Characterization and Stability of Anthocyanins in Purple-fleshed Sweet Potato P40

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Abstract

Purple-fleshed sweet potato P40 has been shown to prevent colorectal cancer in a murine model. This study is to identify anthocyanins by using HPLC/MS-MS and assess the stability during various cooking conditions. P40 possesses a high content of anthocyanins up to 14 mg/g dry matter. Total 12 acylated anthocyanins are identified. Top three anthocyanins, e.g., cyanidin 3-caffeoyl-p-hydroxybenzoyl sophoroside-5-glucoside, peonidin 3-caffeoyl sophoroside-5-glucoside, and cyanidin 3-(6'' –caffeoyl-6''-feruloylsophoroside)-5-glucoside, account for half of the anthocyanin contents. Over 80% of anthocyanins measured by acid hydrolysis were cyanidin derivatives, indicating P40 is unique when compared with other purple-fleshed sweet potatoes that usually contain more peonidin than cyanidin. Steaming, pressure cooking, microwaving, and frying but not baking significantly reduced 8-16% of total anthocyanin contents. Mono-acylated anthocyanins showed a higher resistance against heat than di- and non-acylated. Among of which, cyanidin 3-p-hydroxybenzoylsophoroside-5-glucoside exhibited the best thermal stability. The stable acylated and cyanidin-predominated anthocyanins in P40 may provide extra benefits for cancer prevention.

Keywords: Anthocyanins / purple-fleshed sweet potato / cancer prevention / stability / cooking conditions

1. Introduction

Sweet potato (*Ipomoea batatas*) is known as an excellent source of β-carotene (precursor of vitamin A), vitamin Bs, dietary fiber, minerals, and polysaccharides. In year 2003, the global production of sweet potato was estimated about 122 million metric tons, which was heavily consumed in rural part of China and western African countries (Wu et al., 2008). Purple-fleshed sweet potato (PSP) contains a significantly greater amount of anthocyanin than ordinary orange-fleshed sweet potato. Through several years, a number of PSP varieties with different anthocyanin contents and profiles were bred and grown for their potential health benefits.

Differing from anthocyanins found in berries, PSP anthocyanins primarily exist as acylated forms (Giusti & Wrolstad, 2003; Gould, Davies & Winefield, 2008). Acylation with various phenolic acids makes PSP anthocyanins unique and also provides some advantages in pH and heat resistances, light sensitivity, and overall stability. From a nutritional viewpoint, acylated anthocyanins have been reported to possess elevated antioxidant and anti-mutagenicity activity (Suda et al., 2002). Biological activities of specific acyl groups are still under evaluation, however, it is believed that additional free phenolic hydroxyl groups may raise bio-functionality of anthocyanins. Among six common anthocyanidins peonidin and cyanidin are usually found in PSP, but the most abundant anthocyanins in the reported PSPs were peonidin derivatives (Yoshinaga et al. 1999). In addition, pelargonidin was a negligible anthocyanidin found in a few varieties of PSP such as NC415 and Ayamurasaki (Giusti et al., 1999; Kim et al., 2012; Truong et al., 2010). As an acylated anthocyanin source, PSP has shown excellent coloring properties in numerous acidic to neutral foods that close to the synthetic FD&C red #40 (Suda et al., 2003). In Japan, PSP puree is a popular natural colorant and functional ingredient in the bakery, confectionery, juices, beverages, and dairy food industries (Dyrby et al., 2001; Giusti &

Wrolstad, 2003; Suda et al., 2003). In order to be used as an additive, PSP is usually transformed to cooked puree, dried, and powdered; those processes lead to pigment degradation along thermal treatments and oxygen exposure (Steed & Truong, 2008). Therefore, thermal stability of anthocyanin is recognized as a key property that affects overall quality.

P40 is a variety of anthocyanin-enriched PSP cultured at the John C. Pair Horticulture Research Center, Wichita, KS, by selecting from a large number of parent-seedlings provided by the International Potato Center in Lima, Peru. We previously reported P40 anthocyanins at 10-40 μM significantly inhibited the growth of the human colon cancer SW480 cells by arresting cell cycle phase at G1 (Lim et al., 2013). Mice fed 10-30% of P40 showed less susceptible to azoxymethane-induced colorectal aberrant crypt foci formation, demonstrating a potential cancer prevention (Lim et al., 2013). The objective of this follow-up study is to characterize anthocyanin contents and profile in P40. The thermal stability of them during various cooking conditions is further evaluated.

2. Materials and methods

2.1. *Chemicals*

Acetonitrile, methanol, hexane, and formic acid were either HPLC grade or analytic grade purchased from Thermal Fisher Scientific (Suwanee, GA). Water used in all preparation and analysis was purified through Barnstead E-Pure Deionization System (Dubuque, IA) and filtered using Millipore 0.45 μm membrane (Bedford, MA). Standards of cyanidin-3, 5-diglucoside was obtained from Sigma-Aldrich (St. Louis, MO).

2.2. *Sample preparation and cooking condition*

The PSP P40 is a variety of anthocyanin-rich sweet potato selected and cultured at the John C. Pair Horticulture Research Center, Wichita, KS. Average weight 120-150 g of intact root

tubers were skinned and cut to 6.4 mm thick wedges. Various cooking conditions based upon conventional recipes include baking in conventional oven at 205 °C for 50 min, steaming in Hamilton Beach rice cooker with steaming sleeve at 100 °C for 20 min, pressure cooking in Cuisinart pressure cooker at 121 °C and 15 psi for 17 min, microwave baking in conventional 850 W microwave at 100% power for 5 min, and frying in conventional deep fryer at 177 °C for 5 min. The cooked tubers were peeled, freeze-dried, and powdered using a mortar and pestle. An additional de-fatting procedure was performed on fried samples in case that the residual oil might interfere with the later analyses.

2.3. *Proximate Analysis*

Lyophilized P40 powder was used for analyses of moisture, crude protein, lipid, and ash. Briefly, moisture was removed by hot oven method at 130 °C for two hours; protein was determined by a Leco FP-2000 protein analyzer (Leco Corp, St Joseph, MI, USA) using AOAC method 992.15 with 6.25 as the converting factor (King-Brink & Sebranek, 1993); crude lipid and moisture was determined in CEM Smart Trac system (CEM Corporation, Matthews, NC, USA) by AOAC method 2008.06 (Leffler et al., 2008); and ash content was determined using a muffle furnace according to AOAC method 942.15 (Thiex, Novotny, & Crawford, 2012).

2.4. *Extraction and Quantification*

Defatting: Powdered and fried PSP was extracted in hexane at solid to solvent ratio (1:6, w/v) for one hr, and centrifuged (3000g, 20 min, 4 °C). The procedure was repeated three times and the pellet was air dried overnight.

Anthocyanin Extraction: The extraction and subsequent analysis followed a method of Kim et al., (2012), with minor modifications. Briefly, 1 g of the PSP powder containing internal standard, cyanidin-3, 5-diglucoside, was extracted with 20 mL 5% formic acid water on an

orbital shaker at 40 °C for 12 hrs and centrifuged (4000g, 20 min, 4 °C). The extraction was repeated twice and the supernatants were pooled. A Waters Sep-Pak C_{18} solid phase extraction cartridge (Milford, MA) was activated with 3 mL methanol and 3 mL water, and loaded with 2 mL of supernatant. The colume was washed with 3 mL water and then anthocyanins were eluted with 1 mL methanol, and the cartridge should be free of visible color after elution. The eluent was evaporated in an Eppendorf Vacufuge (Hamburg, Germany) to dryness and re-constituted in 1 mL of 5% formic acid water.

2.5. *HPLC-MS/MS Analysis*

HPLC coupled Electrospray Ionization tandem Mass Spectrometry (ESI/MS/MS) was employed in this study as a proven powerful technique to carry out intact anthocyanin and production-ion analysis. A Shimadzu HPLC system (Kyoto, Japan) was used for chromatographic separation. This system employed a DGU-20A3 built in degasser, a LC-20AB solvent delivery pump, a SIL-20ACHT auto-sampler, a CTO-20AC column holding oven, a CBM-20A communicator module, and a SPD-M20A Photodiode Array Detectors. A Waters (Milford, MA) C_{18} reversed phase column (250 mm length, 4.6 mm diameter) was used for anthocyanin separation. Data was analyzed using LC Solution software (Kyoto, Japan). Elution was performed with mobile phase A (5% formic acid in de-ionized water) and mobile phase B (5% formic acid in acetonitrile/water 1:1 v:v); gradient expressed as mobile phase B volume was 20-40% for 30 min, 40-50% in following five min and held at 50% for 10 min before returning to 20%. The flow rate was maintained as 1 mL/min and column temperature was 25 °C. Detector performed a full spectrum scan between 190-800 nm where 525 nm was used for monitoring anthocyanins. Cyanidin-3, 5-diglucoside was used as an internal standard for quantitation of extraction recovery and anthocyanin contents.

2.6. *Mass Spectrometry Analysis*

Mass spectrometric scan was performed in positive mode with a scanning interval 500-1200 m/z. Nebulization was conducted at 350 °C aided by concurrent N_2 flow at 10 psi; capillary and cone voltages were set at 3.5 kV and 40 V; drying gas flow rate was 5 L/min. Mass of precursor ions and reactions of fragments loss were evaluated. Data were analyzed using BrukerHystar Post Processing software (Bruker, Bremen, Germany). Anthocyanin compounds were identified by HPLC retention time, absorbance spectra pattern, and matching MS fragment database according to previous publications (Giusti et al., 1999; Kim et al., 2012; Tian et al., 2005a; Tian et al., 2005b; Truong et al., 2010).

2.7. *Acid Hydrolysis*

Acid hydrolysis procedure was adopted from Truong et al. (2010). A 50 μL anthocyanin extract was mixed with same volume of 6 N HCl in a sealed HPLC vial. The mixture was heated in boiling water bath for 2 hrs after thorough vortexing. The sample was cooled in ice bath and dried in an Eppendorf Vacufuge before re-constituted in 500 μL of water acidified with 5% formic acid. Mass spectrometric scanning was performed at 100-800 m/z range to identify aglycone anthocyanidins.

2.8. *Statistical Analysis*

The anthocyanin change was analyzed by one-way ANOVA where cooking conditions were main factors. Tukey's post-hoc test was used to assess the multiple differences of individual anthocyanin at various cooking conditions. A probability of $P \leq 0.05$ was considered significant. Statistical procedures were by SAS 9.3 (SAS Institute; Cary, NC).

3. Results

3.1. *Proximate analysis*

Freeze drying reduced the moisture content of raw P40 flesh from 75% to 4.7%. Protein, fat, and ash contents were determined as 8.4%, 0.7%, and 4.3%, respectively. Thermal treatments resulted in leaching and drying effects and affected on moisture contents of fresh sweet potato but did not alter the macronutrients after freeze drying (data not shown).

3.2. *Chromatographic Separation*

As shown in Figure 1, anthocyanin eluents were separated under the experimental conditions. Twelve major peaks, possessed typical anthocyanin spectra of a maximum absorbance at around 520 nm, were separated in addition to internal standard, cyanidin-3,5-diglucoside. Peaks 8, 9 and 10 were the major anthocyanins and they contributed to near half of the total anthocyanin peak areas. Peak number, retention time, and % of total peak areas were summarized in Table 1.

3.3. *Mass Spectrometric Identification*

The m/z ratio of each intact anthocyanin with daughter fragments were captured within the scanning interval ranging. As shown in Figure 2A, the ions of peak 1, i.e., cyanidin 3-sophoroside-5-glucoside (m/z 773), produced three fragments of m/z 611, 449, and 287. Transition 773>611 and 773>449 represented the loss of glucose (m/z 162) and sophorose (m/z 324), respectively, while transition 773>287 produced cyanidin (m/z 287) aglycone due to the loss of both glucose and sophorose. Figure 2B showed another example for mono- and di-acylated anthocyanin, i.e., cyanidin 3-p-hydroxybenzoyl sophoroside-5- glucoside (peak 2). The ions of peak 2 produced transitions of 893 to 731, 449, and 287 m/z, where 893>449 transition indicated the loss of sophoroside and acylation. Identification of the remaining anthocyanins (peaks 3-12) were carried out in a similar fashion. All the m/z ratio of each intact anthocyanin and its daughter fragments were summarized in Table 1. As indicated in Table 1, cyanidin (m/z

287) and peonidin (m/z 301) were two aglycone anthocyanidins detected. Glycosylations with glucose (m/z 162) and sophorose (m/z 324) were found in all 12 anthocyanins. Eleven of them except for cyanidin 3-sophoroside-5-glucoside (peak 1) were acylated at R1, R2, or R3 by caffeic acid (m/z 180), ferulic acid (m/z 194), and/or p-hydroxybenzoic acid (m/z 138), respectively. The chemical structures of skeleton anthocyanin and three acylated phenolic acids were shown in Figure 3.

3.4. *Acid Hydrolysis*

Acid hydrolysis completely removed all the glycosylation attachments and reduced the number of peaks from 9 to 3 (Figure 1). While the 3rd peak was un-identified, peaks 2 and 3 were simple cyanidin and peonidin, respectively, because they presented the m/z ratio as identified by MS at 287 and 301 m/z, respectively (data not shown). Both peak areas of cyanidin and peonidin in the hydrolysate of raw P40 contributed to 90% of total peak area, but near 80% of total anthocyanidins measured by acid hydrolysis were cyanidin derivatives.

3.5. *Effect of Thermal Treatments*

Total contents of anthocyanin in raw P40 and cooked P40 via various cooking conditions were presented in Table 2. HPLC chromatograms of anthocyanins in baked and microwaved P40 in comparison with that in raw P40 were shown in Figure 1. The raw P40 possessed a content of anthocyanins up to 14 mg/g dry matter. The top three major anthocyanins were peak 8 (cyanidin 3-caffeoyl-p-hydroxybenzoyl sophoroside-5-glucoside), peak 9 (peonidin 3-caffeoyl sophoroside-5-glucoside), and peak 10 [cyanidin 3-(6"-caffeoyl-6"-feruloylsophoroside)-5-glucoside], which account for half of the total anthocyanin content. Seven non-, mono-, or di-acylated cyanidin species and five mono- or di-acylated peonidin species contribute for 67% and 33% of total anthocyanins, respectively. While baking did not affect total contents of

anthocyanins significantly, steaming, high pressure cooking, microwaving, and frying reduced 8-16% of total anthocyanin contents. Mono-acylated anthocyanins showed a higher resistance against heat than di- and non-acylated. Among of which, cyanidin 3-p-hydroxybenzoylsophoroside-5-glucoside exhibits the best thermal stability.

4. Discussion

The objectives of this study were focused on characterizing anthocyanin profile in P40 and evaluated their thermal stability. HPLC-MS/MS analysis was applied for anthocyanin extract of P40 before and after various thermal treatments, and then anthocyanin contents were assessed.

A total of 12 anthocyanins were identified and quantitated by HPLC-MS/MS. Eleven of them were acylated with caffeic, ferulic, and/or p-hydrobenzoic acids. When compared to the anthocyanin content of PSPs reported by others (Table 3), total anthocyanin in P40 was the highest (near 1.4% in dry weight). If compared with berry fruits or colored vegetables, P40 (~3000 mg/kg fresh weight) ranked at upper-middle of the ladder, but was still higher to strawberry (350 mg/kg) and red cabbage (250 mg/kg) (Clifford, 2000). It was noteworthy that anthocyanins in P40 were distinguishable from berry anthocyanins because of the unique acylation that was presented in P40 only (Gould, Davies & Winefield, 2008; Neto, 2007).

Identification of anthocyanins was greatly relied on the matching fragment patterns to the mass spectrum database of anthocyanins collected from the published articles. The m/z of the precursor ions were detected by 1^{st} MS after electrospray ionization, and the precursor ions were further dissociated by argon collision for 2^{nd} MS detection. During the collision, the glycosidic bonds joining anthocyanidins and saccharides were vulnerable and cleaved; therefore, each anthocyanin was identified by matching residual fragmentation. Furthermore, tandem MS exhibited a distinct advantage in distinguishing similar isomers. For example, peak 5 [cyanidin

212 3-(6'' –feruloyl sophoroside)-5-glucoside] and peak 9 (peonidin 3-caffeoyl sophoroside-5-
213 glucoside) possessed a same molecular weight (m/z 949), but their identities were revealed by
214 different subsequent fragmentations.

215 PSPs are usually classified into either cyanidin-predominated or peonidin-predominated
216 based upon the ratio of peonidin to cyanidin aglycones (pn/cy). Pn/cy is an important factor to
217 flesh color and maybe some difference in functionalities. Peonidin type (pn/cy>1) sweet potatoes
218 generally have a pink to red flesh color and sometimes they are referred as red-fleshed. As
219 content of cyanidins increase, the color of the flesh shifts to purple and dark purple. Structure-
220 wisely, cyanidin contains more hydroxyl groups than peonidin, leading to a stronger
221 antioxidative activity. Yoshimoto et al. (1999a) reported cyanidin type pigments were superior to
222 peonidin in antioxidant and anti-mutagenicity. However, almost all the PSPs containing a high
223 content of anthocyanins were cyanidin-predominated type (Table 3). P40, as confirmed by acid
224 hydrolysis, was a unique cyanidin-predominated type with exceptionally high anthocyanin
225 content.

226 As a subgroup of polyphenolic flavonoids, anthocyanins are expected to be degraded by
227 heat (Xu & Chang, 2008a). There was a significant impact on anthocyanin contents after various
228 thermal treatments. While baking slightly reduced total contents of anthocyanins, steaming, high
229 pressure cooking, microwaving, and frying significantly reduce 8-16% of total anthocyanin
230 contents. It appeared that each anthocyanin varied greatly in its thermal stability, and most likely
231 the acylation played a role. It appeared that mono-acylated anthocyanin with p-hydroxybenzoic
232 acid possessed the best resistance against heating, followed by ferulic acid and caffeic acid
233 acylation. Mono-acylated anthocyanins generally showed a higher resistance against heat than
234 di- and non-acylated. Among of which, cyanidin 3-p-hydroxybenzoylsophoroside-5-glucoside

exhibits the best thermal stability. Therefore, mono-acylated and cyanidin-predominated anthocyanins in P40 might be more stable in resistance against thermal treatments. Furthermore, thermal treatments may release phytochemicals by destroying and softening bound from food matrices (Xu & Chang, 2009). In the microwave treatment, for example, cyanidin 3-p-hydroxybenzoylsophoroside-5-glucoside (peak 2) increased from 121 to 462 mg/100 g and peonidin 3-p-hydroxybenzoyl sophoroside-5-glucoside (peak 4) was elevated from 19 to 87 mg/100 g. It seemed that microwave treatment might release anthocyanins from physical entrapment in other structures as suggested by Xu & Chang (2008b).

5. Conclusions

Twelve individual anthocyanins were identified and quantified in the newly bred purple sweet potato P40, where eleven were acylated and seven were cyanidin derivatives. Top three main anthocyanins in P40 were cyanidin 3-caffeoyl-p-hydroxybenzoyl sophoroside-5-glucoside, peonidin 3-caffeoyl sophoroside-5-glucoside, and cyanidin 3-(6" -caffeoyl-6"-feruloylsophoroside)-5-glucoside, which account for half of the total anthocyanin contents. Over 80% of total anthocyanins measured by acid hydrolysis were cyanidin derivatives. To the best of our knowledge, P40 seems the first cyanidin-predominated purple-fleshed sweet potato with superior anthocyanin contents. While conventional baking did not reduce anthocyanin content significantly, other thermal treatments facilitated anthocyanin degradation about 8-16% of total anthocyanin contents. Mono-acylated anthocyanins show a higher resistance against heat than di- and non-acylated. Among of which, cyanidin 3-p-hydroxybenzoylsophoroside-5-glucoside exhibits the best thermal stability. Therefore, mono-acylated and cyanidin-predominated anthocyanins in P40 appeared stable in resistance against thermal treatments, which may be an

advantage to the development of a functional sweet potato product for chronic disease prevention.

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338 Figure Legends

339 Figure 1. HPLC chromatograms of anthocyanins in raw, baked, microwaved, and acid
340 hydrolyzed P40.

341 Figure 2. Mass spectra of cyanidin 3-sophoroside-5-glucoside and cyanidin 3-p-hydroxybenzoyl
342 sophoroside-5- glucoside (peak 1 and 2 in Figure 1, respectively). A: a, b and a+b: bond cleavage
343 fragments without glucoside, sophoroside, and glucoside + sophoroside, respectively; B: a, b,
344 and a+b: bond cleavage fragments without glucoside, p-hydroxybenzoyl sophoroside, and
345 glucoside + p-hydroxybenzoyl sophoroside, respectively.

346 Figure 3. Skeleton structures of cyanidin (R_1=H) or peonidin (R_1=CH_3) 3-sophoroside-5-
347 glucoside (a), caffeic acid (b), ferulic acid (c), and p-hydrobenzoic acid (d).

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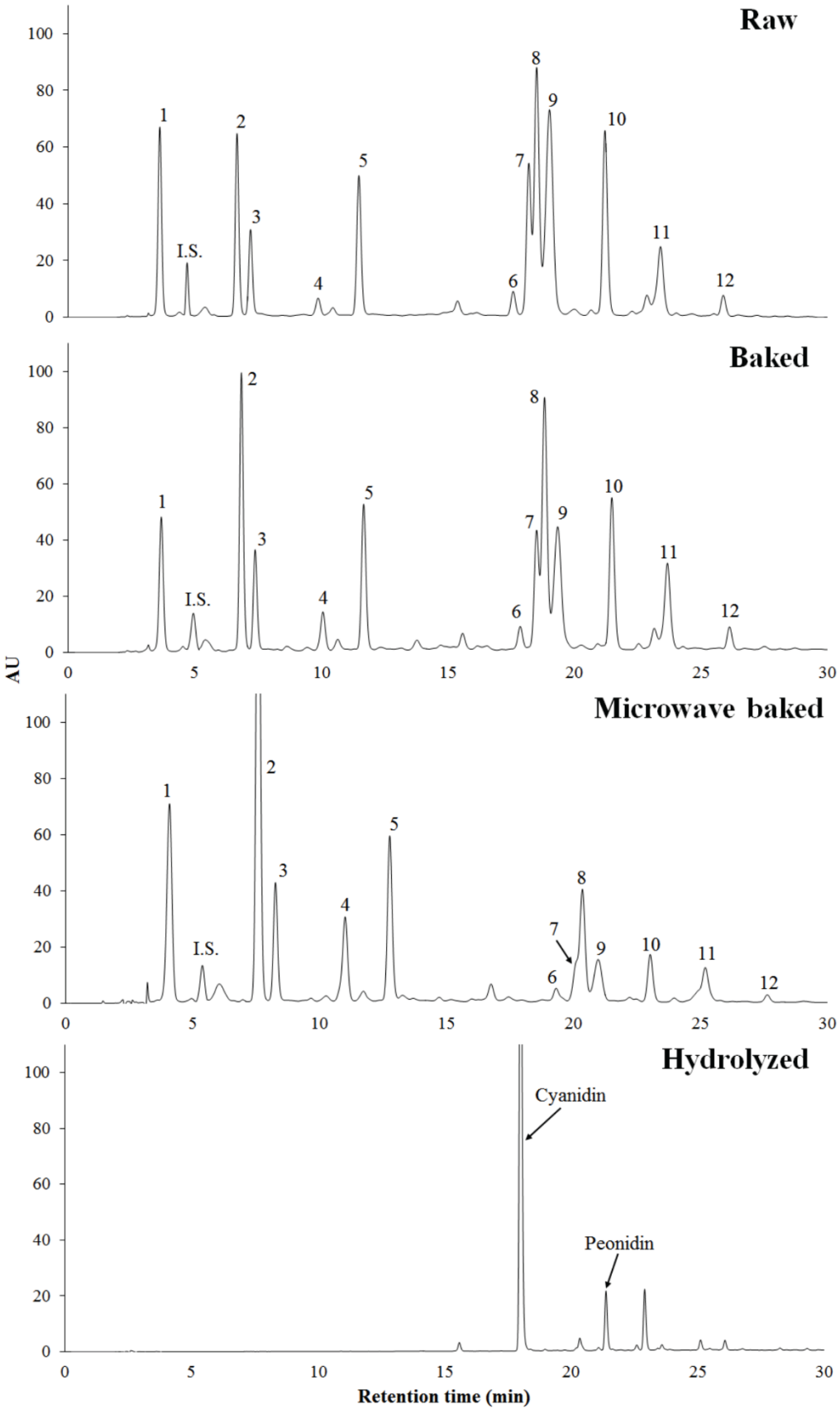
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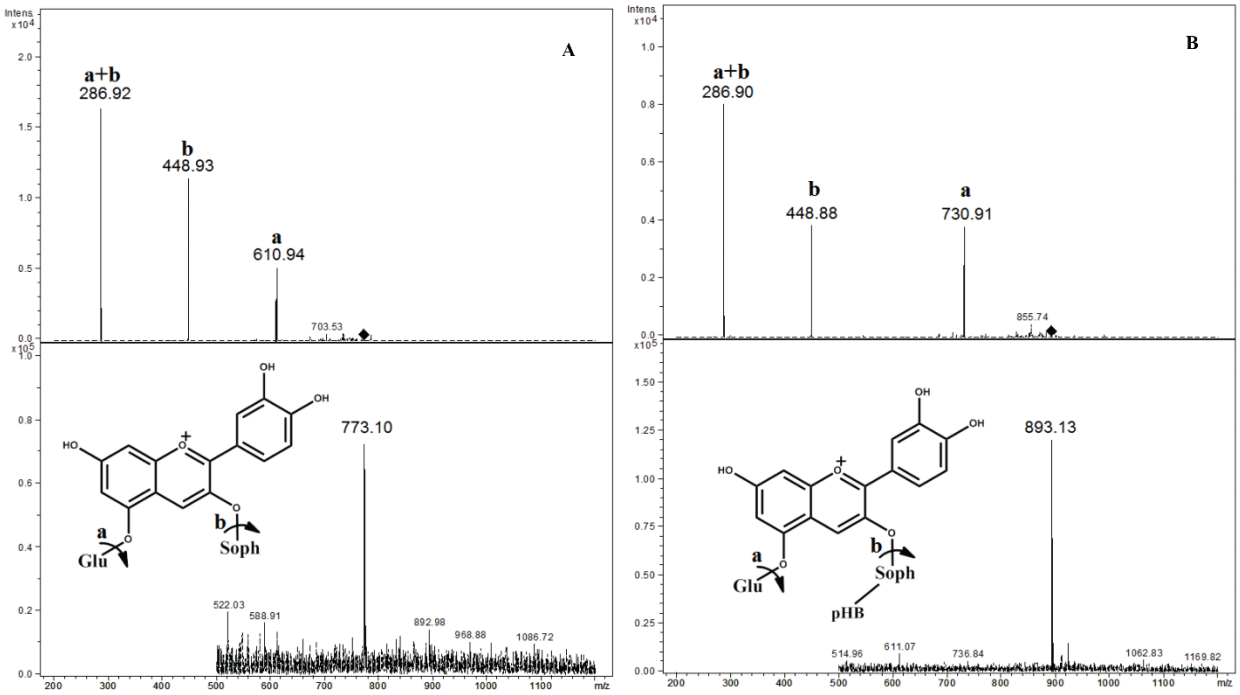
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