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Wathon, MH, Beaumont, N, Benohoud, M et al. (2 more authors) (2019) Extraction of anthocyanins from Aronia melanocarpa skin waste as a sustainable source of natural colorants. Coloration Technology, 135 (1). pp. 5-16. ISSN 1472-3581

https://doi.org/10.1111/cote.12385

Extraction of anthocyanins from Aronia melanocarpa skin waste as a sustainable source of natural colorants

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Abstract

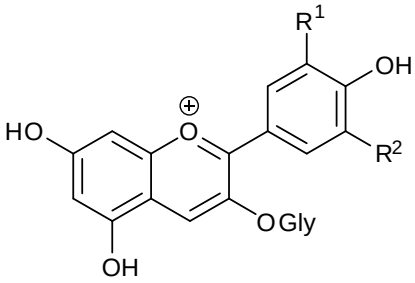
Aronia melanocarpa (Michx.) Elliott (black chokeberry) skin wastes from the production of Aronia fruit juice were extracted using a batch extraction method and a novel integrated extraction-adsorption process. Optimum conditions for batch extraction were: 60 °C, 3 h, acid (0.1% v/v HCl), biomass-solvent ratio of 1:16, and biomass-SPE resin ratio of 1:1. The integrated extraction-adsorption process gave improved anthocyanin yields of higher quality when the process was performed for 3 h without cooling of circulating liquid, and with a flow rate was 1.3 mL s^{-1}. Overall, the new method showed better anthocyanin yield and purity compared to the batch method, increasing the extraction yield by ~20% (5.25→6.34 mg g^{-1} dry weight of pomace) and increasing anthocyanin content by ~40% (19.9%→28.4% w/w dry weight of extract). This method also simplified the process as three steps were eliminated saving time and energy. Furthermore, the integrated extraction-adsorption method is industrially scalable to produce large quantities of anthocyanins. In the batch method, anthocyanins present in A. melanocarpa skins were identified as cyanidin-3-O-galactoside (38.8%), cyanidin-3-O-arabinoside (6.4%), cyanidin-3-O-glucoside (3.6%), cyanidin-3-O-xyloside (0.5%), and the cyanidin aglycon (50.7%); in the continuous method, anthocyanin content was cyanidin-3-O-galactoside (45.7%), cyanidin-3-O-arabinoside (16%), cyanidin-3-O-glucoside (3.6%), cyanidin-3-O-xyloside (2.7%), and the cyanidin aglycon (32%). The integrated extraction-adsorption method was shown to be much less susceptible to acid catalysed anthocyanin decomposition processes. All anthocyanins were derived from only one anthocyanidin parent structure (cyanidin) and only monosaccharide glycosides were identified, which is unusual when compared with other berries that typically have more anthocyanidins and/or greater glycosylation diversity.

Keywords: Black chokeberry, anthocyanin, natural dye, continuous extraction, sustainability.

Introduction

Anthocyanins are responsible for the red, purple and blue colours of fruit, flower petals and some vegetables and are the largest group of polyphenolic pigments in the plant kingdom [1]. More than 20 different anthocyanidins (the aglycons) have been identified in nature, all based on the flavan nucleus, but the six different aglycons shown in Table 1 are the most common components found in foods, leading to many anthocyanins, due to the diversity of glycosylation [2]. In total more than 600 anthocyanins have been identified in nature [3], and their colours are determined by the number of hydroxyl groups (and degree of methylation) and the nature, number and position of sugar moieties [4]. The sugar component(s) usually attached at the 3-O-position may also carry phenolic compounds, notably p-coumaric, sinapic, ferulic or caffeic acids [5]. The great variety of actual colours one observes in flower petals or raw fruit is to a large extent the result of complex formation with metal ions and/or other phenolic substances present in the plant tissues.

Table 1. Structures and absorption maxima for common anthocyanins.



Anthocyanin	**R^1**	**R^2**	**$^*\lambda_{max}$**
pelargonidin	H	H	503
cyanidin	OH	H	517
peonidin	OCH_3	H	517
delphinidin	OH	OH	526
petunidin	OCH_3	OH	526
malvidin	OCH_3	OCH_3	529

$^*\lambda_{max}$ values shown are for corresponding 3-O-glucoside at pH 3.

As shown in Figure 1, in aqueous solution of pH <3, the anthocyanin is red and the flavan nucleus exists mainly as the very stable flavylium cation (**AH^+**). Increasing pH leads to kinetic and thermodynamic competition between two reactions. When pH increases, **AH^+** undergoes a rapid deprotonation reaction (pK_{a1} ~ 3.7) to form the purple quinonoidal base (**A**) as the kinetic product, which leads to formation of the anionic quinonoidal base (**A^-**) at higher pH (pK_{a2} ~ 7) that has a blue colour [6-9]. The alternative thermodynamically favoured colourless hemiketal (**B**) is relatively slowly formed via hydration above pH 2, at position 2 (pK_h 2-3). Ring opening is also slow compared to deprotonation, but typically faster than hydration, and can lead to the formation of yellow E-chalcone (**C_E**), although for many common anthocyanins this is a relatively minor component of the equilibrium; once formed, **C_E** isomerizes to give the Z-chalcone (**C_Z**) [10]. This is activated by UV absorption, as is the reverse process, which allows reversion back to the cyclic hemiketal under

favourable conditions. Although anthocyanins can take on all the forms in aqueous solutions, it has been reported that the colourless hemiketal dominates most of 3-substituted anthocyanins at pH > 4 [11].

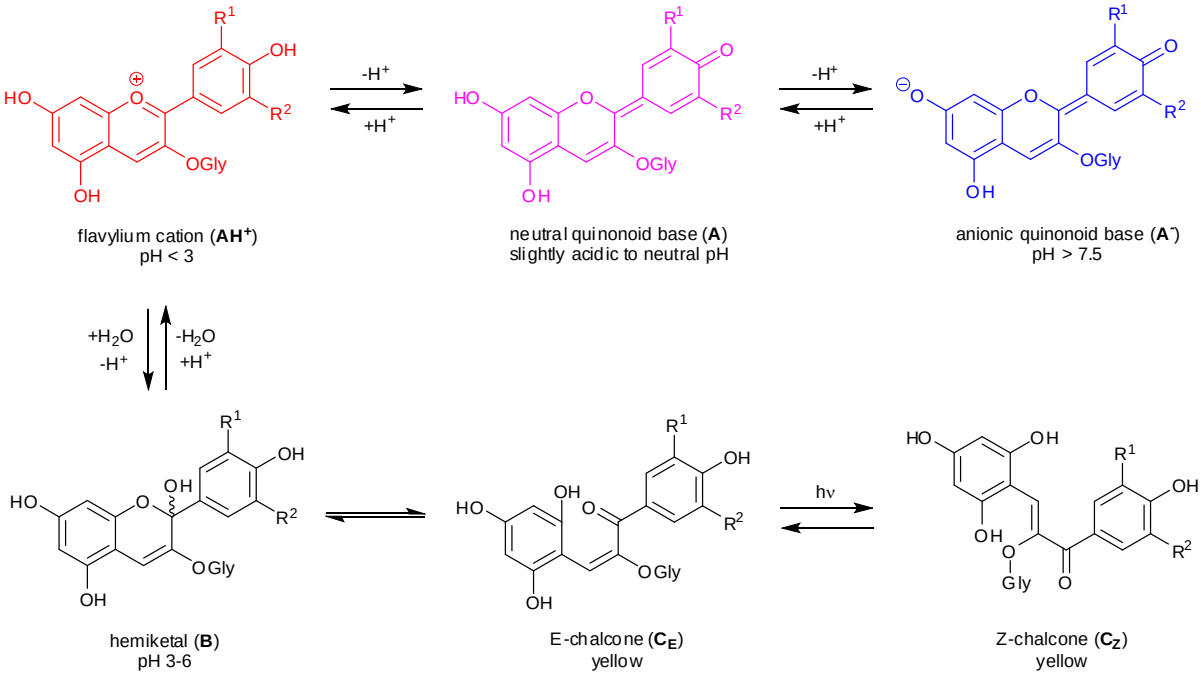


Figure 1. Effect of pH on anthocyanin structure and resultant colour.

Toxicological data on anthocyanins support the view that these pigments pose no threat to human health [12], in fact, dietary consumption of anthocyanins has been associated with many beneficial effects, including anti-inflammatory and anti-carcinogenic activities, reduced incidence of cardiovascular disease, control of obesity and diabetes alleviation [13]. Natural colorants have become preferable to the synthetic ones, as people perceive natural colorants as less toxic, harmless and safe for the environment [14].

Aronia melanocarpa (Michx.) Elliott, commonly known as black chokeberry, is from the Rosaceae family and is native to eastern North America and most commonly found in wet woods and swamps [15]. The berries contain anthocyanins in high concentrations and have a particularly simple anthocyanin profile compared to other anthocyanin-containing berries [16]. Anthocyanin content in A. melanocarpa skin is found to be higher than in the juice, which a key part of the plant's strategy to protect the seeds from the UV radiation and harmful insects [17]. The major anthocyanins found in A. melanocarpa berries are all cyanidin monosaccharides, and have been observed in the following concentrations: cyanidin-3-O-galactoside (Cy3gal; 68.9%); cyanidin-3-O-arabinoside (Cy3ara; 27.5%); cyanidin-3-O-xyloside (Cy3xyl; 2.3%); and cyanidin-3-O-glucoside (Cy3glc; 1.3%) [18,19]. Hence, there are two main anthocyanins (Cy3gal and Cy3ara) and two others present but in low

concentrations. The raw material used herein is waste of A. melanocarpa skins (epicarp) generated as a by-product following pressing of the berries for the production of Aronia fruit juice, which is usually disposed of without any further treatment. This raw material represents a potential natural, sustainable and renewable resource if it can be shown that useful products can be obtained from it in an efficient manner.

Anthocyanins present in A. melanocarpa skin waste can potentially be valorised through a suitable extraction process. Extraction of anthocyanins is influenced by many variables such as their chemical and physical properties, the extraction method employed, sample particle size, extraction time and conditions, as well as presence of interfering substances [20,21]. Solid-liquid extraction is a classical technique to recover anthocyanins from natural resources, but this method is not very selective because many other molecules are typically co-extracted along with anthocyanins [22]. Solid phase extraction (SPE) may be employed as an additional step to purify and separate selected analytes from co-extracted compounds; for anthocyanin purification, Amberlite XAD-7 has been found to be the best adsorbent used for SPE in comparison with other absorbents such as Amberlite XAD-2, Amberlite XAD-4, Amberlite XAD-16, Amberlite IRC-50 and Amberlite CG-50 [23-26].

The purpose of the research described herein is to develop and optimise the extraction and purification of anthocyanins from A. melanocarpa waste skins using a batch method and to compare the yield and quality obtained with a new integrated extraction-adsorption method.

Experimental

Chemicals and materials

Seedless black chokeberry (Aronia melanocarpa (Michx.) Elliott) pomace was provided by GreenField s.c., Warsaw, Poland; the wet A. melanocarpa pomace had been dried in a drum dryer at 75 °C for 15 min. All solvents and reagents were purchased from VWR Chemicals and Fisher Chemicals; solvents were HPLC and analytical grade and were used without further purification. Amberlite XAD-7 was used as SPE adsorbent, which is a moderately polar non-ionic macroreticular acrylic resin that adsorbs and releases ionic species through hydrophobic and polar interactions. Ultrapure water was prepared using an Elga Purelab and resistivity was measured at 18.2 MΩ. Knitted polyester for making extraction bags was provided by The School of Design, University of Leeds.

General procedures and instrumentation

Nuclear magnetic resonance (NMR) spectra were recorded for ^{1}H at 500 MHz on a Bruker DPX500 spectrometer. Chemical shifts are reported in parts per million (ppm) downfield of tetramethylsilane (TMS, singlet at 0 ppm) for proton resonances. Signals are reported as s (singlet), d (doublet), t (triplet), dd (doublet doublet) and m (multiplet). Coupling constants are reported in Hertz (Hz). To

aid characterization 2-D COSY pulse sequences were utilised. In order to maximise flavylium cationic form of the anthocyanins, samples (~20 mg) were dissolved in acidified deuterated methanol (CD_3OD/CF_3COOD 95:5).

UV/Vis analysis was carried out between 200 to 800 nm using an Agilent Cary 100 UV-Vis spectrophotometer and 1 cm quartz cells.

HPLC was carried at 25 °C with an Eclipse XDB C18, 5 μm particle size, 150 x 2.1 mm internal diameter column equipped with pre-column on an Agilent 1200 UHPLC binary pump system with online degasser and photo diode-array detection (DAD). Two solvents were used: solvent A: water (HPLC grade, 0.5% TFA); solvent B: acetonitrile (HPLC grade). A linear gradient programme was applied of 0 minutes 5% B; 0-20 minutes linear increase to 20% B; 20-23 minutes linear increase to 100% B; 23-24 minutes hold at 100% B; 24-25 minutes linear decrease to 5% B; 25-30 minutes hold at 5% B. The flow rate was 1.0 mL min^{-1} and peaks were detected at 520 nm.

LC-MS analyses were carried out at room temperature using a Phenomenex Hyperclone C_{18} column with 5-μm particle size and 250 x 4.6 mm internal diameter which was equipped with a pre-column. Elution was performed with two solvents: solvent A: water (HPLC grade, 0.1% formic acid); solvent B: acetonitrile (HPLC grade). A linear gradient programme was employed: of 0-3 minutes 0-100% increase of solvent B. The flow rate was 1 mL min^{-1} and sample injections were made using a Basic Marathon auto sampler with a 20 μL loop. The experiments were carried out on an Agilent 1200 LC with a Bruker HCT Ultra Ion Trap for MS detection and a photo diode array detector (DAD) for UV/Vis measurements. The electron spray ionisation (ESI) parameters for the positive ionisation (PI) mode were as follows: spray voltage: 4000 V; dry gas flow rate: 10 dm^3 min^{-1}; dry gas temperature: 365 °C; capillary: 60 nA; nebulising pressure: 65 psi; nebulising gas: N_2. Positive mode was found to be more sensitive for the compounds in A. melanocarpa waste skins and hence all analysis was done in positive ionisation mode.

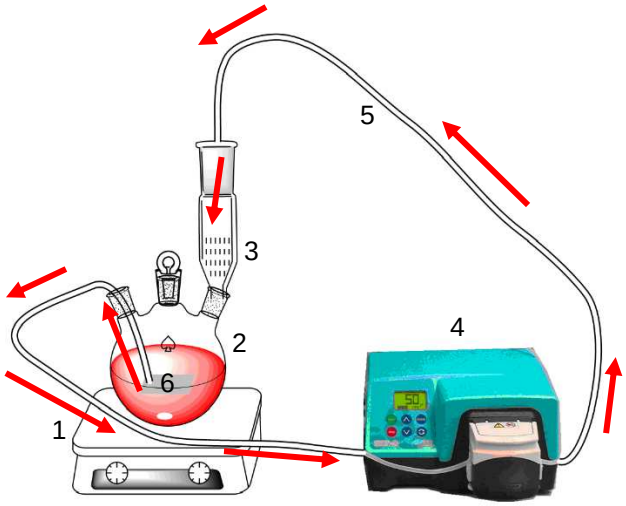
For both HPLC and LC-MS, samples were dissolved in acidified water/ethanol mixture (9:1, 0.1% v/v HCl, 1 mL) and analysed immediately.

Sequential batch extraction – adsorption method

A. melanocarpa skin waste (31.25 g) was enclosed in a polyester ‘tea bag’ (which avoided clogging the extraction apparatus with solids from the biomass) and was extracted with deionised water at pH 5.4, or acidified water at pH 2.4 (by addition of 0.1% v/v HCl) using a solid:solvent ratio of 1:16 or 1:8 (w/v), at 25, 40, 60 or 70 °C, for 3, 6, 24 or 48 h. After extraction, the solution was filtered, cooled down to ~25 °C and loaded onto an SPE column containing Amberlite XAD-7, which had been prepared by soaking in deionised water for 1 h at room temperature to remove any salts and activate the resin; the quantity of resin used was a 1:1 or 1:2 (w/w) ratio by mass of A. melanocarpa waste

skin. The resin loaded with the A. melanocarpa extract was washed with acidified water (0.1% v/v, HCl; 500 mL) to remove free sugars, and then washed with ethyl acetate (250 mL) to elute an initial fraction containing non-anthocyanin phenolics. Finally, the resin was eluted with acidified ethanol (0.1% v/v, HCl; 250 mL) to recover the anthocyanins; the resulting ethanol solution was subsequently evaporated to dryness under reduced pressure on a rotary evaporator without neutralisation. Addition of acid during elution of anthocyanins is required to prevent their degradation and to maintain the extracted anthocyanins in a stable form as the flavylium cation. In the flavylium cation form, anthocyanins have weaker affinity for the resin, which results in higher extraction yields; it was observed with experimentation that acid addition during the elution stage increased anthocyanin extraction yield by 34%, compared to elution without acid (data not shown). The resulting solid was dissolved in acidified (0.1% v/v HCl) water:ethanol (9:1 v/v) for analysis by UV/Vis spectroscopy, HPLC and LC-MS. NMR experiments were run in deuterated methanol-d_4 with addition of TFA-D_1 (CD_3OD/CF_3COOD 95:5) to allow quantitative measurement of the flavylium cation form of the anthocyanin [22].

Integrated extraction-adsorption method



1. Hotplate and magnetic stirrer
2. Three neck round bottom flask
3. SPE column
4. Peristlatic pump
5. Tube
6. Polyester bag

Figure 2. Experimental set-up for integrated extraction-adsorption process. Arrows indicate direction of flow of extract liquid.

The experimental set-up for the integrated extraction-adsorption experiment is presented in Figure 2. The extraction solution was circulated using a Watson Marlow™ peristaltic pump 323 Dz/D in a closed loop around a 1 L round-bottom flask and the resin-packed adsorption column. The circulating tubing used was made of silicone, with wall thickness of 1.5 mm; the length of tubing use for the whole circulation process was 1.5 m. The experiment was carried out using the optimum

conditions obtained from a batch method, specifically 60 °C, 3 h, 0.1% v/v aqueous HCl solution, biomass:solvent ratio of 1:16 (w/v), and biomass-SPE resin ratio of 1:1 (w/w). The cooling process during sampling loading onto an SPE column and flow rate were optimised. At the end of the integrated extraction-adsorption process, the SPE column was eluted as described for batch extraction above.

Results and Discussion

Dried *A. melanocarpa* skin waste was extracted using acidified water followed by concentration using SPE and solvent elution. This was initially carried out using a batch process, and the effect of the main parameters of extraction on yield and anthocyanin content studied. The optimum extraction parameters were then applied to an integrated method where extraction and adsorption were performed continuously as part of the same process.

Table 2. Extraction yield of anthocyanins (dry weight, DW) from various conditions for solid-liquid extraction of *A. melanocarpa* skin wastes (pomace) using a batch method. TMAC is given as % DW of extract (% of extract that is monomeric anthocyanin).

Entry	Temperature (°C)	Time (h)	pH	Biomass to solvent ratio (w/v)	Extraction yield (mg g^{-1} DW pomace)	TMAC (% w/w DW extract)
1	25	24	2.4	1:16	3.4	20.3
2	40	24	2.4	1:16	4.3	22.3
3	60	24	2.4	1:16	10.0	9.5
4	70	24	2.4	1:16	4.5	0.8
5	60	3	2.4	1:16	5.3	18.4
6	60	6	2.4	1:16	7.0	16.5
7	60	48	2.4	1:16	3.8	9.5
8	60	24	5.2[b]	1:16	6.8	11.9
9	60	6	2.4	1:8	4.3	14.4
10	60	6	2.4	1:16	7.8[a]	17.4
11	60	6	2.4	1:16	7.0	16.5

[a]1:2 ratio biomass to resin; [b]deionised water without acid addition.

In the batch method, various extraction parameters were studied to find the optimum condition which give the best extraction yield (Table 2) alongside the quality of the extract (% anthocyanins)

as determined by the HPLC profile of extract at 520 nm. More efficient extraction-adsorption processes that recover anthocyanins from A. melanocarpa waste skins show a higher extraction yield of anthocyanins. Anthocyanin concentration was estimated using the total monomeric anthocyanin content (TMAC) assay [27]. The yield of anthocyanins is then calculated by multiplying the extraction yield (mg g^{-1} dry weight of pomace) by the TMAC (% w/w dry weight of extract, see Table 5 later).

Temperature is one of the most important parameters in extracting natural products, and optimisation is intended to minimise energy consumption during processing and to maximise extraction yield. Batch extraction was investigated at varying temperatures (25, 40, 60 and 70 °C) for 24 h using acidified water as an extraction solvent (pH 2.4), biomass to solvent ratio of 1:16 (w/v), and biomass to SPE resin ratio of 1:1 (w/w) (Table 2, entries 1-4). Extraction progress was monitored using UV-Vis at 520 nm of the extract liquor to help determine optimum extraction time. Figure 3 shows that extraction at 40 and 60 °C was optimal after ca. 6 h, but then decreased gradually over longer times; extraction at 25 °C continually increased with time up to 24 h. The highest absorbance was observed at 70 °C after 3 h, but decreased dramatically after 6 or more hours, suggesting anthocyanin degradation was significant at this temperature. Indeed, Ekici et al. [28] and Galván d'Alessandro et al. [29] both report that anthocyanins degrade more rapidly at 70 °C, resulting in lower yields than the comparable extraction at 60 °C, and anthocyanins are known to show instability at high temperatures in aqueous solutions [14]. Highest absorbance after extraction for 24 h was observed at 60 °C, which suggested this temperature would be a reasonable compromise between extraction efficiency and anthocyanin degradation.

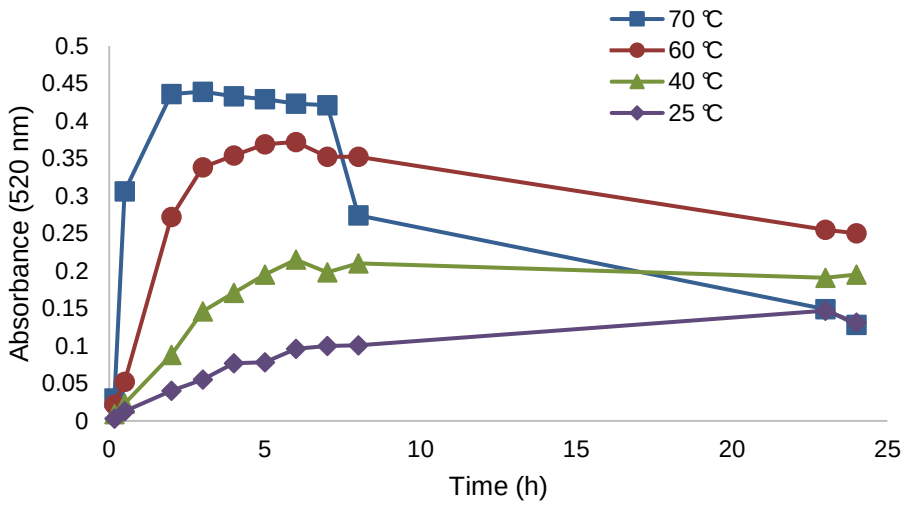


Figure 3. Extraction profiles during a batch extraction of A. melanocarpa skin wastes at various temperatures. The absorbance was monitored using UV-Vis spectrometer at 520 nm.

A quantitative comparison of extraction yield with temperature gives further information on the efficiency of the extraction method, however, it should be appreciated that the extract obtained may contain non-anthocyanin polyphenols that may not absorb at 520 nm, although most would be expected to be removed by the initial ethyl acetate wash of the SPE, prior to using acidified ethanol. Extraction yield after 24 h, post-SPE processing, ranged from 3.4 to 10.0 mg g^{-1} DW of A. melanocarpa waste skins (pomace), and decreased in the following order 60 °C >> 70 °C > 40 °C > 25 °C (Table 2, entries 1-4), similar to findings presented in Figure 3. Lower temperatures are less effective due to the lower solubility of anthocyanins and other phenolic compounds at these temperatures and the energy required to swell and disrupt the cell wall to enable solubilisation of these polyphenols into the extraction solvent. Galván d'Alessandro et al. [30] made similar observations where extraction at 60 °C afforded yields of extracted polyphenols three times higher compared to yields at 20 °C; a rise in extraction temperature can break the phenolic-matrix bonds and influence the membrane structure of plant cell. However, the yield of extracted polyphenols at 70 °C was less than half of the comparative yield at 60 °C, supporting the theory that further increase in extraction temperature may degrade anthocyanins.

Both time and temperature of extraction are particularly important parameters that need to be optimised in order to increase efficiency of the extraction; the longer the extraction time, the higher energy consumed during the extraction. Ćujić et al. [31] reported that at longer extraction periods, the highest total phenolics (TP) and total anthocyanins (TA) were observed. However, longer extraction periods at high temperatures may also give adverse effects due to degradation. To investigate these effects, extraction was carried out in a batch process at 60 °C using acidified water as an extraction solvent (pH 2.4), a biomass to solvent ratio of 1:16 (w/v), and a biomass to SPE resin ratio of 1:1 (w/w). The extraction was conducted for various times (Table 2, entries 3,5-7) and showed that over the first 24 h, the longer the extraction time, the higher the extraction yields, with a range of 3.8-10.0 mg g^{-1} DW of pomace. The highest extraction yield was obtained after 24 h, but decreased significantly when extended to 48 h (ca. 60% reduction in yield), suggesting that longer extraction periods contribute significantly to degradation of anthocyanins; this is in agreement with Galván d'Alessandro et al. [22] who reported that anthocyanin yield decreased with time, especially at high temperatures. A comparison of yields shows that ca. 50% was extracted during the first 3 h whilst another 20% were extracted during the second 3 h (6 h total extraction time). Even though extraction conducted for 6 h has a lower w/w extraction yield compared to 24 h (Table 2), absorbance is higher (Figure 3), indicating a higher anthocyanin content, further suggesting that shorter extraction times can reduce degradation of anthocyanins.

Controlling the pH of the extraction solvent is also an important factor in providing a suitable environment for the anthocyanin extraction. In order to understand the effect of pH on the extraction yield and anthocyanin content, two different extraction solvents were performed at 60 °C (Table 2, entries 3 and 8): one batch extraction used acidified water as an extraction solvent by adding 0.1% v/v, HCl (pH 2.4); whereas another batch reaction used only water with the absence of additional acid (pH 5.2). The extraction yield at pH 2.4 was significantly higher (10.0 mg g^{-1} DW of pomace) than at pH 5.2 (6.8 mg g^{-1} DW of pomace) over 24 h of extraction, confirming that acidification can increase the extraction yield. Anthocyanins are relatively stable in the flavylium cation form at pH < 3 (Figure 1) and have greater aqueous solubility than for example the anhydrobase form present at pH 5.2. This is in an agreement with observations by Ju et al. [32]. It has been documented that above pH 3 the stability of anthocyanins decreases as the red flavylium cation transforms into other less stable forms [33-36]; the colourless hemiketal is slowly formed via hydration above pH 2.5, and ring opening leads to the formation of yellow chalcone forms [10]. However, hydrochloric acid can also cause degradation via hydrolysis of the attached sugar (Figure 4), where optimal absorbance (indicating highest anthocyanin content) at pH 2.4 occurs after 6 h, but a decrease in absorbance is observed over the subsequent 18 h of extraction. The use of acid increases extraction yield, but its application is limited by the extraction time. Interestingly, although lower overall, absorbance after extraction at pH 5.2 increased progressively with time up to 24 h.

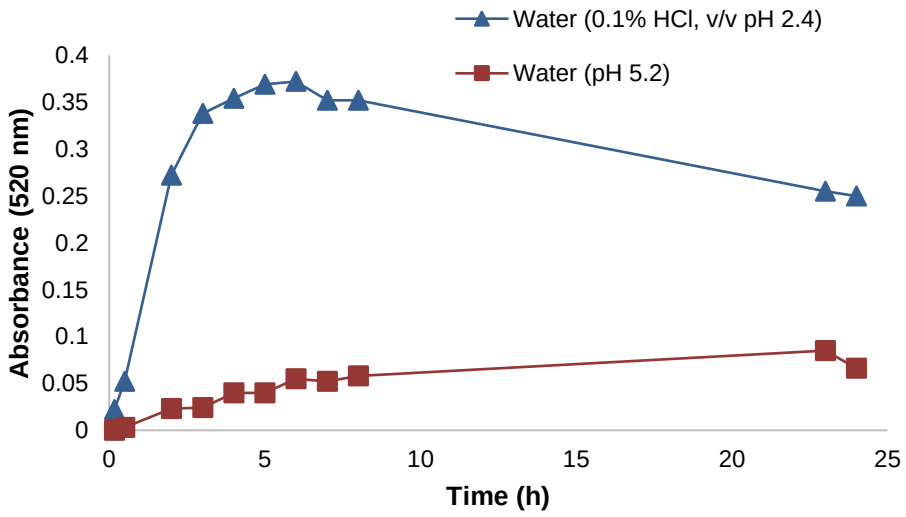


Figure 4. Extraction profiles during a batch extraction of A. melanocarpa waste skins at different pHs (2.4 and 5.2) at 60 °C. The absorbance was monitored using UV-Vis spectrometer at 520 nm.

Extraction of anthocyanins at any given temperature is potentially limited by the solubility of the extract. Changing the biomass to solvent ratio can lead to saturation of anthocyanins in the solvent;

saturation may occur when adding too much biomass. The effect of biomass-solvent ratio on the extraction yield and anthocyanin content was studied at 60 °C with acidified water as a solvent over 6 h. Extraction yields of post-SPE residues of ethanol elute were 4.3 and 7.0 mg g^{-1} DW of pomace for biomass-solvent ratios of 1:8 and 1:16, respectively (Table 2, entries 6 and 9). This is in the agreement with Liu et al. [37] who observed higher anthocyanin yields at a higher biomass-solvent ratio. A higher biomass-solvent ratio allows greater dissolution of phenolic compounds by the solvent and reduces solubility limitations. Although the biomass to liquid ratio is useful for increasing the extraction yield, further increase in the biomass to liquid ratio did not significantly improve the extraction yield. Galván d'Alessandro et al. [30] reported that increasing solid-solvent ratio from 1:20 to 1:40 could slightly decrease the extraction yields from 57.5% to 56.9%.

The effect of biomass-SPE resin ratio on the extraction yield and anthocyanin content was studied at 60 °C by conducting extractions using acidified water as an extraction solvent, using a biomass-solvent ratio of 1:16. The purpose of increasing the ratio of SPE resin was to increase the capacity for adsorbing anthocyanins during resin loading. It is expected that the higher the amount of SPE resin, the more active site that can interact with anthocyanins and other phenolics, which can maximise extraction yield. Extraction yields were 7.03 and 7.77 mg g^{-1} DW of pomace for biomass-SPE resin ratios of 1:1 and 1:2, respectively (Table 2, entries 6 and 10). Although a higher ratio of resin gave a higher extraction yield, as expected, it only afforded a 10% higher yield in comparison with a ratio of 1:1, which has limited benefit given the large increase in SPE resin requirement.

Degradation of thermolabile anthocyanins during batch extraction at relatively high temperature and low pH is unavoidable. An integrated extraction-adsorption was developed to minimise anthocyanin degradation during a batch extraction. Through this continuous process, as soon as anthocyanins are extracted, the solution is continuously circulated for adsorption onto the SPE resin, thus integrating both extraction and adsorption stages and minimising exposure of the anthocyanins to hot aqueous acid. The optimum extraction parameters obtained from a batch method experiment (60 °C, pH 2.4, biomass:solvent ratio 1:16, biomass-SPE resin ratio 1:1) were applied and operating conditions such as cooling during sample loading and liquor flow rate were studied. In the batch method after extraction was complete, the extraction liquor was cooled down and then loaded into an SPE column. In the integrated method, adsorption is integrated with extraction; it was observed that the adsorption phase (resin loading) occurred within the range 56-58 °C, which was sufficiently close to the actual extraction temperature. Different temperatures during adsorption onto the SPE column may affect the adsorption capacity of the resin, therefore, the effect of an applied cooling process during sample loading was studied. An ice bath cooler was added to the system between the extractor and the SPE column in order to maintain the temperature of extraction liquor, which was measured to range between 23-24 °C prior to resin loading. The colour of extraction liquor

exiting the SPE column in the integrated experiments was not always colourless, with some evidence of colour leaching through in most cases. Absorbance of these liquors in the extraction vessel was monitored by UV-Vis spectrophotometer at 520 nm during the extraction process; it was observed that absorbance of the extraction liquor decreased until an equilibrium was attained where no further reduction in absorbance occurred. Elution of anthocyanins from the resin with the acidic ethanol did not achieve 100% removal as evidenced by a residual faint pink colour on the column after elution.

Table 3 shows that the anthocyanin extraction yields were higher when the integrated extraction-adsorption processes was performed without the additional cooling process regardless of the extraction-adsorption time performed (3 h and 24 h); overall the highest yield was found when the extraction-adsorption was run without cooling for 24 h. This finding suggests that the cooling process is not necessarily required for the future extraction-adsorption process, and in fact a higher adsorption temperature may be beneficial to achieving higher yield of extract. Qiu et al. [38] also investigated the effect of resin loading temperature on adsorption of polyphenols at 25, 30, 35, and 40 °C, but found that there was little effect on the adsorption capacity onto an acrylic ester-based resin. Fundamental thermodynamic theory applying Gibbs free energy change states that sorption energy ($-\Delta G$) is directly proportional to temperature ($-\Delta G = R.T.\ln K$) [39]. This is always balanced with solubility of the sorbate in the solvent, but it is understandable why sorption of the anthocyanins onto resin herein at 60 °C was higher than at 25 °C as once they interact with the resin the binding is so strong that there is no subsequent desorption back into solution.

Table 3. Extraction yield of anthocyanins from various conditions for solid-liquid extraction of *A. melanocarpa* skin wastes using an integrated extraction-adsorption method. All extractions were carried out at 60 °C, pH 2.4, biomass:solvent ratio 1:16, biomass-SPE resin ratio 1:1.

Entry	**Time (h)**	**Cooling prior to resin loading?**	**Resin loading flow rate (mL s^{-1})**	**Extraction yield (mg g^{-1} DW pomace)**	**TMAC (% w/w DW extract)**
1	3	Yes	1.0	8.1	19.3
2	3	No	1.0	9.1	18.2
3	24	Yes	1.0	10.7	8.4
4	24	No	1.0	20.0	5.6
5	3	No	0.6	7.8	15.6
6	3	No	1.3	14.4	14.5

Table 3 also shows that the higher the flow rate of sample loading, the higher the extraction yield, with an observed range of 7.8-14.4 mg g^{-1} DW of pomace and highest extraction yield at a flow

rate of 1.3 mL s^{-1}. At higher sample loading flow rates, the residence time of anthocyanins in the extraction cell is shorter while the adsorption of anthocyanins in the SPE resin is faster which therefore minimises the effect of degradation. A greater extract liquor volume throughput obtained by increasing flow rate gave more chance for the resin to interact with extracted anthocyanins. Loading flows above 1.3 mL s^{-1} resulted in overflowing of the packed-resin column and subsequent overpressure, which stopped the SPE system from working effectively. Anthocyanins are expected to be found in the post-SPE residues of ethanol eluent whereas non-anthocyanin phenolic compounds are expected to be found in the post-SPE residues of ethyl acetate eluent. As this research focuses on anthocyanins, detailed characterisation was done only on post-SPE residues of ethanol eluent by using UV-Vis, HPLC, LC-MS and ^{1}H-NMR.

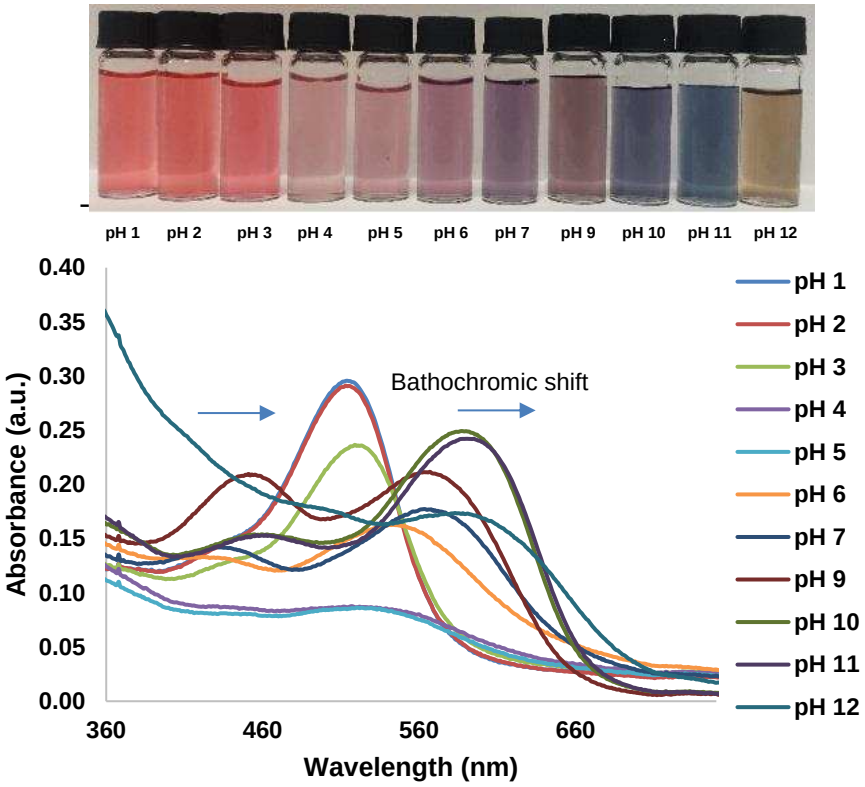


Figure 5. Effect of pH on UV-Vis absorbance of anthocyanins extracted from A. melanocarpa waste skins; measurement was carried out on post-SPE residues of the ethanol elute (1 mg extract in 1 mL acidified water (0.1% v/v HCl)). Top image shows colours of aqueous solutions tested.

In order to understand the colour profile of anthocyanins extracted from A. melanocarpa, the addition of extracted stock solution into buffer solutions was performed and colour changes observed. The results at various pHs ranging from 1 to 12 are presented in Figure 5, where below pH 3 it is observed that the stable red flavylium cation (**AH^{+}**) exists with $\lambda_{max\text{-}vis}$ of 514 nm, in agreement with Jakobek et al. [40] for typical wavelengths of anthocyanins found in A. melanocarpa. Increasing pH

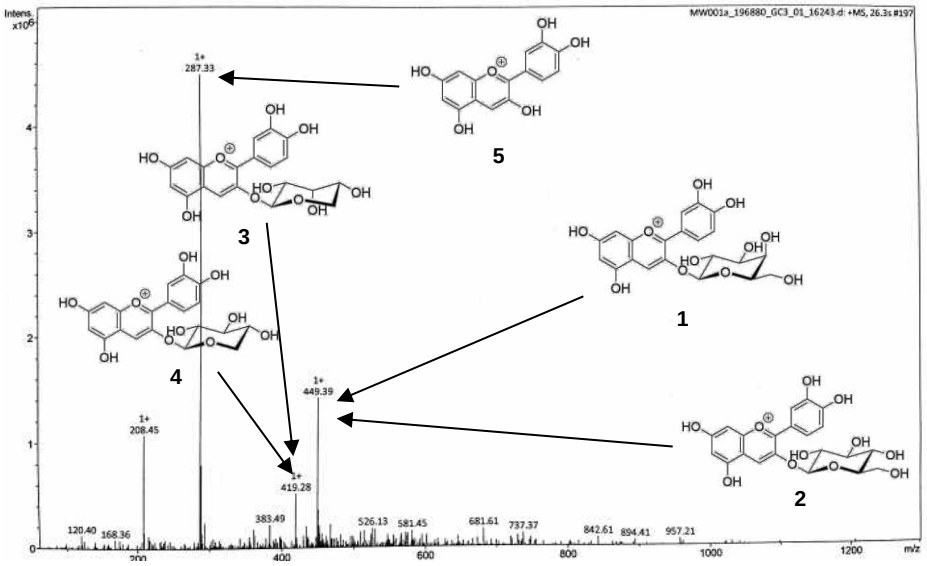
leads to kinetic and thermodynamic competition between two reactions. Deprotonation occurs above pH 3 to form the purple quinonoidal base (**A**) with $\lambda_{max\text{-}vis}$ of 564 nm, and above pH 7 the anionic quinonoidal base ($\mathbf{A}^-$) forms with its characteristic blue colour with $\lambda_{max\text{-}vis}$ of 590 nm. It is noted that the intensities of the colours at pH 4 and pH 5 are lower than at pH 6 and pH 7 and this is most likely due to more rapid formation of the colourless hemiketal (**B**) [11]. The observed blue-shift in $\lambda_{max\text{-}vis}$ that occurs with anthocyanins as pH increases ($\mathbf{AH}^+$ 514 nm → **A** 564 nm → $\mathbf{A}^-$ 590 nm) is in an agreement with the results reported by Fossen et al. [41] for Cy3glc.

HPLC analysis gives information of the anthocyanin profile and is very useful in compound identification. Anthocyanins can be detected at 520 nm using HPLC-UV analysis [16,42], which was chosen as the detection wavelength herein. The elution pattern was consistent with previous work [16,42,43] (see profile in Figure 9 later): Cy3gal (45.7%) was the most abundant anthocyanin, followed by Cy3ara (16%), Cy3glc (3.6%) and Cy3xyl (2.7%). The percentage of the aglycon cyanidin was relatively high (32%) as the consequence of deglycosylation during the extraction, as was previously observed by Chandra et al. [42]. LC-MS was used to further resolve the identification of anthocyanins present in the post-SPE residues of ethanol elute. A full separation of the peaks was achieved using this technique and differentiation between penta-substituted glycosides (Cy3gal and Cy3glc) and tetra-substituted glycosides (Cy3ara and Cy3xyl) of cyanidin was achieved; the aglycon is also detected (Table 4). Cy3gal and Cy3glc were identified with a mass to charge ratio (m/z) of 449.39, Cy3ara and Cy3xyl were identified with m/z of 419.28, and cyanidin was identified with m/z of 287.33, which are consistent with previous research [42,44].

^{1}H-NMR studies were conducted to characterise anthocyanins and other polyphenols extracted from *A. melanocarpa* waste skins. The proton chemical shifts were assigned using 1D and 2D NMR techniques. The ^{1}H-NMR spectra of post-SPE residues of ethyl acetate and ethanol eluents are compared in Figure 6. The region of 8.5-9.2 ppm is particularly diagnostic for anthocyanins and signals are commonly found in this region in the post-SPE ethanol residues, but are notably absent in the ethyl acetate fractions. The ratio of anthocyanins in ethanolic residues is consistent with the results obtained from HPLC experiments as determined by integration of the anthocyanin proton (H-4) diagnostic chemical shifts (singlets, 8.92-9.02 ppm). Evidence for the glycosyl moieties is from the signals in the region of 3.2-4.3 ppm.

Table 4. Chromatogram of anthocyanins obtained from post-SPE residues of ethanol elute by using LC-MS-ESI with accompanying data and compound identification.

Compound	$[M]^+$ m/z (reference) [42,44]	$[M]^+$ m/z (experimental)	Identification
1	449	449.39	Cy3gal
2	449	449.39	Cy3glc
3	419	419.28	Cy3ara
4	419	419.28	Cy3xyl
5	287	287.33	Cyanidin



In this study, a new proposed method namely an integrated extraction-adsorption process was compared to a more conventional batch extraction (Figure 7). In the batch method, the first three steps did not contribute to any purification of anthocyanins extracted from *A. melanocarpa* skin wastes but provide the sample for the SPE. However, the integrated method replaced the first three steps in a batch method which eventually simplified the process and reduced the energy and time consumption during the process. Thus, this process is potentially suitable to be scaled up to an industrial scale. Although extraction yields are important, the quality of that extract, particularly with regard to anthocyanin content is of primary importance in this study. Hence the extraction yield and anthocyanin content of both methods were also compared to find the best method overall, and identify the optimum method for further preparative experiments. The comparison study was done for the experiment run at 60 °C for 3 h using the other optimum parameters.

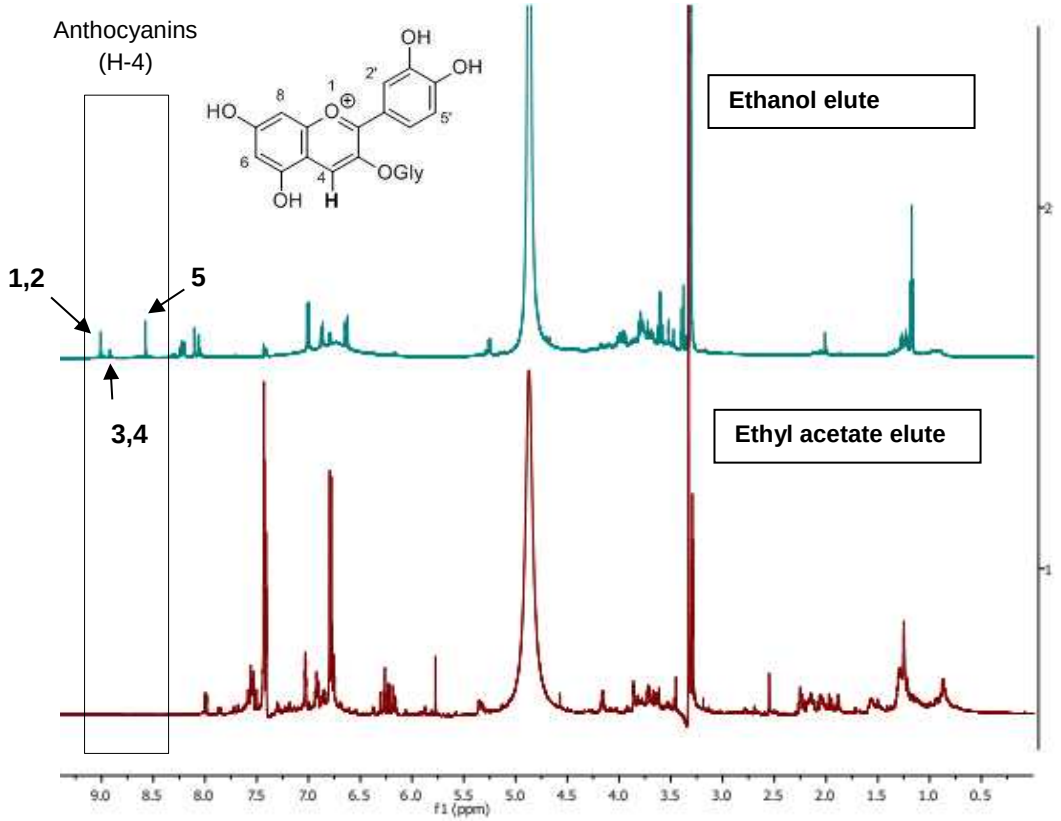


Figure 6. ^{1}H-NMR spectra of post-SPE residues of ethyl acetate elute and ethanol elute. The ^{1}H-NMR spectra were recorded at 500 MHz. Labels **1-5** refer to the five signals seen in the ^{1}H NMR spectra of anthocyanins and correspond to their H-4 protons.

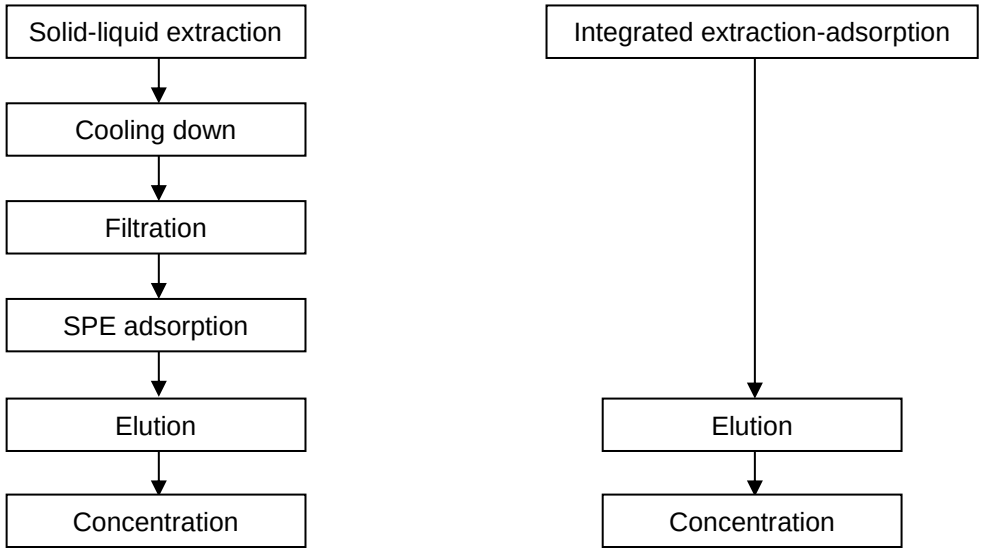


Figure 7. The comparison between a batch method (left) and an integrated extraction-adsorption method (right).

Table 5 shows that the highest yield of anthocyanins was found using the integrated extraction-adsorption method. An integrated extraction-adsorption method could increase the extraction yield up to about 20% and the anthocyanin content up to 40%. The continuous circulating process in a closed loop enriches the anthocyanins adsorbed in the SPE resin. It also avoids further degradation during extraction under the acidic high temperature environment as once anthocyanins were extracted they were circulated and absorbed into a SPE resin.

Table 5. Yield of anthocyanins recovered from A. melanocarpa skin wastes was obtained from post-SPE residues of ethanol wash. The extraction was conducted at 60 °C for 3 h.

Extraction method	**Extraction yield (mg g^{-1} DW pomace)**	**TMAC (% w/w DW extract)**	**Yield of anthocyanins (mg g^{-1} DW pomace)**
Batch	5.25	19.9	1.04
Integrated	6.34	28.4	1.80

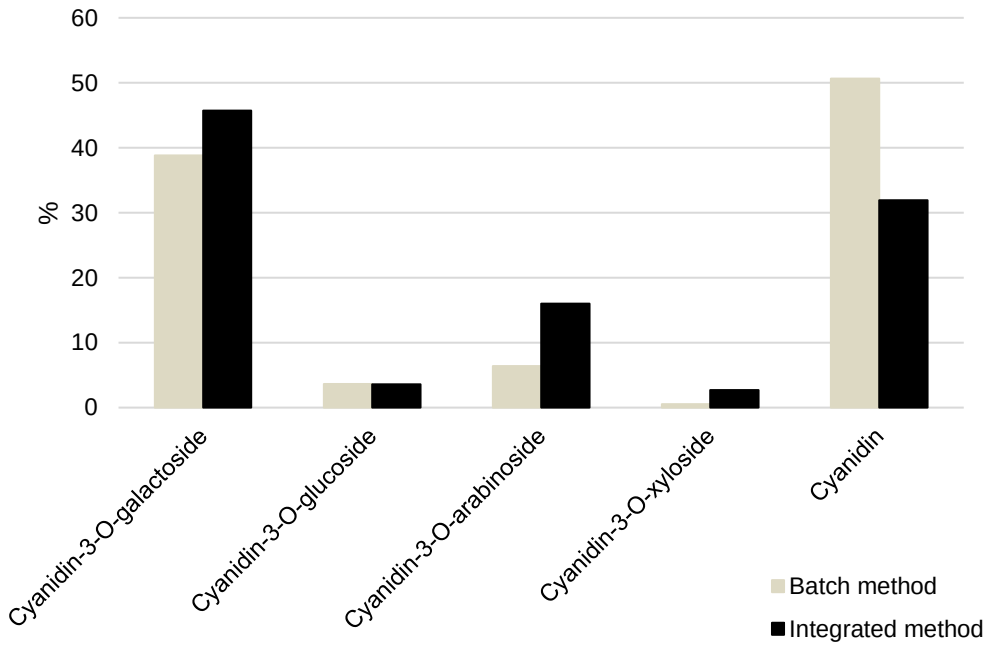


Figure 8. Total anthocyanins calculated according to relative percentages shown in HPLC chromatograms of batch method and integrated method. Solvent **A**: H_2O:TFA (99.5:0.5); solvent **B**: acetonitrile. Detected by DAD at 520 nm.

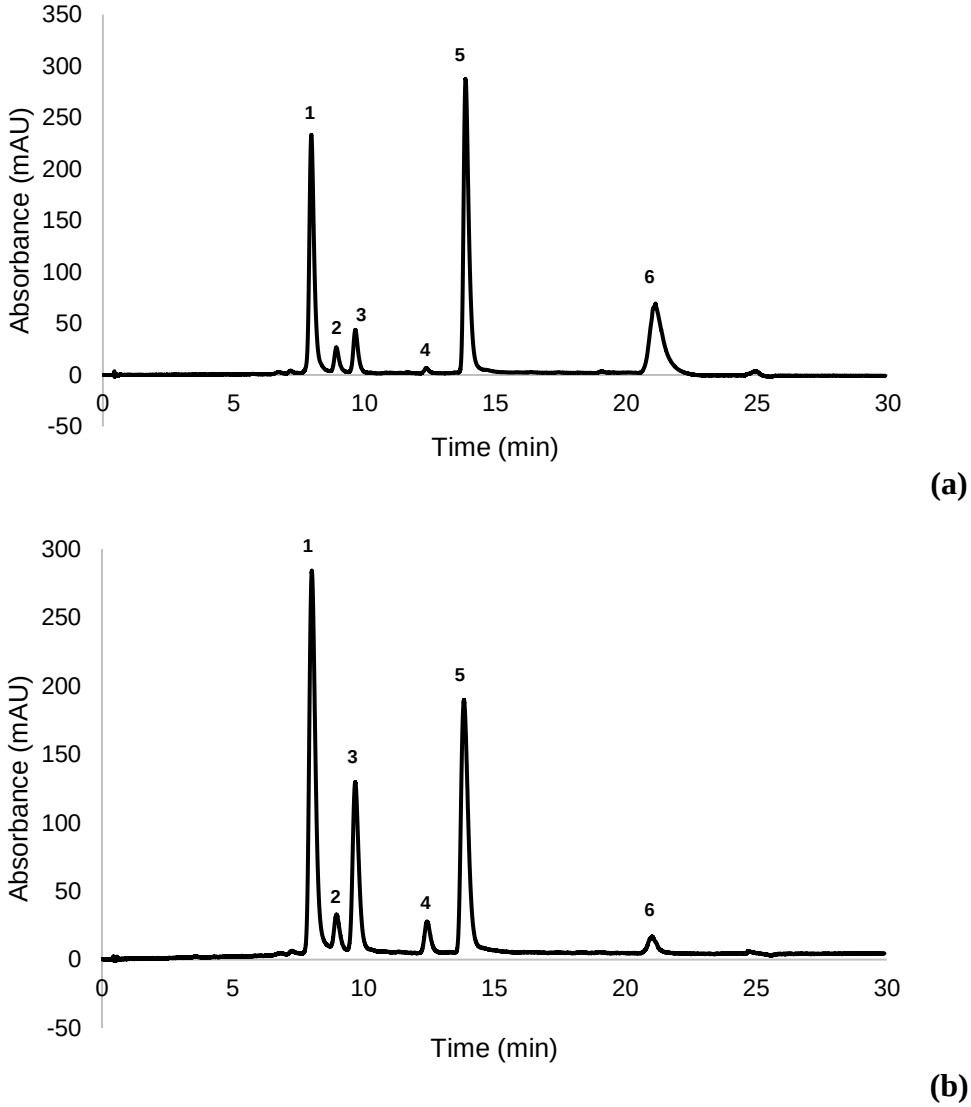


Figure 9. Anthocyanins profile shown in HPLC chromatograms of **(a)** batch method and **(b)** integrated method. Solvent **A**: H_2O/TFA (99.5:0.5); solvent **B**: acetonitrile. Detected by DAD at 520 nm: **1**. Cy3gal; **2**. Cy3ara; **3**. Cy3glc; **4**. Cy3xyl; **5**. cyanidin (aglycon); **6**. polymeric species.

As shown in Figure 8, % cyanidin (aglycon) in the batch method is higher than observed for the integrated method, indicating that greater deglycosylation of anthocyanins occurred during batch extraction, whereas % Cy3gal, Cy3ara and Cy3xyl were higher in integrated method. However, both batch and integrated methods showed the same trend in the levels of individual anthocyanins present (Cy3gal > Cy3ara > Cy3glc > Cy3xyl) (Figure 9). Polymeric anthocyanins, predominantly of (–)-epicatechin, are a major class of polyphenolics compounds found in A. melanocarpa berries and represent 66% of fruit polyphenols [7]. A higher concentration of polymeric species was found in the batch method, compared to integrated method. The reasons for this observation are unclear, but it is possible that the batch method is more effective at extracting both monomeric and polymeric

anthocyanins, or perhaps that the continuous method is more selective for monomeric anthocyanins. Another possibility is that the integrated method could be breaking up the polymeric anthocyanins, so a lower concentration is detected. This requires further investigation as it is preferable to minimise the concentration of polymeric species as this improves application properties for further applications of the extract. Based on this result, it can be concluded that a new proposed method, namely an integrated extraction-adsorption method showed an improvement in terms of yield and purity of anthocyanins compared to a batch method. Thus, this method will be used for obtaining anthocyanins from A. melanocarpa skin wastes in future studies.

^{1}H NMR provides further relevant information for comparing the two extraction methods (Figure 10). The characteristic peak of anthocyanins is shown by a singlet of H-4 at the region of 8.5-9.0 ppm. It is noted that the ratio of the H-4 proton signals for Cy3gal+Cy3glu (**1**+**2**) and Cy3ara+Cy3xyl (**3**+**4**) are similar for both the batch and integrated methods. In the region 6.5-7.5 ppm the peaks are for neutral polyphenol rings, such as those on the anthocyanins and any other polyphenols present. The broad peaks of polymeric species obscured the peaks of aromatics in these regions.

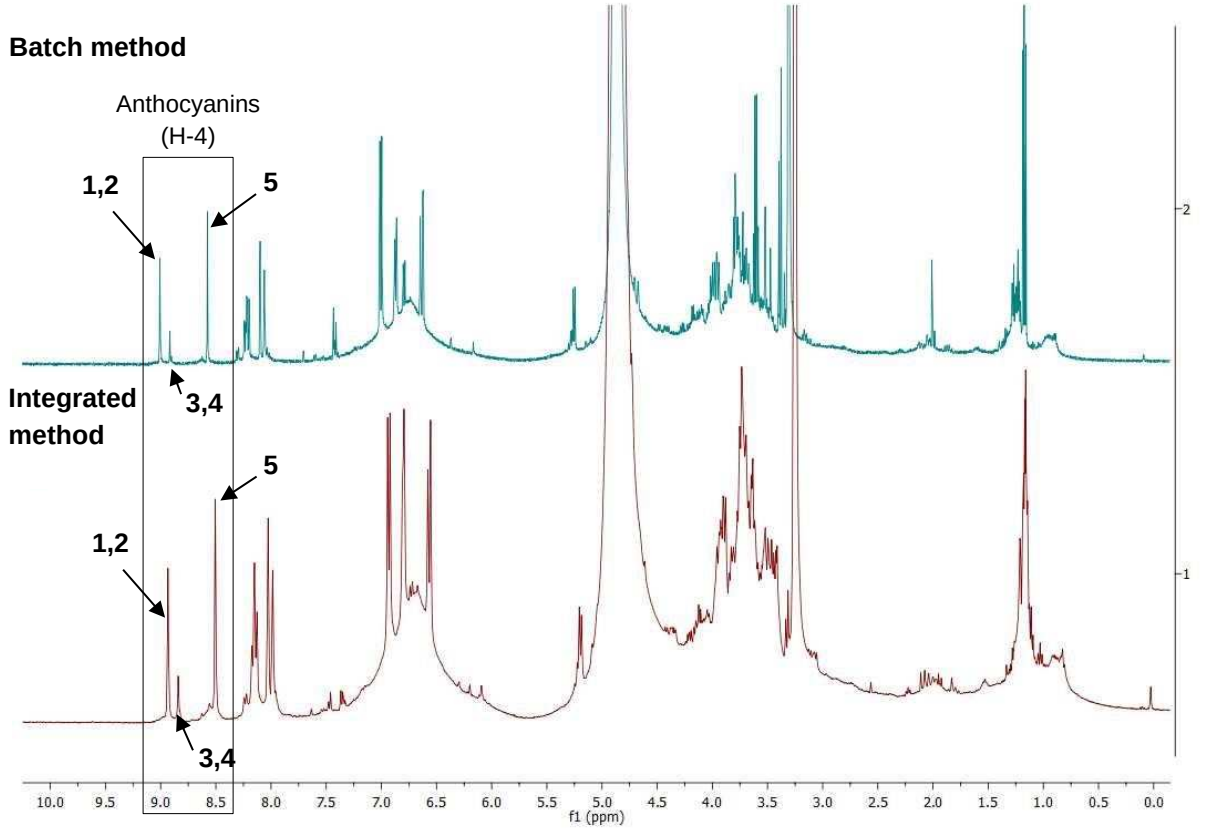


Figure 10. ^{1}H-NMR spectra of post-SPE residues of ethanol wash for batch method and integrated method. The ^{1}H-NMR spectra were recorded at 500 MHz. Labels **1-5** refer to the five signals seen in the ^{1}H NMR spectra of anthocyanins and correspond to their H-4 protons.

Different concentrations of acid were also studied in relation to the formation of cyanidin through deglycosylation for both batch and integrated method (Figure 11). An increase in acid concentration for the batch method clearly correlates to the rate of deglycosylation of anthocyanins in forming the aglycon cyanidin. This can be explained by the fact that extraction at relatively high temperature and acidic condition can promote hydrolysis; furthermore, Cy3ara suffers the deglycosylation more compared to Cy3gal suggesting that pentose sugars are more prone to hydrolysis than hexose sugars. Interestingly, using the integrated method, the formation of cyanidin is only slightly increased by an increase in acid concentration, confirming that this method could avoid any further degradation during the extraction-adsorption process.

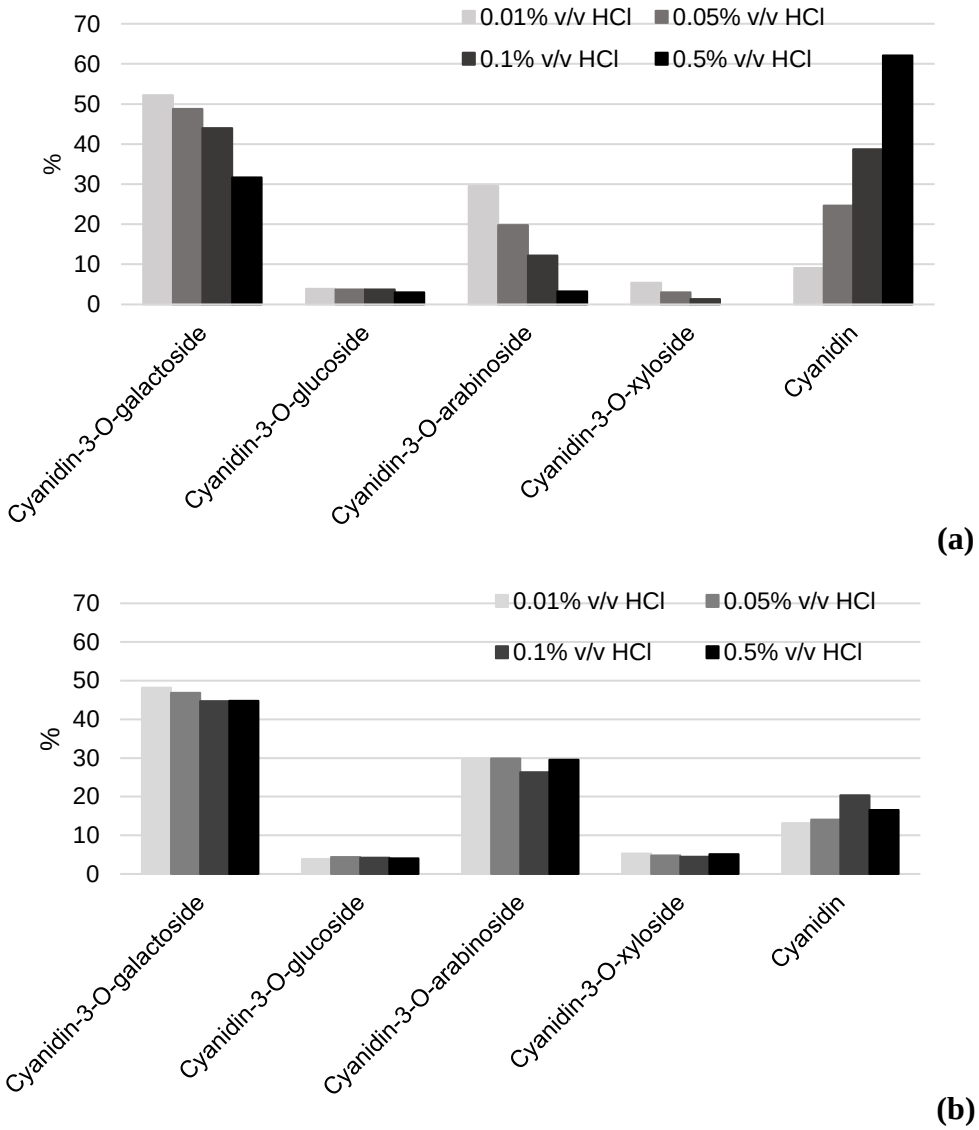


Figure 11. The effect of acid concentration on total anthocyanins calculated according to relative percentages shown in HPLC chromatograms of (a) batch method and (b) integrated method. Solvent **A**: H_2O/TFA (99.5:0.5); solvent **B**: acetonitrile. Detected by DAD at 520 nm.

Conclusions

Extraction conditions have been optimised in order to produce anthocyanins in higher quality and reduce losses during extraction. This study compared a relatively conventional batch method with a new integrated continuous method for recovering anthocyanins from A. melanocarpa skin wastes. The methods investigated consist of two main processes, namely extraction and adsorption. Whilst extraction and adsorption are performed separately in a batch method, the new proposed method offers an integrated extraction-adsorption approach. The optimum conditions used for the batch method are as follows: extraction temperature of 60 °C, extraction time of 3 h, acid additive (0.1% v/v HCl), biomass-solvent ratio of 1:16 and biomass-SPE resin ratio of 1:1. The new method demonstrated the potential to obtain higher anthocyanin yields. The effect of cooling process during sample loading and flow rate of sample loading were investigated. Higher anthocyanin yields were obtained when the process was performed for 3 h without cooling and the flow rate was 1.3 mL/s. Overall, the new integrated method gave better anthocyanin yields and purity compared to the batch method. This method also simplified the process as three steps were eliminated which saves time and energy. The integrated extraction-adsorption also can avoid the degradation of anthocyanins as once anthocyanins were extracted from biomass, they were adsorbed into an SPE resin afterwards. In a batch method, Cy3ara suffers the deglycosylation more compared to Cy3gal during the extraction with an increase of acid concentration. Meanwhile, further hydrolysis can be avoided using the integrated method. Furthermore, a new proposed method is potentially industrially scalable, which potentially will be applied to produce a larger quantity of anthocyanins. Anthocyanins present in A. melanocarpa skins were identified as Cy3gal, Cy3ara, Cy3glc, Cy3xyl and the cyanidin aglycon; Cy3gal and Cy3ara are the major anthocyanins found in A. melanocarpa berries. This is a particularly interesting observation as only one anthocyanin parent structure (cyanidin) and only monosaccharide glycosides were identified in the fruit, which is not typical compared to other berries which have a wider range of anthocyanins and/or more diverse glycosylation.

Acknowledgments and Funding Information

The authors would like to thank Indonesia Endowment Fund for Education (LPDP) for the provision of financial support to MHW to allow him to conduct his PhD studies.

References

1. L-J Chen, G Hrazdina, Phytochemistry, **20** (1981) 297.
2. T P Coultate, Food: The Chemistry of its Components, 6th Edn. (Cambridge: Royal Society of Chemistry, 2016).

3. Ø M Anderson, M Jordheim.. The Anthocyanins. In Flavonoids: Chemistry, Biochemistry and Applications, Eds. Ø M Andersen, K R Markham (Boca Raton: CRC Press, Taylor & Francis Group, 2006).
4. G Mazza, R Brouillard, Phytochemistry **29** (1990) 1097.
5. M M Giusti, R E Wrolstad, Biochem. Eng. J. **14** (2003) 217.
6. R E Wrolstad, J. Food Sci. **69** (2004) C419.
7. K Yoshida, M Mori, T Kondo, Nat. Prod. Rep. **26** (2009) 884.
8. P Trouillas, J C Sancho-García, V De Freitas, J Gierschner, M Otyepka, O Dangles, Chem. Rev. **116** (2016) 4937.
9. A L Maçanita, P Moreira, J C Lima, F Quina, C Yihwa, C Vautier-Giongo, J. Phys. Chem. A **106** (2002) 1248.
10. C Houbiers, J C Lima, A L Maçanita, H Santos, J. Phys. Chem. B, **102** (1998) 3578.
11. F Pina, M J Melo, C A T Laia, A J Parola, J C Lima, Chem. Soc. Rev. **41** (2012) 869.
12. T K McGhie, M C Walton, Mol. Nutr. Food Res. **51** (2007) 702.
13. J He, M M Giusti, Annu. Rev. Food Sci. Technol. **1** (2010) 163.
14. T Coultate, R S Blackburn, Color. Technol. **134** (2018) 165.
15. plants.usda.gov/core/profile?symbol=PHME13, visited 17th June 2018.
16. L Jakobek, M Šeruga, M Medvidović-Kosanović, I Novak, Dtsch. Leb. **103** (2007) 58.
17. J Oszmianski, A Wojdylo, Eur. Food Res. Technol. **221** (2005) 809.
18. J Oszmianski, J C Sapis, J. Food Sci. **53** (1988) 1241.
19. A W Strigl, E Leitner, W Pfannhauser, Z. Lebensm. Unters. Forsch. **201** (1995) 266.
20. I Ignat, I Volf, V I Popa, Food Chem. **126** (2011) 1021.
21. M Naczk, F Shahidi, J. Chromatogr. A **1054** (2004) 95.
22. L Galván d'Alessandro, P Vauchel, R Przybylski, G Chataigné, I Nikov, K Dimitrov, Sep. Purif. Technol. **120** (2013) 92.
23. A. Kraemer-Schafhalter, H. Fuchs, W. Pfannhauser, J. Sci. Food Agric. **78** (1998) 435.
24. E M Silva, D R Pompeu, Y Larondelle, H Rogez, Sep. Purif. Technol. **53** (2007) 274.
25. P M Rose, V Cantrill, M Benohoud, A Tidder, C M Rayner, R S Blackburn, J. Agric. Food Chem. **66** (2018) 6790.
26. S Farooque, P M Rose, M Benohoud, R S Blackburn, C M Rayner, J. Agric. Food Chem. DOI: 10.1021/acs.jafc.8b04373.
27. J Lee, R W Durst, R E Wrolstad, J. AOAC Int. **88** (2005) 1269.
28. L Ekici, Z Simsek, I Ozturk, O Sagdic, H Yetim, Food Anal. Methods **7** (2014) 1328.
29. L Galván d'Alessandro, K Dimitrov, P Vauchel, I Nikov, Chem. Eng. Res. Des. **92** (2014) 1818.
30. L Galván d'Alessandro, K Kriaa, I Nikov, K Dimitrov, Sep. Purif. Technol. **93** (2012) 42.

31. N Ćujić, K Šavikin, T Janković, D Pljevljakušić, G Zdunić, S Ibrić, Food Chem. **194** (2016) 135.
32. Z Y Ju, L R Howard, J. Agric. Food Chem. **51** (2003) 5207.
33. A Castañeda-Ovando, M de L Pacheco-Hernández, M E Páez-Hernández, J A Rodríguez, C A Galán-Vidal, Food Chem. **113** (2009) 859.
34. F Pina, J. Agric. Food Chem. **62** (2014) 6885.
35. F Pina, J Oliveira, V De Freitas, Tetrahedron **71** (2015) 3107.
36. V O Silva, A A Freitas, A L Maçanita, F H Quina, J. Phys. Org. Chem. **29** (2016) 594.
37. G L Liu, H H Guo, Y M Sun, Int. J. Mol. Sci. **13** (2012) 6292.
38. X Qiu, N Li, X Ma, S Yang, Q Xu, H Li, J Lu, J. Environ. Chem. Eng. **2** (2014) 745.
39. P Atkins, J de Paula, *Atkins'* Physical Chemistry, 10th Edn. (Oxford: Oxford University Press, 2014).
40. L Jakobek, M Drenjančević, V Jukić, M Šeruga, Sci. Hortic. (Amsterdam). **147** (2012) 56.
41. T Fossen, L Cabrita, O M Andersen, Food Chem. **63** (1998) 435.
42. A Chandra, J Rana, Y Li, J. Agric. Food Chem. **49** (2001) 3515.
43. K R Määttä-Riihinen, A Kamal-Eldin, P H Mattila, A M González-Paramás, R Törrönen, J. Agric. Food Chem. **52** (2004) 4477.
44. R Taheri, B A Connolly, M H Brand, B W Bolling, J. Agric. Food Chem. **61** (2013) 8581.