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Analysis of total glucosinolates and chromatographically purified benzylglucosinolate in organic and conventional vegetables

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ABSTRACT

The limited availability of foods that are free of pesticides has led Brazil to search for alternative production methods to meet the desires of consumers. Currently, organic cultivation represents a production system that complies with general expectations of producers and consumers. Organic cultivation is particularly interesting mainly because of its effect on plant secondary metabolite content, which may help plants to naturally combat pests; in humans, these substances can also contribute to the prevention of chronic diseases. We report on the extraction of glucosinolates (both as total glucosinolates and as benzylglucosinolate) with trifluoroacetic acid addition in a 70:30 MeOH:water (v/v). Total glucosinolates, determined by a thioglucosidase coupled assay, were measured in different Brassicaceae species and were similar to values reported in the literature. For broccoli, analyses were carried out separately on inflorescences, leaves and stalks; analyses were also conducted on thermally processed samples to simulate cooking. Furthermore, when the analysis was conducted on conventional and organic products, the highest concentrations of these substances were most often found in organically cultivated Brassicaceae. The benzylglucosinolate concentrations were evaluated on the same samples using HPLC. The concentration of benzylglucosinolate was significantly higher in organically cultivated vegetables, as well.

1. Introduction

Recently, many researchers have presented data on organically cultivated foods. These data demonstrate that the concentrations of some compounds can be altered by changing the cultivation procedures. A comparative study on organic and conventional vegetables that utilized a proteomic approach has demonstrated differences in the expression of proteins involved in the metabolism of carbohydrates, polypeptides and secondary metabolites; these protein expression differences were attributed to the cultivation procedures (Nawrocki, Throup-Kristensen, & Jensen, 2011). Among secondary metabolites, scientists have reported on the alteration of phytochemical contents, such as phenolics and carotenoids (Lima & Vianello, 2011), and Williams (2002) has suggested that there is a need for specific studies on the phytochemical and glucosinolate (GL) content in organically and conventionally cultivated plants.

Studies by Verkerk and colleagues demonstrated that plant glucosinolate concentration is related to environmental conditions and cultivation methods and is particularly sensitive to the sulfur content in the soil (Verkerk et al., 2009). Furthermore, authors have previously reported that plants produced by organic cultivation have increased cytochrome P450 concentrations, which contributes to detoxification of xenobiotics (Winkler, Frank, Galbraith, Feyereisen, & Feldmann, 1998).

Glucosinolates belong to a group of thioglycosides, which naturally occur in cruciferous vegetables. The products of the enzymatic or non-enzymatic hydrolysis of these compounds are biologically active compounds with diverse effects on human health (Ciska, Martyniak-Przybyszewska, & Kozlowska, 2000). These substances may also act as antioxidants by scavenging free radicals and reducing oxidative stress, which is responsible for triggering chronic degenerative diseases (Verkerk et al., 2009). Several authors suggest that the ingestion of GL-containing vegetables may reduce the risk of cancer due to an increase in

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detoxifying enzyme activity and by direct inhibition of transcription factors involved in cancer cell signaling pathways (Hu et al., 2006; Tang & Zhang, 2005; Verkerk et al., 2009). Chemically, these compounds are identified as thioglycosides, and they exist in vegetable cell vacuoles with the thioglucosidase enzyme (EC 3.2.3.1), also known as myrosinase. However, this enzyme is compartmentalized in specific myrosin cells and is physically separated from its GL substrates (Andréasson, Jorgensen, Hoglund, Rask, & Meijer, 2001). Any physical or chemical damage to the cellular apparatus such as breaking of the cell membranes, processing, chewing, digestion, and bacterial or fungal infection allows myrosinase to encounter its GL substrates and leads to the production of bioactive compounds. Thus, processing and food preparation can modify the glucosinolate-myrosinase system due to partial or total inactivation of myrosinase (Rungapamestry, Duncan, Fuller, & Ratcliffe, 2006). Other factors such as the cultivation procedure (organic or conventional) may influence the plant glucosinolate content.

The objective of this work was to quantify total glucosinolate concentrations through the utilization of an enzymatic assay and to determine the benzylglucosinolate (glucotropaeolin) content in the plant via higher performance liquid chromatography (HPLC). Quantification of these compounds was conducted on vegetable models that were cultivated either organically or with conventional procedures.

2. Materials and methods

2.1. Materials

All vegetables used in the study belong to the Brassicaceae family, and all were picked at their ideal harvest period. Plants were cultivated in São Paulo State (Brazil – latitude 22°53′09″ South, longitude 48°26′42″ West and 804 m altitude) in organic cultivation areas; manure contained organic compounds were used, and integrated pest management was conducted. The organic cultivation area was separated from the conventionally cultivated plants. Conventional cultivation utilized chemical fertilizers, and chemicals were used for the control of pests and phytopathological diseases. Weeding was carried out in the same manner for both organically and conventionally cultivated plants. For each plant species, (broccoli, watercress, collard green and rocket), planting was carried out manually in a 27 m^2 experimental field for both cultivation procedures. Each experimental unit contained 50 plants. For each cultivar, data were obtained on 10 different plants. For broccoli, plants were transplanted 40 days after sowing; plants had developed to the 4–6 leaf stage (5–10 mm plant diameter and 0.15 m plant height). For broccoli and collard green, the spacing between plants was 50 × 100 cm. Watercress, and rocket cultivations were planted with 20 × 25 cm and 20 × 15 cm spacing, respectively.

During the experiment, mineral fertilizer treatment (120 g m^{-2}) was applied two times (10 days before and 15 days after transplantation), and organic fertilizer (8 kg m^{-2} castor pomace) was applied at planting. The regional climate is mesothermal, humid subtropical and dry during the winter. Irrigation was carried out twice a day.

Broccoli (*Brassica oleracea* L. cv. Italic Ramoso Piracicaba) (Sakata Seed America®) was harvested 90 days after sowing; organic and conventionally grown plants were at the same physiological phase of maturation at the time of harvest. Plants were morphologically separated into inflorescence (I), leaves (L) and stalks (S). A portion of the broccoli was processed raw, and the other portion was treated at 100 °C for 5 min (cooked). The cooking procedure was carried out on the entire broccoli plant (I + L + S), and separate containers were used for organic and conventionally derived vegetables. Immediately thereafter, the broccoli samples were stored at to room temperature, dried with absorbent paper and separated into inflorescence, leaves and stalks, which was similar to the procedures for the raw material; samples were then frozen at −20 °C. Collard green leaves (*B. oleracea* L. cv. Manteiga Cabocla) (Sakata Seed America®) were harvested 80 days after sowing, and rocket (*Eruca sativa* L. cv. Folha Larga) (TopSeed®) and watercress (*Nasturtium officinale* R. Br. cv. Agrião d`Água) (Sakata Seed America®) were harvested at 40 and 60 days after seed germination, respectively. The same procedures described above for broccoli were conducted on the other vegetables.

All samples were previously selected in agreement with the producers and according to cultivation procedures and thermal processing. The samples were washed with water, sanitized with acetic acid (1.201 g L^{-1}) for 10 min and again washed with water. After drying, the samples were rapidly frozen by immersion in liquid nitrogen (SCRIO 22 container) and stored at −20 °C until use.

2.2. Extraction of total glucosinolates

The extraction of total glucosinolates was carried out on Brassicaceae vegetal material (raw and/or cooked) according to Kiddle et al. (2001) with minor modifications. Samples (3 g) were homogenized ($n = 3$, in triplicate for each vegetable and condition) in a porcelain mortar containing 5 mL of 70:30 MeOH (mL):water (mL) in the presence (+) or in the absence (−) of 1.49 g L^{-1} trifluoroacetic acid (TFA) (Sigma). Extracts were transferred to stoppered Erlenmeyer flasks and conditioned in a thermostatic bath under constant agitation. The extraction was carried out at 70 °C for 30 min. After cooling, the extracts were centrifuged at 8000 × g for 20 min. The collected supernatants were filtered with qualitative filter papers (Whatman) and transferred to glass flasks at 40 °C until solvent was completely evaporated (approximately 72 h). The dry glucosinolate-containing precipitate was reconstituted with 1 mL of 0.2 mol L^{-1} HEPES–KOH (pH 7.0) in the same container.

2.3. Enzymatic determination of total glucosinolate concentrations

An extract aliquot (10 μL), which was previously reconstituted in 0.2 mol L^{-1} HEPES–KOH (pH 7.0), was incubated with 5 μL of a thioglucosidase solution (0.12 U). The thioglucosidase solution contained myrosinase purified from *Sinapis alba* L. (Sigma–Aldrich), which was buffered in 0.2 mol L^{-1} HEPES–KOH (pH 7.0) at 37 °C for 24 h; this procedure was in accordance with the methodology of Li and Kushad (2005) which was performed in 3 mL test tubes. In agreement with the degradation reaction of glucosinolates by thioglucosidase, the measurement is accomplished on glucose produced upon glucosinolate hydrolysis.

Glucosinolate content was quantified according to the stoichiometry proposed by Palmieri, Iori, and Leoni (1987), which states that 1 mol of released glucose is equivalent to 1 mol of total glucosinolate.

The enzymatic catalysis was stopped with the addition of 5 μL of 18 mmol L^{-1} perchloric acid solution ($HClO_4$). To detect the background levels of glucose in the samples, a control was prepared. The control contained buffered extract (10 μL) with 18 mmol L^{-1} $HClO_4$ (5 μL), and 5 μL of the thioglucosidase solution was rapidly added. The liberated total glucose was assayed enzymatically by using a glucose oxidase/peroxidase kit (CELM, Brazil). Sinigrin, an allyl-glucosinolate (Sigma), was used as a calibrant and as a positive control.

2.4. Identification and quantification of benzylglucosinolates by HPLC

The sample extraction procedure was identical to the one described for total glucosinolates ($n = 3$, each in triplicate). The

extracts were filtered on Millex™ polyvinylidene fluoride (PVDF) membranes (0.45 μm, Millipore) prior to HPLC injection. The methodology used for the determination of benzylglucosinolate was described by Kiddle et al. (2001) and modified by Rossetto et al. (2008). The calibration curve for benzylglucosinolate and the internal standardization for the sample recovery test were carried out according to Rossetto et al. (2008). A single chromatographic run with an internal standard (50 μL of 12 nmol L^{-1}sinigrinin 1 mL of 70:30 MeOH (mL):water (mL) that also contained 1.49 g L^{-1} TFA) was also completed to determine the sinigrin (allyl-glucosinolate) retention time.

Benzylglucosinolate was isolated by HPLC, which was coupled to an automatic injector and a quaternary pump (HP 1100). The substance was detected by a diode array (PDA) detector at a spectral range of 200–400 nm. A reverse phase column (Luna C18, 250 × 4.6 mm, 5 μm) developed by Phenomenex was used, and the column was coupled to a Security Guard pre-column (Phenomenex). The column temperature was maintained at 25 °C. A gradient mobile phase was used, and it consisted of solvent A (water containing 46 mg L^{-1} formic acid (Sigma–Aldrich)) and solvent B (MeOH (Merck, HPLC grade) containing 46 mg L^{-1} formic acid) as shown in Table 1. The flow rate was 1.0 mL min^{-1}, and the injected volume was 20 μL. The run time for each analysis was 60 min, and 10 min were required for column cleaning and re-equilibration.

2.5. Statistical analysis

The statistical analysis was entirely randomized in groups consisting of 2 treatments: organic and conventional. However, the statistical analysis for broccoli considered two additional treatments: raw and cooked vegetables. Three repetitions were performed, and three producers for each vegetable and cultivation procedure were considered. The analysis of each repetition was accomplished on extractions in triplicate. Variance analysis (F test) was utilized on the data, and averages were compared via the Tukey test ($P < 0.05$) using SAS Version 9 (SAS Institute, Cary, NC).

3. Results and discussion

Glucosinolates and phytoalexins are components of the plant defense system. Reports in the literature have shown that these compounds act as insecticides, fungicides, nematicides and natural herbicides (Chen & Andreasson, 2001; Fahey, Zalcmann, & Talalay, 2001). Consistent with Kiddle et al. (2001), we observed that the extraction efficiency of these substances from vegetal material depends on multiple factors. Compound polarity, which is related to the organic solvent used, and the presence of TFA, which is capable of solubilizing and stabilizing aromatic compounds, polar molecules and peptides, affect the extraction procedure (Matsubayashi, Shiratori, & Kubo, 2010). Furthermore, TFA is widely used due to its low absorptivity in the UV range and because it is highly miscible with most organic solvents (Winkler, Wolschann, Heinz, & Kunz, 1985). More recent data reported that TFA forms complexes with aromatic molecules, which increases the UV absorption of these compounds, e.g. aromatic imide in benzene and cyclobutane formation (Matsubayashi et al., 2010). We have shown that the extraction of total glucosinolates in the presence of TFA was significantly more efficient than the same procedure in the absence of this acid for all vegetables analyzed (Fig. 1). For this reason, all of the subsequent chromatographic analyses were carried out on samples treated with 1.49 g L^{-1} TFA.

Table 1
Elution gradient used for benzylglucosinolate determination by HPLC.

Time (min)	Water containing 0.1% (v:v) formic acid solvent A (%)	MetOH containing 0.1% (v:v) formic acid solvent B (%)	Acetonitrile HPLC grade solvent C (%)
0:0	100	0	0
10:00	100	0	0
15.00	80	20	0
25:00	50	50	0
35:00	0	100	0
45:00	0	100	0
45:01	0	0	100
54:99	0	0	100
60:00	100	0	0

Glucosinolates tend to accumulate in higher amounts in vegetables that were cultivated with organic procedures (Fig. 1); this has been previously reported for flavonoids in tomatoes (Mitchell et al., 2007). Total glucosinolate content, as measured by the thioglucosidase assay, was 2 times greater in organic broccoli inflorescence (0.75 ± 0.05 μmol g^{-1} fresh weight) than in conventional broccoli inflorescence (0.35 ± 0.2 μmol g^{-1} fresh weight). The same trend was observed in broccoli leaves; a 10-fold increase in total glucosinolate concentration was observed in organically cultivated leaved (1.0 ± 0.4 μmol g^{-1}) compared with conventionally produced material (0.1 ± 0.05 μmol g^{-1}) (Fig. 1). The highest concentration of GL was found in the stalks of organic broccoli (1.5 ± 0.4 μmol g^{-1}); this value is similar to values reported by Aires, Rosa, and Carvalho (2006). However, the GL stalk concentration is considerably higher than those reported by Song and Thornalley (2007), which resembled the concentrations we observed in inflorescences.

Some authors have attributed these differences to the type of cultivation, soil conditions, climate, humidity, photoperiod and several other environmental factors (Fahey et al., 2001). The high glucosinolate concentration found in this present study could be due to the extraction medium, which contained TFA. Data reported by other authors (Song & Thornalley, 2007) utilized an extraction method conducted with pure methanol. This hypothesis is supported by the data shown in Fig. 1, which compares the extraction of GL with and without TFA. Another possibility for the discrepancy is the time period used for calculating thioglucosidase activity (24 h). This time duration was optimized for complete GL hydrolysis, and this may have led to the generation of increased amounts of glucose, the product of the hydrolysis reaction. These data are interesting, and we verified some differences in glucosinolate concentrations among different plant parts. We also considered the vegetable parts that are usually discarded by consumers. Some of the discarded plant tissues contain the highest concentration of these substances, which have been reported to have possible positive effects on human health (Tang & Zhang, 2005; Hu et al., 2006). Furthermore, our data suggest that plants cultivated in accordance with organic procedures can be promising sources for elucidating the metabolic synthesis pathways of glucosinolates and for extracting bioactive and natural compounds for industrial use.

The data reported in Fig. 1 show that no significant differences in GL content were observed among various morphological parts of the broccoli grown under conventional cultivation. Furthermore, as first reported by Song and Thornalley (2007), the cooking process did not significantly decrease the total GL content in these conventionally cultivated vegetables. However, this result is controversial and has been discussed by Vallejo, Tomas-Barberan, and Garcia-Viguera (2002). This present work noted a significant decrease in the GL content of organic broccoli following simulated cooking. According to Song and Thornalley (2007), cooking affects glucosinolate composition and content in Brassica vegetables; these changes in composition depending on the processing manner, cooking time, vegetable type and damage to vegetable tissues. In our study, cooking time was short (5 min) and minimized

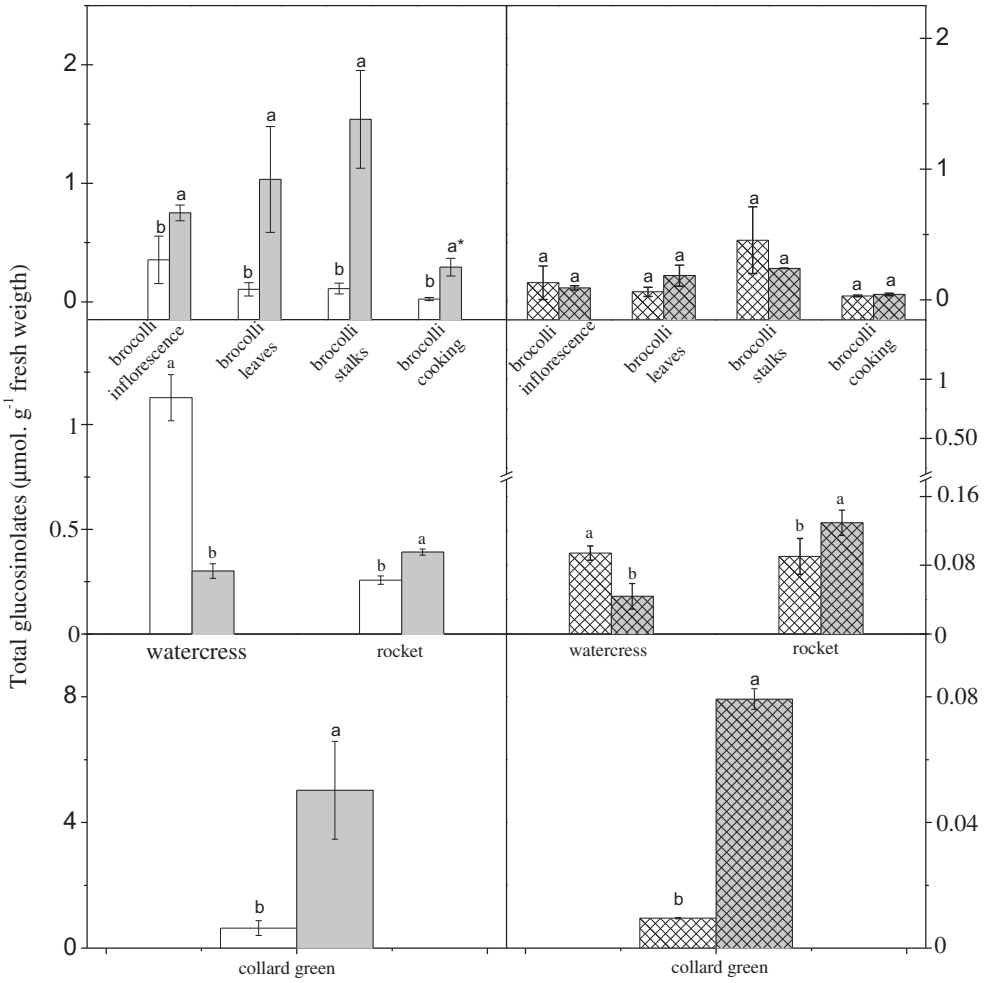

Fig. 1. Total glucosinolate content in Brassicaceaes extracted with 70:30 MeOH (mL):water (mL) without ( ) and with addition ( ) of 1.49 g L^{-1} of trifluoroacetic acid in conventional ( ) and organic vegetables ( ). Data are reported as means ± standard deviation ($n = 3$, three repetitions with 9 measurements). Letters represent statistically significant differences $^{*}P < 0.05$ (Tukey test) and star (*) the same statistically significant differences of total glucosinolates in cooked samples.

the loss of these compounds from conventional vegetables. However, inactivation of myrosinase and tissue damage by the boiling water treatment may have affected the organic broccoli.

Similarly, significant differences in the two cultivation methods were apparent for collard greens, which contained higher GL concentrations (5.02 ± 1.55 μmol g^{-1}) in the organic collard greens when compared to conventionally cultivated plants (0.64 ± 0.24 μmol g^{-1}). The same trend was observed in organic rocket (0.39 ± 0.014 μmol g^{-1}) when compared to conventionally grown rocket (0.26 ± 0.02 μmol g^{-1}). However, a different profile was observed for watercress, which had higher GL contents in conventional leaves (1.13 ± 0.11 μmol g^{-1}) than in organic ones (0.30 ± 0.23 μmol g^{-1}) (Fig. 1). The watercress profile could be due to differences in soil requirements. Additional factors, which include stress level and the presence of plagues and pathogens, can also influence the accumulation of these substances, as was observed and described by Harbone (2001).

We did not observe any evidence of plant disease or pest aggression by visual inspection. One hypothesis that may explain the accumulation of these substances involves the activation of jasmonic acid signaling. This signaling pathway can be induced by the higher bio-availability of sulfur in organic manure, and this has already been observed in *Arabidopsis*, which led to increased gene expression of sulfur-rich defense proteins and enzymes involved in glucosinolate synthesis (Jost et al., 2005).

Little is known about the post-transcriptional and post-transductional modulation of enzymes devoted to the synthesis and metabolism of these compounds (especially myrosinases) when they are subjected to different cultivation procedures. Some plants may be more efficient than others in the accumulation of these compounds, as was observed in conventional watercress.

Benzylglucosinolate (BG), the precursor of benzylisothiocyanate (BITC), is a promising inhibitor of cancer cell proliferation inducers (Hu et al., 2006). BG also has roles in multiple defense mechanisms against plagues and pathogens in papaya (*Carica papaya*) (Seo & Tang, 1982), and it was chromatographically identified at 20 min elution time. The internal standard (sinigrin) was eluted at 6 min. The results reported in Fig. 2 show statistically significant higher BG content in organic vegetables. This relationship was also observed with other secondary metabolites, such as flavonoids (Mitchell et al., 2007) in organic and conventional tomatoes. Conversely, other authors have reported higher myricetin and kaempferol in conventionally cultivated loquat (*Eriobotrya japonica*) when compared to organically cultivated loquats (Lombardi-Boccia, Lucarini, Lanzi, Aguzzi, & Cappelloni, 2004).

Data reported in the present work indicate that all parts of broccoli (*B. oleracea* L. var. italic), collard greens (*B. oleracea* L.) and rocket (*E. sativa* L.) contain statistically significant increased concentrations of BG in organic plants (Fig. 2). A lower concentration of benzylglucosinolate was observed in broccoli inflorescences

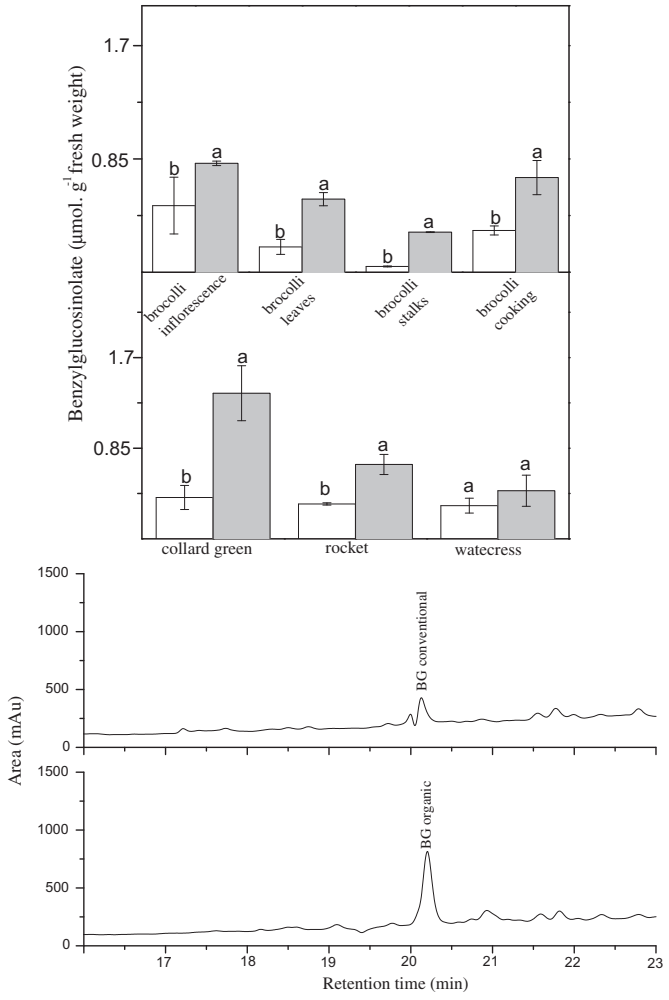

Fig. 2. Benzylglucosinolate content in conventional (▭) and organic (▬) Brassicaceaes and chromatographic profile run of the conventional and organic collard greens (*Brassica oleracea* L.) respectively. Samples were extracted with 70:30 MeOH (mL):water (mL) containing 1.49 g L^{-1} of TFA at 70 °C. Data are reported as means ± standard deviation ($n = 3$ repetition, with 9 measured). Letters represent statistically significant differences at $^{*}P < 0.05$ (Tukey test).

than in leaves and stalks; this was observed for both organic and conventional broccoli. Furthermore, the cooking process did not significantly affect the BG content for either cultivation method, and this was reported previously (Rungapamestry et al., 2006; Verkerk et al., 2009). Among the analyzed vegetables, watercress behaved differently. No significant difference in benzylglucosinolate content was observed between the organically and conventionally cultivated plants. Among the other analyzed Brassicaceaes, organic collard greens had the highest BG content (Fig. 2).

In conclusion, the organic cultivation practice led to increased concentrations of total glucosinolates and benzylglucosinolate in most of the vegetables. These differences were more apparent when the compounds were isolated and separated using HPLC high resolution liquid chromatography.

The acidified methanol extraction of broccoli tissues resulted in significantly higher levels of GLs, which differentiated the two modes of cultivation. This difference was supported by the chromatographic analysis of benzylglucosinolate. The tissue extract analysis without the addition of TFA revealed the same concentration profile, but the concentrations of compounds were much lower.

Among the evaluated Brassicaceaes, watercress exhibited a different profile for benzylglucosinolate and GL concentration; significantly higher concentrations of the compounds were observed in conventionally cultivated watercress. These results suggest that watercress cultivated conventionally is more efficient at sulfur absorption. The highest levels of glucosinolates and benzylglucosinolate were found in Brassica cabbage and broccoli. Furthermore, cooking significantly decreased the GL content of vegetables, but the more accurate HPLC analysis showed that the benzylglucosinolate profile was unaffected.

Thus, we believe that these types of plants, if cultivated organically, may become promising sources of secondary metabolites and may reveal gene targets that could confer resistance against phytopathogenic pests and diseases of agro-economic importance; this would contribute to environmental sustainability without the use of radical agricultural production systems.

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