

## Effects of Novel Processing Techniques on Glucosinolates and Membrane Associated Myrosinases in Broccoli

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High pressure/high temperature (HP/HT) and pulsed electric field (PEF) treatment of food are among the novel processing techniques considered as alternatives to conventional thermal food processing. Introduction of new processing techniques with fast and gentle processing steps may reveal new possibilities for preservation of healthy bioactive compounds in processed food. However, effects on various food components due to autolysis and fast reactions prior to the applied HP/HT or PEF need to be considered as the total contribution of processing steps affects the obtained food quality. The present experiments were performed on broccoli (*Brassica oleracea* var. *Italica*) florets, purée and juice. Specific focus was given to effects of HP/HT and PEF processing on the content of glucosinolates and activities of myrosinase isoenzymes (EC.3.2.1.147) in the broccoli preparations. Certain conditions applied in HP/HT processing of broccoli florets were able to maintain a high level of intact glucosinolates. Treatment at 700 MPa and 20°C for 10 min was found to inactivate myrosinase activity, but also pressure treatments at 300 MPa and 20°C were able to maintain a high level of intact glucosinolates present in the untreated broccoli florets. PEF processing of broccoli purée and juice showed that the myrosinase activities resulted in nearly total glucosinolate transformations as result of autolysis during puréeing and juice making prior to the PEF processing. These data demonstrated that insight into potential effects on myrosinase activities from application of PEF processing implies specific focus on the sample steps preceding the PEF processing.

### INTRODUCTION

Pulsed electric field (PEF) and high pressure / high temperature (HP/HT) processing are among the novel alternatives investigated as supplement to conventional thermal treatment of foods [Andersen *et al.*, 2010b; Gonzales & Barrett, 2010]. The techniques seem promising with respect to improvement of food quality related to texture, flavor, smell, taste, color, nutrients and anti-nutrients/toxicants [Andersen *et al.*, 2010b; Soliva-Fortuny *et al.*, 2009]. In addition, the novel techniques may introduce new possibilities for preparation of convenience food from healthy raw materials with preservation of their bioactive compounds. This is a particular challenge in food systems where traditionally applied processing induces fast chemical reactions as known to occur in autolysis processes as it is seen in the glucosinolate-myrosinase system [Andersen *et al.*, 2007, 2010a; Andersson *et al.*, 2008; Bellos-tas *et al.*, 2009].

The main goal of HP/HT and PEF processing is to ensure a high food quality such as increased freshness and pro-

longed shelf-life by inactivation of micro-organisms and enzymes. HP/HT can at temperatures above 50°C lead to pasteurization using pressure levels from 250–500 MPa [Wilson *et al.*, 2008], while sterilization can be obtained with HP at temperatures higher than 70°C [Oey *et al.*, 2008]. Focus is thus on the application of HP in combination with high temperatures (HT), which may lead to tissue compression, cell wall breakage and cell membrane disruption, depending on the HP and HT levels as well as the type of vegetable or fruit that are treated [Michel & Autio, 2002]. In the present study, where broccoli has been processed, the food quality is particularly connected with the enzyme-substrate (myrosinase-glucosinolate) system, where the different types of non-enzymatic and enzymatic catalyzed hydrolysis products give rise to effects from being health promoting to affecting the taste and odor [Hansen *et al.*, 1995, 1997; Jeffery & Aray, 2009].

PEF is considered a non-thermal treatment, which can lead to pasteurization at ambient temperatures using electrical field strengths of 20–30 kV/cm, and short pulses of 1–10 μs [Mosqueda-Melgar *et al.*, 2008]. Application of short electric pulses may result in pore formation in cell membranes, and depending on the treatment intensity, time, electric field and pulse energy the membrane permeabilisation

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can be reversible or irreversible [Soliva-Fortuny *et al.*, 2009]. However, PEF leads to permeabilisation of both the microbial and the plant cell membranes and disruption of plant cell walls allows for autolysis reactions to occur, which may lower the food quality [Gonzales & Barrett, 2010].

Both PEF and HP have been reported to be able to inactivate several enzymes. HP is described as a technique with ability to induce protein unfolding, leading to loss of enzyme activity [Ludikhuyze *et al.*, 2003], while PEF processing may lead to inactivation of enzymes at certain processing conditions, maybe due to interference from the electric pulses with metal co-factors or due to local Joule heating [Aguiló-Aguayo *et al.*, 2008, 2009]. Only a few studies have dealt with PEF treatment of *Brassica* plants [Gachovska *et al.*, 2010; Guderjan *et al.*, 2007], and none of these has investigated the effects of PEF on glucosinolates and myrosinase.

*Brassicaceae* plants are rich in bioactive compounds including those considered as health promoting phytochemicals such as glucosinolates [Andersen *et al.*, 2010a; Holst & Williamson, 2004; Jeffery & Aray, 2009; Sørensen *et al.*, 2001]. Glucosinolates and their degradation products in broccoli and other cruciferous vegetables have as group attracted much attention owing to their odor and “taste” [Hansen *et al.*, 1995, 1997]. Epidemiological studies indicate that consumption of broccoli can reduce the risk of cancer [Jeffery & Aray, 2009] and this biological effect is considered to be related to sulforaphane, the highly reactive isothiocyanate produced from the aliphatic glucosinolate glucoraphanin [Herr & Buchler, 2010; Juge *et al.*, 2007; Zhang *et al.*, 1994].

The glucosinolates are biosynthetically derived from amino acids and they all have a basic structure consisting of an amino acid derived alkyl aldoxime-O-sulphate ester with a  $\beta$ -D-thioglucopyranosyl group *cis* to the sulphate ester group (Figure 1) [Bellostas *et al.*, 2007].

In green broccoli cultivars the glucosinolates are quantitatively dominated by tryptophane derived indol-3-ylmethylglucosinolates and methionine derived aliphatic glucosinolates [Hansen *et al.*, 1995; Kushad *et al.*, 1999]. In the undamaged broccoli plant tissue, glucosinolates are separated from myrosinases (EC.3.2.1.147) by membranes, preventing the myrosinase isoenzymes from catalyzing the glucosinolate transformations to different bioactive products [Bellostas *et al.*, 2008a; 2009]. Disruption of cell membranes, due to cell damage, processing or chewing, leads thus to contact between glucosinolates and the myrosinase isoenzymes, and in the presence of water this results in many different

kinds of transformation products, depending on the type of glucosinolates present and the reaction conditions [Bellostas *et al.*, 2007, 2009; Bones & Rossiter, 2006].

At neutral pH the alkylglucosinolates are generally transformed into isothiocyanates, while nitriles are the main products at low pH [Bellostas *et al.*, 2009; Bones & Rossiter, 2006; Sørensen, 1990].

The isothiocyanates are very reactive compounds, and fast reactions occur with nucleophiles, as amines, thiols and hydroxy groups, resulting in a range of different compounds as *e.g.* dithiocarbamates and thioureas [Bellostas *et al.*, 2007, 2008 a,b, 2009]. It is therefore likely that the results from many studies concerning the low recovery of sulforaphane found in *Brassica* autolysates also could be explained by a further reaction of the isothiocyanate with nucleophiles to form dithiocarbamates or thioureas [Andersen *et al.*, 2010a].

Concerning the indol-3-ylmethylglucosinolates, these compounds may be transformed into a complex range of different products upon myrosinase catalyzed hydrolysis including indol-3-ylcarbinols, indol-3-ylacetonitriles, ascorbigens and various indol-3-yloligomers [Agerbirk *et al.*, 1998; Bones & Rossiter, 2006; Buskov *et al.*, 2000 a, b; Jeffery & Aray, 2009]. Although indol-3-ylmethylglucosinolates are quantitatively dominating compounds in broccoli, only limited information is available on the potential biological effects of the individual indolyl derivatives [Bonnesen *et al.*, 1999; Jensen *et al.*, 1991; Loft *et al.*, 1992; Vang *et al.*, 2001].

Glucosinolates may also be transformed by non-enzymatic reactions, which can take place under reducing conditions and acidic pH, leading to the production of nitriles, or thionamides for 2-hydroxy substituted glucosinolates [Bellostas *et al.*, 2008b; Bones & Rossiter, 2006].

The effects from HP/HT and PEF processing of food based on cruciferous vegetables as broccoli are thus relevant to study in relation both to enzymatic and non-enzymatic transformation of glucosinolates.

## MATERIALS AND METHODS

### Chemicals

Sodium cholate hydrate and sulfatase from *Helix pomatia* were purchased from Sigma, while di-sodiumtetraborate-decahydrate was from VWR – Bie & Berntsen. Benzylglucosinolate and sinalbin were from the laboratory collection [Bjerg & Sørensen, 1987] as well as other pro analyses chemicals [Sørensen *et al.*, 1999].

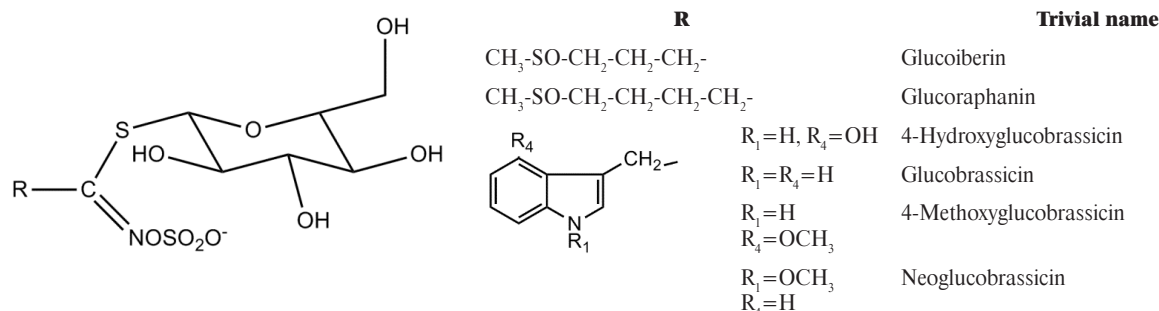


FIGURE 1. Structures of glucosinolates.

### Sample preparation

The processing conditions used for HP/HT and PEF treatment of broccoli florets and purée varied from mild to more intensive treatments. The low intensity treatment, which results in cell damage and thus autolysis reactions, was applied to study the potential release of membrane-associated myrosinase isoenzymes and vacuole-accumulated glucosinolate substrates. High intensity treatments, where enzyme inactivation is expected, were performed at ambient and at thermal conditions. Two types of reference samples were prepared, an untreated and a thermally treated. Temperature, pressure and electric field conditions applied are given below. All treatments were performed in triplicates. The conditions used for PEF treatment of broccoli juice focused on shelf-life stability and only covered treatment conditions where enzyme inactivation is expected.

### High pressure and temperature treatment (HP/HT)

High pressure and temperature treatment (HP/HT) of broccoli was performed by Dr. Iesel van der Plancken, Katholieke Universiteit Leuven, Belgium. Florets of broccoli (*Brassica oleracea* var. *Italica*, unknown cultivar) were prepared by cutting the flowers of broccoli from the stem, and florets from three broccolis were mixed. The broccoli florets were HP/HT processed for 10 mins in triplicates at the following conditions; A= 20°C, 300 MPa; B= 20°C, 700 MPa; C= 82°C, 0.1 MPa; D= 82°C, 700 MPa. Processing treatments were performed in triplicate during 2 days. Following processing, samples were kept at -40°C until lyophilization. Throughout the experiment 6 samples were collected (3 samples each day) to represent the reference material (U= untreated).

### Pulsed electric field treatment (PEF)

Pulsed electric field (PEF) treatments of moderate intensity were performed on broccoli purée by Dr. Ana Balasa and Dr. Anne Grohmann, Technical University of Berlin, TUB, Germany. Broccoli (*Brassica oleracea* var. *Italica* cv. *Ironman*) was crushed in a cube dicer and afterwards puréed in a Raetz-mill. The PEF conditions performed at room temperature were: A: 3 kV/cm, 15.6 ms; B: 10 kV/cm 3 ms; C: 20 kV/cm, 4.8 ms and D: 20 kV/cm, 15.6 ms.

PEF treatments of broccoli juice were performed at higher intensity in the facilities of the Novel Food Processing Technologies research group of the University of Lleida, Spain. Broccoli juice was obtained by chopping and crushing broccoli florets (*Brassica oleracea* var. *Italica*) and the resulting juice was filtered through cheesecloth and degassed for 10 min. A continuous-flow bench-scale PEF system (OSU-4F, Ohio State University, Columbus, OH) that holds square-wave pulses was used. The treatment system consisted of eight co-field flow chambers in series, each one containing two stainless steel electrodes separated by a gap of 2.92 mm. Temperatures of inlet and outlet of each pair of chambers were monitored during PEF treatment and never exceeded 35°C. The temperature was regulated by means of cooling coils connected between each pair of chambers that were submerged in an ice-water shaking bath. The treatment flow was controlled by a variable-speed pump (model 75210-25, Cole Parmer Instruments Company, Vernon Hills, IL, USA).

Treatment conditions comprised variable generated polarity in both monopolar and bipolar modes with a treatment time of 500, 1250 or 2000  $\mu$ s with electric field strength of 15, 25 or 35 kV/cm.

### HPCE determination of desulfoglucosinolates

The glucosinolate composition of each sample was determined in triplicate by extraction followed by High Performance Capillary Electrophoresis (HPCE) of desulfoglucosinolates. Freeze-dried broccoli powder (500 mg) was extracted with boiling methanol-water (7:3, V:V) for 1 min. and centrifuged for 3 min at 2750 $\times$ g. The supernatants from three subsequent extractions were evaporated and then dissolved in 4.00 mL MilliQ-water (crude extract). Benzylglucosinolate and sinalbin were used as internal standards [Sørensen *et al.*, 1999]. The crude extract (1 mL) was applied to a DEAE Sephadex A-25 column (1 mL), followed by washing with 2 mL water and 1 mL 0.02 mol/L acetate buffer, pH 5.0. Sulfatase (75  $\mu$ L; 35 mg/mL) was added to the column and left overnight for reaction. Desulfoglucosinolates were eluted with 3 $\times$ 1 mL MilliQ water, evaporated to dryness and re-dissolved in 100  $\mu$ L MilliQ water. The samples were analysed by HPCE (Agilent, Waldbronn, Germany) using the following conditions; 60°C, 12 kV, capillary dimensions 64.5 cm  $\times$  75  $\mu$ m with on-column detection at 230 nm. The separation buffer consisted of: sodium cholate (250 mmol/L) and borate (200 mmol/L), pH 8.5. The quantitative amounts of individual glucosinolates were determined from the electropherogram using normalized peak areas and response factors as described elsewhere [Sørensen *et al.*, 1999].

### Myrosinase activity

Freeze-dried broccoli powder (2 g) was extracted with Milli-Q water and the myrosinase isoenzymes were isolated on a Concanavalin A-Sepharose column according to methods described elsewhere [Sørensen *et al.*, 1999].

### Statistical analyses

Statistical significance was accepted at  $p < 0.05$ , and the analysis was performed by one-way ANOVA.

## RESULTS AND DISCUSSION

### Preparation of broccoli prior to processing

The present studies have comprised quantitative determination of the individual glucosinolates in different samples prior to HP/HT and PEF processing; broccoli florets, broccoli purée and broccoli juice as well as the broccoli powder used for juice preparation (Table 1). Large differences are seen in the glucosinolate content between the different types of investigated samples, but florets, purée and juice have profiles which are quantitatively dominated by indol-3-ylmethylglucosinolates, and the aliphatic glucosinolates glucoiberin and especially glucoraphanin. Other aliphatic glucosinolates only account for a minor percentage of the total glucosinolate content. This corresponds with previous findings on glucosinolate composition, and quantitative glucosinolate levels in the widely produced green broccoli cultivars are dominated by these compounds [Hansen *et al.*, 1995; Kushad *et al.*, 1999].

TABLE 1. Average content ( $\mu\text{mol/g}$  dry matter (DM)) of intact glucosinolates in different batches of unprocessed broccoli florets (6 batches), purée (4 batches) and juice (6 batches) including broccoli powder of the same material as juice.

Glucosinolate		Broccoli florets	Broccoli purée	Broccoli juice	Broccoli powder
To be treated with		(Belgium) HP/HT	(Germany) PEF	(Spain) PEF	(Spain)
Aliphatic glucosinolates	Glucoiberin	n.d.	$0.3 \pm 0.2$	n.d.	$1.3 \pm 0.1$
	Glucoraphanin	$1.1 \pm 0.4$	$2.1 \pm 0.7$	n.d.	$12.2 \pm 0.4$
	4-Hydroxyglucobrassicin	$0.5 \pm 0.2$	$0.2 \pm 0.2$	n.d.	$0.34 \pm 0.3$
Indol-3-ylmethyl-glucosinolates	Glucobrassicin	$2.5 \pm 1.3$	$1.1 \pm 0.6$	$0.04 \pm 0.0$	$1.5 \pm 0.7$
	4-Methoxyglucobrassicin	$0.9 \pm 0.4$	$0.3 \pm 0.2$	n.d.	$1.5 \pm 1.5$
	Neoglucobrassicin	$3.9 \pm 2.0$	$2.0 \pm 1.1$	$0.03 \pm 0.03$	$0.4 \pm 0.1$
<b>TOTAL</b>		<b><math>8.9 \pm 4.4</math></b>	<b><math>6.0 \pm 1.8</math></b>	<b><math>0.08 \pm 0.03</math></b>	<b><math>17.2 \pm 0.6</math></b>

The total amount of glucosinolates in the broccoli heads, purée, juice and powder ranged from 0.08 to 17.2  $\mu\text{mol/g}$  DM. This level is usually between 5 and 30  $\mu\text{mol/g}$  DM, but it can be as high as *ca.* 90  $\mu\text{mol/g}$  DM in green broccoli [Hansen *et al.*, 1997; Kushad *et al.*, 1999; Van Eylen *et al.*, 2009]. Variations in concentration can be related to differences in varieties, plant parts, growth conditions, climatic conditions, storage time and storage conditions [Ciska *et al.*, 2000; Hansen *et al.*, 1995; Kushad *et al.*, 1999; Vang *et al.*, 2001; Verkerk *et al.*, 2001]. However, important knowledge can also be gained from the glucosinolate profile and the relative concentration between the aliphatic glucosinolates and indol-3-ylmethylglucosinolates. In the present study the broccoli powder, related to the broccoli juice, has a predominance of aliphatic glucosinolates, which corresponds well with the typical profile found for broccoli [Hansen *et al.*, 1995; Kushad *et al.*, 1999; Vang *et al.*, 2001], as also found for other cruciferous vegetables [Cartea *et al.*, 2008; Ciska *et al.*, 2000]. However, the relatively low content of aliphatic glucosinolates detected in the florets, purée and juice in the present study indicates that myrosinase-catalyzed glucosinolate hydrolysis has already occurred, since especially aliphatic glucosinolates usually accounts for more than 50% of total glucosinolate levels in broccoli [Hansen *et al.*, 1995, 1997; Kushad *et al.*, 1999].

Unprocessed broccoli florets contained the highest concentration of glucosinolates of the three investigated unprocessed materials (florets, purée, juice), as the plant cells in this material only are damaged at the cross sectional cuts; however, the average data from the 6 different unprocessed samples did have a high standard deviation ( $8.9 \pm 4.4$   $\mu\text{mol/g}$  DM; Table 1). Each sample was analyzed by extraction and HPCE in triplicate resulting in relative standard deviations of an average of 13% originating from this step of analysis. Figure 2 shows examples of electropherograms of two different unprocessed samples indicating the large variation in glucosinolate content between the expectedly similar samples. Glucosinolate contents in the 6 samples thus ranged from a total glucosinolate level of 2.3  $\mu\text{mol/g}$  DM to 13.4  $\mu\text{mol/g}$  DM. This difference cannot be ascribed to variations in plant materials, as the samples were collected from the same batch of mixed florets from three broccoli florets. However, the difference could be explained by difference in storage time at room temperature, *e.g.* before freezing or lyophilization, resulting in higher enzymatically-catalyzed hydrolysis of gluco-

sinolates in the cutting places. This is in good agreement with other studies, where slicing of broccoli followed by storage for 48 h in particular decreased the concentration of aliphatic glucosinolates [Michaelsen *et al.*, 1991]. The results emphasize the importance of temperature control during preparation, as well as reduced handling or processing time.

The broccoli purée had a lower concentration of glucosinolates than the broccoli florets, due to more extensive cell damage during puréeing. However, the detection of intact glucosinolates in the purée indicates the presence of some intact cells in the purée. The reference material (broccoli purée) had a glucosinolate content of approx. 6  $\mu\text{mol/g}$  DM (Table 1), which is a relatively low level, but not unexpected as the puréeing is likely to have damaged a great part of the cells depending on the grinding efficiency.

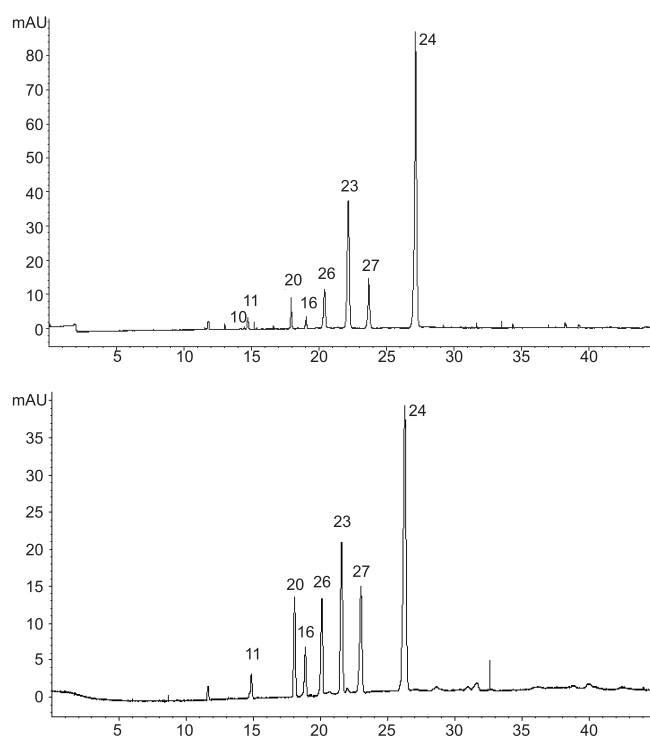


FIGURE 2. Content of glucosinolates in different batches of untreated broccoli florets analysed as desulfoglucosinolates by HPCE. 10: glucoiberin; 11: glucoraphanin; 26: 4-hydroxyglucobrassicin; 23: glucobrassicin; 27: 4-methoxyglucobrassicin; 24: neoglucobrassicin; Internal standards: 20: sinalbin, 16: glucotropaeolin.

The last broccoli material evaluated was broccoli juice, and the results in Table 1 clearly show that the juice processing had a dominating effect on the content of intact glucosinolates. The broccoli used for juice originally contained  $17.2 \pm 0.6 \mu\text{mol}$  glucosinolates/g DM of which the aliphatic amount was  $13.4 \pm 0.4 \mu\text{mol/g}$  DM and the level of indol-3-ylmethylglucosinolates was  $3.8 \pm 0.9 \mu\text{mol/g}$  DM. In the broccoli juice the remaining glucosinolates only constituted 0.5 to 1.0% of the original content. No aliphatic glucosinolates were detected and only the indol-3-ylmethylglucosinolates 4-methoxyglucobrassicin and neoglucobrassicin were present (Table 1).

The results presented in Table 1 demonstrate that pre-processing of plant material can have major effects on the glucosinolate levels. The problems with autolysis processes are especially of concern in relation to PEF processing, where the processing is preferably performed on liquids or purées.

### High pressure treatment of broccoli florets

The effect on HP/HT treatment of broccoli florets on glucosinolate contents was analyzed as shown in Figure 3, with application of pressure (300 or 700 MPa) and/or temperature (20°C or 82°C). As a reference for pressure application, samples were tested at 82°C at atmospheric pressure (0.1 MPa). This reference with broccoli florets treated at 82°C for 10 min at 0.1 MPa contained a significantly higher level of glucosinolates than the unprocessed broccoli florets ( $p < 0.001$ ), which is probably due to glucosinolate degradation during handling of the unprocessed samples as discussed above.

The thermally-treated samples (0.1T) contained  $21 \pm 4 \mu\text{mol/g}$  DM, whereas untreated samples contained from 2.32–13.4  $\mu\text{mol}$  glucosinolates/g DM (Figure 3). Five different glucosinolates were detected (Table 1), and thermally-treated samples had the following average glucosinolate composition; glucoraphanin (2.85  $\mu\text{mol/g}$  DM), 4-hydroxyglucobrassicin (0.65  $\mu\text{mol/g}$  DM), glucobrassicin (5.71  $\mu\text{mol/g}$  DM), 4-methoxyglucobrassicin (1.45  $\mu\text{mol/g}$  DM) and neoglucobrassicin (10.00  $\mu\text{mol/g}$  DM). The thermal treatment alone is thus seen to have an effect on pres-

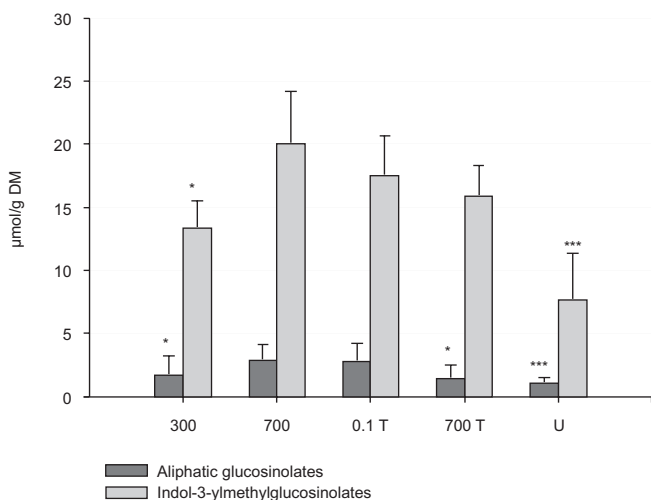


FIGURE 3. Total glucosinolate content in HP/HT processed broccoli florets (10 min treatment); 300: 300 MPa, 20°C; 700: 700 MPa, 20°C; 0.1T: 0.1 MPa, 81°C; 700T: 700 MPa, 80°C, and U: untreated. Statistical analysis with sample 0.1T as reference; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

ervation of intact glucosinolates. This is in agreement with data produced by Van Eylen *et al.* [2008], who found broccoli myrosinase activity to be reduced by 95% when heated at 70°C for 10 min.

The highest pressure treatment of broccoli florets did have some effect on the glucosinolate level. Samples treated with pressure level of 700 MPa (20°C) had a similar content of intact glucosinolates to that in the thermally-treated broccoli florets (0.1 MPa). There was no significant difference between the glucosinolate level determined in the thermally-treated broccoli with the samples treated at 700 MPa at 20°C ( $P = 0.18$ ) for indol-3-ylmethylglucosinolates and for aliphatic glucosinolates ( $P = 0.87$ ). However, the sample treated at 700 MPa at 82°C had a significantly lower level of aliphatic glucosinolates ( $P = 0.035$ ), but not of the indol-3-ylmethylglucosinolates ( $P = 0.25$ ). The sample treated at 700 MPa at 20°C also had a significantly higher level of indol-3-ylmethylglucosinolates ( $P = 0.02$ ) and aliphatic glucosinolates ( $P = 0.013$ ) than when treated at 700 MPa and 82°C. Application of HP at 700 MPa (20°C) is thus found to be equally effective for myrosinase inactivation as thermal treatment at 82°C and atmospheric pressure, and with improved performance in relation to preservation of intact glucosinolates.

Application of 20°C, 300 MPa leads to significantly lower glucosinolate concentrations of both aliphatic and indol-3-ylmethylglucosinolates than detected for the thermally-treated samples ( $P < 0.05$ ). However, some inactivation of myrosinase may still have occurred at 300 MPa as the glucosinolate level detected in these samples was higher than in the unprocessed samples.

Reduction of myrosinase activity in a buffer system at pressure levels of 200–450 at 20°C MPa has been shown [Ludikhuyze *et al.*, 2000, 2003]. In another study on HP treatment of broccoli, myrosinase inactivation in broccoli juice was obtained with slightly higher pressure levels and in this study myrosinase was isolated by the ammonium sulfate precipitation and the myrosinase activity measured from released glucose by use of the enzyme coupled assay [Van Eylen *et al.*, 2008], thus supporting our findings that HP treatment is able to inactivate myrosinase.

The relative content of glucosinolates was comparable for all the processed samples (Figure 3), and glucosinolate hydrolysis was largely prevented with pressure levels at 700 MPa (20°C) as discussed above. However, as aliphatic glucosinolates usually account for more than 50% of total glucosinolate levels in broccoli [Hansen *et al.*, 1995, 1997; Kushad *et al.*, 1999] some degree of glucosinolate degradation is expected to have occurred prior to the HP/HT treatment. A study of the effect of HP/HT treatment on the naturally occurring pool of intact glucosinolates in broccoli florets is therefore needed to confirm the positive effect of pressure treatment at ambient temperature found in this work.

### Pulsed electric field processing of broccoli purée and broccoli juice

Broccoli purée was prepared shortly before the PEF treatments. The purée was processed with different field strength of 3, 10 or 20 kV/cm and with varying number of pulses (Figure 4). Each batch of puree was divided into two, one

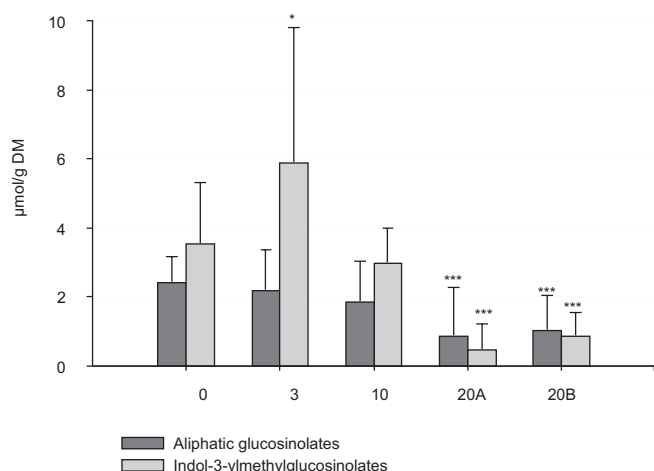


FIGURE 4. PEF processed broccoli purée; Electric field strength: 0, 3, 10 and 20 kV/cm. Samples denoted 3 and 20 (B) were treated for 16  $\mu$ s, whereas the others were treated for 3–5  $\mu$ s. Statistical analysis with\*:  $P < 0.05$  and \*\*\*:  $p < 0.01$ , where samples are related to the untreated purée.

sample which was PEF treated and one blank sample. Generally the production of broccoli purée may result in varying amounts of intact glucosinolates of the theoretical possible amount of glucosinolates depending on the grinding efficiency. For the mildest treatment conditions, 3 and 10 kV/cm, there was a further tendency to a decrease in glucosinolate concentration compared to the untreated broccoli purée, while the treatments with 20 kV/cm lead to a marked decrease in glucosinolate content regardless of the pulse number. The high electric field strength may have resulted in a higher degree of membrane permeabilisation, and thus a decrease in the level of intact glucosinolates, as a consequence of their contact with myrosinase. There was a significant reduction ( $P < 0.01$ ) in the amount of aliphatic glucosinolates and indol-3-ylmethylglucosinolates when samples were treated with 20 kV/cm at room temperature. Even though most of the intact glucosinolates were degraded during puréeing, further degradation was observed when applying 20 kV/cm to the purée. This indicates that PEF processing at this electric field strength does not negatively affect the conformation and activity of myrosinase, since these samples contained a lower concentration of glucosinolates. However, it could be expected that the electric field strength would affect  $Zn^{2+}$  ion, which is found to be a co-factor for myrosinase [Burmeister, 2000]. As previously mentioned, pasteurization is achieved with electric field strength of 20–35 kV/cm. If the intention additionally is to obtain a product where glucosinolates are kept intact it will be essential to inactivate myrosinase prior to the PEF processing.

Broccoli juices were PEF-processed at different conditions; 15, 25 and 35 kV/cm, in time ranges of 500–2000  $\mu$ s with either a monopolar or a bipolar mode. A sample of untreated broccoli powder was analyzed in triplicate for the content of glucosinolates, and contained glucoiberin, glucoraphanin, 4-hydroxyglucobrassicin, glucobrassicin, 4-methoxyglucobrassicin and neoglucobrassicin (amounts presented in Table 1). The unprocessed broccoli juice contained only trace amounts of glucobrassicin and neoglucobrassicin

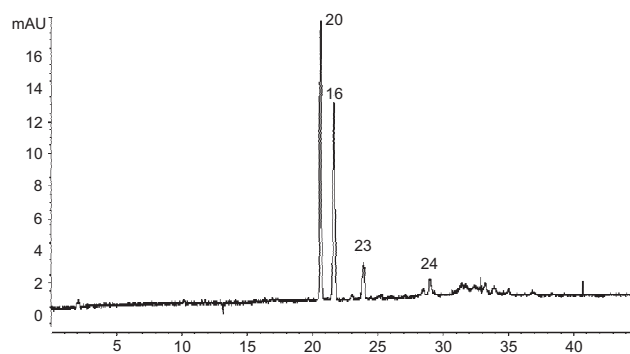


FIGURE 5. Content of glucosinolates in broccoli juice separated as desulfoglucosinolates by HPCE. 23: glucobrassicin; 24: neoglucobrassicin. Internal standards: 20: sinalbin, 16: glucotropaeolin.

and no detectable amounts of aliphatic glucosinolates were found (Figure 5). However, a high variation was observed in the level of these two glucosinolates, as the analyzed level from 6 different samples ranged from 0–0.12  $\mu$ mol/g DM for glucobrassicin and 0–0.05  $\mu$ mol/g DM for neoglucobrassicin. Analysis of the PEF-processed juice clearly demonstrated that the main effects on glucosinolate concentrations occurred during juice preparation and hence prior to the PEF processing. There was no observed difference in glucosinolate contents relating to the type of voltage waveforms used or in the treatment time, however, there did seem to be an effect of the electric field strength. The level of glucobrassicin significantly increased when 35 kV/cm was applied, but not when 15 or 25 kV/cm was applied (Figure 6). It is thus likely that the high electrical field strength inactivates myrosinase, which also has been found for other metallo enzymes. All of the aliphatic glucosinolates (glucoiberin and glucoraphanin) have thus been degraded during the juice preparation and this has also been the case for the majority of the indol-3-ylmethylglucosinolates. The application of PEF might have an effect of myrosinase at high electric field strength, however, it would be interesting to examine this further.

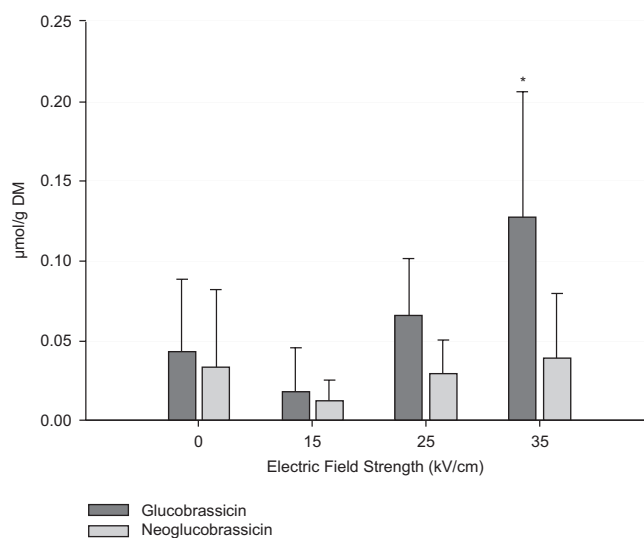


FIGURE 6. Content of glucosinolates in PEF processed broccoli juice. Electric field strength: 0, 15, 25 and 35 kV/cm.

The results from the present study on broccoli illustrate that initial myrosinase inactivation is crucial if glucosinolates are intended to be kept intact during PEF processing. The problem with autolysis reactions should therefore be addressed in order to reduce a negative impact on the food preprocessed prior to application of novel processing techniques, where reactions catalyzed by enzymes like PPO (polyphenoloxidase EC 1.10.3.2.), POD (peroxidase EC.1.11.1.7), CS-lyase (cysteine lyase EC 4.4.1.10) and S-thiolmethyltransferase (thiol methyltransferase EC 2.1.1.9.) can cause unwanted color formation, taste and flavor problems. Several studies have shown that PEF and HP are effective in inactivating several enzymes as PPO and POD [Aguiló-Aguayo *et al.*, 2009; Cano *et al.*, 1997; Huang *et al.*, 2012; Ludikhuyze *et al.*, 2003], however, as seen in the present study autolysis reaction can cause severe damage to the product in the steps prior to the PEF or HP processing.

Glucosinolates from broccoli have also been the subject of a great deal of studies focused on their positive bioactive effects, which have especially been associated with the presence of sulforaphane produced from glucoraphanin [Gasper *et al.*, 2005; Matusheski & Jeffery, 2001; Razis *et al.*, 2010]. Indol-3-ylcarbinol, the hydrolysis product of glucobrassicin, has as well been investigated for bioactivities [Jeffery & Aray, 2009]. The carbinols from indol-3-ylmethylglucosinolates are, however, starting point for complex groups of products when they occur in digesta and conditions as in the stomach [Buskov *et al.*, 2000 a,b,c]. They call for special attention in relation to structure and concentration defined bioactivities [Bellostas *et al.*, 2008 a,b; Matusheski *et al.*, 2004, 2006; Mithen *et al.*, 2003]. Moreover, the autolysis may also result in indolyl-compounds that have a negative effect on smell and taste as well as on color [Drewnowski & Gomez-Carneros, 2000]. During and prior to PEF or HP/HT processing it is important to control the glucosinolate degradation which occurs in non-enzymatic and/or myrosinase catalyzed hydrolysis reactions [Bellostas *et al.*, 2007, 2009; Mithen *et al.*, 2003]. Several studies have focused on the effects of traditional food processing of broccoli with respect to maintain the potential health properties of sulforaphane. Most of these studies aim to produce a material where myrosinase is active, but still separated from the glucosinolates, which would give basis for the highest possible sulforaphane production, as found for raw broccoli or lightly blanched broccoli [Conaway *et al.*, 2000; Jones *et al.*, 2010], rather than cooking where glucosinolates may leach into the cooking water and myrosinase is inactivated [Howard *et al.*, 1997].

## CONCLUSION

The results now obtained clearly show that the handling to avoid autolysis reactions of *Brassicaceae* plants prior to the novel processing (HP/HT and PEF) is a crucial step. The myrosinase-catalyzed transformations of broccoli glucosinolates are found to be a special part of these autolysis reactions, leading to a complex group of bioactive products. These products are considered to be dominating factors in relation to quality of broccoli products, and it is, therefore, important

to have processing control of their transformation. HP/HT and PEF have now been investigated for their potential value for such types of processing. High pressure treatment of broccoli florets at 700 MPa for 10 min inactivates myrosinase at room temperature. PEF treatment at 20 kV/cm resulted in enhanced cell membrane permeabilization, while PEF treatment at 35 kV/cm seemed to inactivate myrosinase. However, since most of the glucosinolates were degraded prior to the PEF processing any possible myrosinase inactivation seems less relevant. Initial myrosinase inactivation by temperature or HP treatment is thus required for success with PEF processing of *Brassicaceae* plants.

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