keap1/Nrf2/ARE pathway and it manifests a variety of properties in cell culture systems and animal models, including antioxidative, anti-inflammatory, antiangiogenic and neuroprotective activities (Calabrese et al., 2012). Moreover, a recent human feeding study with fresh intact broccoli sprouts has brought to light a bactericidal activity against *Helicobacter pylori* infections, which are strongly associated with gastric cancer (Yanaka et al., 2009).

Although the main focus has been on broccoli and sulforaphane, several studies support the beneficial role of radish in the human diet. Radish sprouts are known to contain two unsaturated

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analogues of GRA, namely thio-functionalised GLs glucoraphasatin (GRH, 4-methylsulfanyl-3-butenyl GL) and glucoraphenin (GRE, 4(*R*)-methylsulfinyl-3-butenyl GL) (Hanlon & Barnes, 2011), and their breakdown products raphasatin (4-methylsulfanyl-3-butenyl ITC) and sulforaphene (4(*R*)-methylsulfinyl-3-butenyl ITC) were recently shown to have selective cytotoxic/apoptotic activity on three human colon carcinoma cell lines (Papi et al., 2008). *In situ* generated raphasatin was shown to be a potent inducer of phase II enzymes in precision-cut rat liver slices treated with natural purified GRH and myrosinase (Abdull Razis, De Nicola, Pagnotta, Iori, & Ioannides, 2012a). Furthermore, a study performed *in vivo* showed that Japanese radish (Daikon) sprouts administered as a lyophilised powder have the potential to alleviate hyperglycaemia in diabetes cases and are effective in the primary prevention of diabetes mellitus in animal models (Taniguchi et al., 2006).

In addition to ITCs, phenols may contribute to the protective effect associated with the intake of brassica vegetables. In particular, epidemiological and experimental studies indicate that anthocyanins, the most important group of coloured flavonoids within phenols, protect against the risk of cardiovascular disease, cancer and other chronic degenerative conditions. Moreover, anthocyanins and ITCs may influence the same signalling pathways in their chemopreventive activity, namely the induction of antioxidant responsive elements (Fimognari, Lenzi, & Hrelia, 2008).

In this study, we investigated seven-day old sprouts of two *B. oleracea* varieties, broccoli and Tuscan black kale (*B. oleracea* (L.) ssp. *acephala* (DC) var. *Sabellica* L.), and two *R. sativus* varieties, Sango and Daikon, with the aim of evaluating their nutritional quality by quantifying GLs, myrosinase activity, total ITC release, phenols, flavonoids, anthocyanins and chlorophyll as constituents related to human health, as well as the oxygen radical absorbance capacity (ORAC) as a measure of their *in vitro* antioxidant potential.

Bearing in mind that ITCs are recognised as being in great part responsible for the beneficial health effects of eating brassica vegetables, and with a view to comparing the four investigated cultivars, we have particularly focused here on the potential level of release of ITCs resulting from the catalytic action of plant endogenous myrosinase on GLs.

The four brassica sprouts showed a high content of phytochemicals proving them suitable as nutritious food for fresh consumption or as food ingredients to be employed in several nutraceutical applications.

2. Materials and methods

2.1. Chemicals

Benzene-1,2-dithiol (CAS Registry No. 17534-15-5), Folin–Ciocalteu reagent and (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (CAS Registry No. 53188-07-1) were obtained from Sigma–Aldrich Chemie (Steinheim, Germany); methyl isothiocyanate (CAS Registry No. 556-61-6) was purchased from Fluka Chemie (Buchs, Switzerland). 2,2′-Azobis(2-amidinopropane)dihydrochloride (AAPH) (CAS Registry No. 2997-92-4) was purchased from Polysciences Inc. (Warrington, PA). Acetonitrile and methanol were HPLC grade from Sigma–Aldrich Chemie, whereas other chemicals were of analytical grade. Glucoraphenin was isolated from ripe Daikon seeds and purified through a sequential ion exchange and size exclusion chromatography as reported (Baasanjav-Gerber et al., 2011).

2.2. Plant source and sprouting process

The four cultivars of the Brassicaceae family investigated in our study were: broccoli (*B. oleracea* (L.) spp. *italica* cv. 0D22), Tuscan black kale (*B. oleracea* (L.) ssp *acephala* (DC) var. *Sabellica* L. cv. 0D74), Daikon (*R. sativus* (L.) major cv. 0P38) and Sango (*R. sativus* (L.) cv. 0P153). Ripe seeds were supplied by Suba Seeds (Longiano, FC, Italy) and stored in a dry and dark place at room temperature. Seeds were identified by a lot number and guaranteed by the producer for the quality and the homogeneity of the product. Seeds were surface sterilised by soaking for 30 min in 1% sodium hypochlorite and rinsed with tap water. Sprouts were grown at room temperature by using an automatic sprouter VitaSeed (Suba Seeds, Longiano, FC, Italy) under an 8/16 h light/dark cycle. Seven-day old sprouts were gently washed with tap water, whole frozen, freeze-dried and ground to fine powder.

2.3. Glucosinolate analysis

2.3.1. Extraction, desulfation and HPLC–PDA analysis of glucosinolates

GLs were extracted from samples (200 mg) of finely powdered freeze-dried sprouts, as previously reported (De Nicola et al., 2012). Each extract (1 ml) was loaded onto a mini-column filled with DEAE Sephadex A-25 anion-exchange resin (0.6 ml; GE Healthcare, Freiburg, Germany), conditioned with 25 mM acetate buffer (pH 5.6). After washing with the same buffer (3 ml), purified sulfatase (200 μl, 0.35 U/ml) was loaded onto the mini-column which was then left overnight at 30 °C. The desulfo-GLs (DS-GLs) were then eluted with ultrapure water (3 ml) and finally injected into an HPLC Agilent 1100 system equipped with a PDA detector and an Inertsil ODS-3 column (250 × 3.0 mm, 5 μm) thermostated at 30 °C. The chromatography and the quantification were achieved as reported (De Nicola et al., 2012). All extractions and desulfation procedures were carried out in duplicate. GLs values are reported as μmol per g of freeze-dried sprouts.

2.3.2. Optimisation of glucoraphenin desulfation and quantification

Because of the instability of desulfoglucoraphenin (DS-GRE) (Iori et al., 2008) the desulfation procedure for the quantification of GRE in Sango and Daikon sprouts was modified as follows: samples of the hydro-alcoholic extract (0.2 ml) were loaded onto a mini-column as described above; after 3 h of incubation with sulfatase, the mini-columns were first eluted with ultrapure water (3 ml) and left a further 3 h. Then, a second water elution (1 ml) was performed. The two eluates were then analysed by HPLC, as previously described.

2.4. Myrosinase activity determination

Soluble and total myrosinase activity was determined in the four freeze-dried sprout samples. A crude myrosinase vegetable extract was obtained by crushing sprout powder (1 g) with 50 mM phosphate buffer pH 6.5 (10 ml) using an Ultra-Turrax T25 homogeniser in an ice bath. After centrifugation, the activity of soluble myrosinase was determined by spectrophotometric analysis performed with a computerised Varian Cary 300 Bio UV/vis spectrophotometer equipped with a dual cell Peltier accessory, as previously described (Palmieri, Leoni, & Iori, 1982). Soluble myrosinase activity is reported as the mean ± SD of two replicate experiments with three samples analysed per replicate. Total myrosinase activity was determined using the pH-STAT method with a Mettler Toledo DL50 titrator according to the procedure described by Finiguerra, Iori, and Palmieri (2001). Results of total myrosinase activity are the mean ± SD of six independent measurements. Both activities are expressed as units of myrosinase per gram of freeze-dried sprouts. One myrosinase unit was defined as the amount of enzyme able to hydrolyse 1 μmol of sinigrin per min at pH 6.5 and 37 °C.

2.5. Synthesis of benzo[d]-1,3-dithiole-2-thione

The benzo[*d*]-1,3-dithiole-2-thione is the standard for quantitative measurements of ITCs by means of the cyclocondensation assay (see Section 2.6). As this standard is not commercially available, it must be synthesised. To this end we applied the procedure of Kristensen et al. (2007) with slight modifications. Briefly, to a solution of benzene-1,2-dithiol (3.6 mmol) in methanol/0.5 M phosphate buffer pH 8.5 (1:2, v/v, 36 ml), a methanolic solution of methyl ITC (6.5 mmol, 12 ml) was added dropwise over 30 min. After overnight reaction at room temperature under nitrogen atmosphere, the mixture was extracted with chloroform and the organic phase dried over Na_2SO_4. After filtration, the solvent was removed by evaporation on a rotary evaporator Laborota 4002 (Heidolph Instruments, Schwabach, Germany). Benzo[*d*]-1,3-dithiole-2-thione was purified by using Partisil PLKC18F reversed phase thin TLC preparative plates (Whatman International Ltd, Maidstone, UK). The crude product was dissolved in chloroform and charged onto the plates which were then eluted with hexane/dichloromethane (5:1, v/v). After revelation by UV-light (254 nm), the silica was scraped from the plates and the product was recovered by extraction with chloroform, filtration and evaporation of the solvent. The yellow solid benzo[*d*]-1,3-dithiole-2-thione was characterised by ^{1}H and ^{13}C NMR spectroscopy and GC–MS analysis.

2.6. Autolysis and cyclocondensation assay

The cyclocondensation assay is the analytical method for measuring total ITCs. In brassica vegetable extracts, ITCs exist as free and as protein-bound ITCs, due to their high affinity for protein thiol functions. The assay is based on the reaction of free and bound ITCs, in the presence of an excess of benzene-1,2-dithiol, to produce benzo[*d*]-1,3-dithiole-2-thione (Zhang, 2012). Briefly, the autolysis was performed as follows: finely powdered samples of freeze-dried sprouts (200–400 mg) were stirred for 5 min in 0.1 M phosphate buffer pH 6.5 (5 ml) or in the same volume of ultrapure water at 37 °C. After addition of EtOH (5 ml), the resulting mixture was vortexed 1 min and centrifuged 30 min (17,000*g* at 10 °C). The residue was then extracted again by vortexing 5 min with pure EtOH (5 ml), and centrifuged as before. The two solutions were kept separated and analysed with the cyclocondensation assay as already reported (Conaway et al., 2000) by adding each sample (100 μl) to a 10 mM solution of benzene-1,2-dithiol in isopropanol (600 μl) and 0.1 M phosphate buffer pH 8.5 (500 μl). Mixtures were incubated for 2 h at 65 °C, then left to cool at room temperature, centrifuged 20 min at 13,000*g* with a 5415C centrifuge (Eppendorf, Hamburg, Germany) and the supernatants (20 μl) were analysed (see Section 2.7). The autolysis process was performed twice and each cyclocondensation assay in triplicate. ITCs values are reported as μmol per g of freeze-dried sprouts.

2.7. HPLC analysis of total isothiocyanates

The cyclocondensation product benzo[*d*]-1,3-dithiole-2-thione was analysed on an Agilent 1100 HPLC system equipped with a Zorbax SB-C18 column (150 × 4.6 mm, 3.5 μm) thermostated at 30 °C and a PDA detector. The chromatography was performed at a flow rate of 1 ml/min eluting with a gradient of 1% formic acid in water (A) and methanol (B) following the program: 7 min 80% B; 1 min linear gradient up to 100% B; 4 min 100% B. Benzo[*d*]-1,3-dithiole-2-thione was detected monitoring the absorbance at 365 nm. Total ITCs were quantified by using a calibration curve (ranging from 0.004 to 0.189 mM) of benzo[*d*]-1,3-dithiole-2-thione standard. The calibration curve was verified during each analysis session.

2.8. Total phenols assay

Phenolic compounds were assayed with the Folin–Ciocalteu method according to Singleton, Orthofer, and Lamuela-Raventos (1999); values are the mean ± SD of three independent measurements and they are expressed as mg of caffeic acid equivalents per g of freeze-dried sprouts. This assay also accounts for ascorbic acid, which reacts with the Folin–Ciocalteu reagent under the same conditions as the phenolic compounds.

2.9. Total flavonoids assay

Flavonoids were determined according to Eberhardt, Lee, and Liu (2000) as follows: the aqueous extract sample (500 μl) was added to water (1 ml) and 5% $NaNO_2$ (75 μl). After 5 min at room temperature, a 10% solution of $AlCl_3{\cdot}6H_2O$ (150 μl) was added, followed 6 min later by the addition of 1 M NaOH (500 μl). The mixture was then adjusted with water (up to 2.5 ml) and the absorbance was read at 510 nm against a blank. Flavonoid concentration is the mean ± SD of three independent measurements and is expressed as mg of catechin equivalents per g of freeze-dried sprouts.

2.10. Anthocyanin assay

The anthocyanin content of samples was determined using pH differential and bisulfite bleaching methods (Giusti & Wrolstad, 2001). A UV–visible spectrophotometer (Perkin-Elmer Lambda EZ-201) and 1-cm path length glass cells were used for spectroscopic measurements at 420, 520, and 700 nm. The anthocyanin content, expressed as the mean ± SD of three independent measurements, was calculated as cyanidin 3-glucoside equivalents and the results were expressed as mg cyanidin-3-glucoside equivalent per g of freeze-dried sprouts.

2.11. Total chlorophyll assay

The total chlorophyll amount was assayed spectrophotometrically according to Lichtenthaler (1987). Briefly, acetone extracts of freeze-dried sprouts were read at 645 and 663 nm and the mass of chlorophyll was calculated according to the following formula: $20.2 \times Abs_{645\ nm} + 8.02 \times Abs_{663\ nm}$. Results are the mean ± SD of three independent measurements and they are expressed as mg of chlorophyll per g of freeze-dried sprouts.

2.12. Oxygen radical absorbance capacity (ORAC) assay

Antioxidant activities were determined by the ORAC assay, as reported by Ninfali, Mea, Giorgini, Rocchi, and Bacchiocca (2005). The assay was carried out using a Fluostar Optima plate reader fluorimeter (BMG Labtech, Offenburg, Germany) equipped with a temperature-controlled incubation chamber and an automatic injection pump. Incubator temperature was set at 37 °C. The reaction mixture for hydrophilic assay was 0.096 μM fluorescein sodium salt (200 μl) in 0.075 M sodium phosphate buffer pH 7.0, with the sample or Trolox (20 μl). A calibration curve was made each time with the Trolox standard (50, 100, 200, 250, 400, 500 μM). The blank was 0.075 M sodium phosphate buffer, pH 7.0. The reaction was initiated with 0.33 M AAPH (40 μl). Fluorescence was read at 485 nm excitation and 520 nm emission until complete extinction. ORAC values are expressed as μmol Trolox equivalents (TE) per g of freeze-dried sprouts and each value is the mean ± SD of six independent measurements.

2.13. Statistical analysis

Data of GLs, myrosinase activity, ITCs, phenols, flavonoids, anthocyanins, chlorophyll and ORAC (Tables 1–4) were expressed as the mean ± standard deviation (SD) of different independent replicates, as indicated in previous subsections. Student's *t* test was performed using Microsoft Excel. The level for accepted statistical significance was $p < 0.05$.

3. Results and discussion

3.1. Qualitative and quantitative analysis of glucosinolates

3.1.1. Glucosinolates in broccoli and Tuscan black kale sprouts

Individual and total content of GLs in the two *B. oleracea* sprouts are reported in Table 1. In both sprouts, aliphatic GLs bearing a thio-functionalised side chain accounted for 92% of the total content with glucoraphanin (GRA predominant, followed by glucoerucin (GER, 4-methylsulfanylbutyl GL) and a minor quantity of glucoiberin (GIB, 3(*R*)-methylsulfinylbutyl GL). 4-Hydroxyglucobrassicin (4-OHGBS), glucobrassicin (GBS), 4-methoxyglucobrassicin (4-OMeGBS) and neoglucobrassicin (NeoGBS) were also detected in both *B. oleracea*, together accounting for the remaining 8% of total GLs. The same GLs profile with aliphatic and indole present in a 92:8 ratio was reported by Rychlik and Adam (2008) in seven-day old sprouts of an unknown broccoli cultivar. Tuscan black kale showed a 1.6-fold higher amount of beneficial GRA compared with the selected broccoli cv., which mainly determined the difference in total GLs. The large amount of GRA, 3.4% in freeze-dried matter, contained in those kale sprouts should be highlighted since this GL is the precursor of *R*-sulforaphane, a highly promising protective agent in many chronic conditions, including neurodegeneration. ITCs from several other aliphatic GLs have been reported to have a similar effect on detoxifying enzyme induction, whereas care should be taken with GLs containing an indole moiety, since they have also been found to exert an inducing effect on phase I enzyme systems, which are known to activate some procarcinogens. It is worth noting that both broccoli and Tuscan black kale sprouts were progoitrin-free and contained small amounts of indole GLs, which instead predominate in the mature vegetable. Indeed, it was demonstrated that indole GLs accounted for only 3% of total GLs in three-day old sprouts of seven broccoli cultivars, whereas the level in the mature plant rose to 68% (Fahey, Zhang, & Talalay, 1997). The same trend was shown in our analysis of mature Tuscan black kale freeze-dried leaves which indicated GRA (4–5 μmol/g) and GBS (8–9 μmol/g) as the main GLs in line with previously reported findings (D'Antuono, Elementi, & Neri, 2007), representing about 35% and 65% of the total GLs, respectively. As a matter of fact, hydrolysis of indole GLs may result in an undesired bioactivity and prove detrimental to health; a recent study on broccoli juice has thus shown NeoGBS to be a potent mutagen in *Salmonella typhimurium* upon activation by myrosinase (Baasanjav-Gerber et al., 2011). Based on current knowledge, it seems therefore advisable to prefer vegetables containing mainly aliphatic GLs, as occurs in these sprouts.

Table 2
Glucosinolate content (μmol/g DW) in Daikon (*Raphanus sativus* (L.) major cv. 0P38) and Sango (*Raphanus sativus* (L.) cv. 0P153) seven-day old sprouts.

Variety	Aliphatic GLs		Indolic GLs		Total GLs
	GRE	GRH	4-OHGBS	4-OMeGBS	
Daikon	46.8 ± 2.3[a]	65.9 ± 2.1[b]	1.0 ± 0.4[a]	3.6 ± 0.9[a]	117.3 ± 5.7
Sango	102.9 ± 2.4[b]	43.7 ± 0.7[a]	1.2 ± 0.1[a]	4.4 ± 0.1[a]	152.2 ± 3.3

The data represent the mean ± SD of two replicates experiments with 2 samples analysed per replicate ($n = 4$).
GRE, glucoraphenin; GRH, glucoraphasatin; 4-OHGBS, 4-hydroxyglucobrassicin; 4-OMeGBS, 4-methoxyglucobrassicin.
[a,b] Different letters within the same column indicate statistically significant differences, $p < 0.05$.

3.1.2. Optimisation of glucoraphenin desulfation

Although desulfation is recognised as a valuable tool for GLs analysis, there are challenges to overcome when considering the quality of the final analytical results (Agerbirk & Olsen, 2012). The correct quantification of GRE by means of HPLC analysis of its desulfo counterpart is affected by the instability of desulfoglucoraphenin (DS-GRE), which has been reported to undergo progressive degradation in water and produce a number of more polar compounds (Iori et al., 2008). The same products are detected by HPLC analysis after overnight desulfation on DEAE-Sephadex A-25 anion-exchange resin of pure GRE, or vegetable extract containing GRE.

The standard overnight desulfation performed with a sample of purified GRE yielded only 18–21% of the expected DS-GRE. With the aim of improving this result and to reduce the observed degradation, we set up different experiments to optimise the sample volume charged on the resin and the incubation time with sulfatase. The best results were obtained charging the mini-column with 0.30–0.60 μmol pure GRE and making a two-step water elution of the mini-column, the first one (3 ml) being performed after 3 h desulfation followed by an additional one (1 ml) after a further 3 h. In this way, we were able to reduce the undesired transformation and the yield of recovered DS-GRE rose to 99%. Twofold removing of DS-GRE limited its degradation on the resin and no polar compounds were detected in the two eluates. On the basis of these results obtained with pure GRE, we modified the analyses of Daikon and Sango hydro-alcoholic extracts accordingly. Charging a lesser volume (200–300 μl) of extract (20:1 w/v) instead of the usual one (1 ml) permitted sufficient contact between the enzyme and GRE to complete the desulfation process within just 6 h.

3.1.3. Glucosinolates in Daikon and Sango sprouts

Four GLs were detected in the two *R. sativus* varieties: two aliphatic, GRE and GRH, representing 96% of the total, and two indol-

Table 1
Glucosinolate content (μmol/g DW) in broccoli (*Brassica oleracea* (L.) spp. *italica* cv. 0D22) and Tuscan black kale (*Brassica oleracea* (L.) ssp *acephala* (DC) var. *sabellica* L. cv. 0D74) seven-day old sprouts.

Variety	Aliphatic GLs			Indolic GLs				Total GLs
	GIB	GRA	GER	4-OHGBS	GBS	4-OMeGBS	NeoGBS	
Broccoli	1.6 ± 0.1[a]	43.6 ± 0.6[a]	13.1 ± 0.1[a]	2.7 ± 0.1[a]	0.7 ± 0.0[a]	0.8 ± 0.0[a]	1.0 ± 0.1[a]	63.5 ± 1.0
Tuscan black kale	4.5 ± 0.4[b]	72.1 ± 3.2[b]	14.1 ± 0.8[b]	5.0 ± 0.3[b]	1.1 ± 0.0[b]	0.8 ± 0.0[a]	1.2 ± 0.1[a]	98.8 ± 4.8

The data represent the mean ± SD of two replicates experiments with two samples analysed per replicate ($n = 4$).
GIB, glucoiberin; GRA, glucoraphanin; GER, glucoerucin; 4-OHGBS, 4-hydroxyglucobrassicin; GBS, glucobrassicin, 4-OMeGBS, 4-methoxyglucobrassicin; NeoGBS, 1-methoxyglucobrassicin.
[a,b] Different letters within the same column indicate statistically significant differences, $p < 0.05$.

Table 3
Myrosinase activity and total isothiocyanates determined with the cyclocondensation assay in seven-day old sprouts of (*Brassica oleracea* (L.) spp. *italica* cv. 0D22), Tuscan black kale (*Brassica oleracea* (L.) ssp *acephala* (DC) var. *sabellica* L. cv. 0D74), Daikon (*Raphanus sativus* (L.) major cv. 0P38) and Sango (*Raphanus sativus* (L.) cv. 0P153).

Variety	Myrosinase activity		GLs	Autolysis pH 6.5		Autolysis in H_2O	
	Soluble[a] (U/g DW)	Total[b] (U/g DW)	Total aliphatic[c] (μmol/g DW)	Total ITCs[d] (μmol/g DW)	Conversion (%)	Total ITCs[e] (μmol/g DW)	Conversion (%)
Broccoli	2.5 ± 0.1[g]	14.4 ± 0.6[g]	58.3 ± 0.8[f]	10.9 ± 0.3[f]	18.7 (10.5)[j]	8.2 ± 0.5[f]	14.1 (3.4)[j]
Tuscan black kale	1.1 ± 0.1[f]	12.2 ± 0.8[f]	90.7 ± 4.4[g]	62.1 ± 1.1[g]	68.5	29.1 ± 0.4[g]	32.1
Daikon	1.2 ± 0.1[f]	19.9 ± 0.7[h]	112.7 ± 4.4[h]	108.8 ± 5.0[h]	96.5 (30.0)[j]	106.6 ± 4.2[h]	94.6 (31.1)[j]
Sango	4.0 ± 0.2[h]	21.7 ± 1.4[h]	146.6 ± 3.1[i]	131.9 ± 2.5[i]	90.0	137.8 ± 2.7[i]	94.0

[a,d,e] The data represent the mean ± SD of two replicates experiments with three samples analysed per replicate ($n = 6$).
[b] The data represent the mean ± SD of six independent determinations ($n = 6$).
[c] The data represent the mean ± SD of two replicates experiments with two samples analysed per replicate ($n = 4$).
[f,g,h,i] Different letters within the same column indicate statistically significant differences, $p < 0.05$.
[j] The value in parenthesis is the level of conversion determined without the use of ethanol.

Table 4
Phenols, flavonoids, anthocyanins, chlorophyll and ORAC determined in seven-day old sprouts of (*Brassica oleracea* (L.) spp. *italica* cv. 0D22), Tuscan black kale (*Brassica oleracea* (L.) ssp *acephala* (DC) var. *Sabellica* L. cv. 0D74), Daikon (*Raphanus sativus* (L.) major cv. 0P38) and Sango (*Raphanus sativus* (L.) cv. 0P153).

Variety	Phenols[a] (mg CAE/g DW)	Flavonoids[a] (mg CAE/g DW)	Anthocyanins[a] (mg CGE/g DW)	Chlorophyll[a] (mg/g DW)	ORAC[b] (μmol TE/g DW)
Broccoli	9.8 ± 0.4	5.6 ± 0.1	n.d.[d]	0.3 ± 0.0	314 ± 39
Tuscan black kale	10.4 ± 0.6	5.3 ± 0.2	n.d.[d]	0.7 ± 0.0	333 ± 43
Daikon	12.7 ± 1.0	5.5 ± 0.1	0.7 ± 0.0	0.7 ± 0.5	405 ± 53
Sango	19.4 ± 1.0[c]	9.7 ± 0.7[c]	5.8 ± 0.3[c]	0.2 ± 0.0	746 ± 98[c]

[a] The data represent the mean ± SD of threee independent determinations ($n = 3$).
[b] The data represent the mean ± SD of six independent measurements ($n = 6$).
[c] Significantly different from the other three sprouts, $p < 0.05$.
[d] n.d.: Not determined.

ic, namely 4-OHGBS and 4-OMeGBS, accounting for the remaining 4% (Table 2). Hanlon and Barnes (2011) examined 8 different radish varieties with a GL content ranging from 76.9 to 133.9 μmol/g with almost the same GL profile and some additional small amounts of GER and GBS. Total GL concentration in Sango was about 30% higher than in Daikon and the two varieties also showed significant differences in the relative abundance of aliphatic GLs, with GRE being predominant in the first, with a relative concentration of 70%, whereas GRH was the major GL in Daikon, representing 58% of the sum of the two GLs.

Consumed under the name Kaiware Daikon in Japan, Daikon sprouts, are already known for their high GL content, whereas data on Sango sprouts are reported here for the first time. GRE and GRH were expected in Sango sprouts, because a fair amount of sulforaphene and raphasatin had recently been detected while characterising a freeze-dried juice prepared using seven-day old sprouts of this radish (Matera et al., 2012). Daikon sprouts contain GRH in higher quantities compared to the mature white radish and this occurrence had already been exploited to set up an improved chromatographic methodology for the purification of GRH on the gram scale (Barillari et al., 2005). The possibility to obtain pure GRH in large amounts has made it possible to initiate a series of studies on the biological activity of this GL and thus on the role of *R. sativus* sprouts as a good source of GRH in the human diet. In the aforementioned study, GRH is reported to have a protective role and good redox properties against oxidative stress. Moreover, our ongoing research on the biological activities of GLs and ITCs has recently highlighted that a Daikon sprout extract upregulated hepatic glutathione S-transferase activity in rats, at low dose to simulate dietary intake (Abdull Razis, De Nicola, Pagnotta, Iori, & Ioannides, 2012b).

3.2. Glucosinolates autolysis and total isothiocyanate determination

The results of soluble and total myrosinase activity, total ITCs released by endogenous myrosinase and the percentage of GLs conversion into ITCs for the four investigated sprouts are presented here for the first time in Table 3. All determinations were made on the freeze-dried powder according to Keck, Qiao, and Jeffery (2003), who demonstrated that the freeze-drying process does not alter myrosinase hydrolysis product formations. Sprouts were first analysed for myrosinase activity and then, for total ITCs released by autolysis. The sprout powder was rehydrated in a suitable volume of pH 6.5 buffer or water, taking into account the amount of water removed in the process of drying that corresponds to 94–95% of the weight as reported for seven-day old sprouts of broccoli (Williams, Critchley, Pun, Nottingham & O'Hare, 2008) and for Daikon and radish (Hanlon & Barnes, 2011).

Preliminary experiments showed that all GLs were completely hydrolysed within 5 min incubation at 37 °C in both aqueous media without adding exogenous myrosinase. After the autolysis reaction, ITCs may exist as free and, in part, as conjugates, due to their strong affinity for protein thiol functions. Therefore, the total ITCs level was determined according to the widely used cyclocondensation assay, based on the quantitative reaction of ITCs and their conjugates with 1,2-benzenedithiol to produce benzo[*d*]-1,3-dithiole-2-thione (Zhang, 2012). For quantitative determination, a fourteen-point standard calibration curve in the 0.004–0.189 mM range was constructed and the linear regression analysis of the peak area responses (y) vs. the concentration value (x) gave the following equation: $y = 23{,}618x$. The correlation coefficient value ($r^2 = 0.9999$) demonstrated a very high linearity of the method over the explored concentration range.

Radish sprouts exhibited the highest level of ITCs with an almost complete transformation of GLs by the action of endogenous myrosinase. In phosphate buffer pH 6.5, Daikon showed the greatest level of conversion of 96.5%, followed by Sango with 90.0%, Tuscan black kale reached the value of 68%, whereas broccoli displayed the lowest conversion with 18.7%. In a recent study, Hanlon and Barnes (2011) measured the total ITCs in aqueous extracts of eight different radish sprout varieties reporting a mean value of conversion of GLs to ITCs of 38% after 15 min of hydrolysis, but

these authors did not extract ITCs after autolysis by means of a protic solvent like methanol or ethanol (Conaway et al., 2000).

Our results on Daikon sprouts autolysed in buffer or water, and avoiding the use of ethanol, were in line with those previous findings (Hanlon & Barnes, 2011), which showed 30% and 31% conversion in the two media, respectively (Table 3), probably due to the modest water solubility of ITCs. Neither the addition of exogenous myrosinase nor a prolonged autolysis time altered these results. It is worth noting instead, that the introduction of the extraction step with ethanol affected the level of ITCs, both free and conjugates, drawn from the autolysed vegetable material improving analytical quantification of total ITCs. The addition of ethanol after 5 min autolysis and further extraction of the residual solids with the same solvent allowed the value of 96.5% to be reached (Table 3).

Broccoli sprouts recorded a low level of total ITCs and as for Daikon, neither the addition of exogenous myrosinase, nor a prolonged time of autolysis improved the conversion level. Also in this case, the use of ethanol in the assay increased the total ITC released values from 10.5% to 18.7% in buffer, and from 3.4% to 14.1% in water.

In the case of Daikon and Sango, ITCs were the only GL breakdown products, with raphasatin and raphenin being detected by GC–MS analysis performed on both hydrolysates obtained in phosphate buffer pH 6.5 and ultrapure water (data not shown). Conversely, nitriles rather than ITCs were found to be largely produced in both aqueous media by the broccoli sprouts used in this study. Under conditions similar to that of consumption of raw sprouts, ITCs should theoretically be the only compounds formed through enzymatic hydrolysis of GLs at a pH of 6.5–7, which is also the saliva pH of healthy people. Broccoli sprouts contain a myrosinase cofactor, the epithiospecifier protein (ESP) (O' Hare et al., 2009), that exerts influence on the formation of products during the myrosinase-dependent hydrolysis, by directing GRA into 5-methylsulfinylpentanenitrile, also known as sulforaphane nitrile, and GER to 5-methylsulfanylpentanenitrile. The bioactivity of sulforaphane nitrile is different from that of sulforaphane and it is known to be a far less potent inducer of phase II detoxification enzymes quinone reductase and glutathione *S*-transferase (Matusheski & Jeffery, 2001).

Interestingly, GRA-rich Tuscan black kale sprouts yielded a fair amount of total ITCs with a GLs conversion yield of 68.5%, despite the presence of nitriles in the autolysis extract. Actually, it is well known that the activity of ESP depends on genotypes and cultivars and that it can be altered by breeding, leading to lines with enhanced ITC production ability. Thus, Mithen et al. (2003) developed broccoli lines with a tenfold increased GL content which also showed a 95% level of conversion into ITCs, whereas only a 20% level was reached by the standard commercial broccoli cv. Marathon.

Beside GL quantification, total ITC determination provides important information, given that bioavailability is strongly connected to intake. Thorough mastication of fresh sprouts creates optimal conditions for GLs to be exposed to myrosinase. It was thus shown in a human study that the excretion of dithiocarbamates was higher when broccoli sprouts were chewed and swallowed, than when they were swallowed without chewing (Shapiro et al., 2001). Moreover, Vermeulen, Van Den Berg, Freidig, Van Bladeren, and Vaes (2006) showed that after consumption of several raw brassica vegetables and condiments, an average of 61% of the ingested ITCs was recovered in urine as mercapturic acids, whereas this value decreased to 22% when the cooked vegetable was consumed.

The selection of brassica sprouts for optimum phytochemical composition is the key to optimise new fresh foods enriched in health-bioactive compounds (Baenas, Moreno, & García-Viguera, 2012). On the other hand, knowledge concerning the bioavailability of plant ITCs is essential to predict the potential level of exposure after intake of brassica, as GL determination alone may not accurately reflect how much of the final active ITC can be formed from sprouts. To maximise the uptake of the nutritional benefits of ITCs, preferential conversion of GL to ITC is necessary and consumption of raw brassica vegetables containing ESP may not be the best way to enhance the intake of ITCs, as in the case of the broccoli cultivar examined here.

3.3. Antioxidant properties

Table 4 shows total phenols and the main phytochemicals detected in the four sprouts. It is worth noting that the reported phenol values incorporate the contribution of total GLs (Tables 1 and 2) as well as that of vitamin C, which accounts for 50% of the total compounds reacting with Folin–Ciocalteu reagent (Vale, Rodríguez-Bernaldo de Quirós, & López-Hernández, 2010). In Table 4, we reported also the total antioxidant capacity, which results from the contribution expressed by the whole mixture, namely phenolic compounds with the flavonoid fraction, anthocyanins, GLs and vitamin C. As observed by Cabello-Hurtado, Gicquel, and Esnault (2012), the ORAC method allows the expression of the maximal contribution of antioxidant activity of the glucosinolates, with respect to other methods for antioxidant capacity assay (Cabello-Hurtado et al., 2012). The strongest antioxidant capacity was found in Sango, with ORAC values at more than double those measured for broccoli and Tuscan black kale. In spite of the varied composition of the phenolic pool, we observed a linear correlation of ORAC values with total phenols: the regression line shows the following equation $y = 45.62x - 147.03$ and r^2 was 0.9915 (data not shown). As far as chlorophyll is concerned, it is worth noting that this isoprenoid plant lipid may be both pro-oxidant, in the presence of light, and antioxidant in the dark. Endo, Usuki, and Kaneda (1985) reported that chlorophyll *a* shows the highest antioxidant activity with regard to chlorophyll *b* and their derived compounds. In our sprouts, the chlorophyll values were not related to antioxidant capacity and therefore we consider that the ORAC value was not dependent on the contribution of chlorophyll.

4. Conclusion

There is a general awareness of the need for a vegetable-based diet to achieve intake of phytochemicals related to chronic disease prevention. The results of our investigation have shown that brassica sprouts can provide a high content of GLs and considerable antioxidant properties. To compare the four investigated sprouts we particularly focused on determining the extent to which GLs were converted, by endogenous myrosinase, into ITCs, as the latter are recognised as the main bioactive compounds responsible for health effects associated with brassica consumption. Our findings clearly show that Tuscan black kale, Sango and Daikon sprouts can provide many more ITCs than broccoli sprouts. Moreover, data related to these three sprouts, here reported for the first time, show that they contain a unique pattern of bioactive molecules which make these vegetables attractive functional foods for a health-promoting diet.

To sum up, the potential beneficial effects of the consumption of brassica vegetables may be particularly expressed by eating raw sprouts of Tuscan black kale and radish, where the bioavailability of ITCs is higher than in raw broccoli sprouts.

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