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A review of influence of environment and process parameters on glucosinolate-myrosinase system from *Brassica*

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ABSTRACT

The family *Brassicaceae* has been very studied due to the pharmacologic properties of the glucosinolates (GLS) and their hydrolysis products, which are associated with the action of an endogenous thioglucosidase myrosinase. Factors such as climate, soil, genotype, seasonal variation, processing, extraction quantification can affect the enzyme activity and stability, leading to increase or decrease the hydrolysis of GLS. Based on this aspect, the main objective of this work is present a review concerning the glucosinolate-myrosinase system, influence of climate and genotype to seasonal variation in the glucosinolate-myrosinase system, effect of thermal and high hydrostatic pressure treatments on the GLS content, as well as, the isolation and quantification of GLS from *Brassica*.

INTRODUCTION

The family *Brassicaceae* includes about 400 genus and 4,000 species. In Brazil is found 7 genus and 50 species. Through the genetic improvement have several horticultural varieties that present economic interest, mainly *B. oleracea* var. *acephala* (kale), *B. oleracea* var. *capitata* (cabbage), *B. oleracea* var. *gemmifera* (Brussels sprouts), *B. oleracea* var. *borytis* (cauliflower) and *B. oleracea* var. *italica* (broccoli). Other species economically important are *Brassica nigra* and *Sinapis* spp. (mustards), *Raphanus sativus* (radish), *Armoracia rusticana* (horseradish), *Rorippa nasturtium-aquaticum* (watercress) and *Eruca sativa* (rocket). Some species are cultivated as ornamental, mainly *B. oleracea* var. *acephala* (ornamental cabbage), *Lobularia maritime* (Alyssum) and *Cleome hassleriana* (spider flower). Many *Brassicaceae* are weeds, including *Brassica rapa*, *Rapistrum rugosum and Sinapis arvense* (turnip mustard), *Capsella bursa-pastoris* (shepherd's-purse), *Cardamine bonariensis* (wild watercress), *Coronopus didymus* (Lesser swine-cress), *Lepidium* spp. (peppercress) and *Raphanus raphanistrum* (wild radish) (Souza and Lorenzi, 2005). The family *Brassicaceae* has been very studied due to the pharmacologic properties of its main metabolites, the glucosinolates (GLS). These metabolites, as well as, their hydrolysis products (isothiocyanate and nitriles) are powerful antioxidants and anti-carcinogenic agents (Paulino, 2008). The GLS are one of the greatest metabolic groups containing sulfur, which are found mainly in comestible vegetables. Until the year of 2004, approximately 120 GLS were identified from species of *Brassicaceae* and other families (Falk *et al.*, 2004). The chemical structure of GLS consists of ester of (Z) hydroxylamine sulphate that had an atom of sulfur bonded to a β-$_D$-glucopyranose that is a side chain derived from an amino acid. The chemical side chain is highly variable and can contain groups aliphatic (alkyl, alkenyl, hydroxy alkenyl, ω-methyl alkyl, ω-sulphinyl and ω-sulphinyl alkyl), aromatic (benzyl, substituted benzyl) or heterocyclic (indolic groups), depending of the amino acid precursor

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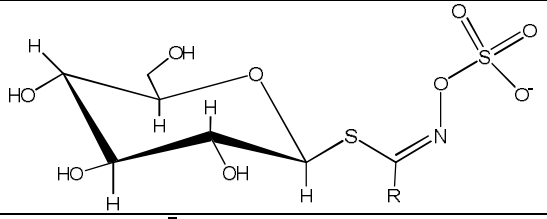
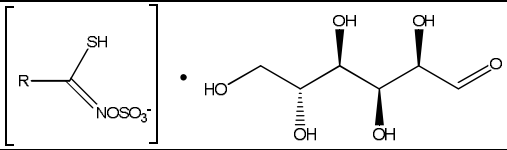
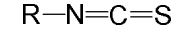
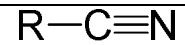
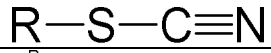
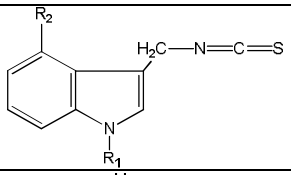
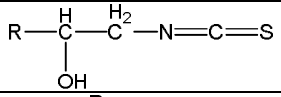
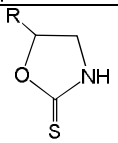
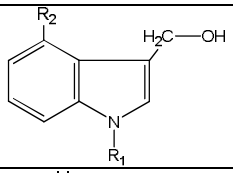
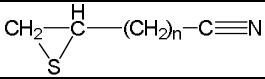
(Holst and Williamson, 2004; Padilla *et al.*, 2007). The presence of sulphate in the molecule confers properties strongly acid. In this way, the GLS are not volatile and occur as salt (Holst and Williamson, 2004). The hydrolysis of GLS occurs due to action of an endogenous thioglucosidase myrosinase when the plant tissues are disrupted during processing, chewing, and digestion, since this enzyme is located within the vacuoles of the plant matrix. The hydrolysis generates an unstable aglycone intermediate, thiohydroxamate-O-sulfonate, which is spontaneously converted to different classes of breakdown products including isothiocyanates, thiocyanates, nitriles, epithionitriles, hydroxynitriles, and oxazolidine - 2 - thiones (Fenwick and Heaney, 1983b). In Table 1 are presented the chemical structures of the main compounds 76 detected in Brassica family. **Table 1**

The extent of hydrolysis of glucosinolates and the nature and composition of the breakdown products formed are known to be influenced by various characteristics of the hydrolysis medium. Intrinsic factors such as coexisting myrosinase and its cofactors ascorbic acid, epithiospecifier protein (ESP), or ferrous ions (Bones and Rossiter, 1996) and extrinsic factors such as pH and temperature (Ludikhuyze *et al.*, 2000) can affect the hydrolysis of glucosinolates, because these factors present influence on myrosinase activity and stability, leading to increase or decrease the efficiency of hydrolysis of glucosinolates.

Table. 1: Chemical structure of some glucosinolates.

Compound	Chemical structure
Glucosinolate	
Thiohydroxamate-sulfonate	
Stable isothiocyanate	 R= $(CH_2)_4S(=O)CH_3$ R= $(CH_2)_4SCH_3$ R= $(CH_2)_3S(=O)CH_3$
Nitrile	
	R= $CH_2CHOHCH{=}CH_2$
Thyocianate	
Indolylmethil-Isothiocyanate	
β-OH-isothiocyanate	
5-oxazolidine -2- thione	 R= $CH{=}CH_2$
Indole-3-carbinol	
Epithioalkylnitrile	

By this reason, processing, extraction and quantification methods are likely to influence the extent of glucosinolate hydrolysis and the ratio of the derivatives produced (Verkerk *et al.,* 1997). Although the related variables influence the myrosinase activity, the literature is focused mainly on the effects on glucosinolase content. There is a well documented review in the literature (Cartea and Velasco, 2008) that reports the influence of environmental conditions, processing and storage on the glucosinolates content of *Brassica* sp. and their effects on human nutrition and health. Based on these aspects, the present work reports the influence of climate, genotype and seasonal variation in the glucosinolate-myrosinase system, effect of heat treatment and high hydrostatic pressure on the stability of myrosinase as well as in the GLS content, and additionally, this work reports the isolation and quantification of these compounds, focusing in the methods of isolation and quantification of glucosinolates from biological matrices.

Influence of climate and genotype to seasonal variation in the glucosinolate-myrosinase system

Cultivar, location, and growing conditions play important roles in the production of bioactive compounds in *Brassica* sp. (Rosa *et al.,* 1997). The concentration and composition of GLS, phenolics, and vitamin C in *Brassica* sp. is genotype dependent (Sarikamis *et al.,* 2009). Moreover, climatic factors such as temperature, irradiation, and water supply also have an important influence on the phytochemical content in *Brassica* sp. (Martinez-Villaluenga *et al.,* 2009). According to Schmidt *et al.,* (2010), GLS breakdown product levels are due to the combination of GLS content in the plant and myrosinase activity (Ludikhuyze *et al.,* 2000). The activity of this enzyme depends on the genetic variation (Rask *et al.,* 2000), on some intrinsic (metal ions, ascorbic acid, pH) and on some extrinsic factors (temperature) (Bones and Rossiter, 1996; Ludikhuyze *et al.,* 2000). Therefore, cultivar selection should be tailored to specific environmental factors at each location to achieve optimization in phytochemical content of *Brassica* sp. In addition, selected white cabbages with an optimized bioactive compound content could be used as raw material for sauerkraut production, enhancing the human dietary intake in health promoting compounds.

Brassicas are economically important crops that show high intra-specific variation in morphological and chemical traits (Hanson *et al.,* 2009). Previous studies have shown that concentrations and profiles of GLS show considerable variation within species and that they vary with environmental conditions and developmental stage (Potter *et al.,* 2000; Rosa and Rodrigues, 2001; Castro *et al.,* 2004; Poelman *et al.,* 2008; Hanson *et al.,* 2009).

Among the cultivated *Brassicaceae*, broccoli attracted attention after the discovery that it contains high levels of the isothiocyanate sulforaphane [1-isothiocyanate-(4R)-(methylsulfinyl) butane], and of other glucosinolate derivatives thought to have anticarcinogenic properties (Beecher, 1994; Zhang *et al.,* 1992; Cover *et al.,* 1998). The ability of sulforaphane or indole-3-carbinol to protect against tumorigenicity is dose and time dependent. Therefore, selection of cultivars accumulating high levels of isothiocyanates may be important. Based on the perceived beneficial effects, broccoli has received widespread attention as a medicinally significant food, its consumption being recommended throughout the year. To meet this requirement, and because it is a very perishable vegetable, producers tend to grow suitable cultivars under mild climatic conditions in spring and summer, principally for the fresh market, although some cultivars are more suited to freezing.

Variation in GLS has been of interest to ecologists and nutritional chemists alike. Ecological studies have investigated the effects of variation in GLS concentrations and profiles of *Brassica oleracea* on above ground plant-insect interactions. Both generalists and specialists herbivores can be influenced by GLS (Gols *et al.,* 2007; Poelman *et al.,* 2008).

Members of the *Brassicaceae* vary considerably in GLS quantity and composition (Rask *et al.,* 2000; Fahey *et al.,* 2002) and insect herbivores specialized on *Brassicaceae* differ in their performance on different members of this plant family (Fox *et al.,* 1996; Ohsaki and Sato, 1999; Sznajder and Harvey, 2003). Differences in GLS quantity and composition between crucifer species may thus contribute to differences in herbivore performance. *Plutella xylostella* L. (Lepidoptera, Plutellidae) is a specialist herbivore that is known to feed on a number of species in the *Brassicaceae*. Adult females and larval stages use GLS as oviposition and feeding stimulants (Pivnick *et al.,* 1994). However, an increase in GLS concentration does not always positively correlate with larval performance (Li *et al.,* 2000).

Some GLS may have anticarcinogenic effects (Lund, 2003; Moreno *et al.,* 2006) and nutritional studies have mainly focused in the aerial parts of the plants. Root GLS levels, although less studied in the context of human health, are important for resistance against soil pests and may be used for biofumigation (Smetanska *et al.,* 2007). In aerial parts, GLS concentrations depend on a variety of factors including temperature, time of day, water content, and nutrient supply (Rosa *et al.,* 1994; Rosa, 1997; Pereira *et al.,* 2002; Gols *et al.,* 2007). Cole (1980) observed, based on measurements of volatile myrosinase hydrolysis products, a substantial decline in levels of aliphatic glucosinolates over the first few days of development in seedlings of turnips, Chinese cabbage, fodder rape (*B. campestris* L), cauliflower (*B. oleracea* var *botrytis*) and radish (*Raphanus sativus* L). A thorough understanding of glucosinolate metabolism in plants requires intensive studies of their distribution between plant organs and changes during the various development stages. Major differences in the relative amount of individual GLS have been observed between the different parts of developing rapeseed plants McGregor (1988), indicating that them have defined distribution patterns. Furthermore, large differences between seed, leaf and root glucosinolate profiles of several brassicas have been described by Sang *et al.,* (1984). Rosa *et al.,* (1994) reported that under mild conditions, GLS in the leaves of young cabbage plants showed significant variation throughout a single day. Although the

data suggested a rapid metabolism of GLS, the causes of this variation are not fully understood. Even under controlled greenhouse conditions, GLS concentrations in leaves of Brassica species fluctuate when sown over a time period of several months, which was ascribed to abiotic seasonal changes (Gols *et al.,* 2007). Soil characteristics like pH influenced GLS concentrations in leaves of kale (Petersen *et al.,* 2002; Velasco *et al.,* 2007; Gerendas *et al.,* 2008; Pongrac *et al.,* 2008). Variation on the amount and pattern of GLS has been attributed to genetic and environmental factors, including plant age, temperature, water stress, and soil type (Fenwick *et al.,* 1983; Rosa *et al.,* 1997; Farnham *et al.,* 2004). Distribution of the GLS varies depending on plant part, with both quantitative and qualitative differences among roots, leaves, stems, and seeds. Van Dam *et al.,* (2009), in his review article reports that the roots usually have a higher concentration of glucosinolates compared with other parts of the plant. Agronomic factors, such as soil type, moisture, and mineral nutrient availability, are known to exert a significant effect on GLS content. Soil fertility has significant effects on levels of specific GLS in the growing plants (Rosa *et al.,* 1997). Total and indolic GLS concentrations have been correlated with climatic factors in several crops of *B. oleracea* (Charron *et al.,* 2005). Winter seasons seem to induce lower GLS levels due to short days and cool temperatures accompanied by frost (Rosa *et al.,* 1997).

In addition, the profile and concentration of GLS are affected by developmental stage of the plant (Petersen *et al.,* 2002) and by biotic interactions. Above ground plant-animal interactions can cause up regulation of specific GLS, depending on the tissues attacked and the identity of the herbivore (Textor and Gershenzon, 2009). In *B. oleracea* herbivory by the generalist *Myzus persicae* up regulates indolic GLS(Kim and Jander, 2007), whereas damage resulting from the specialist *Pieris rapae* also increases aliphatic GLS (Agrawal and Kurashige, 2003). There is especially little information on root GLS.

Effect of thermal treatment on the glucosinolate-myrosinase system of *Brassica*

Glucosinolate hydrolysis products, and in particular isothiocyanates and indoles, have received a special interest in food research because of their anticarcinogenic properties (Verhoeven *et al.,* 1997; Wallig *et al.,* 1998). Moreover, glucosinolates and their hydrolysis products are associated with important taste, aroma and flavour attributes in *Brassica* vegetables (Van Doorn *et al.,* 1998; Drewnowski and Gomez-Carneros, 2000; Coogan *et al.,* 2001). Like other vegetables, most *Brassica* vegetables are heat processed before consumption. This leads to myrosinase inactivation and hence stops the hydrolysis of GLS into beneficial breakdown products. GLS can be hydrolyzed by myrosinases existing in the human gut, but the production level of isothiocyanates is three times greater when GLS are hydrolyzed by plant myrosinase (Conaway *et al.,* 2001). Controlling myrosinase activity during processing is, therefore, of particular interest. Earlier investigations showed different thermal stability of myrosinase based on *Brassica* source (Table 2). This table shows that broccoli myrosinase has the lowest thermal stability compared to other myrosinase sources, whereas it is the highest in the case of rapeseed myrosinase. However, Matusheski *et al.,* (2004) found that heating fresh broccoli florets to 60 ºC prior to homogenization simultaneously increased sulphoraphane formation, which indicates a much higher thermal stability of broccoli myrosinase than what has been reported by Ludikhuyze *et al.,* (1999). However, some of the differences could be due to the limitation of heat transfer to whole vegetable. Earlier work has indicated that the glucosinolate-myrosinase system is modified during the processing of *Brassica* vegetables due to partial or total inactivation of myrosinase, thermal breakdown of GLS and their hydrolysis products, loss of enzymatic cofactors, leaching of GLS and their derivatives into the cooking medium, or volatilization of the derivatives (Dekker *et al.,* 2000). The extent of these losses probably depends on the duration and type of heat treatment, the degree of material disintegration, and the vegetable matrix itself (Rosa and Heaney, 1993). High hydrostatic pressure (HHP) is a non-thermal technology used in the food industry to inactivate microorganisms and spoilage enzymes without affecting the quality of fresh products (San Martin *et al.,* 2002). Moreover, the application of pressure processing has been found to retard thermal inactivation (Hendrickx *et al.,* 1998). HHP stability has been investigated for broccoli and mustard seed myrosinases (Ludikhuyze *et al.,* 1999; Van Eylen *et al.,* 2008). However, a very wide difference in pressure stability has been reported. Green cabbage is widely consumed as either cooked or processed products; however, no published data on the thermal and pressure inactivation of myrosinase from green cabbage are yet available in the literature. Vegetables are primarily consumed in the cooked form and are processed by various techniques. Blanching is a short heat treatment that is typically applied to vegetables prior to further processing with the aim of enhancing both safety and quality attributes. Blanching imparts benefits, such as destruction of surface microflora of vegetables and enhancing the colour and texture and also the keeping quality of vegetable products. The quality of blanched product depends significantly on the time and temperature of blanching and also on the size of vegetable to be blanched. Under-blanching speeds up the activity of enzymes and is worse than no blanching. Over-blanching causes loss of texture, colour, phytochemicals and minerals. Industrial blanching processes involve temperatures ranging from 70 to 95 °C and times usually no higher than 10 min (Morales-Blancas *et al.,* 2002) whereas for domestic purposes vegetables are generally blanched for 10–12 min in boiling water (98–100 °C). A considerable amount of research has been done to understand the effects of blanching on texture, colour, phytochemical content and antioxidante activity of different vegetables. Volden *et al.,* (2008) showed the effects of blanching of red cabbage on the levels of glucosinolates, polyphenols and anthocyanins, as well as for the antioxidant potential by the ferric reducing ability power (FRAP) and oxygen radical absorbance capacity (ORAC) assays. Data on the effect of blanching on physicochemical properties of cabbage is scarce (Amin and Lee, 2005; Volden *et al.,* 2008).

Table. 2: Thermal stability of myrosinase from different *Brassica* sources.

Source of myrosinase	Stability (ºC)
Broccoli myrosinase (crude extract)	Up to 30ºC
Broccoli myrosinase (juice)	Up to 40ºC
Red cabbage myrosinase (crude extract)	Up to 60ºC
Red cabbage myrosinase (crude extract)	Up to 40ºC
White cabbage myrosinase (crude extract)	Up to 50ºC
Mustard seed (crude extract)	Up to 60ºC
Rapeseed myrosinase (crude extract)	Up to 65ºC
Rapeseed myrosinase (intact and flaked seeds)	Up to 90ºC

Source: Ghawi et al. (2012)

Table. 3: List of glucosinolates identified in *Brassica* family, extraction methods and characterization.

Plant species	Extraction method	Characterization method	Compounds identified	References
Brassica napus *Brassica juncea*	Headspace-solid phase microextraction	GC-MS	dimethyl sulfide, 3-methyl-3 butenenitrile 1-isothiocyanato-butane 4-isothiocyanato-1-butene	Wey et al. (2012)
Sinapis alba	decoction	RP-HPLC	sinigrin, sinalbin, allyl- and benzyl isothiocyanates	Herzallah and Holley (2012)
Brassica oleracea L. var. botrytis L. *Brassica* rapa *ruvo*	extraction with methanol	HPLC-PDA, ESI-MS NMR	sinigrin, glucoiberin, glucoiberverin, gluconapin, glucobrassicanapin and gluconasturtiin	Toribio et al. (2011)
Brassica oleracea L. *var. capitata* f. *rubra* DC	extraction with methanol	LC/MS	sulforaphane	Koo et al. (2011)
Brassica oleracea L. var. *costata* DC	headspace-solid phase microextraction	GC/ITMS	allyl isothiocyanate dimethyl disulfide dimethyl trisulfide	Pinho et al. (2009)
Brassica oleracea var. *capitata* f. *alba*	extraction with methanol	HPLC-UV	glucobrassicin, sinigrin	Kusznierewicz aet al. (2008)
Brassica oleracea var. italica	aqueous extract	CPC	sinalbin and glucoraphanin	Toribio et al. (2007)

GC-MS: Gas chromatography coupled with mass detector; RP-HPLC: Reverse phase liquid chromatography; HPLC-PDA: liquid chromatography- photo diode array detector; ESI-MS: electrospray ionization-mass spectrometry; NMR: Nuclear magnetic resonance; LC/MS: liquid chromatography–mass spectrometry; GC/ITMS: chromatography- ion trap mass spectrometry; HPLC-UV: liquid chromatography- ultraviolet detector; CPC: Centrifugal partition chromatography

Isolation and quantification of glucosinolates from *Brassica*

Due to their physicochemical properties, the separation and the isolation of GLS is an extremely difficult task. The presence of the sulfate group and of the thioglucose moiety cause the octanol–water partition coefficient of GLS (log Po/w) to fall in the low value domain (−4.30 for glucoiberin to −1.38 for neoglucobrassicin), thus suggesting they are very hydrophilic and always water-soluble entities (Holst and Williamson, 2004). The characterization and quantification of GLS, either in pure state or within mixtures, is possible through the desulpho-glucosinolates technique (Wathelet *et al.*, 1995; Kiddle *et al.*, 2001).

Moreover, many ion pair chromatography based methods have been developed after the initial work published by Helboe *et al.,* (1980) to purify GLS (Prestera *et al.,* 1996; Toribio *et al.*, 2007). They exploit the well-known property of alkyl-ammoniums (tetramethyl ammonium, tetraoctyl ammonium, tetradecylammonium, etc.) to form ion pairs with sulfate groups (Prestera *et al.,* 1996). This is the strategy used by plants to transport GLS. Indeed, a GLS anion is sometimes associated with an aromatic choline ester cation, such as sinapine, in sinalbin from mustard or glucoraphanin from broccoli (Butzenlechner, 1996).

Recently, Fahey *et al.,* (2002) successfully resolved glucoraphanin and glucoiberin from crude plant homogenates using high speed counter-current chromatography (HSCCC) in the elution mode and a highly-salted and highly polar biphasic solvent system: 1-propanol/acetonitrile/saturated aqueous ammonium sulfate/water (1:0.5:1.2:1).This protocol was optimized and scaled up by Fisher *et al.,* (2005) where 15 g of enriched glucoraphanin extract were injected in a MIDI-dynamic extraction centrifuge apparatus equipped with a 928mL column.

There are different methods of extraction and characterization of glucosinolates in Brassica family. As can be seen in Table 3, extraction with methanol is widely reported, but other methods such as decoction, headspace solid-phase microextraction are also employed.

Among the methods for detection and quantification, there is a wide variation, although liquid and gas chromatography coupled or not the mass detector predominated, since they allowed confirmation of the compounds analyzed. Regarding the isolated glucosinolates, each species has its particularity in relation to them, which can also vary within the same species due to climate change, seasonal variation, aspects that were discussed earlier. Compounds that exhibit prominent, ie appear in a large number of species are sinigrin and sinalbina addition of sulfur compounds, allyl, benzyl isothiocyanates and dimethyl sulfide. Herzallah and Holley (2012), developed a method to reversed phase-high performance liquid chromatography method was to quantify sinigrin, sinalbin, allyl isothiocyanate and benzyl isothiocyanate

present in aqueous and freeze-dried yellow and Oriental (brown) mustard extract samples using two pre-treatment methods (autoclaving, boiling) to prevent degradation by myrosinase. Toribio *et al.,* (2007) purified the glucosinolates sinalbin and glucoraphanin by strong ion-exchange displacement centrifugal partition chromatography (SIXCPC). The optimized conditions involved the biphasic solvent system ethyl acetate/n-butanol/water, the lipophilic anion-exchanger. Amounts as high as 2.4 g of sinalbin and 2.6 g of glucoraphanin were obtained in starting from 12 and 25 g of mustard and broccoli seed aqueous extracts

CONCLUSIONS

From this review it was seen that the vegetables of the *Brassica* family are rich in glucosinolates, which have a valuable anticarcinogenic action. Glucosinate-myrosinase system may be influenced by factors such as climate, soil, genotype, with seasonal variation. Therefore, cultivar selection should be tailored to specific environmental factors at each location to achieve optimization in phytochemical content of *Brassica* sp. In addition, selected white cabbages with an optimized bioactive compound content could be used as raw material for sauerkraut production, enhancing the human dietary intake in health promoting compounds.

The study of the effects of processing on the concentrations of GLS and the parameters related to their hydrolysis in *Brassica* vegetables has a pivotal role in complementing research on the epidemiology of the consumption of *Brassica* vegetables and chemoprevention. An understanding of the physical and biochemical changes occurring before the ingestion of processed *Brassica* vegetables may help to interpret the metabolic fate of GLS in experimental studies in animals and humans and inform the subsequent formulation of dietary strategies to optimize the uptake of isothiocyanates in vivo. Hydrolysis of vegetables at high temperatures leads to destruction of myrosinase, an enzyme responsible for converting glucosinate in active substances, as well as treatment at high pressures, which is used in the food industry leads to destruction of this enzyme. Therefore, when one wishes to obtain constant characteristics in a plant of the *Brassica* family all these aspects should be taking in account.

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