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Use of unconventional mixed Acetone-Butanol-Ethanol solvents for anthocyanin extraction from Purple-Fleshed sweetpotatoes

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ABSTRACT

Anthocyanins from purple-fleshed sweetpotatoes constitute highly valued natural colorants and functional ingredients. In the past, anthocyanin extraction conditions and efficiencies using a single acidified solvent have been assessed. However, the potential of solvent mixes that can be generated by fermentation of biomass-derived sugars have not been explored. In this study, the effects of single and mixed solvent, time, temperature, sweetpotato genotype and preparation, on anthocyanin and phenolic extraction were evaluated. Results indicated that unconventional diluted solvent mixes containing acetone, butanol, and ethanol were superior or equally efficient for extracting anthocyanins when compared to commonly used concentrated extractants. In addition, analysis of anthocyanidins concentrations including cyanidin (cy), peonidin (pe), and pelargonidin (pl), indicated that different ratios of pn/cy were obtained depending on the solvent used. These results could be useful when selecting processing conditions that better suit particular end-use applications and more environmentally friendly process development for purple sweetpotatoes.

1. Introduction

Transition from a petroleum dependent society to a bio-based economy has become a key focus of scientific developments in recent years. The effective use of crops and plants for sustainable production of energy and industrial goods will require the design of processing steps that can be integrated for enhanced performance and cost reduction. Emerging crops central to such integrated biorefinery efforts are purple-fleshed sweetpotatoes (PSP). Their short growth cycle, wide geographical distribution, and adaptability to marginal lands and drought (Truong, Avula, Pecota, & Yencho, 2018) makes them attractive feedstocks for two highly valued bio-based products, namely anthocyanins and fermentable sugars.

Anthocyanins are among the natural pigments considered as plausible synthetic dye replacements with the potential to provide health benefits when incorporated into diet (Krga & Milenkovic, 2019). Solid-liquid extraction using polar solvents like methanol, ethanol and acetone under acidic conditions has been a popular methodology for the recovery of anthocyanins and phenolics from plant materials including PSP (Silva, Costa, Calhau, Morais, & Pintado, 2017). However, PSP starches have been largely overlooked during extraction processes even though they constitute an invaluable source of fermentable sugars that can be converted through fermentation to renewable solvents applicable to anthocyanin recovery. For instance, several solventogenic strains of genus *Clostridium* have the ability to produce three different solvents, acetone, butanol, and ethanol (ABE) through fermentation of biomass sugars (Sandoval-Espinola, Chinn, & Bruno-Barcena, 2015; Zuleta-Correa, 2018). Other fermentation products like acetic and butyric acids are also obtained during this biological transformation of PSP sugars, which could provide the acidic environments required for anthocyanin cationic form stabilization (He & Giusti, 2010). Investigating the feasibility of a future coupling of ABE fermentation and anthocyanin extraction as either separate unit operations or a simultaneous process was the motivation for this study.

Previous studies have reported PSP total monomeric anthocyanin content in the range of 10 to 417 mg cyanidin-3-glucoside/100 g fresh

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Table 1
Acidified solvents (7%v/v acetic acid) and conditions used for anthocyanin and total phenolics extraction.

Solvent (% v/v)		Designation	Temperature (°C)	Sweetpotato preparation	Time (min)
Experiment 1	Acidified 80% methanol	M80	37, 65, 85	Flour and fresh preparation of NC-413 and P06-020	10, 30, 60,180, 1440
	Acidified 80% butanol	B80			
	Acidified 7% butanol	B7			
	Acidified 80% ethanol	E80			
	Acidified 80% acetone	A80			
	Acidified water	W			
Experiment 2	Acidified 48% butanol	B48Up B48Bot	37, 65	Flour preparation of NC-413 and P06-020	10, 60, 180
	Acidified ABE 1 (1.1% acetone, 1.4% butanol, 0.1% ethanol)	ABE1			
	Acidified ABE 2 (5.2% acetone, 7% butanol, 0.41% ethanol)	ABE2			
	Acidified acetone butanol (5.2% acetone, 7% butanol)	AB			
	Acidified butanol ethanol (7% Butanol, 0.41% Ethanol)	BE			
	Acidified acetone ethanol (5.2% acetone, 0.41% ethanol)	AE			

weight (FW) (≈33–1390 mg/100 g dry weight (DW)), while total phenolics content varied from 47.7 to 1483.7 mg chlorogenic acid equivalents/100 g FW (≈152–5152 mg/100 g DW) (Bridgers, Chinn, & Truong, 2010; Cevallos-Casals & Cisneros-Zevallos, 2003; de Aguiar Cipriano, Ekici, Barnes, Gomes, & Talcott, 2015; Diaz, Veal, & Chinn, 2014; Fan, Han, Gu, & Chen, 2008; Grace, Yousef, Gustafson, Truong, Yencho, & Lila, 2014; Padda & Picha, 2008; Steed & Truong, 2008; Teow, Truong, McFeeters, Thompson, Pecota, & Yencho, 2007; Truong et al., 2010; Xu et al., 2014). Despite advances in solid–liquid extractions of PSP and other plant-based anthocyanins, some opportunities remain unaddressed: 1) previous reports evaluating process conditions have only studied effects for a single extraction solvent; 2) uncommon solvent mixes (e.g. that can be generated by fermentation of biomass-derived sugars such as acetone-butanol-ethanol) have not been explored; and 3) there are few studies that simultaneously evaluate the interaction effect of different solvents, temperatures, PSP genotypes and PSP preparations on anthocyanins and phenolics extractions. Pre-processing steps of PSP roots (e.g. fresh vs. flour) are of particular importance since they influence anthocyanin extraction yields and efficiencies, but also dictate storage and infrastructure needs, and year-round supply capabilities in non-temperate regions. Effective integration of fermentation steps and anthocyanin extraction warrants further study to support development of a sustainable bio-based manufacturing process with a real eco-friendly supply chain where PSP sugars, anthocyanins, and phenolics could be captured as valuable co-products.

The objective of this work was to evaluate and compare the extraction performance of conventional solvents (i.e. methanol, ethanol, acetone) and uncommon diluted solvent mixes (containing acetone, butanol, and ethanol) in the extraction of anthocyanins and phenolics from different PSP genotypes (peonidin-rich vs cyanidin-rich) and PSP preparations (flour vs fresh). Extractions were performed at different temperatures (37, 65 and 85 °C) and samples were taken at multiple incubation times. The effect of extraction conditions on anthocyanidin composition including proportions of peonidin, cyanidin and pelargonidin in PSP extracts were also evaluated. The results of this study can guide selection of appropriate process conditions for anthocyanin and phenolic extraction from different preparations and genotypes of PSPs, and demonstrate the effectiveness of using mixed solvents that can enable opportunities to bridge bioprocesses and enhance sustainability.

2. Materials and methods

2.1. Chemicals

Glacial acetic acid (Fisher A35-500, CAS 64-19-7, 99.9%), methanol (CQ concepts Inc CAS 67-56-1, 99%), butanol (Fisher A339-4, CAS 71-36-3, 99.9%), ethanol (Kopte, 190 proof CAS 64-17-5, 95%), acetone (Fisher A18-4, CAS 67-64-1, 99.9%) and distilled deionized water (DDI) were used for solvent stock solutions preparation. Cyanidin-HCl and Peonidin-HCl were purchased from Chromadex (Irvine, CA) and Pelargonidin-HCl was obtained from Thomas scientific (Swedesboro, NJ).

2.2. Sweetpotato genotypes and preparation

Two purple sweetpotato (PSP) genotypes, developed by the North Carolina State University sweetpotato breeding program, were used in this study. PSPs NC-413 and NCPUR06-020 (P06-020) (Yencho, Pecota, & Driscoll, 2014) were harvested in 2016 season, cured (29 °C, 85% relative humidity (rh), 7 days), and stored (15 °C, 80–85% rh) until use. Roots were washed, towel dried and stored at 15 °C for 48 h before anthocyanin extraction. For flour preparations, roots were sequentially sliced (≈2 mm), dried (70 °C, 60 h) and grounded (2-mm screen, Wiley mill). Flour was stored in sealed, airtight plastic bags until use. Fresh samples were prepared by placing diced roots in a food processor until sizes of approximately 1.0 × 3.0 × 5.0 mm (height × width × length) were obtained. PSP moisture content was measured by drying fresh or flour material at 105 °C in an oven for 24 h.

2.3. Solvent extraction of anthocyanins and phenolics

Two independent experiments were performed under the extraction conditions described in (Table 1). Experiment 1 evaluated extraction efficiency of individual solvents on both flour and fresh preparations. Experiment 2 was performed with flour preparations and assessed efficiency of solvent mixes containing different combinations of acetone, butanol and ethanol (ABE). Temperature levels selection corresponds to those implemented in the liquefaction (85 °C), saccharification (65 °C) and fermentation (37 °C) steps of sweetpotato processing for butanol production through acetone butanol ethanol fermentation (ABE fermentation). They allow to assess the potential to simultaneously capture sugars, solvents/acids and anthocyanins as valuable co-products. As a result of solvent evaporation at 85 °C, 210 min was the maximum time at this temperature.

The solvent ABE1 concentrations (Table 1) were selected to represent mean acetone, butanol, and ethanol levels generated during ABE fermentation by the solventogenic strain *Clostridium beijerinckii* NCIMB 8052. Solvent ABE 2 concentrations (Table 1) represented a concentrated version (4X, concentrated) of an ABE fermentation performed by *C. beijerinckii* SA-1 (Sandoval-Espinola, 2013). Solvent

concentrations in ABE 2 solution were used in binary solvent mixes, namely AB, AE, and BE. Butanol solutions below 7% v/v and above 80% v/v represent complete miscible solutions at all evaluated temperatures, while 48% butanol shows a two immiscible phase formation.

All experiments were performed in 50 mL conical Falcon tubes and PSP preparations were added to achieve a final dry solid loading of 5% w/v (dry g/total solvent). Glacial acetic acid, methanol, butanol, ethanol, acetone and distilled deionized water were used to prepare solvent stock solutions at the desired concentrations. All solvents were acidified with acetic acid (7 %v/v). For instance, 1L of acidified 80% methanol stock solution contained 70.1 mL of acetic acid, 808.1 mL of methanol and DDI water was added to reach a final volume of 1 L. A stock solution was not prepared for butanol 48% (immiscible two phase formation), instead 40 mL of the extraction mix was prepared in separate Falcon tubes, preheated, and then poured into the vessels containing the sweetpotato material. Two dry g of PSP were added to tubes and moisture content of PSP preparation was accounted for in the determination of mass added. Pre-heated solvent stock solutions were added (40 mL) to the appropriate tubes and contents were homogenized for 1 min. Extraction slurries were placed in a shaking water bath at the desired temperature (100 rpm) and samples (2 mL aliquots) were taken at specified times (Table 1) and stored at −80 °C until further analysis. For 48% butanol, 1 mL samples were taken from each phase and anthocyanins were measured from both top (Up) and bottom (Bot) phases. A minimum of three independent experimental units were prepared for each treatment (combination of temperature, solvent, PSP genotype, and preparation) and samples were removed at each sampling time as repeated measures from three different randomly selected experimental units, giving three samples replicates for each time level.

2.4. Total monomeric anthocyanin and total phenolic determination

Total monomeric anthocyanin (TMA) content was determined following the pH-differential method (Lee, Durst, & Wrolstad, 2005) and results reported as mg cyanidin-3-glucoside (cya-3-gluc) equivalents per 100 g of dry PSP or dry weight (DW) (or fresh weight, FW, as necessary to make comparisons). Briefly, anthocyanin extract was diluted in buffers at pH 1.0 (potassium chloride, 0.025M) and pH 4.5 (sodium acetate, 0.4M) and absorbances were determined at both 520 and 700 nm after 20 min of equilibration. TMA was then calculated using the molecular weight for cya-3-gluc (449.2 g/mol) and its molar extinction coefficient (26 900 t, in L/mol * cm).

Total phenolics (TPH) were determined by a modified Folin-Ciocalteau (FC) method and reported as milligrams of chlorogenic acid equivalents (CAE) per 100 g of dry PSP (mg CAE/100 g dry SP). Briefly, chlorogenic acid solutions at different concentrations were prepared and a standard curve was constructed. Reaction mixtures contained 0.25 mL of sample, 4 mL of distilled deionized water, and 0.25 mL FC reagent. After 3 min of reaction at room temperature, 0.5 mL of sodium carbonate (1 N) was added. The absorbance of the resulting blue mixture was measured at 725 nm after 1 h of incubation at room temperature (Folin & Ciocalteau, 1927).

2.5. HPLC analysis of hydrolyzed extracts

Anthocyanin extracts were hydrolyzed using HCl as described by Truong et al. (2010) and analyzed in a Thermo Finnigan HPLC System equipped with a UV6000LP photodiode array detector, AS3000 autosampler, SCM1000 degasser, P2000 binary pump and ChromQuest software version 4.1 (Thermo Electron Corp., San Jose, CA). In brief, 200 to 300 μL of anthocyanin extract and 300 μL 6 N HCl were combined and the hydrolysis was performed at 100 °C for a period of 30 min for acetone and 1 h for other solvents. After hydrolysis, samples were immediately cooled and analyzed by HPLC using a YMC C18, ODS-AM column (5 μm particle size, 150 × 4.6 mm) with a gradient elution solvent: 3% to 45% (acetonitrile containing 1% formic acid) from 0 to 20 min, and a 10 min post run at 3% (acetonitrile containing 1% formic acid) for column equilibration. The eluent flow rate was 1 mL/min with an injection volume of 10–20 μL. The autosampler tray temperature was maintained at 6 °C and the column oven at 35 °C. Identification of anthocyanidin compounds were based on the retention time with reference to cyanidin-HCl, peonidin-HCl and pelargonidin-HCl standards. Peak areas were used to determine concentration (expressed in mg/g dry PSP).

2.6. Statistical analysis and model development

A linear mixed effect model was analyzed using the *lme* package (Version 3.1) in R. Sweetpotato preparation, sweetpotato variety, temperature, solvent, and time were entered as fixed effects. The random variable was the experimental unit (treatment tube) from which repeated measures were taken over time. Statistic model for each TMA and TPH response was described by:

$$y = X\beta + Zu + \epsilon$$

Where y is a $N \times 1$ column vector containing the response variable (either TPH or TMA) of N observations; X is a $N \times p$ matrix of the predictor variables (p), p could include preparation, genotype, time, temperature and solvent with their interactions; β is a $p \times 1$ column vector of the fixed-effects regression coefficients; Z is the $N \times q$ design matrix for the q random effects; u is a $q \times 1$ vector of the random effects; and ε is a $N \times 1$ column vector of the residuals.

For experiment 1 several models including all possible predictor variables (preparation, sweetpotato clone, time, temperature and solvent) and their interactions were compared. Model selection was performed using different covariance structures for the random effects: independent, AR1, and Compound Symmetry. The random effects were modeled assuming independence among them. The best model was chosen according to the likelihood ratio tests. The normality for residuals was evaluated in all cases.

For experiment 2, the main and interaction effects of temperature and solvent were selected as predictor variables for TMA and TPH. Solvent mixes results were compared with those of acidified 80% methanol and acidified water obtained in experiment 1. SAS 9.4 (SAS ®, Cary, NC) GLM procedure was used to perform analysis of variance (classic ANOVA or Welch ANOVA) and *t*-test comparisons were made to determine if means differences were statistically significant (*t*-test, $\alpha = 0.05$). Levene and Shapiro Wilk were used to test for homoscedasticity and normality, respectively.

3. Results and discussion

3.1. Total monomeric anthocyanin (TMA) and phenolics (TPH) extraction using conventional acidified solvents

Fig. 1 shows the multiple comparisons (Tukey $\alpha = 0.05$) of the TMA and TPH least square means (LSM) across preparation, solvent and genotype. The full experimental data set for TMA and TPH content for P06-020 and NC-413 are presented in Supplementary Figs. 1 and 2. Anthocyanin and phenolic extractions were influenced by preparation, temperature, solvent, and genotype. The Analysis of Variance (ANOVA) for TMA showed that main and interaction effects of selected predictor variables were statistically significant (p-value < 0.05) including the full interaction (Supplementary Table 1). In the case of TPH, third order interactions were statistically significant and contained all predictor variables (Supplementary Table 2). Descriptive statistical analysis confirmed that extraction time did not affect TMA or TPH and it was included as a random effect in the final models. This implies that anthocyanin and phenolic extraction can be performed in shorter times within each respective treatment and similar statistical results will be obtained.

Phenolics and anthocyanin recovery were greatly influenced by PSP

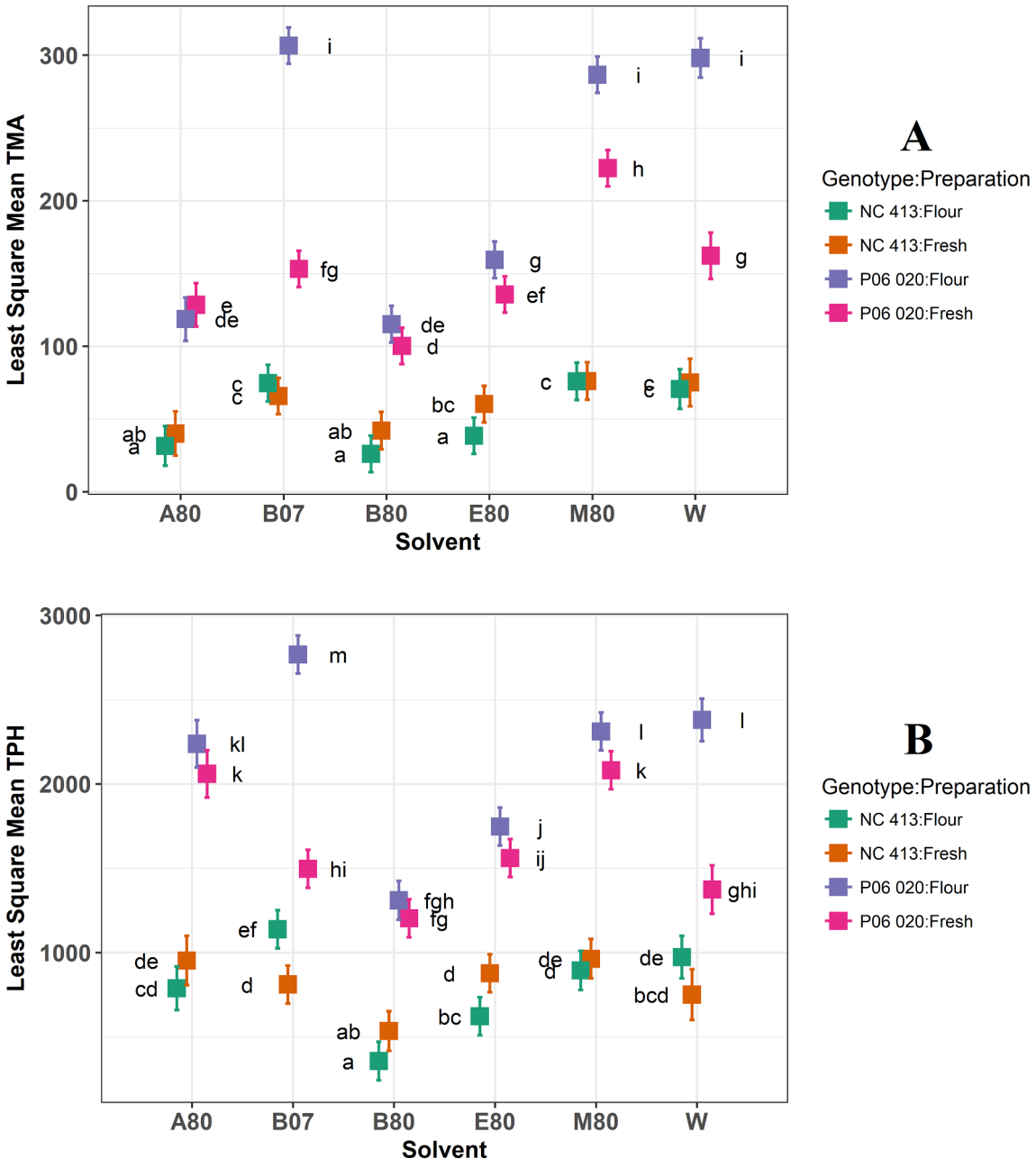

Fig. 1. Total monomeric anthocyanin (TMA) and total phenolics (TPH) per preparation, clone and solvent. A = TMA; B = TPH. Boxes indicate the least square mean (LSM) estimated by the linear mixed model at a fixed temperature level (62.3 °C, average temperature of evaluated levels). Error bars indicate the 95% confidence interval for the LSM. Means sharing a letter are not significantly different (Tukey-adjusted comparisons α = 0.05).

genotype (Fig. 1). On average, TMA and TPH values for P06-020 were at least 50% higher than those from the NC-413 for a given solvent and preparation combination. For instance, the combination of fresh-acidified acetone in NC-413 presented a TPH least square mean of 952.7 mg CAE/100 g dry PSP, while the same combination of preparation and solvent yielded an average of 2059.6 mg CAE/100 g dry PSP in P06-020 (Fig. 1B). Previous reports have shown that PSP antioxidant activity is linearly correlated (R^2 = 0.937) with TPH values (Teow, Truong, McFeeters, Thompson, Pecota, & Yencho, 2007). Therefore, P06-020 as a purple sweetpotato genotype has greater potential to provide antioxidant compounds than NC-413.

PSP preparation also influenced anthocyanin and phenolics recovery. Interestingly, among NC-413 TMA and TPH extractions, significant differences were not found between flour and fresh preparations for most solvents (Fig. 1). On the other hand, P06-020 flour preparations yielded significantly larger TMA and TPH values than fresh roots for most treatments. Smaller particles sizes of flour compared to fresh roots increase surface area available for mass transfer processes and consequently the diffusion rate of anthocyanins from the solid material into the solvent. Additionally, flour preparation steps (e.g. drying, shear from milling) could promote disruption of cellular structures encapsulating the pigments allowing for enhanced extraction. NC-413 peonidin-based anthocyanins can be more susceptible to thermal degradation during drying steps than cyanidin-based compounds found in the P06-020 genotype. The differences in anthocyanidin composition between the two PSP genotypes and thermal degradation differences among the compounds could explain the preparation effect observed.

For P06-020 flour preparations, acidified 7% butanol, acidified 80% methanol, and acidified water showed high potential in extracting both

TMA and TPH. P06-020 flour extractions with acidified 7% butanol generated the statistically highest TPH yield out of all treatment combinations examined ($P < 0.05$) (Fig. 1B). However, when fresh P06-020 materials were used, 80% acidified methanol performance was superior and statistically different than all the other solvents. Methanol efficiency in both flour and fresh preparations was anticipated given its wide use in PSP anthocyanin recovery and satisfactory results in previous works (Bridgers et al., 2010; Truong, Hu, Thompson, Yencho, & Pecota, 2012). Interestingly, acidified water and 7% butanol solutions showed similar performances to those of 80% methanol when applied to flour preparations but were significantly lower when treating fresh materials. This could be an indication that concentrated methanol has a higher ability to penetrate and disrupt the fresh PSP tissue than diluted solvents, or it has a detrimental effect on polyphenol oxidase activity, preserving polyphenolic integrity. However, the use of diluted solvents could benefit the economics of anthocyanin recovery by reducing the high solvent consumption associated with traditional solid–liquid extractions.

The lowest TMA values of either flour or fresh preparations of both P06-020 and NC-413 were found with acidified 80% acetone and acidified 80% butanol. For instance, P06-020 extractions with 80% methanol, water and 7% butanol were above 286.5 mg cya-3 gluc/100 g dry PSP, representing a minimum 2.2-fold increase in anthocyanin recovery compared to 80% butanol. Concentrated butanol results were expected since its lower polarity, compared to water and methanol, limits the dissolution process of polar molecules like anthocyanins. The lower acetone results, on the other hand, were surprising since it is widely used for anthocyanin extraction due to its high polarity and low evaporation point that facilitates concentration of resulting extracts. These results indicated that PSP anthocyanins were negatively affected by this particular combination of solvent and temperature. Interestingly, acetone performance for phenolics extraction was among the highest and suggests that acetone is an adequate extractant for total phenolics but potentially has a detrimental effect on anthocyanins at given temperatures. Significantly higher heat sensitivity of anthocyanins compared to other phenols has been reported before (Zorić, Dragović-Uzelac, Pedisić, Kurtanjek, & Garofulić, 2014). It is reasonable to assume that structural anthocyanin degradation occurred resulting in the generation of smaller phenolic compounds. That could be a reason why, conversely to anthocyanin extraction, total phenolic recovery benefited from higher temperatures in most treatments with both PSPs, even though the extent of the positive effect depended on the solvent (Supplementary Table 3). For instance, phenolic extractions with acetone, ethanol, and methanol, increased in 8.83 mg CAE/100 g dry PSP for each degree Celsius rise (0.013 standardized units/°C). This indicates that a maximum of 423.84 mg CAE/100 g dry PSP could be added to TPH values when the incubation temperature changes from 37 °C to 85 °C. A slight decline of 0.001 standardized units (0.63 mg CAE/100 g dry PSP) was observed for acidified 7% butanol extractions with both sweetpotatoes.

Temperature effect on TMA extraction varied according to clone and solvent treatment combinations (Supplementary Table 1). Temperature did not have a statistically significant effect on TMA using NC-413 materials (p-value > 0.05). Therefore, the lower temperature level, representing less energy consumption, can be ideal for pigment recovery from this sweetpotato genotype. In the case of P06-020, different treatments showed different responses to temperature changes (p-value < 0.05). Most solvents showed negative coefficient values ($\Delta\mu/\Delta T < 0$) indicating a reduction of TMA values when the temperature is increased: standardized TMA values decreased in 0.021, 0.006, 0.009, and 0.005 units when acidified 80% acetone, acidified 7% butanol, acidified 80% butanol, and acidified 80% ethanol were used for P06-020 extractions, respectively (Supplementary Table 3). These values correspond to a reduction of 1.84, 0.50, 0.83, and 0.48 mg cya 3-gluc/100 g dry PSP per degree Celsius increase, respectively, and point to acetone-P06-020 combinations as particularly temperature sensitive treatments. Reduced anthocyanin recovery in grape extracts using acetone at elevated temperature was also reported by Vatai, Škerget, Knez, Kareth, Wehowski, & Weidner (2008). High kinetic energy of acetone molecules at mild temperatures (boiling point at 60 °C) likely induced anthocyanin degradation in that study and in this work. Heat sensitivity is a well-known characteristic of anthocyanins, but our results indicate that other factors such as the type of solvent and PSP genotype and preparation also had significant effects. Water and methanol extractions seemed to benefit, at different extents, from higher temperatures. Experiments performed using acidified 80% methanol showed the highest increase in TMA (0.63 mg cya 3-gluc/100 g dry PSP *°C) per degree Celsius. Higher efficiencies using methanol at higher temperatures agree with previous results (Bridgers, Chinn, & Truong, 2010) and confirm the importance of selecting adequate extraction conditions for specific combinations of solvents and PSP genotypes as obtained by other authors working with different commodities (Wang, Jung, Tomasino, & Zhao, 2016).

Overall, our statistical results suggest that the lowest evaluated temperature level (37 °C) and extraction time (10 min) could be selected for all anthocyanin extractions with the different solvents, except perhaps for acidified 80% methanol where changing temperature from 37 °C to 85 °C resulted in a TMA increase of 30.04 mg cya-3 gluc/100 g dry PSP. Use of mild temperatures and short times do present advantages from an economic standpoint allowing energy savings and increased potential profitability. Similarly, if the primary extractant needs to be a rich-phenolics solution from PSP, acidified 7% butanol at 37 °C would be functional extraction conditions. Diluted solvents allow savings in costs associated with more concentrated solutions which has been one of the main drawbacks of the traditional process for extracting anthocyanins. Additionally, since 37 °C is the typical temperature of fermentation broths using *Clostridium* species for butanol production, there is potential to combine anthocyanin and phenolics extraction with the conversion of PSP starch to ABE. Even though 7% (v/v) butanol is not achieved by simple ABE fermentation, it can be obtained by in-situ butanol recovery techniques that increase butanol concentrations after microbial transformation (Jiménez-Bonilla & Wang, 2018).

The anthocyanin and phenolics values obtained in this study are similar to those reported by several authors for different PSPs genotypes under different extraction conditions (Table 2). Lower extraction yields compared to Cevallos-Casals & Cisneros-Zevallos (2003) and Xu et al. (2014) may be attributed to cultivar superior intrinsic characteristics, but also to differences in extraction times, temperatures, acid selection, and PSP preparation. For instance, fresh root storage (≈5 months) before processing could have led to a significant decline in anthocyanin and phenolic content as evidenced by Grace Yousef Gustafson Truong Yencho & Lila (2014). Although freeze drying used by Xu et al. (2014) preserves desired characteristics and bioactivity of compounds of interest, this preprocessing step can significantly increase production costs because of the specialized equipment and energy required. Low extraction temperatures (4 °C) reported by Cevallos-Casals & Cisneros-Zevallos (2003) may decrease polyphenol oxidase activity, but extraction time was prolonged (24 h). Additionally, use of strong acids such as HCl (pKa = 1) and formic acid (pKa = 3.75) in other studies compared to acetic acid (pKa = 4.75) could increase the disruption of enclosed cell compartments, and release of colorants, as well as the anthocyanin protonation that leads to higher solubility and stability (Khoo, 2017). However a disadvantage to using strong acids, is that they are more aggressive and can have implications in material selection for pipes, valves and vessels. In addition, different fermentation processes can generate weak acidic compounds like acetic acid making it more easily attainable, even from renewable feedstocks, than stronger acids like hydrochloric acid.

Table 2
Experimental results for solid–liquid extraction of anthocyanins and phenolics from different purple-fleshed sweetpotatoes genotypes.

TMA*	TPH#	Extraction conditions	Reference
618	3220	Acidified 95% ethanol (0.225 N HCl), 24 h, 4 °C	(Cevallos-Casals & Cisneros-Zevallos, 2003)
79–161	152–296	Acidified 95% ethanol (0.225 N HCl), 24 h, 4 °C	(Teow, Truong, McFeeters, Thompson, Pecota, & Yencho, 2007)
NA	470	80% methanol, 10 min, 80 °C, FDP.	(Padda & Picha, 2008)
318–607	1733–5152	Acidified 80% methanol (7% acetic acid), 15 min, 100 °C, FDP.	(Steed & Truong, 2008)
158§	NA	Acidified ethanol (1.15 mol/l HCl), 60 min, 80 °C, 1:32 solid ratio	(Fan, Han, Gu, & Chen, 2008)§
186§	501§	Acidified 70% methanol, 80 °C	(Bridgers, Chinn, & Truong, 2010)§
33–328	828–1992	Acidified 80% methanol (7% acetic acid), 10 min, 100 °C, FDP.	(Truong et al., 2010)
517–657	3419–4008	80% methanol (1% acetic acid), 10 min, 80 °C, FDP	(Grace, Yousef, Gustafson, Truong, Yencho, & Lila, 2014)
45–64	NA	Ethanol (produced over fermentation)	(Diaz, Chinn, & Truong, 2014)
1390	NA	5% formic acid, 40 °C, 12 h, FDP.	(Xu et al., 2014)
40–130†	100–400†	Preheat (90 °C, 10 min); citric acid, 1 h, 70 °C	(de Aguiar Cipriano, Ekici, Barnes, Gomes, & Talcott, 2015)†
191–373	1661 to 988	Acidified 80% methanol (7% acetic acid)	This paper¶
92–163	1125–1629	Acidified 80% butanol (7% acetic acid)	This paper¶
264–331	2650–2895	Acidified 7% butanol (7% acetic acid)	This paper¶
125–221	1135–2207	Acidified 80% ethanol (7% acetic acid)	This paper¶
76–190	1634–3242	Acidified 80% acetone (7% acetic acid)	This paper¶
272–335	2254–2672	Acidified water (7% acetic acid)	This paper¶
295–355	2158–2558	Acidified ABE 1 (1.1% Acetone, 1.4% Butanol, 0.1% Ethanol)	This paper¶
250–298	2651–2840	Acidified ABE 2 (5.2% Acetone 7% Butanol 0.41% Ethanol)	This paper¶
262–321	2810–3005	Acidified Acetone Butanol (5.2% Acetone 7% Butanol)	This paper¶
298–354	2569–2775	Acidified butanol ethanol (7% Butanol 0.41% Ethanol)	This paper¶
276–341	2387–2786	Acidified acetone ethanol (5.2% Acetone 0.41% Ethanol)	This paper¶

FPD: Freeze dried powder
* Total monomeric anthocyanins (mg cyanidin-3-glucoside equivalents/ 100 g dry weigh t(DW))
# Total phenolics (mg chlorogenic acid equivalents/ 100 g dry weight (DW))
§ Optimized conditions
† Data in fresh-weigh (FW) basis.
¶ Results for Flour-P06-020 across different temperatures and times. Values for Flour-NC-413 are around 50% of those for P06-020

3.2. Total anthocyanins and phenolic compounds using unconventional solvent mixes

Diluted solutions of acetone butanol and ethanol (ABE) mixes (simulating extractions using fermentation derived solvents) were tested for effectiveness in extracting anthocyanins and total phenolics from flour preparations of NC-413 and P06-020. Results were compared to 80% methanol, a popular solvent applied to anthocyanin and phenolics recovery from PSPs.

ANOVA showed that the interaction of solvent and temperature was statistically significant for both TMA and TPH in the two PSP evaluated, and time was not a significant factor (Supplementary Tables 4 and 5). Estimated means for anthocyanin and phenolics extraction using unconventional mixes of solvents are presented in Table 3.

Extraction temperature did not have a significant effect with most diluted solvent mixes, or it led to a decline in TMA values (i.e. combination of AB-P06-020). Conversely, increase in temperature benefited TMA recovery from P06-020 and NC-413 when methanol and acidified water were used as extraction solvents as previously observed. Another important result can be gathered by making comparisons across temperature (columns in Table 3): diluted solvents had a similar or even higher performance in pigment recovery compared to 80% methanol, even for those at the lowest mix concentrations as ABE 1 (1.1% Acetone, 1.4% Butanol, 0.1% Ethanol). For instance, ABE 1 showed an anthocyanin recovery yield of 327.2 mg/g at 37 °C with P06-020 flour, while 80% methanol reached significantly lower extraction (245.3 mg/100 g) under the same process conditions. ABE 1 results are of particular importance because it represents a typical ABE fermentation, with the final solvent concentrations achieved (≈8.7 g/L acetone, 10.3 g/L butanol, 0.9 g/L ethanol) by several authors with different renewable substrates (Li, Tang, Chen, Liu, & Lee, 2019). ABE 2 and all binary mixes represented a 4-fold concentrated version of the typical fermentation solvent products. Nevertheless all treatment solvent concentrations were below 7% v/v which in comparison to 80% v/v methanol would significantly decrease the use of pure solvents.

Similar to TMA results, most TPH values with unconventional mixes were not affected by temperature selection, except for the AE (acetone ethanol)-P06-020 combination where the higher incubation temperature led to higher TPH values, and BE (butanol ethanol)–NC-413 where the increased temperature decreased total phenolics recovery. Methanol, as discussed previously, showed better TPH results at elevated temperatures. Interestingly, TPH results with unconventional mixes were as good as methanol or even better at all temperature levels. For example, AB solution extraction at 65 °C reached 2898.3 mg CA/

Table 3
Least square means of anthocyanins and phenolics recovery from purple-fleshed sweetpotatoes using unconventional mixes of solvents.

PSP	Solvent	TMA				TPH			
		37 °C		65 °C		37 °C		65 °C	
P06-020	ABE1	327.2	A	316.0	abc	2431.0	CD	2365.5	c
	ABE2	280.4	B	264.9	d	2729.8	AB	2730.1	b
	AB	312.5	A*	279.6	cd	2849.4	A	2898.3	a
	AE	309.9	A	299.0	abc	2487.3	C*	2656.6	b
	BE	321.3	A	323.0	a	2665.0	B	2726.6	b
	M80	245.3	C*	298.3	bc	1981.3	E*	2286.6	c
	W	279.5	B*	313.0	abc	2281.1	D	2350.4	c
NC-413	ABE1	72.4	A	76.2	ab	893.4	C	924.3	c
	ABE2	71.6	A	68.3	c	1117.6	A	1059.9	b
	AB	73.1	A	73.2	abc	1148.4	A	1143.9	a
	AE	70.2	AB	70.5	bc	1011.1	B	947.3	c
	BE	74.5	A	73.5	abc	1128.0	A*	963.1	c
	M80	56.7	C*	76.5	ab	674.1	D*	799.8	d
	W	64.6	B*	75.1	ab	934.1	BC	916.1	c

ABE = Acetone Butanol Ethanol; AB = Acetone Butanol ; BE = Butanol Ethanol; AE = Acetone Ethanol; AM = acidified methanol; W = acidified water.
In each column for each PSP, letters indicate differences across solvents for a given temperature. LS means sharing the same letter are not significantly different. Upper case, differences among solvents at 37 °C; Lower case, differences among solvents at 65 °C.
* Indicate significant difference across rows (same solvent at 37 °C VS. 65 °C).

100 dry PSP compared to 2286.6 mg in 80% methanol. Indeed, AB showed to be a particular good TPH extractant at 65 °C with both sweetpotato materials. Solvents like butanol and acetone have been shown to have a negative effect in bacterial membranes and cell walls at low concentrations, even below 1% v/v, due to their ability to remove lipopolysaccharides (Bowles & Ellefson, 1985; Fletcher, Pilizota, Davies, McVey, & French, 2016; Vollherbst-schneck, Sands, & Montenecourt, 1984). In fact, butanol leads to adverse changes in phospholipid and fatty acid composition in solventogenic bacteria that changes membrane permeability and can limit ABE fermentation (Kumar & Gayen, 2011). Even though composition of cell walls and membranes varies between species, it is reasonable to assume that a similar process is taking place in anthocyanin removal with diluted solvents in sweetpotatoes: even at the low concentrations used in this study, the results suggest that evaluated solvents had the capacity to disrupt vacuoles where anthocyanins and pigments are normally concentrated after their biosynthesis in the cytosol (Zhang, Butelli, & Martin, 2014).

Overall, these results suggest that diluted solvents could present similar results or even outperform some solvents typically used for pigment extractions like methanol and ethanol in flour materials (Table 2). The evaluated ABE solvent blends, especially ABE1, can be expected during fermentation of soluble sugars including biomass-derived sugars by butanol producing *Clostridium* species (Sandoval-Espinola, Chinn, & Bruno-Barcena, 2015; Zuleta-Correa, 2018). Application of raw post-fermentation mixes in anthocyanin/phenolic extractions (such as ABE 1 mix), without the need of expensive purification steps, could increase both process profitability, due to savings in pure solvent acquisition, and extraction friendliness with environment, by using renewable bio-products as extraction agents. Although the benefits are promising, drawbacks are also expected from using fermentation-derived solvents. Some limitations would include low solvent and acid concentrations compared to those implemented in conventional anthocyanin extractions; variability in fermentation products; and need of a microorganism separation step. Additionally, the potential of diluted solvent mixes in extracting fresh PSP samples should be evaluated since flour preparation is a costly processing step (Kemp, 2012) and mass transfer processes are more challenging when particles sizes are increased (Silva, Costa, Calhau, Morais, & Pintado, 2017).

3.3. Anthocyanidin composition of different solvent extracts

The HPLC results for acid hydrolysis of anthocyanin extracts are shown in Fig. 2. These analyses were performed with samples taken after 1 h of extraction at 65 °C. Results indicated that anthocyanins from NC-413 were mainly composed of peonidin glucosides. In contrast, cyanidin was shown to be the main anthocyanidin extracted from P06-020 roots. Peonidin and cyanidin have been reported as primary PSP anthocyanidins by other investigators (de Aguiar Cipriano, Ekici, Barnes, Gomes, & Talcott, 2015; Li et al., 2013; Truong et al., 2010; Xu et al., 2014) and have shown higher electron scavenger activity than all other anthocyanins (Suda et al., 2008). Between cyanidin and peonidin, the former possesses more hydroxyl groups in its structure and it has been associated with larger antioxidant and antimutagenic activity (Yoshimoto, Okuno, Yamaguchi, & Yamakawa, 2001). Cyanidin-based anthocyanins have also been used for dyeing cotton fabric (Wang, Tang, & Zhou, 2016). In agreement with previous publications (Truong et al., 2010), pelargonidin was detected in all PSP extracts in lower concentrations compared to cyanidin and peonidin. Acetone hydrolyzed extracts had significantly lower anthocyanidins and as a result of sample evaporation during compound analysis, the acidified acetone data was not considered in statistical analysis.

Anthocyanidin ratios were calculated (peonidin/ cyanidin, pn/cy) and the results are shown in Table 4. The ratios agree with phenotypical characteristics of the tested PSPs: NC-413 (pn/cy $>$ 1) is closer to a red-fleshed range, whereas P06-020 (pn/cy $<$ 1) is a dark purple-fleshed genotype. Ratios were compared to evaluate if the solvent had any effect on the final pn/cy ratios regardless of anthocyanin extraction efficiency.

Results show that pn/cy ratios increased for NC413 extractions with acidified 80% butanol and the values are statistically higher from the other solvents for fresh preparations and for all solvents except methanol for flour preparations. Significant differences on pn/cy ratios were also shown for flour preparations of P06-020. For instance, acidified 80% butanol and acidified 80% ethanol had significant higher pn/cy ratios than all other extracts, while acidified water and acidified 7% butanol samples were significantly lower (p-value $<$ 0.05). Methanol was in the middle range and different to all other tested solvents. These trends point to different solvent affinity for a given type of anthocyanin over another. Particularly, butanol showed a high affinity to dissolve peonidin-based anthocyanins than cyanidin-based. Indeed, experiments with butanol 48% (data not shown), where two immiscible layers formed, confirmed that the pn/cy value was significantly larger in the upper phase (butanol-rich reddish layer) than in the bottom phase (water-rich purplish layer). While a pn/cy ratio of 4.481 $\pm$ 0.089 was determined for the bottom phase of NC-413 butanol extractions, it increased to 5.960 $\pm$ 0.060 in the upper phase. Similarly, pn/cy ratio increased from 0.352 $\pm$ 0.003 to 0.441 $\pm$ 0.006 when bottom and upper phases of P06-020 extractions were evaluated, respectively. The higher affinity of butanol for peonidin-based anthocyanins was reasonably expected. Peonidin has been associated with less polarity than cyanidin because of the decreased number of hydroxyl groups (Khoo, 2017; Yoshimoto, Okuno, Yamaguchi, & Yamakawa, 2001). These properties support the larger levels of peonidin in concentrated butanol solvent and solvent fractions, and the generally lower pn/cy ratios in the more polar solvents such as 80% acetone, 80% methanol, 7% butanol, and water where cyanidin based pigments showed to be more dissolved. Interestingly, acidified 7% butanol showed significantly lower pn/cy ratios than 80% methanol in P06-020 and NC-413 flour extractions but no statistically significant differences in TMA measurements. These results suggest that methanol is less selective in dissolving specific types of anthocyanins than diluted butanol. Due to the higher stability and antioxidant capacity of cyanidin, extracts with lower pn/cy ratios would be preferred and these results indicate that 7% butanol would show an improved performance in comparison to 80% methanol in the evaluated PSP flour preparations. Overall, these results point to anthocyanidin solvent-dependent affinity characteristics that can be used to select specific solvents and further separate/concentrate the desired anthocyanins intended for specific uses.

4. Conclusions

Anthocyanin and total phenolic compounds in fresh roots and flour preparations of two different purple sweetpotato genotypes were extracted with commonly used acidified solvents and unconventional acetone-butanol-ethanol mixes simulating biomass fermentation. Statistical analysis found significant interactions between sweetpotato genotype, sweetpotato preparation, solvent, and temperature, on anthocyanin and phenolic yields. HPLC analysis further revealed that different solvents lead to different anthocyanidin compositions of the extracts. Particularly, butanol-rich extractions presented larger peonidin/cyanidin ratios than other solvents. These results indicate that different extraction conditions should be applied for specific clones and preparations in order to obtain higher anthocyanin and phenolic values and particular compositions. Although flour preparation showed the highest extraction values, selection of fresh or flour forms will depend on storage capacities, infrastructure availability, and final intended application.

Different extraction yield capacities were also found among diluted solvent mixes of acetone, butanol, and ethanol. Solvent blends may be a sustainable alternative for anthocyanin and phenolic extraction by considering the possibility of producing them by fermentation of

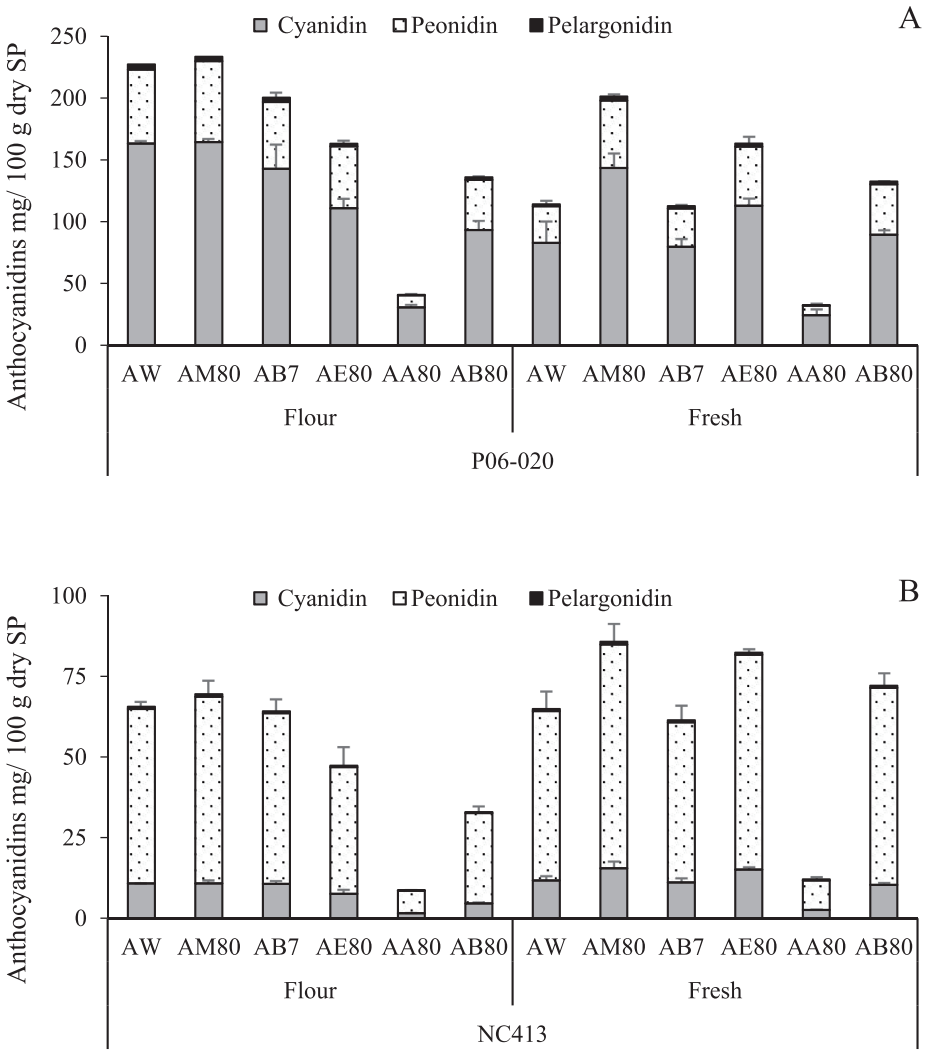

Fig. 2. Cyanidin, peonidin and pelargonidin extracted with different solvents from flour preparations of P06-020 (A) and NC-413 (B.)

Table 4
Peonidin/Cyanidin ratio for different solvent extractions.

	Average Peonidin/Cyanidin											
	NC413						P06-020					
Solvent	Flour			Fresh			Flour			Fresh		
A80	4.625	±	0.611	3.568	±	0.655	0.321	±	0.004	0.318	±	0.010
B7	4.973	±	0.045 ^{C}	4.476	±	0.086 ^{B}	0.378	±	0.002 ^{C}	0.390	±	0.009 ^{AB}
B80	6.142	±	0.086 ^{A}	5.909	±	0.109 ^{A}	0.435	±	0.007 ^{A}	0.456	±	0.020 ^{A}
E80	5.186	±	0.080 ^{BC}	4.420	±	0.132 ^{B}	0.451	±	0.009 ^{A}	0.424	±	0.058 ^{AB}
M80	5.372	±	0.229^{AB}	4.501	±	0.255 ^{B}	0.398	±	0.005 ^{B}	0.380	±	0.012 ^{AB}
W	5.011	±	0.191 ^{BC}	4.478	±	0.020 ^{B}	0.366	±	0.003 ^{C}	0.359	±	0.025 ^{B}

Tukey-adjusted comparisons ($\alpha = 0.05$). Comparisons were made within columns. Means sharing a letter are not significantly different.

biomass-derived sugars. Fermentation of sweetpotato sugars to produce solvents applicable to anthocyanin extraction would support current environment friendly manufacturing practices and increase spectrum of marketable products from pigment-rich roots.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.125959.

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