**Headspace components that discriminate between thermal and high pressure high
temperature treated green vegetables: identification and linkage to possible process-
induced chemical changes**

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Abstract

For the first time in literature, this study compares the process-induced chemical reactions in three industrially relevant green vegetables: broccoli, green pepper and spinach treated with thermal and high pressure high temperature (HPHT) processing. Aiming for a fair comparison, the processing conditions were selected based on the principle of equivalence. A comprehensive integration of MS-based metabolic fingerprinting techniques, advanced data preprocessing and statistical data analysis has been implemented as untargeted/unbiased multiresponse screening tool to uncover changes in the volatile fraction. For all vegetables, thermal processing, compared to HPHT, seems to enhance Maillard and Strecker degradation reaction, triggering the formation of furanic compounds and Strecker aldehydes. In most cases, high pressure seems to accelerate (an)aerobic thermal degradation of unsaturated fatty acids leading to the formation of aliphatic aldehydes and ketones. In addition, both thermal and HPHT processing accelerated the formation of sulfur-containing compounds. This work demonstrated that the approach is effective in identifying and comparing different process-induced chemical changes, adding depth to our perspective in terms of studying a highly complex chemical changes occurring during food processing.

Keywords

green vegetables; thermal processing; high pressure high temperature processing; sterilization; MS-based chemical fingerprinting; process induced chemical reaction.

48 1. INTRODUCTION

49 Thermal processing of foods is based on the application of an increased temperature for a
50 particular length of time to inactivate microorganisms and enzymes to a targeted level in order to
51 stabilize the product during subsequent storage (Awuah, Ramaswamy, & Economides, 2007).
52 Since all reactions are accelerated by temperature, when enhanced stability (i.e. ambient shelf-
53 life) is targeted, the quality (e.g. nutrient content, flavor, process-induced contaminants) of the
54 product is (mostly) negatively affected. Fortunately, the difference in temperature sensitivity
55 between the inactivation of microorganisms and the destruction of quality attributes creates
56 space for thermal processing optimization. In cases where heat transfer is not a limiting factor
57 (e.g. to convection-heating foods), this optimization resulted in the high-temperature short-time
58 or the ultra-high temperature processes. However, it is difficult to apply this principle to
59 conduction-heating foods (Silva, Hendrickx, Oliveira, & Tobback, 1992). Due to these
60 limitations, combined with the increased consumer perception towards more healthy and
61 convenient foods, alternative approaches resulting in gentle preservation of foods have been
62 investigated by food industries and researchers: (i) advanced heating techniques resulting in
63 reduced heating and cooling times (Awuah et al., 2007); and (ii) novel processing technologies
64 introducing a processing variable other than heat (Knorr, Froehling, Jaeger, Reineke, Schlueter,
65 & Schoessler, 2011).

66
67 In the context of both approaches, in the last two decades, high pressure high temperature
68 (HPHT) has been cited as an interesting processing technology to produce shelf-stable, low-
69 acidic, foods (de Heij, van Schepdael, Moezelaar, Hoogland, Matser, & van den Berg R., 2003).
70 HPHT has two main unique characteristics. Firstly, it relies on fast compression heating and
71 decompression cooling to create fast temperature changes throughout the food product. This
72 phenomenon could allow for the application of the high-temperature short-time principle to

73 conduction-heating foods (Torres, Pedro, Laura, rez, & Marleny, 2009; Grauwet, Van der
74 Plancken, Vervoort, Hendrickx, & Van Loey, 2011; Bravo et al., 2012). Secondly, the sensitivity
75 of any reaction to pressure ultimately depends on the partial activation volume (V_a) (Le
76 Chatelier's principle). Consequently, reaction rates will be either reduced (when characterized by
77 a positive V_a) or enhanced (when characterized by a negative V_a), creating an extra dimension for
78 process design and optimization (Valdez-Fragoso, Mujica-Paz, Welte-Chanes, & Torres, 2011).
79 Although HPHT is still at the research stage, progress has been made in understanding the
80 impact on food safety and quality attributes. For instance, the mechanism of spore (e.g.
81 *Clostridium botulinum* and *Bacillus*) inactivation by HPHT has been widely studied (Wilson,
82 Dabrowski, Stringer, Moezelaar, & Brocklehurst, 2008; Shao & Ramaswamy, 2011;
83 Ramaswamy, 2011). However, the effect of HPHT on chemical changes responsible for
84 chemical safety (e.g. process-induced contaminants, allergens) and food quality (e.g. texture,
85 flavor, color, enzymatic activity, vitamins), seems to depend on reaction type (Oey, Van der
86 Plancken, Van Loey, & Hendrickx, 2008b; De Roeck, Mols, Duvetter, Van Loey, & Hendrickx,
87 2010; Verbeyst, Oey, Van der Plancken, Hendrickx, & Van Loey, 2010; De Vleeschouwer, Van
88 der Plancken, Van Loey, & Hendrickx, 2010; Knockaert, De Roeck, Lemmens, Van
89 Buggenhout, Hendrickx, & Van Loey, 2011; Van der Plancken et al., 2012; Vervoort et al.,
90 2012b). For instance, Oey et al. (2006) and Verbeyst et al. (2012) reported increased degradation
91 of folates (vitamin B9) and vitamin C respectively due to HPHT-enhanced oxidation reactions.
92 Valdez-Fragoso et al. (2011) reported that the rate of chemical reactions responsible for the
93 formation of undesirable compounds (e.g. formation of aldehydes in milk) can be significantly
94 increased with high pressure. De Vleeschouwer et al. (2010) observed a reduction in acrylamide
95 formation as a result of HPHT compared to thermal processing. Moreover, since the knowledge
96 on the effect of HPHT on these reactions is still limited, there is a need for a thorough approach

to investigate the potential of HPHT processing impact on chemical reactions, in different food matrices, in comparison to thermal processing.

Food processing triggers complex chemical reactions and therefore it has been a challenge to identify, characterize and model at least the most significant reaction pathways. Nevertheless, recently, improvements in the detection capabilities of analytical instruments and data mining softwares have enhanced food quality investigation. For example, a comprehensive combination of metabolomics-like approaches (chemical fingerprinting) and statistical tools (chemometrics) enabled investigating topics that were previously considered unapproachable (Issaq, Van, Waybright, Muschik, & Veenstra, 2009; Herrero, Simo, Garcia-Canas, Ibanez, & Cifuentes, 2012). For instance, a mass spectrometry (MS)-based chemical fingerprinting platform can be mentioned under this decree. In this platform, a robust sample extraction step (i.e. headspace-solid phase microextraction; HS-SPME) has been integrated with a high-resolution separation step (i.e. gas chromatography (GC)) and MS detection. Chemical fingerprinting can be defined as a high-throughput identification and characterization of small molecule chemicals (Dettmer, Aronov, & Hammock, 2007; Wishart, 2008) and can be a powerful screening tool, for example, to investigate process-induced chemical changes in a specific food fraction (e.g. volatilizable fraction). To reduce the huge and complex data sets, obtained from chemical fingerprinting, the potential of pattern recognition programs and data mining tools (e.g. multivariate statistical techniques) has been introduced in food analysis.

The present work aims at comparing process impact on chemical changes in three industrially relevant, low-acid, green vegetables: broccoli, green pepper and spinach treated with thermal and HPHT processing. The novelty of this study relies on the integrated approach where: (i) for a fair quantitative cross-comparison between thermal and HPHT processing, matrices were treated

aiming for a particular processing value ($F_0 = 5$ min, based on microbial spore inactivation); (ii) MS-based chemical fingerprinting platform (HS-SPME-GC-MS) was used as an untargeted/unbiased multiresponse screening tool to uncover changes in the volatile fraction of treated vegetables; (iii) the obtained data were analyzed. Firstly, the data were analyzed with interference removing and peak deconvoluting software (i.e. Automated Mass Spectral Deconvolution and Identification System; AMDIS) followed by filtering and peak alignment software (i.e. Mass Profiler Professional; MPP) and finally with pattern recognition and data mining software (i.e. Multivariate Data Analysis; MDA) to compare the processing impact and to identify possible discriminant headspace components. Until today only very few comparative studies considered impact comparison from an equivalent point of view, e.g. Knockaert et al. (2011) and Vervoort et al (2012b). However, these studies followed a targeted approach, focusing on particular compounds (e.g. specific (micro)nutrient, process-induced contaminant) which might result in overlooking unexpected changes. To the best of our knowledge there is not a single study that compared the impact on process-induced chemical reactions in green vegetables treated with equivalent HPHT and conventional thermal processing, from an untargeted point of view using a comprehensive integration of MS-based chemical fingerprinting techniques and advanced data preprocessing (i.e. AMDIS/MPP) and statistical data analyses (MDA) (**Fig.1**).

Fig. 1. Schematic overview of the performed quantitative cross-comparison among thermal and HPHT processing. Starting from sample preparation to the equivalent thermal and HPHT treatment, HS-SPME-GC-MS fingerprinting and finally data analyses. For the data analyses, as a preprocessing step, the obtained data were analyzed with interference removing and peak deconvoluting software (i.e. Automated Mass Deconvolution and Identification System; AMDIS) followed by filtering and peak alignment software (i.e. Mass Profiler Professional; MPP). The preprocessed data were analyzed with pattern recognition and data mining software (i.e. Multivariate Data Analyses; MDA).

2. MATERIALS AND METHODS

2.1. Sample preparation

Fresh vegetables: a single batch of broccoli (*Brassica oleracea* cv. *Southern comet*), green pepper (*Capsicum annuum* cv. *California wonder*) and spinach (*Spinacia annuum* cv. *Falcon*) were purchased at a local market. The vegetables were carefully washed and cut into standardized shapes: broccoli was cut into small pieces (2.5 cm flower and 1 cm stem), for the green pepper the petiole and the seeds were discarded and cut in the length into strips of 0.5 cm – 1 cm thickness and for the spinach only the petiole was removed. The vegetables were then put into low density polyethylene bags. To prevent all enzymatic reactions during processing, storage and thawing and to assure only changes during thermal and HPHT processing, the vegetables were blanched at 95 °C for 8 min in a water bath (Haak W15 DC-10, Germany). The blanched plastic bags were immediately cooled in ice water for 10 minutes, frozen in liquid

nitrogen and stored in a freezer at - 40 °C. The blanching conditions were validated using a qualitative and quantitative peroxidase (POD) test (Adebooye, Vijayalakshmi, & Singh, 2008; Vervoort et al., 2012b). Prior to the treatments (section 2.2), a Buchi mixer (B-400, BUCHI, Switzerland) was used to blend the blanched vegetables.

2.2. Treatment

Aiming fair comparison of the process impact, an equivalent industrially relevant process value $F_{121.1^{\circ}\text{C}}^{10^{\circ}\text{C}}(F_0) = 5$ min was put forward for both thermal and HPHT processing targeting inactivation of spores of *Clostridium botulinum*. A constant holding temperature (T_h) of 117 °C was selected for both processing treatments. Each treatment was repeated 6 times. Due to the lack of reliable kinetic data as a result of incomplete understanding of the combined effect of pressure and temperature on *Clostridium botulinum* spore inactivation (Van der Plancken et al., 2012), in the present work, the HPHT was considered as pressure assisted thermal processing. Due to their inert nature, glass jars for the thermal and Teflon sample holders for the HPHT processing were selected.

2.2.1. Thermal processing

The thermal treatment was carried out in a static steriflow pilot retort (Barriquand, Paris, France). The glass jars (370 ml volume, 99 mm height and 80 mm diameter) were filled with 85 ± 0.5 g of vegetable puree and then closed with metal lids. Next, they were loaded into the retort and sterilized during processing times of 80 min. Temperature profiles in the retort and at the coldest point of the product were recorded using type T thermocouples (Ellab, Denmark) (**Fig. 2, thin red solid line**).

2.2.2. High pressure high temperature treatment

The HPHT treatment was carried out in laboratory-scale HPHT equipment (custom-made, Resato, The Netherlands). The equipment consists of six vertically oriented individual vessels ($Volume = 43 \text{ cm}^3$ and $diameter = 2 \text{ cm}$). The vessels were jacketed with a heating coil connected to a temperature controlling unit. The HPHT equipment allows computer controlled pressure build-up to 800 MPa, temperature control up to 120 °C and data logging of both sample pressure (**Fig. 2, thick blue solid line**) and temperature (**Fig. 2, red dashed line**). The pressure medium was 100 % propylene glycol (PG fluid, Resato, The Netherlands). During a HPHT treatment, a preheating at atmospheric pressure, pressure build-up, a holding and cooling steps were established (Grauwet, Van der Plancken, Vervoort, Hendrickx, & Van Loey, 2010; Grauwet et al., 2012; Van der Plancken et al., 2012). Teflon (polytetrafluoroethylene) sample holders (12 mm inner diameter, 85 mm length, 4 mm wall thickness, Vink, Belgium) were filled with vegetable puree and closed with a movable cap and vacuum sealed with double plastic bags. The sample holders were pre-equilibrated at 10 °C in a cryostat and soon after that were loaded (**Fig. 2, ②**) into the HP vessels that were equilibrated at the T_h (**Fig. 2, ①**). Starting from room temperature, using only compression heating, the product temperature cannot be raised to the point where inactivation of spores under high pressure is feasible. Therefore, prior the actual HPHT treatment, the samples were preheated at atmospheric pressure to experimentally determined initial temperature (T_i) ~ 75 °C (**Fig. 2, ③**). When the desired T_i was achieved, the pressure in the vessels was increased via pumping the pressure medium to the vessels (through indirect compression) (**Fig. 2, ④**). During the pressure build-up by the intensifier, two consecutive stages can be identified: (i) instantaneous pressure increase from 0.1 to 150 MPa; (ii) further pressure increase until 600 MPa at a rate of 10 MPa/s. After reaching 600 MPa, the individual vessels were isolated and an equilibration time of 1 min was taken into account (**Fig. 2, ⑤**). Due to the pressurization and isolation processes, the temperature inside the product was

increased from T_i to T_h through compression heating. The product temperature was recorded online and the holding time was corrected to achieve the targeted F_0 value. On average, the pressure was held for 15 min (**Fig. 2, ⑥**). At the end of the holding time, the pressure was released from the vessels, which was accompanied with temperature drop inside the product (decompression cooling) (**Fig. 2, ⑦**).

Fig. 2. The profiles of thermal treatment with product temperature (thin red solid line) and HPHT treatment with product temperature (dashed red line) and pressure (thick blue solid line). For the HPHT treatment, sequential steps were followed: ① equilibration of the pressure vessel to the holding temperature, T_h ; ② insertion of sample in the isolated sample holder in the vessel; ③ preheating of the sample to initial temperature, T_i (experimentally determined and dependent on pressure and required T_h); ④ fast pressure build-up accompanied by compression heating; ⑤ The sample temperature is allowed to equilibrate to T_h during 1 min isolation time; ⑥ the process pressure was maintained during a holding time; ⑦ the pressure is released, which is associated with a fast temperature drop. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

2.3. Post treatment sample handling

Following treatments, samples were immediately transferred to ice water to stop any further reaction. Consequently, treated samples were emptied in a cooling room and transferred to a

223 small volume (10 ml) polyethylene terephthalate tubes with a polyethylene cap. Hereafter, the
224 tubes were frozen in liquid nitrogen and stored at -40 °C until analysis.

225 **2.4. HS-SPME-GC-MS analysis**

226 Each sample was thawed overnight in the cooling room (4 °C). 2.5 g of thawed sample and 2.5
227 ml saturated NaCl solution were mixed into a 10 ml amber glass vial (10 ml, VWR International,
228 Radnor, USA). The vials were then tightly closed using screw-caps with PTFE/silicon septum
229 seal (GRACE, Columbia, MD, USA), homogenized and transferred to the cooling tray of the
230 auto-sampler which was maintained at 10 °C. Headspace fingerprinting was conducted on a gas
231 chromatography (GC) system (6890N, Agilent technologies, Diegem, Belgium) coupled to a
232 mass selective detector (MSD) (5973N, Agilent Technologies, Diegem, Belgium) and equipped
233 with a CombiPAL autosampler (CTC analytics, Zwingen, Switzerland). Targeting detection of a
234 wide range of volatiles in a particular food extract, a HS-SPME-GC-MS method of analysis was
235 optimized beforehand. The method includes incubation, extraction using an appropriate type of
236 fiber coating and GC-MS parameters. In the selected method, the samples were incubated at 40
237 °C during 20 minutes under agitation at 500 rpm. Next, extraction of the volatiles was performed
238 using HS-SPME fiber coated with 30/50 µm divinylbenzene/carboxen/polydimethylsiloxane
239 (DVB/CAR/PDMS) (StableFlex, Supelco, Bellefonte, PA, USA) at 40 °C during 10 min.
240 DVB/CAR/PDMS was recommended for analyzing a wide range (semi)volatiles. The SPME
241 fiber was inserted into the heated (230 °C) GC-injection port for 2 min to desorb the headspace
242 components. Prior to extraction, the fibers were conditioned and regenerated according to the
243 manufacturer's guidelines in the conditioning station of the autosampler. A single fiber per
244 vegetable was used. Injection of the samples to the GC-column was performed in split (1/5)
245 mode. Chromatographic separation was carried out on HP-5MS capillary column (30 m ×0.25
246 mm i.d., 0.25 µm film thickness, Agilent Technologies J&W, Santa Clara, CA, USA) having

5%-phenyl-methylpolysiloxane as a stationary phase and helium as a gas phase at a constant flow of 1.5 mL/min. This non-polar column is excellent for trace level analyses, e.g. (semi)volatiles. The GC-oven temperature was programmed from a starting temperature of 40 °C, which was retained for 2 min, to 172 °C at 4 °C/min, then ramped to 300 °C at 20°C/min and kept constant at 300 °C for 2 min before cooling back to 40 °C. The mass spectra were obtained by electron ionization (EI) mode at 200 eV with a scanning range of 35 to 400 m/z and a scanning speed of 3.8 scans per second. MS ion source and quadrupole temperatures were 230 °C and 260 °C, respectively.

2.5. Data preprocessing and multivariate analysis

As commonly observed in GC-MS analysis, co-eluting compounds were present in the obtained chromatograms. Therefore, all chromatograms were analyzed with Automated Mass Spectral Deconvolution and Identification System (AMDIS) (Version 2.66, 2008, National Institute of Standards and Technology, Gaithersburg, MD, USA) to extract “pure” component spectra from complex chromatograms. In addition, AMDIS can be configured to build retention index calibration file and to use retention index data along with the mass spectral data. The deconvoluted spectra were then analyzed with Mass Profiler Professional (MPP) (Version 12.0, 2012, Agilent Technologies, Diegem, Belgium) aiming filtering and peak alignment. The MPP obtained a spreadsheet containing peak areas, which was used as an input for the statistics. The multivariate data were analyzed with a multivariate statistical data analysis (MDA) which was carried out in Solo (Version 6.5, 2011, Eigenvector Research, Wenatchee, WA, USA). As a preprocessing step, all data were mean-centered and the variables were weighed by their standard deviation to give them equal variance. In a first step, unsupervised classification, Principal Component Analysis (PCA), was conducted. Following that, to compare the treatment impact, regression based supervised classification technique, namely Partial Least Squares

Discriminant Analysis (PLS-DA) was implemented. For PLS-DA, the headspace components were considered as X-variables and the three classes (blanched (reference), thermal and HPHT) as categorical Y-variables. Determining the complexity of the model, the lowest number of latent variables (LVs) resulting in a class separation were used. In PLS-DA, to qualitatively investigate impact differences among the classes, bi-plots were plotted. To quantitatively select discriminant headspace components, Variable IDentification (VID) coefficients were calculated (Vervoort et al., 2012a). These values correspond to the correlation coefficient between each original X-variable and Y-variable (s). Variables with an absolute VID value higher than 0.800 were considered to be important. These discriminant components were plotted individually as a function of treatment. In these plots, the mean areas and the standard errors calculated from the six replicates were depicted. All plots were made using OriginPro 8 (Origin Lab Corporation, Northampton, MA, USA). A Duncan's multiple comparison was used to test for significant differences between the mean peak areas ($p < 0.05$) of the discriminant components. Identification of these compounds was performed by comparing the deconvoluted mass spectrum with the reference mass spectra from the NIST spectral library (NIST08, version 2.0, National Institute of Standards and Technology, Gaithersburg, MD, USA). A threshold match of 90 % was implemented and for confirmation further visual inspection of the spectral matching was conducted.

3. RESULTS AND DISCUSSION

The discussion will be initiated with the review on major reaction pathways in the respective vegetables (sections 3.1). From section 3.2 on, step by step, the followed HS-SPME-GC-MS fingerprinting procedures and results will be discussed: (i) from chromatogram to data table (data preprocessing); (ii) qualitative classification of impact differences; (iii) quantitative selection of

discriminant headspace components. In the last section, 3.5, an interpretation of the identity of the selected discriminant components will be described.

In the present work, prior to treatment, vegetables were blanched (see section 2.1). Therefore, enzymatic activities were not expected to have a significant impact on the formation of volatiles and consequently changes will be related to non-enzymatic process-induced chemical reactions.

3.1. Review on major process-induced reactions responsible for volatile formation

3.1.1. Broccoli

Cruciferous vegetables, such as broccoli, are rich sources of compounds called glucosinolates, which are secondary plant metabolites. With regard to their chemical structure, glucosinolates commonly contain a β -D-thioglucose group, a sulphonated oxime moiety and a variable side chain derived from amino acids. This side chain determines whether the glucosinolates are aliphatic or aromatic (Van Eylen et al., 2009; Aires, Carvalho, & Rosa, 2012). The major reactions responsible for the formation of characteristic volatiles in broccoli can be categorized as: (i) enzymatic hydrolyses of glucosinolates and sulfur-containing amino acids ensuing from loss of cellular integrity; (ii) thermal-induced glucosinolate and sulfur-containing amino acid degradation; (iii) unsaturated fatty acid degradation; and (iv) Maillard and its consecutive side reactions (Bones & Rossiter, 2006).

3.1.2. Green pepper

Capsicum is a plant that belongs to the genus *Solanaceae*. The bell pepper is actually the fruit of the *Capsicum* plant. Bell peppers differ from most other members of the genus in that they do not express the 'hot' taste since they contain a recessive gene that eliminates the non-volatile capsaicin in the fruit (Faustino, Barroca, & Guine, 2007). Bell peppers exist in a wide variety of colors, but in this work, focus will be given to the green pepper. Green pepper possesses a

pleasant characteristic aroma that enhances the flavor of many other raw and cooked foods. The major reactions responsible for the formation of characteristic volatiles, in green pepper, can be categorized as: (i) enzymatic hydrolyses of terpenoids and sulfur-containing amino acids following loss of cellular integrity; (ii) thermal-induced terpenoid and sulfur-containing amino acid degradation; (iii) unsaturated fatty acid degradation; and (iv) Maillard reaction together with its consecutive side reactions (Luning, Ebbenhorstseller, Derijk, & Roozen, 1995).

3.1.3. Spinach

Spinach, which is an edible flowering part, is mostly consumed as a slightly cooked vegetable. It is reported with respect to high amount of vitamin A and C, oxalic acid and last but not least an appreciable flavor (Naf & Velluz, 2000). In spinach, the major reactions responsible for the formation of characteristic volatiles could be categorized as: (i) enzymatic hydrolyses of sulfur-containing amino acids ensuing from loss of cellular integrity; (ii) thermal-induced sulfur-containing amino acid degradation; (iii) unsaturated fatty acid degradation; and finally (iv) Maillard reaction together with its consecutive side reactions.

3.2. From chromatogram to data table (data preprocessing)

As discussed in section 2.4, in order to detect wide range of volatiles, an HS-SPME-GC-MS fingerprinting procedure was optimized. Although following this procedure only a certain extract could be analyzed, as more than 100 headspace components (see **Table 1**) were detected (per vegetable), the procedure can be considered as untargeted for that particular extract. A total ion chromatogram of a particular volatile fraction of the reference broccoli, green pepper and spinach obtained by HS-SPME-GC-MS fingerprinting is shown in **Fig. 3**. As described in section 2.5, the complex GC-MS data files were analyzed with a sequence of data preprocessing techniques (i.e. AMDIS and MPP). The number of headspace components, in the three

340 vegetables, selected by each data preprocessing step is exemplified in **Table 1**. The MPP
341 obtained a spreadsheet containing peak areas, which was used as an input for the next statistical
342 analysis.

343
344 **Fig. 3.** Total ion chromatogram of a particular volatile fraction of reference (blanched) (a) broccoli, (b) green pepper and (c)
345 spinach obtained by HS-SPME-GC-MS fingerprinting. The chromatogram contains complex spectra with co-eluting peaks.

3.3. Qualitative classification of impact differences

Per vegetable, the data set obtained using MPP was analyzed with MDA. From the Principal Component Analysis (PCA), no outliers were detected and a clear separation was observed within the reference, thermal and HPHT classes (result not shown). Next, Partial Least Squares Discriminant Analysis (PLS-DA) was implemented. Since adding an additional LV did not improve the separation, two LVs were selected as optimal.

Table 1

Maximum number of headspace components selected by the applied data preprocessing, i.e. AMDIS and MPP, in broccoli, green pepper and spinach.

Vegetable type	HS-SPME-GC-MS fingerprinting	AMDIS	MPP
Broccoli	174	230	52
Green pepper	170	212	48
Spinach	107	132	22

Fig. 4 shows a bi-plot with LV1 and LV2 for broccoli, green pepper and spinach. The X- and Y-variance explained by each LV is indicated in the respective axes. The bi-plots graphically illustrate the relation between classes and also contain information about the importance of each headspace component for the classification. On the bi-plot, classes that are close to each other are considered similar and classes that are far away from each other are considered different. In that context, there is a clear separation between the three classes with two LVs. In broccoli, LV1 describes the classification between the reference and the treated (thermal and HPHT) classes, whereas LV2 classifies thermal from the other two classes. In green pepper, LV1 explains the classification between the reference and the treated classes, whereas, LV2 classifies HPHT from reference and thermal classes. In spinach, LV1 describes the classification between HPHT and the other two classes, whereas, LV2 classifies thermal from the reference and HPHT classes. In addition to illustrating the relation among the classes, depending on the location of the headspace

components on the bi-plot, their importance for classification can be qualitatively represented. For instance, if a component is found between the two ellipses, more than 70 % of its variability is explained with two LVs (inner and outer ellipses in the plot represent correlation coefficients of 70 and 100 %). In addition, the components' importance increases as a function of distance from the center. In other words, components projected far away from the center and close to a certain group of classes or far away at the opposite side of the plot, are respectively highly positively or highly negatively correlated to the corresponding class (Vervoort et al., 2012a).

Fig. 4. A bi-plot based on PLS-DA describing impact comparison between the reference (■), thermal (●) and HPHT (▲) treated broccoli, green pepper and spinach. The open circles represent the headspace components, where only the selected discriminant components (bold open circles) are named (Table 2). The vectors represent the correlation loading for the categorical Y-variables. The X- and Y-variance explained by each LV is indicated in the respective axes.

3.4. *Quantitative selection of discriminant headspace components*

Even though the bi-plot provides graphical illustration of headspace components important for classification, it is not straightforward to identify and rank components which are particularly relevant for a specific class compared to the other classes - in other words to select discriminant components. Therefore, VID coefficients were calculated (section 2.5). The discriminant components selected for each class, per vegetable, are presented in **Table 2**. The retention index of each component is also listed. These components are plotted individually as a function of class and the significant differences among the three classes is tested by means of pair-wise comparisons (result not shown). In this way, the importance of the discriminant components for the classification observed in **Fig. 4** is tested. To clearly show the most important of the selected discriminant components, those with a total peak area difference of $\geq 90\%$, compared to at least one of the other classes, are put in italic. In addition, components detected only in one class, which could be a potential marker of that class, are put in bold italic. Positive VID coefficient indicates a higher concentration of a component in the corresponding class and vice versa. In all the vegetables, components selected in the reference class have a negative VID; whereas, components selected in thermal (except 1-pentanol; in broccoli) and HPHT classes have a positive VID, implicating possible increase in the concentration of existing and/or formation of new components as a result of the treatments. This observation can also be visualized, in **Fig. 4**, as more headspace components are projected towards the treated classes.

3.5. *Interpretation of the identity of the selected discriminant components*

In the following sections, per vegetable, a discussion about the identity of the selected discriminant components and the possible reaction pathways responsible for their formation will be postulated based on research findings.

406 **Table 2**

407 Discriminant headspace components, selected in broccoli, green pepper and spinach based on the VID method, for
 408 each class; reference, thermal and HPHT. The headspace components are listed in a decreasing order of VID
 409 coefficient, where a positive VID coefficient illustrates a higher concentration of a component for that class and vice
 410 versa. The retention index (RI) of components is listed. Components with a total peak area difference of $\geq 90\%$
 411 compared to at least one of the other classes and components selected in only one class were put in italic and bold
 412 italic, respectively.

	VID	BLANCHED IDENTITY	RI	VID	THERMAL PROCESSING IDENTITY	RI	VID	HPHT PROCESSING IDENTITY	RI
BROCCOLI	-0.812	pentanal	749	-0.872	1-pentanol	789			
	-0.846	<i>dimethyl disulfide</i>	775						
	-0.848	<i>dimethyl tetrasulfide</i>	1217						
	-0.860	<i>(Z)-2-heptenal</i>	956						
	-0.861	<i>(E)-2-pentenal</i>	781						
	-0.890	<i>heptanonitrile</i>	980						
	-0.890	<i>carbon disulfide</i>	708						
	-0.943	<i>dimethyl trisulfide</i>	968						
	-0.978	<i>hexanenitrile</i>	881						
	-0.983	<i>benzenepropanenitrile</i>	1243						
	-0.988	<i>4,4-dimethyl-3-oxopentanenitrile</i>	844						
				0.986	<i>3-methylbutanal</i>	733	0.981	<i>(E)-2-octenal</i>	1060
				0.979	<i>2-methylbutanal</i>	736	0.968	<i>(E)-2-hexenal</i>	857
				0.949	<i>borane-dimethylsulfide</i>	705	0.968	<i>1-penten-3-one</i>	745
				0.871	<i>dimethyl disulfide</i>	775	0.967	<i>(Z)-2-heptenal</i>	956
				0.822	<i>2-heptanone</i>	891	0.962	<i>5-(methylthio)-pentanenitrile</i>	1203
				0.805	<i>heptanonitrile</i>	980	0.960	<i>1-octen-3-one</i>	978
							0.956	<i>octanal</i>	1003
							0.942	<i>(E)-2-pentenal</i>	781
							0.902	<i>carbon disulfide</i>	708
							0.866	<i>heptanal</i>	902
							0.843	<i>hexanal</i>	812
							0.803	<i>2-butenal</i>	732
GREEN PEPPER	-0.819	<i>2,5,9-trimethyl-decane</i>	1036						
	-0.819	<i>2-bornene</i>	1232						
	-0.920	<i>linalol</i>	1102						
	-0.932	<i>2-heptanone</i>	891						
	-0.937	<i>d-limonene</i>	1029						
				0.985	<i>benzaldehyde</i>	959	0.920	<i>2,2,6-trimethyl-6-vinyltetrahydropyran (2H-pyran)</i>	971
				0.983	<i>3-methylbutanal</i>	733	0.881	<i>3,3-dimethyl-octane</i>	1010
				0.979	<i>2-octen-4-one</i>	1128	0.862	<i>terpenol</i>	1192
				0.978	<i>2-methylbutanal</i>	763	0.839	<i>2-bornene</i>	1232
				0.978	<i>vinyl hexanoate</i>	1065			
				0.977	<i>2-nonen-4-one</i>	1079			
				0.929	<i>trans-ocimene</i>	1040			
				0.914	<i>borane-dimethylsulfide</i>	705			
				0.902	<i>3-hepten-2-one</i>	936			
				0.893	<i>carbon disulfide</i>	708			
				0.888	<i>2-pentylfuran</i>	992			
				0.887	<i>3-methylfuran</i>	721			
				0.830	<i>tricyclene</i>	920			
				0.821	<i>2-methylpropanal</i>	710			
SPINACH	-0.814	<i>2-methylbutane</i>	717						
	-0.838	<i>2-methylfuran</i>	721						
	-0.887	<i>2,4-dimethyl-1-heptene</i>	846						
	-0.922	<i>pentanal</i>	749						
	-0.945	<i>2,2,4,6,6-pentamethylheptane</i>	989						
				0.976	<i>3-methylbutanal</i>	733	0.986	<i>5-(1,1-dimethylethyl)-1,3-cyclopentadiene</i>	848
				0.940	<i>2-methylbutanal</i>	736	0.979	<i>benzaldehyde</i>	960
				0.927	<i>borane-dimethylsulfide</i>	705	0.967	<i>1,3-dimethylbenzene</i>	871
							0.952	<i>2-methylbutane</i>	717
							0.910	<i>toluene</i>	789
							0.902	<i>2,2,5,5-tetramethyltetrahydrofuran</i>	786
							0.857	<i>octanal</i>	1004
							0.844	<i>1-octanol</i>	7073

3.5.1. Broccoli

From the individual plots (result not shown), it seems that sulfur-containing compounds (i.e. dimethyl disulfide, dimethyl tetrasulfide, carbon disulfide and dimethyl trisulfide) and nitriles (i.e. heptanonitrile, hexanenitrile, benzenepropanenitrile and 4,4-dimethyl-3-oxopentanenitrile) are formed due to the treatments since in the reference samples their concentration is below the detection limit. In (1999), Jin and co-workers also implicated that dimethyl sulphide and methyl (methylthio)methyl disulfide are one of thermal degradation products of sulforaphane, which is an enzymatic hydrolysis product of broccoli glucosinolates. Another study performed by Kubec et al. (1998) indicated that dimethyl disulfide and dimethyl trisulfide are respectively the major and minor thermal degradation products of sulfur-containing amino acids in *Brassica*. Contrary to the above two studies, in the work by Hanschen et al. (2012) where the influence of temperature on the degradation of sulfur-containing aliphatic glucosinolates in broccoli sprout was investigated, nitriles are reported to be the main thermal-induced breakdown products. A comparable observation was also previously reported by MacLeod et al. (1981). Based on these scientific discussions, it can be concluded that the thermal impact in both thermal and HPHT processing induces the degradation of glucosinolates and sulfur-containing amino acids. Consequently, in the treated samples, the formation of sulfur-containing compounds can be linked to the degradation of both glucosinolates and sulfur-containing amino acids, whereas nitriles can be formed due to degradation of glucosinolates.

With respect to the thermal class, 7 components are selected using the VID procedure. Borane-dimethylsulfide, dimethyl disulfide and heptanonitrile are selected with a positive VID. This is logical since, in the above discussion, it was hypothesized that formation of sulfur-containing compounds and nitriles might increase as a function of temperature. Strecker aldehydes, i.e. 3-methylbutanal and 2-methylbutanal, (which are products of Strecker degradation, side reaction of Maillard reaction) are also selected with a positive VID. From the individual plots (result not

shown), the concentration of the Strecker aldehydes is significantly higher in thermal class compared to HPHT class, whereas in the reference class their concentration is below the detection limit. In that context, conventional thermal processing seems to enhance Maillard and its side reactions in comparison to HPHT processing. This observation is in agreement with the report by De Vleeschouwer et al. (2010), where HPHT retarded overall Maillard reaction in comparison to conventional thermal processing in model systems. However, there is a need for increased insight in the way HPHT process parameters affect the formation of these reaction products. The concentration of 2-heptanone, which is a ketone, also appears to be significantly higher in thermal class compared to other classes, which can possibly be linked to thermal-induced unsaturated fatty acid degradation reactions. Different to the above discussions, the concentration of 1-pentanol is significantly lower in thermal class compared to other classes. A possible explanation can be that this alcohol, which can be one of oxidative unsaturated fatty acid degradation products (lipoxygenase pathways), is formed during tissue disruption before blanching and its amount is decreased or is converted to another compound (mainly) during thermal processing only.

Discriminant components selected in HPHT class can be categorized as sulfur-containing compounds (carbon disulfide), nitrile (5-(methylthio)-pentanenitrile), aldehydes ((E)-2-octenal, (E)-2-hexenal, (Z)-2-heptenal, octanal, (E)-2-pentenal, heptanal, hexanal and 2-butenal) and ketones (1-penten-3-one, 1-octen-3-one). As discussed in the introduction, since the research on the impact of HPHT on important chemical reactions is still in its infancy, it is challenging to discuss the identity of components selected in HPHT class and to link them with specific reaction pathways. With respect to sulfur-containing compounds and nitriles, a similar hypothesis provided in the reference class can be used. From the individual plots (result not shown), the concentration of aliphatic aldehydes and ketones is significantly higher in treated classes compared to reference. In addition, among the treated classes, the concentration is

significantly higher in HPHT class. In general, several studies have already implicated that oxidative chemical reactions are enhanced under increased pressure, specifically during the dynamic compression heating phase (Oey et al., 2008b; Verbeyst, Bogaerts, Van der Plancken, Hendrickx, & Van Loey, 2012). For instance, high pressure-induced oxidation of free fatty acids, such as linoleic and linolenic acid, seems to increase the concentration of many aldehyde and ketone volatile compounds, which are important for the green and grass-like notes (Oey, Lille, Van Loey, & Hendrickx, 2008a; Van der Plancken et al., 2012). Therefore, it can be hypothesized that high pressure accelerates (an)aerobic thermal degradation of unsaturated fatty acids. However, there is a need for further understanding of the mechanism and kinetics of these chemical reactions under HPHT treatment.

3.5.2. Green pepper

Headspace components selected in reference class are: (i) terpenoid degradation products (d-limonene, linalool and 2-bornene); (ii) ketone (2-heptanone); and (iii) 2,5,9-trimethyl-decane. From the individual plots (result not shown), the concentration of terpenoid degradation products is significantly higher in thermal and HPHT classes compared to reference. It looks that the treatments enhanced the degradation and release of naturally existing green pepper terpenoids. The concentration of 2-heptanone and 2,5,9-trimethyl-decane appears to be significantly higher in treated classes compared to reference, unsaturated fatty acid degradation reactions which are highly enhanced as a result of the treatments can be the possible reason.

Components selected in thermal class can be categorized as: (i) furanic compounds (2-pentylfuran and 3-methylfuran); (ii) Strecker aldehydes (3-methylbutanal, 2-methylbutanal, 2-methylpropanal and benzaldehyde); (iii) ketones (2-octane-4-one, 2-nonen-4-one and 3-hepten-2-one); (iv) ester (vinyl hexanoate); (v) sulfur-containing compounds (borane-dimethylsulfide and carbon disulfide); and (vi) terpenoid degradation products (tricyclene and trans-ocimene).

487 From the individual plots (results not shown), it is observed that the concentration of furanic
488 compounds and Strecker aldehydes is significantly higher in thermal class compared to HPHT
489 class, whereas in the reference class their concentration is below the detection limit. The furanic
490 compounds can be generated by different degradation reactions and/or recombination of reaction
491 fragments. For instance, in green pepper two major reaction pathways can be identified: (i)
492 Maillard reaction (Cremer & Eichner, 2000; Limacher, Kerler, Davidek, Schmalzried, & Blank,
493 2008); and (ii) recombination of fragments obtained from various precursors such as sugars,
494 amino acids and ascorbic acid (Limacher, Kerler, Conde-Petit, & Blank, 2007). In addition,
495 thermal treatment seems to accelerate one of the major side reactions of Maillard reaction, i.e.
496 Strecker degradation, leading to the formation of Strecker aldehydes. Luning et al. (1995)
497 reported that hot air drying increased the level of 2-methylpropanal, 2- and 3-methylbutanal in
498 different Dutch bell pepper cultivars. A comparative hypothesis was also reported by Aragon et
499 al. (2005) in their investigation of changes in the aromatic fraction of paprika that occurs during
500 traditional drying processes. Taking this hypothesis as a basis, it can be concluded that, in the
501 present work, the conventional thermal processing enhances Maillard reaction and its side
502 reactions in comparison to HPHT processing. A comparable hypothesis was also postulated by
503 De Vleeschouwer et al (2010). Nevertheless, further investigation on the way that HPHT
504 processing controls these chemical reactions is vital. From the individual plots (results not
505 shown), 2-nonen-4-one and 3-hepten-2-one appear to be significantly higher in thermal class
506 compared to HPHT class. Luning et al. (1995) also implicated that the concentration of 4-octen-
507 3-one increased during hot air bell peppers drying. In that context, it can be concluded that, in
508 the present work, thermal treatment accelerates the formation of these possible unsaturated fatty
509 acid degradation products compared to HPHT. The increased formation of vinyl hexanoate can
510 also be linked to the same reaction pathway. Borane-dimethylsulfide and carbon disulfide also
511 show to be significantly higher in thermal class compared to HPHT class. Since sulfur-

containing amino acids are naturally present in green peppers, thermal-induced degradation of these amino acids can be the possible explanation for increased formation of these sulfur-containing volatiles. With respect to terpenoid degradation products, a similar hypothesis provided for the reference class can be used.

Components selected in HPHT class are: 2,2,6-trimethyl-6-vinyltetrahydropyran; 3,3-dimethyloctane; terpenol and 2-bornene. In literature, no paper can be found with respect to the effect of HPHT on chemical reaction in green pepper or even in general in bell peppers. Therefore, in the present work, it is challenging to discuss the identity and the possible reaction pathway for components selected in HPHT class. 2,2,6-trimethyl-6-vinyltetrahydropyran (2H-pyran), which is a monocyclic monoterpene, is of special interest, because it is only detected in HPHT class, which can make it an excellent marker for the type of processing. Since an equivalent temperature history was applied for thermal and HPHT treatments, the extra processing variable, i.e. high pressure, in HPHT processing can be the reason for the formation of this cyclic aromatic compound. Nevertheless, there is a need for further research investigating the origin, possible reaction pathways and the effect of this compound from quality and safety point of view. The concentration of 3,3-dimethyloctane is significantly higher in HPHT class compared to thermal class and below the detection limit in the reference class. During lipid autocatalytic degradation, the primary degradation products (hydroperoxides) will further decompose to subsequent degradation products where hydrocarbons are one of those. Therefore, increased unsaturated fatty acid degradation reactions during HPHT can be a possible reaction pathway. With respect to terpenoid degradation products a similar hypothesis provided for the reference class can be used.

534 3.5.3. Spinach

535 Components selected in reference class are 2-methylbutane; 2-methylfuran; 2,4-dimethyl-1-
536 heptene; pentanal and 2,2,4,6,6-pentamethylheptane. Based on the individual plots (result not
537 shown), it looks that 2-methylbutane and 2-methylfuran are formed due to the treatments since in
538 the reference samples their concentration is below the detection limit. The concentration of 2,4-
539 dimethyl-1-heptene; pentanal and 2,2,4,6,6-pentamethylheptane is significantly higher in thermal
540 and HPHT treated samples compared to the reference. Both phenomena can be explained as
541 outcomes of thermal-induced chemical reactions.

542 Components selected in thermal class are: 3-methylbutanal, 2-methylbutanal and borane-
543 dimethylsulfide. 3-methylbutanal and 2-methylbutanal (Strecker aldehydes), seem to be formed
544 as a result of thermal and HPHT treatments, since in the reference class their concentration is
545 below the detection limit, whereas among the treated classes, their concentration is significantly
546 higher in thermal class. Borane-dimethylsulfide is formed in all classes, but the concentration
547 appears to be significantly higher in thermal class compared to other classes. A comparable
548 observation was reported by Masanetz et al. (1998) when evaluating the highly volatile potent
549 odorants on raw, boiled and dried spinach samples. They reported that 3-methylbutanal and 2-
550 methylbutanal significantly contributed to the flavor of dried spinach and also observed a strong
551 increase in the concentration of sulfur-containing compounds, such as dimethyl sulphide,
552 following boiling and drying. Therefore, in the present work, the conventional thermal
553 processing, compared to HPHT processing, seems to accelerate one of the major side reactions
554 of Maillard reaction, i.e. Strecker degradation, leading to the formation of Strecker aldehydes. In
555 addition, it can also be hypothesized that thermal-induced degradation of sulfur-containing
556 amino acids possibly increases formation of sulfur-containing volatiles in thermal class.

557 Components selected in the HPHT class are: 5-(1,1-dimethylethyl)-1,3-cyclopentadiene;
558 benzaldehyde; 1,3-dimethylbenzene; 2-methylbutane; toluene; 2,2,5,5-

tetramethyltetrahydrofuran; octanal and 1-octanol. Considering the fact that the knowledge on the impact of HPHT on chemical reaction is still limited, it is challenging to describe the identity of the components selected in HPHT class and to link them with specific reaction pathways. Octanal and 1-octanol are of special interest, because they are detected only in HPHT class, which could make them an excellent marker for the processing. The formation of these aromatic aldehydes and alcohols can be linked to HPHT-induced unsaturated fatty acid degradation reactions. This observation is in agreement with reports that high pressure enhances oxidation of free fatty acids leading to increased concentration of aldehyde and ketone volatile compounds (Oey et al., 2008a; Van der Plancken et al., 2012). The increased formation of 2-methylbutane and benzaldehyde can also be linked to the same reaction pathway. The concentration of 1,3-dimethylbenzene; 2-methylbutane; toluene; 2,2,5,5-tetramethyltetrahydrofuran and 5-(1,1-dimethylethyl)-1,3-cyclopentadiene is significantly higher in HPHT class compared to thermal class. There is a need for further investigation on the origin, possible reaction pathways of these headspace components and their effect from safety and quality point of view.

4. CONCLUSION

In the present work, the effectiveness of a comprehensive integration between MS-based chemical fingerprinting, advanced data preprocessing and statistical data analysis to identify and compare different process-induced chemical changes is demonstrated. Aiming for a fair comparison, three industrially relevant, low-acidic, vegetables, i.e. broccoli, green pepper and spinach, were treated with conventional thermal and HPHT processing technologies targeting an equivalent microbial safety.

Thermal processing, compared to HPHT, seems to enhance Maillard and Strecker degradation reactions, triggering formation of furanic compounds (2-pentylfuran and 3-methylfuran in green pepper and 2-methylfuran in spinach) and Strecker aldehydes (e.g. 3-methylbutanal, 2-

583 methylbutanal in all vegetables). In most cases, high pressure seems to accelerate (an)aerobic
584 thermal degradation of unsaturated fatty acids leading to the formation of aliphatic aldehydes and
585 ketones. In addition, both thermal and HPHT processing accelerated formation of sulfur-
586 containing compounds. 2,2,6-trimethyl-6-vinyltetrahydropyran (2H-pyran) (in green pepper) and
587 octanal and 1-octanol (in spinach) are of special interest, because they are detected only in
588 HPHT class, which can make them a potential marker for the type of processing.

589 Next to comparison of the treatment impact per vegetable, it was questioned if any trend on the
590 process-induced chemical reactions over all selected vegetables could be detected. In that case,
591 two components are selected in thermal class, i.e. 2 and 3-methylbutanal. It is noteworthy that
592 these headspace components are consistently selected, in all three vegetables, in thermal class
593 compared to other classes. The fact that these components also discriminate between the three
594 treatment classes in a comprehensive assessment of the vegetables combined confirms their
595 importance.

596 Even though the approach followed in this work provides a broader perspective into complex
597 chemical reactions, in order to fully understand the effect of HPHT on safety and quality of food
598 products, further attention should be given to the following aspects: (i) identity confirmation
599 (using a pure standard sample) and quantification of the selected discriminant components; (ii)
600 characterizing headspace components with respect to nutritional value, metabolism and level of
601 undesirable substances (i.e. toxicity); (iii) detailed kinetic study, in order to increase insight to
602 the potential of a process parameter to control a particular process-induced reaction; (iv)
603 chemical changes during post-treatment storage; and finally (v) impact on different extract of the
604 volatile fraction or on other fraction (e.g. liquid fraction) of the same or in different matrices.

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