Effect of storage on the content of indole-glucosinolate breakdown products and vitamin C of sauerkrauts treated by high hydrostatic pressure

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Running Title: Storage of pressurized sauerkrauts

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ABSTRACT

The effect of refrigerated storage for three months on the content of indole glucosinolate (GLS) breakdown products (ascorbigen -ABG-, indole-3-carbinol -I3C- and indole-3-acetonitrile -I3ACN-) and vitamin C in sauerkrauts treated by high hydrostatic pressure (HHP) was investigated. Sauerkrauts were produced either by spontaneous fermentation (NF) or by using a mixed-starter culture (*Lactobacillus plantarum* and *Leuconostoc mesenteroides*) (PMF) at 0.5g/100g and 1.5g/100 g NaCl concentrations and they were pressurized in order to prolong their shelf life. HHP-sauerkrauts were a good source of vitamin C (143-161 mg/100g d.m.) and ABG was the main indole GLS derivative (37-65 μmol/100g d.m), followed by I3C (5-17 μmol/100g d.m) and I3ACN (1.5-3 μmol/100g d.m). NF-HHP sauerkrauts presented higher I3C and I3AC and lower vitamin C content than PMF-HHP sauerkrauts. Refrigerated storage led to a gradual decrease of ABG and vitamin C (losses of 33-67% and 96-98%, respectively, after 3 months) while slight changes of I3C and I3ACN were observed.

Keywords: cabbage, high hydrostatic pressure, indole glucosinolate breakdown products, sauerkraut, storage, vitamin C.

1. Introduction

Brassicaceous crops are among the most grown vegetables worldwide and over the last decades their consumption has been associated with health benefits (Bjorkman et al., 2011). These benefits are largely attributed to their high content of antioxidant compounds and glucosinolates (GLS) breakdown products formed by the myrosinase action when the plant cells are broken (Kusznierewicz, Bartoszek, Wolska, Drzewiecki, Gorinstein, & Namiesnik, 2008). In cabbage, glucobrassicin is one of the predominant GLS, and once it is fermented, bioactive indole glucobrassicin derivatives such as ascorbigen (indol-3-ylmethyl-ascorbate, ABG), indole-3-carbinol (I3C) and indole-3-acetonitrile (I3ACN) are some of the most important GLS hydrolysis products found in sauerkrauts (Ciska & Pathak, 2004; Martinez-Villaluenga et al., 2009; Peñas, Frias, Sidro, & Vidal-Valverde, 2010a).

Several studies have demonstrated the anticarcinogenic effect of ABG due to its ability to induce activation of xenobiotic-metabolizing enzymes and apoptosis of tumoral cells. In addition, ABG exerts a protective action against DNA and decreases the estrogen pool, thereby reducing the possibility of generating genotoxic compounds (Sepkovic, Bradlow, Michnovicz, Murtezani, Levy, & Osborne, 1994; Sparnins, Venegas, & Wattenberg, 1982) and conferring protection to the skin against oxidative stress (Wagner et al., 2008). I3C is a potent anticarcinogen in mammals by induction of enzymes involved in the carcinogen metabolism, inhibition of steroid hormone binding, scavenging of electrophiles and protection against oxidative damage (Takahashi, Dashwood, Bjeldanes, Williams, & Bailey, 1995). This compound has been shown to inhibit the proliferation of cancer cells from different human tissues *in* vitro (Kim et al., 2006; Sarkar, & Li, 2004) at a concentration of 30-100 μM. Regarding I3ACN, it has been shown to inhibit chemical-induced neoplasia in rodents (Wattenberg, & Loub,

1978) and to increase the activity of glutathione-S transferase, which has the capacity to detoxify chemical carcinogens (Sparnins et al., 1982). On the other hand, sauerakraut contains a high concentration of vitamin C, a potent antioxidant which may exert its action directly to scavenge free radical species, by metabolyzing peroxides to non-radical products and by chelating metal ions to prevent generation of oxidizing species (Duthie, Ma, Ross, & Collins, 1996). Due to their health promoting properties, the increase of these bioactive compounds in sauerkrauts may have a beneficial impact on the consumer's health.

Sauerkraut is a popular white cabbage fermented product in Central and Eastern Europe and, after its production it is usually kept in domestic refrigerators or it is pasteurized until consumption. Recently, high hydrostatic pressure (HHP) has been successfully applied to minimise the microbial load of sauerkraut, improving its microbiological quality and extending its shelf-life (Peñas, Frias, Gomez, & Vidal-Valverde, 2010b). HHP is a non-thermal technology that satisfies the demand for minimally processed products, particularly avoiding the need of antimicrobial agents (Mújica-Paz, Valdez-Fragoso, Tonello-Samson, Welti-Chanes, & Torres, 2011). To the best of our knowledge, HHP may be a valuable processing alternative to lengthen to shelf-life of sauerkrauts maintaining their health promoting properties. Although the content of GLS breakdown products is high at the end of the fermentation period, there are no data documenting the amount of GLS derivatives and vitamin C after HHP and after further refrigerated storage. Therefore, the objective of the present work was to determine the content of indole GLS breakdown products and vitamin C in HHP-treated sauerkrauts produced either by natural or induced fermentation with different salt concentrations and to follow their content for 1, 2 and 3 months at 4 ºC.

2. Materials and methods

2.1. Starter culture preparation

L. plantarum (CECT 748) and *L. mesenteroides* (CECT 219) strains were provided by the Spanish Type Culture Collection (CECT, Valencia, Spain) and multiplied following the procedure indicated by Peñas et al. (2010a). A starter culture containing equal proportions of both strains was inoculated at approximately 10^6 colony-forming units /g of cabbage.

2.2. Cabbage fermentation process

Fresh white cabbages (*Brassica oleracea* L. var. *capitata* cv. Bronco) grown in the Eastern region of Spain (Levante) were provided by Bejo Iberica S. L. (Madrid, Spain). The edible cabbage parts were shredded into strips (~2 mm) using a domestic shredder (Moka Express, Barcelona, Spain). Different batches with two concentrations of NaCl (1.5 and 0.5g/100g) were prepared. Cabbage and brine were then transferred to autoclaved polyethylene vessels (8 L) and were tightly pressed together to remove air. Then, two types of fermentations were performed: natural fermentation using the indigenous microbiota naturally present in raw white cabbage, and induced fermentation using the mixed starter culture of *L. plantarum & L. mesenteroides* previously prepared. Each type of fermentation was run in 3 parallel batches (4 Kg per batch) at room temperature (22-25 ºC) for 7 days.

2.3. High hydrostatic pressure processing

Several lots of approximately 25 g of natural and inoculated sauerkraut were vacuum-packed and pressurised at 300 MPa at 40 ºC for 10 min in a discontinuous high pressure machine (ACB GEC, Alsthom, Nantes, France) according to Peñas et al.

(2010b). After the HHP treatment, pressurized naturally produced sauerkrauts (NF-HHP) or inoculated with *L. plantarum & L. mesenteroides* (1:1) sauerkrauts (PMF-HHP) were immediately opened, freeze-dried and analysed to quantify the content of indole GLS degradation products and vitamin C. Simultaneously, pressurized packed sauerkrauts were stored for 1, 2 and 3 months at 4 ºC. Afterwards, bags were opened, freeze-dried, and analysed to determine the content of indole GLS degradation products and vitamin C. The treatment was carried out in triplicate.

2.4. Chemical analysis

2.4.1. Analysis of indole GLS hydrolysis compounds

The content of ABG, I3C and I3ACN in HHP-treated sauerkrauts and after storage for 3 months at 4 ºC was quantified as in Peñas et al. (2012). Quantification was performed by HPLC using an Alliance Separation Module 2695 (Waters, Milford, USA), a Photodiode Array detector 996 at 280 nm (Waters, Milford, USA) and a computer running the Empower 2 chromatographic software (Waters). 20 µL of sample were injected into an ODS-2 column 150 x 4.6 mm i.d., 5 µm size column (Waters) at 30 ºC. The chromatogram was developed at a flow rate of 1.2 mL/min using a gradient of mobile phase A (0.1 M ammonium acetate pH 5.7 containing 10% acetonitrile) and mobile phase B (0.1 M ammonium acetate, pH 5.7 containing 80% acetonitrile) as follows: linear gradient of 100% A-100% B for 25 min, isocratic 100% B for 5 min, linear gradient of 100% B-100% A for 5 min and, finally, equilibrate for 5 min.

Standard I3C and I3ACN (Sigma-Aldrich, Steinheim, Germany) were used to identify these compounds in sauerkraut. Standard ABG was synthesised according to Kiss and Neukon (1966) with the modifications described by Peñas et al. (2010a). The

134 purity of standard ABG was determined by HPLC and it was frozen under nitrogen and
135 protected from light.
136 Calibration curves were made with the standard compounds, then plotted and
137 adjusted by using the method of least squares. The regression coefficients of ABG, I3C
138 and I3ACN curves were greater than 0.990.
139
140 *2.4.2. Determination of vitamin C*
141 The determination of vitamin C content in HHP-sauerkrauts before and after
142 refrigeration for 3 months was performed by capillary electrophoresis using a fused
143 silica capillary TSP075375 (47 cm x 75 μm) purchased from Composite Metal Services
144 LTD (The Chase, Hallow, Worcester, UK). A P/ACE system 2050 (Beckman
145 Instruments, Fullerton, CA, USA) equipped with UV detection at 254 nm was used for
146 the analysis (Frias, Miranda, Doblado, & Vidal-Valverde, 2005). Ascorbic acid was
147 quantified from a calibration curve built with the pure ascorbic acid standard (Fluka)
148 and with a response factor relative to the internal standard; the regression coefficients
149 were greater than 0.990.
150
151 *2.5. Statistical analysis*
152 Data were expressed as means of three experiments. Results were compared by
153 one-way analysis of variance (ANOVA) using the least significant differences ($P \leq$
154 0.05) (Statgraphic 5.0 software, Statistical Graphics Corporation, Rockville, MD, USA).
155
156 **3. Results and discussion**
157 HHP technology satisfies the demand for minimally processed products and can
158 provide quality superiority over products obtained by conventional technologies.

159 Since glucobrassicin is the most abundant indole GLS found in raw white
160 cabbage cv. Bronco (Peñas, Frias, Martinez-Villaluenga, & Vidal-Valverde, 2011), the
161 content of their main breakdown products, ABG, I3C and I3AC, were quantified in
162 pressurised sauerkrauts (NF-HHP and PMF-HHP) and stored for 1, 2 and 3 months
163 (Tables 1-2). Figure 1 represents the effect of storage in those indole GLS derivatives
164 expressed as retention percentage.

165 Table 1 shows the main indole GLS breakdown products found in the 0.5 and
166 1.5% NaCl NF-HHP sauerkrauts and their content storage. ABG was the most abundant
167 indole GLS degradation compound (65 μmol/ 100g d.m.), followed by I3C (17
168 μmol/100g d.m.), while I3ACN was present in the lowest amount (3 μmol/100g d.m.).
169 The presence of these three compounds in just fermented cabbages is in the range
170 reported previously (Ciska, Verkerk, & Honke, 2009; Peñas et al., 2012). In NF-HHP
171 sauerkrauts produced with the highest NaCl level, the content of ABG (37μmol/100g
172 d.m.) and I3C (13μmol/100g d.m.) was lower than in those obtained with the lowest salt
173 concentration, while no difference was observed between the two NF-HHP sauerkrauts
174 for I3ACN content. These results are within the range of those obtained recently by
175 Ciska and Honke (2012) for pasteurized naturally produced sauerkraut for 30 min.

176 Refrigerated storage affected the content of the indole GLS derivatives on NF-
177 HHP (Table 1, Figure 1). ABG was quite stable during the first month of storage, but
178 decreased significantly during the second one (28-58%) and reached larger losses (64-
179 67%) at the end of the storage period. However, I3C was very stable during the first two
180 months of storage, and it was only during the third month when a slight but significant
181 ($P \leq 0.05$) decrease (7%) in the NF-HHP obtained with the lowest level of NaCl was
182 found. Similarly, I3ACN underwent very small changes during the storage period.

183 Table 2 shows the content of indole GLS derivatives in just processed PMF-
184 HHP sauerkraut and after storage in refrigeration for three months. PMF-HHP
185 sauerkrauts obtained at both salt levels showed similar content of ABG and I3ACN (40
186 and 2 μmol/100g d.m., respectively), while I3C was significantly ($P \leq 0.05$) higher in
187 0.5g/100g NaCl pressurised sauerkraut. These concentrations were significantly
188 ($P \leq 0.05$) lower than those obtained in NF-HHP sauerkrauts at 0 time of storage (Table
189 1).

190 During storage at 4 ºC, ABG gradually decreased in 0.5g/100g NaCl PMF-HHP
191 and losses of 17, 23 and 52% after 1, 2 and 3 months of storage, respectively, were
192 found. I3C experienced a slight but significant ($P \leq 0.05$) reduction during the first month
193 (12%), which was maintained during the rest of the refrigerated period. On the contrary,
194 I3ACN did not change significantly ($P \leq 0.05$) throughout refrigeration (Figure 1).
195 However, the effect of storage on the concentration of the indole breakdown GLS
196 compounds in NaCl PMF-HHP sauerkrauts obtained with 1.5 g/100g of NaCl, was
197 variable. The content of ABG was kept during the first month, and losses around 23 and
198 33% were obtained after 2 and 3 months of storage, respectively. I3C, however,
199 suffered a significant ($P \leq 0.05$) decrease (9 and 23% during the first two months,
200 respectively) and no further changes were observed. In contrast, I3ACN showed no
201 significant ($P \leq 0.05$) loss during the first month of storage and afterwards experienced a
202 drop of 14% (Table 2, Figure 1).

203 The content of ABG in the just processed NF-HHP and PMF-HHP sauerkrauts
204 presented here were lower than those previously reported in the corresponding non-
205 HHP sauerkrauts obtained from the same harvested material (white cabbage cv.
206 Bronco), in which amounts of ~100 μmol/ 100g d.m were found in 0.5g/100g NaCl
207 sauerkrauts, than in those manufactured with 1.5g/100g NaCl (~75 μmol/ 100g d.m)

208 (Peñas et al, 2010a). Taking in account these numbers, it works out that HHP treatment
209 led to ABG reductions of 35% and 60% for 0.5 g/100g NaCl sauerkrauts obtained by
210 either natural or induced fermentations respectively, and of ~60% for both 1.5 g/100g
211 NaCl sauerkrauts. These findings clearly indicate that HHP technology produces a
212 marked reduction in ABG content. In contrast with these results, Van Eylen et al. (2009)
213 reported a small increase of ABG in broccoli heads treated with pressures in the range
214 of 100-500 MPa for 35 min at 20 ºC. The differences between these studies can be
215 attributed not only to the difference in the vegetable material studied, but also to the
216 different conditions used during the pressurization experiment (time, temperature and
217 pressure intensity). However, from the results presented here, it can be speculated that
218 the ABG stability is fairly low at the HHP conditions used in this study. However,
219 Hrncirik, Valusek, and Velisek (1998), reported that ABG was relatively stable at 25 ºC
220 in solutions of pH 3 to 6, conditions at which only 3-5% of this compound was
221 degraded, but they observed higher degradation of ABG at 40 ºC. Although the pH of
222 the sauerkrauts after pressurization is within the range studied by Hrncirik et al. (1998)
223 in which ABG was stable, the temperature of the treatment used in the present study
224 was 40 ºC, thus it may be speculated that this temperature, together with the high level
225 of pressure intensity used, promoted the degradation of ABG. In addition, the loss of
226 ABG content might be partially due to the hydrolysis of this compound to I3C induced
227 by HHP, as well as to the formation of ABG dimers and trimers, as previously reported
228 (Hrncirik et al., 1998).

229 No information has been found on the effect of HHP on the concentration of I3C
230 and I3ACN. These compounds are present in pressurised sauerkrauts at concentrations
231 in the ranges reported in the literature for pasteurized sauerkrauts (Ciska and Honke,
232 2012), and even in non-pressurized sauerkrauts (Ciska & Pathak, 2004; Peñas et al.,

233 2011), thus suggesting the possible non adverse influence of pressurisation on both
234 indole GLS derivatives. However, the confirmation of this hypothesis requires further
235 studies which should monitor the content of these compounds during sauerkraut
236 production.

237 Regarding the impact of refrigerated storage on the content of the indole GLS
238 derivatives in HHP-sauerkrauts, our results are consistent with those reported by our
239 group for ABG in stored non-pressurised sauerkrauts (Peñas et al., 2010a). In the latter
240 work, ABG content was found to decline gradually in sauerkraut stored for three
241 months at 4 ºC (decreases of 53-74% after 3 months), reductions in the same range to
242 those obtained in the present study. On the other hand, Ciska and Pathak (2004) reported
243 no changes in the content of I3C and I3ACN in naturally produced sauerkraut stored for
244 17 weeks at 5 ºC, results in agreement with those obtained in the present work.

245 The concentration of vitamin C in HHP treated sauerkrauts and stored at 4 ºC for
246 1, 2 and 3 months is also shown in Tables 1-2 and Figure 1. NF-HHP sauerkrauts
247 obtained at both NaCl concentrations exhibited amounts of vitamin C of 143 mg/100 g
248 d.m and 149 mg/100 g d.m., respectively. Storage at 4 ºC led to a sharp decrease in
249 vitamin C (85-80% for 0.5 and 1.5g/100g NaCl NF-HHP sauerkrauts, respectively) for
250 the first month and only 2% was retained after 3 months of storage (Table 1).
251 Significant differences ($P \leq 0.05$) were not observed for vitamin C content between the
252 two salt levels during the storage period.

253 The content of vitamin C in PMF-HHP sauerkrauts, produced with both levels of
254 NaCl, at 0 time of storage was 149 and 161 mg/100g d.m., respectively (Table 2).
255 During refrigerated storage, vitamin C showed a noticeable diminution comparable to
256 that observed in NF-HHP sauerkrauts (Tables 1, Figure 1), and reductions of ~98%
257 were observed at the end of the storage period. Recently, Peñas et al. (2010a) reported

vitamin C contents of 243 mg/100 g d.m. and 277 mg/100 g d.m., respectively, in the corresponding non-HHP sauerkrauts obtained from the same vegetable material (white cabbage cv. Bronco) by either natural fermentation or by induced fermentation with *L. plantarum & L. mesenteroides*. Compared with the results of the present work, it can be observed that the HHP treatment may have reduced the vitamin C content of sauerkrauts by almost 50%, depending on the fermentation conditions. To date, no information has been found about the influence of HHP treatment on vitamin C content of fermented cabbage. Most studies performed in the last decade have evaluated the effect of HHP on fruit juices and vegetable purees and most of them concluded that HHP induced no or only insignificant losses of vitamin C compared with the unpressurised fruit and vegetable products. In this sense, Sánchez-Moreno, Plaza, De Ancos, Martin, and Cano (2005) found a retention of ascorbic acid of 91% in orange juice after HHP at 400 MPa, 40ºC, 1 min, while Patras, Brunton, Da Pieve, and Butler (2009) observed that pressurisation at 600 MPa, 10-20 ºC, 15 min of strawberry puree preserved around 94% of the content of vitamin C. Barba, Esteve, and Frigol (2010) found losses of vitamin C that did not exceed 9% in a vegetable beverage treated by 100-400 MPa for 420-540 s at 30ºC. It can be speculated that the high vitamin C diminution observed in the present work with sauerkraut as a consequence of HHP treatment could be due to a longer time period or a higher temperature during pressure exposure and also to the different type of food matrix studied. The HHP conditions used in the present work were previously selected by our group on the basis that they noticeably improved the microbial quality of sauerkraut by decreasing the populations of aerobic mesophillic and lactic acid bacteria compared to untreated fermented cabbages (Peñas et al., 2010b). However, the selected HHP conditions that enhanced the microbial safety of sauerkraut and did not significantly modify the content of I3C and I3ACN, had a negative impact on the

283 vitamin C and ABG contents. No information has been found on the effect of
284 refrigerated storage on vitamin C of pressurized sauerkrauts, and the results presented in
285 this work show a sharp decrease during storage at 4ºC.
286
287 **4. Conclusions**
288 Pressurized sauerkrauts are a good source of indole GLS breakdown products
289 and refrigeration for 3 months led to a gradual decrease of ABG (33-67%) and to non-
290 significant changes of I3C and I3ACN. However, vitamin C underwent a sharp decline
291 (96-98%). Therefore, although HHP treatment can be considered an efficient technology
292 to improve the microbial quality of sauerkrauts, the conditions used in this work
293 followed by refrigerated storage led to a decrease in some of their bioactive compounds.
294
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FIGURE CAPTIONS

Figure 1. Effect of storage on the retention of indole GLS breakdown products and vitamin C of HHP-sauerkrauts.