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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