Effect of sodium chloride concentration on off-flavor removal correlated to glucosinolate degradation and red radish anthocyanin stability

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Abstract Anthocyanin-rich concentrates from different red radish can be used as natural food colorants. However, the development of off-flavor during extraction has been major challenge in processing industries. This work aimed to evaluate the effect of sodium chloride (NaCl) concentration in phosphoric acidified medium pH 2.5 on removal of off-flavor from red radish anthocyanin. The effect of NaCl concentration on anthocyanin properties was also evaluated. Results showed that the total glucosinolate was highly degraded at high NaCl concentration (< 500 mM) compared with control, leading to higher off-flavor development. Additionally, the glucosinolate degradation was positively and significantly correlated to isothiocyanate, while was negatively and significantly correlated with dimethyl di-, trisulfide, cedrol, triacetin, 6-methyl-5-hepten-2-one. Moreover, total monomeric and color properties of extracted anthocyanins were degraded at high NaCl concentration (< 500 mM) compared with control. The tentative anthocyanin identification by UPLC–TQ–MS showed 12 glycosylated anthocyanins substituted at C3 and C5 in tested anthocyanin extracts. In conclusion, higher NaCl concentration (< 500 mM) could not be useful for red radish off-flavor removal and anthocyanin properties.

Keywords Red radish · Sodium chloride · Glucosinolate degradation · Monomeric anthocyanins · Off-flavor formation

Introduction

Anthocyanins are a sub-class of secondary plant metabolites belonging to the flavonoids. They are water-soluble pigments imparting bright colors from red to blue hues to all kinds of plant tissue including flowers, fruits, leaves, roots, and stems (Müller-Maatsch et al. 2016). During the last decade, the restrictions in the use of synthetic colorants in foods have generated interest in the potential use of natural anthocyanins as food color additive in beverages, syrups, fruit juices, jellies, jams, ice-creams, pastries, candies and yoghurts, as well as in tooth paste, pharmaceuticals, cosmetics and similar products (Durante et al. 1995).

Red radish (*Raphanus sativus* L.) cultivars, belonging to the Brassicaceae family, also known as cruciferous vegetables are cultivated in many countries, especially in China. Red radish anthocyanins are considered medicinal, nutritious and a potential economic source of natural food coloring (Xu et al. 2015). However, its use as natural food colorant has drawbacks due to the development of off-flavor. The off-flavors are result of the degradation of glucosinolates by myrosinase during harvesting, cooking, chewing, processing and storage (Force et al. 2007). Glucosinolates are sulphur-containing compounds in cruciferous vegetables. They are hydrolyzed under moist conditions by the coexisting endogenous enzyme myrosinase (Thioglucoside glucohydrolase, EC 3.2.3.1) to a variety of compounds (isothiocyanates, nitriles, thiocyanates, epithionitriles, oxazolidines), depending on pH, metal ions and other protein elements. These products are

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generally responsible for the odor (off-flavor) and taste of red radish anthocyanins (Bones and Rossiter 2006).

During the last decade, many efforts have been made in order to improve the quality of red radish anthocyanin during extraction. Gao et al. (2014) found that the use of adsorptive chitosan could reduce glucosinolate content resulting in the removal of off flavor from red radish. Kottman extracted color from premium radish skin by abrasive action or steam then treated the extracted colored juice by filtration, evaporation, salt and acid addition followed by heat. The results of sensory evaluation showed that samples extracted with salt, acid or steam had lower aroma intensity but with lower pigment yields (Kottman 2011). Chen et al. (2016) reported that the lower phosphoric acidified hexane (pH 2.5) could significantly reduce the red radish anthocyanin off flavor and improve the anthocyanin content compared with lower citric acidified hexane (pH 2.5) and water extracts.

Goto et al. (1976) and Dangles and Brouillard (1992) concluded that a decrease in water activity due to the anion solvation will hinder the hydration of the flavylium form, thus displacing the equilibrium toward, with the consequent gain of color. Therefore, suggested the use of salt solutions for the extraction of pigments from plant material. Figueiredo and Pina (1994) reported that ionic salts enhanced the color through the formation of an ion-pair between the charged flavylium cation and the anion, which displaces the equilibria towards the colored flavylium form. On contrary, Hubbermann et al. (2006) reported that the color stability of anthocyanin decreased with increasing salt concentration due to the altered solvation characteristics of aqueous solutions with exception to the studies on effect of salting stress (NaCl) on anthocyanins content and glucosinolate-myrosinase system at growth stages (Guo et al. 2013; Pang et al. 2012; Yuan et al. 2010), there are no detailed work about the effect of salts concentration (NaCl) on red radish anthocyanin off-flavor removal and its anthocyanin properties during processing. Therefore, our goal was to study the effect of different NaCl concentration on red radish anthocyanin off-flavor removal and its anthocyanin characteristics.

Materials and methods

Red Radish were harvested in earlier November, 2016 from a farm in Chongqing province and kept frozen at -40°C prior to their use. Food grade citric acid and phosphoric acid were purchased from Sigma (Shanghai, China). Dimethyl disulfide, tetrahydrothiophene, dimethyl trisulfide, methyl isothiocyanate, 3-buten-1-yl-isothiocyanate, 3-(methylthio) propyl isothiocyanate, 4-(methylthio)-3-butenyl isothiocyanate, 2,4-dimethyl thiazole, nonanal,

1-octanol, naphthalene, furfuryl methyl sulfide and benzaldehyde, all authentic internal standards were purchased from ANPEL Laboratory Technology (Shanghai) Inc. 1,2-dichloro-benzene internal standard was purchased from Sigma (Shanghai, China). The n-alkane standard (C7–C30) was provided by Sigma-Aldrich Chemical Co. (St. Louis, MO). Analytical grade ethanol 99.7% (v/v) was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). All other reagents used in this study were of analytical grade.

Red radish anthocyanin extraction

Twenty-five grams of fresh frozen red radish were immersed in 100 mL in phosphoric acidified water at pH 2.5, the samples were homogenized in fruit juicer (Joyoung JYL-D020, Joyoung Co., Ltd, China) into radish juice according to Chen et al. (2016). Appropriate amount of NaCl was added to the juice to make the final mixture concentration to 100 mM, 300 mM, 500 mM, 1000 mM and 2000 mM. The mixtures were placed into double wall reactor glasses and heated at 50°C for 2 h for pigment extraction, then cooled at room temperature in ice water bath. After extraction, the mixture was passed on filter paper (Whatman no. 1) and stored at 4°C prior to further analysis. Red radish pigment extracted in phosphoric acidified water pH 2.5 without NaCl was used as control.

Determination of total glucosinolate

Extraction of glucosinolate (GSL)

Glucosinolate was extracted according to the method described in previous study (Chen et al. 2016). Fifty milligrams of lyophilized red radish anthocyanin extracts powder were placed in glass tubes, and each test tube was pre-incubated in a hot water bath at 75°C for 1 min. After cooling, 4.8 mL of 80% methanol and the GSLs extraction was carried out. The tubes were kept at 25°C for 30 min and then shaken reciprocally (120 r/min) for 30 min in a shaker. The tubes were centrifuged at $1600\times g$ for 10 min. The supernatant was used as a crude extract.

Palladium colorimetric analysis of the total glucosinolate content

Colorimetric analysis of the total GSL content was performed by simplifying the method described by Møller et al. (1985). Purification with ion-exchange chromatography was omitted. To 0.2 mL of crude GSL extract, 0.3 mL of distilled water and 3 mL of 2 mM palladium (II) chloride were added and mixed. After incubation at 25°C for 1 h, absorbance at 452 nm was measured using a

spectrophotometer A360 (AOE Instrument (Shanghai) CO., Ltd, China). Absorbance was shown by an average of three measurements. After subtraction of value of a blank, as obtained using 0.2 mL of distilled water as a sample, differences of absorbance were used for estimation of total GSL contents according to the linear regression equation of sinigrin (standard) to absorbance $y = 2.636x + 0.023$ ($R^2 = 0.995$) and the concentration was estimated as μmol sinigrin per g dry weight (DW).

Evaluation of off-flavor removal

Headspace solid-phase microextraction (HS-SPME)

The volatile compounds of red radish extracts before and after n-hexane treatment were investigated by headspace solid-space microextraction (HS-SPME) combined with gas chromatography–mass spectrometry according to previous study (Chen et al. 2016). The SPME device for manual extraction included a holder assembly with a 50/30- μm divinyl-benzene/carboxen/polydimethylsiloxane (DVB/CAR-PDMS) fibre (Supelco, Madrid, Spain). The fibre was equilibrated at 250 °C for 30 min prior to use as recommended by the manufacturer. Radish extracts (5 mL) were transferred into vials (15 mL) and 50 μL of internal standard were added. A proper mixing of the solutions during the SPME was achieved with a magnetic stirrer. The syringe assembly unit was lowered into the vial with the fibre suspended in the headspace above the liquid layer of extracts for 30 min at 40 °C. Then, the fibre was immediately retracted back into the needle and transferred immediately to the injection port of gas chromatography. A time period of 8 min was adopted for desorption and conditioning at the desorption temperature of 250 °C.

Gas chromatography/mass spectrometry (GC/MS)

The GC–MS was performed with an SCION-SQ-456-GC–MS, (Bruker Daltonics., Billerica, MA, USA). An injector in a splitless mode was maintained at 250 °C. The separation was achieved on a DB-Wax fused silica capillary (50 m * 0.32 mm, 1 μm film thickness, from J&W Scientific, Folsom, CA, USA). Helium was used as the carrier gas at a flowrate of 1.0 mL/min. The inlet temperature was 250 °C. The column temperature was initially maintained at 40 °C for 1 min, increased at the rate of 5 °C per min to 200 °C, maintained at 200 °C for 5 min, increased at the rate of 10 °C per min to a final temperature of 240 °C and maintained at 240 °C for 5 min. The MSD transfer-line and ion source temperatures were 230 and 250 °C, respectively, with an electron-impact ionization potential of 70 eV. A mass range from m/z 35–400 was recorded in a

full scan mode. Data was collected and processed using a HP-Chem station system.

Identification and quantitative determination of components

Individual peaks from GC were identified by comparing their linear retention indices (LRI) and mass spectra (MS) with spectra in our homemade spectral library, as well as by computer matching against the Wiley 275-library spectra database, NIST Mass Spectral Search Program (National Institute of Standards and Technology, Washington, DC, USA) and comparison of the mass spectra with literature data (Blažević and Mastelić 2009; Chen et al. 2016). Spectra and LRI values of sulfides and isothiocyanates were compared with those of authentic compounds obtained commercially.

Determination of linear retention indices (LRI)

A standard mixture of *n*-alkane (C6–C20) in ethanol was analyzed each day before GC runs to allow a check of the instrument performance and the calculation of retention indices of each component in the samples. The standard (1.0 μL) was injected to the trap and the solvent was removed by purging with oxygen-free nitrogen (40 mL min^{−1}) for 5 min. These alkanes were used as external standard references in LRI calculations. LRI of each compound was calculated from the standard alkane retention time and the peak retention time using the following formula:

$$LRI = \frac{(RT_x - RT_n + n)}{(RT_{n+1} - RT_n)} \times 100$$

where: LRI = linear retention index, RT_x = retention time of compound, RT_n = retention time of *n*-alkane before peak, RT_{n+1} = retention time of *n*-alkane after peak, *n* = carbon number of *n*-alkane before peak.

The standard curve of each authentic volatile chemical was constructed using six different concentrations, which were contained the concentration of volatile compounds. The standard volatiles for making standard curves were a mixture of different concentrations of authentic materials in ethanol and they were diluted to different concentration using Ultra- pure water. A plot of authentic internal standard concentration (mg/L), against the peak area was linear, with R^2 value > 0.9. Each sample was analyzed in triplicate and the concentration was calculated following below formula:

$$C_x = \left(\frac{A_x}{A_i} - b \right) \div a \times c_i \text{ mg/L}$$

where C_x is the amount of each volatile compound; A_x is the area of the peak of each volatile compound; A_i is the area of the peak of the internal standard; C_i is the concentration of the internal standard (mg/L); a and b were the slope factor and intercept in the standard curves of each volatile compound standard.

Anthocyanins identification

Prior to Ultra Pressure Liquid Chromatography Triple Quadruple Mass Spectrometry (HPLC–MS) analysis, anthocyanin containing extracts were passed through a (1 g, 6 mL) LC-C18 SEP cartridge (ANPEL Laboratory Technology, Shanghai Inc.), previously activated with 3.8 mL of methanol followed by 4.8 mL of acidified water (0.5% TFA) through the sorbent bed (Wentian et al. 2016). Anthocyanins and other phenolics were adsorbed onto the C-18 cartridge, and sugars, acids and other water-soluble compounds were eluted with 20 mL of acidified water (0.5%, v/v, TFA). The non-anthocyanin phenolics were eluted from the cartridge using 20 mL of ethyl acetate, and anthocyanins were subsequently eluted with 40 mL of acidified methanol (0.5% v/v, TFA). The methanolic extracts were then concentrated using a vacuum rotary evaporator at 40 °C.

Mass spectrometry (MS) analysis was performed on UPLC-TQ-MS (Waters, Tokyo, Japan). Mass spectrometry was performed with a Waters TQD system for data collection. Compounds were analyzed under the positive ion (PI) mode with electrospray ionization (ESI) source. The major MS parameters were as follows: mass range 100–1000 m/z, ion source temperature 200 °C, heated block temperature 200 °C, nebulization gas flow 1.5 L/min, detector voltage 1.75 kV. Data acquisition and processing were conducted using the LCMS Solution software (Mass Lynx V4.1, Waters). Anthocyanins were identified by comparing the mass data with published data (Jing et al. 2012).

Determination of total anthocyanin content

Total anthocyanin content was determined using the pH-differential method according to our previous study Chen et al. (2016), using two buffer systems: potassium chloride buffer, pH 1.0 (0.025 M), and sodium acetate buffer, pH 4.5 (0.4 M). A 0.1 mL aliquot of the pigment extract was transferred to a 10 mL volumetric flask and filled with corresponding buffer, and the absorbance was measured at 520 and 700 nm. The dilution factor (DF) was determined by diluting each extract in 0.025 M potassium chloride (Fisher Scientific, Fair Lawn, NJ) buffer at pH 1.0, until the absorbance was within the appropriate spectrophotometer

range; greater than 0.1 and less than 1.2. Total anthocyanins were calculated as cyanidin-3-glucoside according to the following equation:

$$\text{Total anthocyanins (mg/100 g FW)} \\ = A \times M_w \times DF \times 1,000 / (\epsilon \times 1),$$

where A (absorbance) = $(A_{510} - A_{700})_{\text{pH}1.0} - (A_{510} - A_{700})_{\text{pH}4.5}$; M_w and ϵ were calculated as pelargonidin-3-glucoside, where the $M_w = 433.2$ g with an extinction coefficient of $31,600 \text{ cm}^{-1} \text{ mg}^{-1}$; DF = dilution factor; 1 = path length in cm; 1000 = conversion from g to mg. all analyses were done in triplicate ($n = 3$).

Determination of color density and percentage polymeric color of anthocyanins

A bisulfate bleaching method used to determine percentage polymeric color of extracted red radish anthocyanins (Chen et al. 2016). 0.2 mL of 20% $\text{K}_2\text{O}_5\text{S}_2$ and 0.2 mL of distilled water were added to two different 2.8 mL samples of extracted anthocyanins separately to make bleached and unbleached samples. Then, spectrophotometer A360 (AOE Instrument (Shanghai) CO., Ltd, China) was used to read and record the absorbance of bleached and unbleached samples of anthocyanin at the λ_{max} (512 nm), 420 nm and at 700 nm (for correction of turbidity). Percentage polymeric anthocyanins of red radish were calculated using following equations:

$$\text{Color density sample} = (\lambda_{512\text{nm}} - A_{700\text{nm}}) \\ + (A_{420\text{nm}} - A_{700\text{nm}}) \times 10$$

$$\% \text{ Polymeric color} = (\text{polymeric color/color density}) \\ \times 100$$

Determination of color properties

Color was evaluated with a WSC-S color difference meter (Shanghai, China). For color measurements of radish red pigment in aqueous system, 2 mL of samples were placed in 2.5 cm diameter test tube for measurement (Wentian et al. 2016). The CIELAB parameters (L^* , a^* , b^* , C , h) were determined. The ΔE , C , h representing the total color differences between the L^* , a^* , and b^* , chroma and hue angle of the sample respectively was shown as followings

$$\Delta E = \left((\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2 \right)^{1/2}$$

$$C = \left((a^*)^2 + (b^*)^2 \right)^{1/2}$$

$$h = \tan^{-1}(b^*/a^*)$$

Statistical analysis

Data from the descriptive analysis was evaluated by analysis of variance (ANOVA) using SPSS V.24 (IBM, Armonk, NY, USA). Analysis of variance was used to compare means with Tukey multiple range tests for post hoc analysis. $P \leq 0.05$, $P \leq 0.001$ or $P \leq 0.01$ was considered significant. Partial least squares regression (PLSR) analysis was performed using the Unscrambler X version 10.4 (CAMO ASA, Oslo, Norway). PLSR was performed with GC–MS as the X-matrix and glucosinolate degradation as the Y-matrix. The correlations between flavor compounds (GC–MS data) and glucosinolate degradation were analyzed by PLS1, and PLS2 was applied to illustrate correlations among flavor compounds and glucosinolate degradation datasets. Data reported in this work were the means of duplicate (GC–MS) or triplicate (anthocyanin yield and color change) experiments.

Results and discussions

Effect of NaCl concentration on total glucosinolate degradation

Glucosinolates degradation and turnover are carried out by the activity of myrosinases (Chen et al. 2016). As shown in Fig. 1, total glucosinolate significantly degraded with increasing NaCl concentration. Total glucosinolate was degraded by 44.83% at 2000 mM, NaCl concentration compared to the control; indicating that myrosinase activity could not be inhibited at higher NaCl concentration. Del Carmen Martínez-Ballesta et al. (2013) reported that the

Fig. 1 Total glucosinolate degradation as affected by NaCl concentration. Values represent the means of three replicate samples with standard error of the means

increase in glucosinolates degradation was a consequence of membrane damage by salt stress indicated by high relative electrolyte leakage. Pang et al. (2012) also reported that NaCl treatment caused a significant variation in the patterns of aliphatic, aromatic, and indole glucosinolate concentrations, resulting in varying levels of total glucosinolates in different organs at three different developmental stages.

Effect of NaCl concentration on red radish anthocyanin off-flavor

The effect of NaCl concentration on the content of off-flavor was assessed by HS-SPME combined with GC–MS. Headspace analysis is one of the options for instrumental determination of volatile compounds in a sample as the headspace contains all the volatiles that are responsible for the odor sensation. As shown in Table 1, a total of 57 volatile compounds were identified and grouped according to their chemical structure: 6 sulfur containing compounds, 4 isothiocyanates, 4 nitriles, 10 aldehydes, 15 alcohols, 1 carboxylic acid, 4 esters, 4 ketones, 2 terpenes and 8 others.

The content of sulfur containing compounds increased with increasing NaCl concentration compared to the control. Dimethyl sulfide, tetrahydrothiophene, dimethyl trisulfide and dimethyl sulfoxide were identified as the major sulfur containing compounds (Table 1). The increase in dimethyl disulfide and dimethyl trisulfide may be derived from subsequent degradation of some volatiles derived from glucosinolates via oxidation of methanethiol (Blažević and Mastelić 2009). Dimethyl disulphide and dimethyl trisulphide are derived from (+)-*S*-methyl-L-cysteine sulfoxide, an amino acid found in Brassica. Tetrahydrothiophene was identified in *Eruca sativa* rocket salad from Austria with *Allium*- and cabbage-like odor characteristic (Jirovetz et al. 2002). Dimethyl sulfoxide was previously identified in commercial blanched peas, and it might be formed via the catalytic autoxidation of dimethyl sulfide (Ralls et al. 1965). The higher concentration of the sulfur containing compounds might increase the off-flavor characteristic of red radish pigment extracts due to their odor characteristic and lower odor threshold.

As shown in Table 1, four isothiocyanates were identified including two methylthio-substituted isothiocyanates (3-(methylthio)-propyl-isothiocyanate and 4-(methylthio)-3-butenyl isothiocyanate) and two alkenyl substituted isothiocyanates (methyl isothiocyanate, 3-butenyl isothiocyanate). The isothiocyanates content decreased with increasing NaCl concentration compared to the control. These results are in agreement with the findings of glucosinolate degradation. 4-(methylthio)-3-butenyl isothiocyanate was identified as the pungent principle of radishes and it might probably be produced from 4-(methylthio)-3-

Table 1 Effect of NaCl concentrations on red radish off flavor characteristics (mg/L)

RI	Sulfur containing compounds	Code	Control	100 mM	300 mM	500 mM	1000 mM	2000 mM	ID
1028	Dimethyl disulfide	S1	0.993 ± 0.00	8.380 ± 0.00	5.783 ± 0.00	1.636 ± 0.00	10.764 ± 0.00	12.895 ± 0.00	MS, LRI, Std
1071	Tetrahydrothiophene	S2	5.858 ± 0.00	2.946 ± 0.00	4.113 ± 0.01	6.062 ± 0.00	8.502 ± 0.00	10.674 ± 0.00	MS, LRI, Std
1353	Dimethyl trisulfide	S3	0.082 ± 0.00	0.864 ± 0.00	0.469 ± 0.00	0.164 ± 0.00	0.928 ± 0.00	1.035 ± 0.01	MS, LRI, Std
1578	Dimethyl sulfoxide	S4	0.011 ± 0.00	0.037 ± 0.00	0.015 ± 0.00	0.019 ± 0.00	0.055 ± 0.00	0.110 ± 0.00	MS, LRI
1660	S-methyl 2-thiofuroate	S5	0.089 ± 0.00	0.545 ± 0.00	ND	ND	ND	1.025 ± 0.00	MS, LRI
1887	S-methyl methanethiosulphonate	S6	ND	ND	0.503 ± 0.00	ND	ND	ND	MS, LRI
			7.033	12.772	10.883	7.881	20.249	25.739	
<i>Isothiocyanates</i>									
1229	Methyl isothiocyanate	T1	3.468 ± 0.00	0.000 ± 0.00	2.005 ± 0.01	3.280 ± 0.03	0.889 ± 0.00	0.565 ± 0.01	MS, LRI, Std
1433	3-Butenyl isothiocyanate	T2	1.243 ± 0.01	0.609 ± 0.00	0.787 ± 0.00	0.951 ± 0.00	0.475 ± 0.00	0.176 ± 0.00	MS, LRI, Std
1894	3-(Methylthio)propyl isothiocyanate	T3	1.785 ± 0.00	1.420 ± 0.00	1.506 ± 0.00	1.603 ± 0.00	0.941 ± 0.00	0.855 ± 0.00	MS, LRI, Std
1993	4-(Methylthio)-3-butenyl isothiocyanate	T4	1.521 ± 0.00	1.049 ± 0.00	1.150 ± 0.00	1.381 ± 0.01	0.798 ± 0.00	0.619 ± 0.00	MS, LRI, Std
			8.017	3.078	5.448	7.215	3.103	2.215	
<i>Nitriles</i>									
1170	Pyridine	N1	0.108 ± 0.00	0.151 ± 0.00	0.112 ± 0.00	0.129 ± 0.00	0.181 ± 0.00	0.265 ± 0.00	MS, LRI
1492	2,3-Diethyl-5-methylpyrazine	N2	0.027 ± 0.01	ND	ND	ND	ND	ND	MS, LRI
1881	Benzothiazole	N3	0.053 ± 0.00	0.100 ± 0.00	0.063 ± 0.00	0.051 ± 0.00	0.146 ± 0.00	0.000	MS, LRI
1890	Tetrahydro-2-thiopheneacetoneitrile	N4	0.051 ± 0.00	0.364 ± 0.00	0.149 ± 0.00	0.120 ± 0.00	0.539 ± 0.00	1.989 ± 0.01	MS, LRI
			0.239	0.615	0.324	0.300	0.866	2.254	
			15.289	16.465	16.655	15.396	24.218	30.208	
<i>Aldehydes</i>									
1137	Heptanal	AL1	0.268 ± 0.00	0.753 ± 0.00	ND	ND	1.523 ± 0.00	2.064 ± 0.00	MS, LRI
1253	Octanal	AL2	0.248 ± 0.00	ND	ND	0.423 ± 0.00	2.585 ± 0.01	ND	MS, LRI
1288	(E)-2-heptenal	AL3	0.782 ± 0.00	0.642 ± 0.00	ND	ND	0.443 ± 0.00	0.159 ± 0.00	MS, LRI
1384	Nonanal	AL4	0.263 ± 0.00	0.906 ± 0.00	1.115 ± 0.00	0.765 ± 0.00	0.590 ± 0.00	1.489 ± 0.00	MS, LRI, Std
1413	2-Octenal	AL5	ND	ND	0.032 ± 0.00	ND	0.211 ± 0.00	ND	MS, LRI
1497	Decanal	AL6	ND	ND	0.584 ± 0.00	0.267 ± 0.00	0.463 ± 0.01	ND	MS, LRI
1519	Benzaldehyde	AL7	ND	ND	0.359 ± 0.00	0.326 ± 0.00	0.918 ± 0.01	ND	MS, LRI, Std
1535	(Z)-6-nonenal	AL8	ND	ND	0.179 ± 0.00	0.051 ± 0.00	0.251 ± 0.00	ND	MS, LRI
1590	(E,Z)-2,6-nonadienal	AL9	ND	ND	0.067 ± 0.00	0.069 ± 0.00	ND	ND	MS, LRI
1793	2,4-Dimethyl benzaldehyde	AL10	0.431 ± 0.00	ND	2.904 ± 0.00	2.340 ± 0.01	4.027 ± 0.01	ND	MS, LRI
			1.992	2.300	5.238	4.240	11.009	3.712	
<i>Alcohol</i>									
1144	1-Penten-3-ol	OL1	0.034 ± 0.00	ND	ND	ND	0.076 ± 0.00	0.130 ± 0.00	MS, LRI
1173	2,4-Hexadien-1-ol	OL2	0.067 ± 0.00	0.093 ± 0.00	ND	ND	ND	0.180 ± 0.00	MS, LRI
1295	(Z)-2-penten-1-ol	OL3	ND	ND	ND	0.003 ± 0.00	0.092 ± 0.00	ND	MS, LRI

Table 1 continued

RI	Sulfur containing compounds	Code	Control	100 mM	300 mM	500 mM	1000 mM	2000 mM	ID
1337	Hexanol	OL4	0.058 ± 0.00	ND	0.072 ± 0.00	0.052 ± 0.00	0.199 ± 0.00	ND	MS, LRI
1439	(E)-2-undecen-1-ol	OL5	0.989 ± 0.00	ND	ND	ND	0.203 ± 0.00	ND	MS, LRI
1488	2-Ethyl-1-hexanol	OL6	ND	ND	0.647 ± 0.00	0.500 ± 0.00	0.622 ± 0.00	ND	MS, LRI
1494	Decanol	OL7	ND	ND	0.475 ± 0.00	ND	ND	ND	MS, LRI
1516	L-Camphor	OL8	0.026 ± 0.00	0.158 ± 0.00	0.083 ± 0.00	0.057 ± 0.00	0.090 ± 0.00	0.610 ± 0.00	MS, LRI
1550	Linalool	OL9	0.096 ± 0.00	0.157 ± 0.00	0.248 ± 0.00	0.205 ± 0.00	0.384 ± 0.00	0.504 ± 0.00	MS, LRI
1563	1-Octanol	OL10	0.033 ± 0.00	0.193 ± 0.00	0.268 ± 0.00	0.204 ± 0.00	0.506 ± 0.00	0.285 ± 0.00	MS, LRI, Std
1615	2-Methyl-hepta-1,6-dien-3-ol	OL11	0.364 ± 0.00	0.026 ± 0.00	0.023 ± 0.00	ND	ND	0.270 ± 0.00	MS, LRI
1702	(-)-Beta-fenchol	OL12	0.620 ± 0.00	1.004 ± 0.00	ND	ND	ND	0.998 ± 0.00	MS, LRI
1900	Phenol	OL13	ND	ND	0.039 ± 0.00	0.016 ± 0.00	0.030 ± 0.00	ND	MS, LRI
1960	Cedrol	OL14	0.5645 ± 0.00	9.032 ± 0.01	6.368 ± 0.01	3.783 ± 0.00	9.833 ± 0.00	11.302 ± 0.01	MS, LRI
2027	2,4-Di- <i>tert</i> -butyl phenol	OL15	0.544 ± 0.00	0.754 ± 0.00	0.626 ± 0.00	0.559 ± 0.00	1.980 ± 0.00	2.202 ± 0.00	MS, LRI
			3.394	11.416	8.848	5.378	14.013	16.480	
<i>Carboxylic acids</i>									
1448	Acetic acid	AC	ND	ND	0.846 ± 0.00	1.039 ± 0.01	0.539 ± 0.00	2.948 ± 0.01	MS, LRI
<i>Esters</i>									
887	Ethyl lactate	ET1	20.075 ± 0.04	ND	ND	ND	ND	23.890 ± 0.01	MS
1703	Linyl propionate	ET2	ND	ND	0.083 ± 0.00	0.027 ± 0.00	1.110 ± 0.00	ND	MS
1930	Triacetin	ET3	9.775 ± 0.01	20.768 ± 0.01	19.820 ± 0.01	14.250 ± 0.07	21.828 ± 0.06	24.380 ± 0.03	MS, LRI
2130	Diisobutyl phthalate	ET4	0.376 ± 0.00	0.370 ± 0.00	0.385 ± 0.00	0.131 ± 0.00	0.977 ± 0.00	1.351 ± 0.00	MS, LRI
			30.226	21.138	20.288	14.408	23.915	49.621	
<i>Ketones</i>									
1308	6-Methyl-5-hepten-2-one	ONE1	0.014 ± 0.00	0.644 ± 0.00	0.340 ± 0.00	0.153 ± 0.00	1.217 ± 0.00	1.380 ± 0.00	MS, LRI
1458	(±)-Isomenthone	ONE2	0.574 ± 0.00	ND	ND	0.151 ± 0.00	ND	ND	MS, LRI
1735	Carvone	ONE3	0.076 ± 0.00	ND	0.394 ± 0.00	0.214 ± 0.00	0.438 ± 0.00	ND	MS, LRI
1818	(E)-geranyl acetone	ONE4	0.069 ± 0.00	0.628 ± 0.00	0.292 ± 0.00	0.093 ± 0.00	0.533 ± 0.00	0.265 ± 0.00	MS, LRI
			0.733	1.272	1.026	0.610	2.188	1.645	
<i>Terpenes</i>									
1225	Para-cymene	TN1	0.533 ± 0.00	0.776 ± 0.00	ND	ND	ND	0.648 ± 0.00	MS, LRI
1248	Ortho-cymene	TN2	ND	0.727 ± 0.00	ND	ND	ND	ND	MS, LRI
			0.533	1.503	ND	ND	ND	0.648	
			36.345	36.126	36.246	25.675	51.664	74.406	
<i>Others</i>									
< 700	2,5-Dimethoxy-4-(methylsulfonyl)amphetamine	DMSA	0.060 ± 0.00	ND	0.245 ± 0.00	0.090 ± 0.00	ND	0.266 ± 0.00	MS
890	Nitrosomethane	NM	ND	6.595 ± 0.01	1.478 ± 0.01	1.040 ± 0.00	4.229 ± 0.01	ND	MS

Table 1 continued

RI	Sulfur containing compounds	Code	Control	100 mM	300 mM	500 mM	1000 mM	2000 mM	ID
977	Trichloromethane	TM	0.128 ± 0.00	0.121 ± 0.00	ND	ND	ND	0.162 ± 0.00	MS, LRI
999	Toluene	TO	0.058 ± 0.00	0.265 ± 0.00	ND	ND	0.329 ± 0.00	ND	MS, LRI
1434	1-Methyl-4-prop-1-en-2-ylbenzene	MPB	0.135 ± 0.00	0.290 ± 0.00	0.350 ± 0.00	ND	ND	ND	MS
1738	Azulene	AZ	ND	0.137 ± 0.00	ND	ND	0.010 ± 0.00	ND	MS, LRI
1741	Naphthalene	NA	ND	ND	0.079 ± 0.00	0.046 ± 0.00	0.103 ± 0.00	0.100 ± 0.00	MS, LRI, Std
			0.380	7.407	2.152	1.175	4.670	0.527	

Reliability of the identification proposal was indicated by the following: ^(A), mass spectrum and Kovats index according to literature; ^(B), mass spectrum compared with NIST98 and Wiley mass spectral databases. KI Kovats index calculated for the DB-Wax capillary column. nd: Not detected. Values are represented as mean ± SD (n = 2)

butenyl-glucosinolate by rapid enzymatic hydrolysis (Friis and Kjaer 1966). 3-(Methylthio)-propyl-isothiocyanate (radish isothiocyanate) is considered as products of enzymatic hydrolysis of glucoside progenitors (glucoibervirin) in various cruciferae. Kjær et al. (1978) demonstrated the formation of 3-(methylthio)-propyl-isothiocyanate and isolated it from natural mustard oil, fresh Japanese and Kenyan radish. In addition, 3-butenyl isothiocyanate and methyl isothiocyanate are considered as product of alkenyl glucosinolates (Gluconapin) hydrolysis through rearrangement to give 1-cyanoepithioalkanes by sulfur migration (Kirk and Macdonald 1974) and glucocapparin degradation respectively (Concurso et al. 2016). 3-butenyl isothiocyanate was identified during vacuum isolation of the volatile oil from cabbage and cauliflower without cooking prior to extraction (Buttery et al. 1976). Methyl isothiocyanate has pungent mustard horseradish odor characteristic, and is considered as characteristic aroma fraction of capers (Concurso et al. 2006).

Four nitrogen-containing compounds were also identified including pyridine, 2,3-diethyl-5-methylpyrazine, benzothiazole and tetrahydro-2-thiopheneacetonitrile. Concentration of nitrogen containing compounds increased with increasing NaCl concentration compared to the control. These compounds might contribute to the development of red radish anthocyanin off-flavor due to their lower odor thresholds and characteristic odor. Pyridine might be mainly generated from the interactions of ammonia, one of the thermal degradation product of *S*-methyl-L-cysteine sulfoxide, and aldehydes (Kubec et al. 1998, Bernhard and Wyllie 2008). However, tetrahydro-2-thiopheneacetonitrile was first identified in red radish anthocyanin extracts. The formation of nitrogen containing compounds is favored by low pH values or by the presence of ferrous ions or other cations and the elimination of elemental sulfur (Rizzi 2002).

Oxygen containing compounds were also identified including aldehydes, alcohols, ketones, carboxylic acids and esters. They are the most dominating compounds identified in this study and their contents were higher at higher NaCl concentration. Nonanal, L-camphor, linalool, 1-octanol, cedrol, 2,4-di-*tert*-butyl phenol, triacetin, diisobutyl phthalate, 6-methyl-5-hepten-2-one and (E)-geranyl acetone were the only oxygen containing compounds identified in all extracts. Alcohols, aldehydes, esters, acids and ketones are common volatiles in processed vegetables, and are formed by oxidative breakdown of fatty acids by the action of lipxygenase (Buttery et al. 1976; Concurso et al. 2016). Nonanal and 1-octanol were identified as the major components of cauliflower and broccoli volatiles (Buttery et al. 1976).

Fig. 2 **a** Correlation loading plot for red radish anthocyanin extracts. The model was derived from GC–MS isolated compounds as the X-matrix and glucosinolate degradation as Y-matrix, respectively. **b** PLS1 prediction model for glucosinolate degradation

Relationship between glucosinolate degradation and flavor volatiles as affected by NaCl concentration

The correlation between glucosinolate degradation and red radish anthocyanin off-flavor compounds was analyzed with ANOVA-PLSR. The X-matrix was designed as GC–MS data (34 volatile compounds), while the Y-matrix was designed as glucosinolate degradation (Fig. 2). The derived

PLSR model included 4 significant principal components (factors) and Fig. 2a was plotted by Factor 1 versus Factor 2, while other factors did not show any additional information compared to Factor 1 versus Factor 2. The derived PLSR model included two significant Factors explaining 71% of the cross-validated variance. For X variables, the explained variance was Factor 1 = 57% and Factor 2 = 14%, respectively. For Y variables, the explained variance for the model was Factor 1 and Factor 2 with 100

Fig. 3 Qualitative analyses of anthocyanins presenting in red radish extracts as affected by NaCl concentration: Molecular ion fragmentation

and 0%, respectively. The two big circles in the plot indicated 50 and 100% explained variances, respectively. The estimated regression coefficients from the jackknife uncertainty test (Fig. 2) indicates that the majority of compounds and glucosinolate degradation were located between the inner and outer ellipses, $r^2 = 0.5$ and 1.0, respectively, indicating they were well explained by the PLSR model.

To further investigate which volatile compounds are cause of glucosinolate degradation, PLS1 regression analysis was carried out to determine the significance of volatile compounds to glucosinolate degradation (Fig. 2). The significant variables were identified by calculating estimated regression coefficients from the jackknife uncertainty test (Karangwa et al. 2014). As shown in Fig. 2b, 3-butenyl isothiocyanate, 3-(methylthio) propyl isothiocyanate and 4-(methylthio)-3-butenyl isothiocyanate were positively and significantly correlated to the glucosinolate degradation. Methyl isothiocyanate, benzothiazole and toluene were positively but not significantly correlated to the glucosinolate degradation. Furthermore, all sulfur containing compounds showed negative correlation on glucosinolate degradation. Dimethyl disulfide, dimethyl trisulfide, cedrol, triacetin, 6-methyl-5-hepten-2-one and naphthalene were negatively and significantly correlated to glucosinolate degradation. On the other side, all other oxygen containing compounds were negatively but not significantly correlated to glucosinolate degradation. These results indicate that they might be resulted from lipid degradation. According to Paré and Tumlinson (1999), fatty acids serve as volatile precursors for several

compounds through autolytic oxidative breakdown, such as saturated and unsaturated six-carbon alcohols, aldehydes, and esters. These compounds are responsible for the green-leaf odour of the leaves.

Characterization of red radish anthocyanins

Identification of anthocyanin as affected by NaCl concentration

The use of HPLC coupled with mass spectroscopic analyses for characterization of individual anthocyanins has successively been used over the past decade (Wu and Prior 2005). As shown in Fig. 3 and Table 2, a tentative identification of red radish anthocyanins as affected by NaCl concentration was carried out by UPLC-TQ-MS analysis. Twelve acylated pelargonidin derivative anthocyanins were identified in all 3 extracts. Pg-3-(*p*-coumaroyl)-diglu-5-glu with molecular ion at m/z 903 was detected in NaCl extracts but was not identified in our previous study (Wentian et al. 2016). Three red radish anthocyanin extracts (100 mM, 500 mM and 2000 mM) were selected to study the effect of NaCl concentration on red radish anthocyanin. Pg-3-(caffeoyl) (*p*-coumaroyl) diglu-5-glu with molecular ion at m/z 1065 was the only anthocyanin not detected in NaCl treated extracts compared with the control. Moreover, identified anthocyanin showed similar fragmentation pattern as in our previous study by which the glycosylated substitutes at positions C3 and C5 of the flavylum ring were cleaved. all anthocyanins were characterized as 3-mono- or dihydroxycinnamoyl (*p*-coumaric, caffeic, and/or ferulic acid)-diglucoside-5-glucoside, 3-mono- or dihydroxycinnamoyl (*p*-coumaric, caffeic, and/or ferulic acid)-diglucoside-5-malonylglucoside of pelargonidin. The acyl fragmentation patterns are in

Table 2 Qualitative analyses of anthocyanins presenting in red radish extracts as affected by NaCl concentration

Rt (min)	Compounds	m/z	100–500–2000 mM
8.717	Pg-3-(caffeoyl)-diglu-5-glu	919	✓
9.497	Pg-3-(caffeoyl)-diglu-5-(malonyl)-glu	1005	✓
10.087	Pg-3-(<i>p</i> -coumaroyl)-diglu-5-glu	903	✓
10.468	Pg-3-(feruloyl)-diglu-5-glu	933	✓
10.589	Pg-3-(caffeoyl)(feruloyl)-diglu-5-glu	1095	✓
11.456	Pg-3-(<i>p</i> -coumaroyl)-diglu-5-(malonyl)-glu	989	✓
11.907	Pg-3-(feruloyl)-diglu-5-(malonyl)-glu	1019	✓
12.115	Pg-3-(feruloyl)(<i>p</i> -coumaroyl)-diglu-5-glu	1079	✓
12.375	Pg-3-(feruloyl)(feruloyl)-diglu-5-glu	1109	✓
12.722	Pg-3-(caffeoyl)(feruloyl)-diglu-5-(malonyl)-glu	1181	✓
13.554	Pg-3-(feruloyl)(<i>p</i> -coumaroyl)-diglu-5-(malonyl)-glu	1165	✓
13.849	Pg-3-(feruloyl)(feruloyl)-diglu-5-(malonyl)-glu	1195	✓

Pg pelargonidin, Diglu diglucoside, Glu glucoside, Mal malonic acid

Fig. 4 Effect of NaCl concentration on **a** total monomeric anthocyanin content (mg/100 g FW), and percentage polymeric color (%), **b** lightness and *a*-value, **c** Chroma and Hue angle. Values represent the means of three replicate samples with standard error of the means

accordance with previous studies on red radish anthocyanin structures (Giusti and Wrolstad 2003; Wu and Prior 2005) who reported that red radish major anthocyanidin was

Pelargonidin, which mono-acylated with *p*-coumaric acid or ferulic acid up to 30%, while 70% of di-acylation occurred with *p*-coumaric and malonic acids or ferulic and malonic acids.

Effect of NaCl concentration on total monomeric anthocyanin, polymeric color and color characteristics

The effect of NaCl concentration on red radish total anthocyanin content is shown in Fig. 4. NaCl displayed a destabilizing effect on total red radish monomeric anthocyanin, which significantly decreased with increasing NaCl concentration compared to control. The decrease in monomeric anthocyanin content was up to 52.5% at 2000 mM, NaCl concentration. The lower red radish anthocyanin yield at higher NaCl concentration might be attributed to the altered solvation characteristics of aqueous solutions. These results agree with the findings of Hubermann et al. (2006) who reported that salt ions increase the solvation capacity of the solvent and thereby the dissociation of the anthocyanin molecule to the intermediately formed carbenium-ion, which promotes the hydration reaction. This may have resulted in enhanced monomeric anthocyanin loss of the concentrates in the presence of salt.

Following the degradation of monomeric anthocyanin as function of NaCl concentration, polymeric colors were formed. The change in percentage polymeric color is an indicator of the anthocyanins polymerization during processing. As shown in Fig. 4, the percentage content of the polymeric anthocyanin significantly increased with increasing NaCl concentration. The percentage of polymeric color was 3.5 fold higher in 2000 mM extracts compared to control. The higher increase in polymeric color at high NaCl concentration clearly confirmed the monomeric anthocyanin reduction. The possible mechanism for anthocyanin polymerization involves condensation reactions of anthocyanins with other phenolic compounds, including flavan-3-ols or polyflavan-3-ols, that can be mediated by acetaldehyde and furfural or occur via direct anthocyanin–tannin reactions (Es Safi et al. 2000).

In addition, the concentration of NaCl significantly destabilized the color properties of red radish anthocyanin extracts Fig. 4. The lightness (*L*) and the redness (*a*-value) of the extracts significantly increased and decreased with increasing NaCl concentration compared to control (Fig. 4b). However, the hue angle (*h*) and chroma (*C*) significantly decreased with increasing NaCl concentration compared to control (Fig. 4c). The lower lightness and great chroma indicates darker and more vivid. While, the increase of lightness followed by the decrease of chroma indicate that the color became lighter and duller in a gradual manner. Rodriguez-Saona et al. (1999) reported that the increase in anthocyanin content of red radish might

be correlated to the increase of the pigment chroma and hue angle and low lightness of the red radish pigment extracts at pH 3.

Conclusion

The total glucosinolate degradation and total anthocyanin yield were largely affected by the NaCl concentration. Overall, increasing NaCl concentration yield a decay in total glucosinolate, anthocyanin concentration and color. Around 45% and 53% of total glucosinolate and total monomeric anthocyanins were degraded at 2000 mM NaCl concentration compared to control. The higher glucosinolate degradation lead to the higher off-flavor formation. The correlation study between glucosinolate degradation and volatile compounds revealed that 3-butenyl isothiocyanate, 3-(methylthio) propyl isothiocyanate and 4-(methylthio)-3-butenyl isothiocyanate were positively and significantly correlated to the glucosinolate degradation, while methyl isothiocyanate, benzothiazole were positively but not significantly correlated to glucosinolate degradation. On the other side, sulfur and oxygen containing compounds showed negative but not significant correlation with glucosinolate degradation. Dimethyl disulfide, dimethyl trisulfide, cedrol, triacetin, 6-methyl-5-hepten-2-one and naphthalene were negatively and significantly correlated to glucosinolate degradation. Moreover, the tentative anthocyanin identification by UPLC-TQ-MS showed 12 glycosylated anthocyanins substituted at C3 and C5 in all extracts. In summary, 500 mM NaCl concentration could be the higher NaCl concentration limit to extract red radish anthocyanin.

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