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Characterization and purification of anthocyanins from black peanut (*Arachis hypogaea* L.) skin by combined column chromatography

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ABSTRACT

Black peanut skins as a byproduct from peanut industry contain abundant anthocyanins, evaluated as 8.61 ± 0.27 mg/g dry black peanut skins, are currently poorly exploited. In this work, four anthocyanins and three major flavonols were detected and identified by HPLC-PDA-ESI–MS/MS from the acidified water extract of black peanut skins of *Arachis hypogaea* L. After preliminary removal of flavonols by ethyl acetate (EtOAc), further purification of the anthocyanins was conducted using a combination of Amberlite XAD-7HP and ODS-AQ-HG column chromatography methods. Two most abundant monomeric anthocyanins cyanidin-3-*O*-sophoroside (5.77 ± 0.42 mg) and cyanidin-3-*O*-sambubioside (4.10 ± 0.17 mg) were eventually obtained from 2 g dry black peanut skins, and their purities were determined by HPLC-PDA as 97.29% and 98.28% at the yields of 87.47% and 64.27% on the basis of their total amount in the crude extracts, respectively. These sequential treatments can be easily adapted to large-scale fractionation of pure anthocyanin monomers.

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1. Introduction

Anthocyanins are a group of pigments in plants which have been considered a promising natural pigment to replace artificial food colorants due to the bright color and high water solubility [1]. Besides the coloring functions, anthocyanins in food also possess potent antioxidant capacity and health promoting properties [2,3]. There are reports of more than 600 types of anthocyanins identified from fruits, vegetables, flowers and other plant materials. The structure of these anthocyanins differs in types of anthocyanidins, sugar molecules and numbers, and types of acylation groups [4].

Peanut, taxonomically classified as *Arachis hypogaea* L., is widely grown in the tropics and subtropics mainly for its edible seeds. Over 750,000 tons of peanut skins are generated annually worldwide as a byproduct of peanut processing, mainly used as animal feed with a commercial value of only 12–20 US$ per ton [5,6]. Black peanut is a variety of peanut. In recent years, it has been discovered that black peanut skin is rich in anthocyanins [7,8], such as cyanidin 3-sambubioside [9]. However, studies on individual anthocyanins, major flavonols, especially the chemical structure of anthocyanins in black peanut skins are still incomplete.

HPLC coupled with electrospray ionization (ESI) mass spectrometer (MS), especially the tandem mass spectrometer can provide mass spectrum of intact molecular ion and fragment ions [10]. NMR spectroscopy has also been widely applied in the complete structural elucidation of anthocyanins [11,12]. Therefore, the combination of both MS and NMR spectroscopies leads to unequivocal identification of the individual anthocyanins.

The purification and fractionation of anthocyanins from plants has been studied using high-speed counter-current chromatography [13], solid-phase extraction [14,15], preparative high-performance liquid chromatography [16,17]. The best results were obtained using the latters. The mixed-mode RP/ion-exchange stationary phase might be useful to provide improved selectivity which permitted the separation of cis-trans anthocyanins isomers from *Lycium ruthenicum* Murr. [16]. The main shortcomings of this method is that laborious pre-purification steps such as AB-8 resin, SCX solid-phase extraction and again AB-8 resin were needed to remove impurities and improve resolution, prior to chromatography. Wang et al. [17] used the isolation of anthocyanin mixtures and monomers from wild blueberries using a combination of Amberlite XAD-7HP column, Sephadex LH-20 column and a semi-preparative

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HPLC, but the method is tedious, time and solvent consuming, and requires multiple chromatographic steps. In addition, the highly acidic conditions and poisonous residual solvents (methanol, acetic acid, formic acid, trifluoroacetic acid, and acetonitrile, ionic buffer, etc.) of these two methods also introduce risks into food and pharmaceutical use of the products. Macroporous resin, because of their relatively low cost and easy regeneration, emerges as an alternative candidate for the pre-separation of anthocyanins from plant materials. In our previous work [7], various macroporous resins were evaluated for their adsorption properties, and finally an efficient method was developed for the large-scale production of black peanut skin anthocyanins mixtures. However, the detailed composition of the anthocyanins in black peanut skin has not yet been reported and value-added high-purity anthocyanins from black peanut skins are not yet commercially available.

In this study, skins were collected from black peanut and subjected to acidified water extraction and followed by analyses with HPLC-PDA-ESI–MS/MS for anthocyanins and flavonols characterization. A novel combination of Amberlite XAD-7HP and ODS-AQ-HG column chromatograph methods is proposed for the fractionation of monomeric anthocyanins. One of two major anthocyanins was further characterized by NMR spectroscopy.

2. Materials and methods

2.1. Plant material

Black peanuts were supplied by Qingdao Pengyuan Kanghua Natural Products Co. Ltd. (Qingdao, China). This plant belongs to a variety of peanut (*Arachis hypogaea* L.), bred by the Chinese Academy of Agricultural Sciences and cultivated in many area of China, especially Shandong and Hunan Provinces.

2.2. Chemicals

Analytical grade ethanol, EtOAc and HCl were purchased from Tianjin Chemistry Factory (Tianjin, China). All other solvents were of HPLC grade and all chemicals were of analytical grade (>99%). Methanol and formic acid were purchased from Merck (Darmstadt, Germany). Deionized water was produced by a Milli-Q unit (Millipore Co., MA, USA). Macroporous adsorption resin Amberlite XAD-7HP (20–60 mesh) and YMC*Gel ODS-AQ-HG (50 μm) were purchased from Aldrich Chemical Co. (St. Louis, MO, USA). Cyanidin-3-glucoside (purity ≥ 98%) was purchased from Beijing Tanmo Quality Testing Technology Co. Ltd. (Beijing, China).

2.3. Peeling of black peanut skin

Black peanuts were peeled using an oven drying method in an electrically-heated drum wind-drying oven before extracting anthocyanins. However, anthocyanins are highly unstable molecules and susceptible to degradation at high temperature. Thus, three different drying temperatures (100 °C, 70 °C and 40 °C) were explored respectively to minimize anthocyanin degradation during the drying process. The optimal condition for peeling was determined by comparison of the total anthocyanin content (TAC) in black peanut skin peeled at the above three temperatures.

2.4. Extraction of anthocyanins

Extraction procedure was adapted from the method of Chang, et al. [18]. 2.0 g black peanut skin obtained in the optimal peeling condition from approximately 66.7 g black peanut seeds was extracted with 40 mL deionized water acidified with HCl (pH 2.0) at 70 °C for 10 min. Then the extracts were filtered quickly through a vinylon filter cloth and cooled in ice-water for 15 min in the dark. After the extracts were filtered, the residue was dissolved again with 40 mL deionized water acidified and extracted for 10 min using the same conditions previously described. The ice-water cooled liquid from two extractions was combined and centrifuged at 4800 rpm for 15 min (TD5B centrifuger, Xiangzhi, Changsha, China), and the clear supernatant was collected and measured as 74 mL. The resulting supernatant was sequentially concentrated to a volume of 40 mL using a rotary evaporator (RE52, Yarong, Shanghai, China) at temperatures not exceeding 45 °C.

2.5. Purification by combined column chromatography

A schematic of the proposed method for obtaining high-purity anthocyanin monomers is illustrated in Fig. 1. First, the above extract (40 mL) was purified sequentially by partitioning (three times) with 40 mL of EtOAc. The resulting aqueous phase was then concentrated to 20 mL in order to remove EtOAc residuals and loaded onto a column of Amberlite XAD-7HP resin (50 × 1.0 cm) with a bed-volume (BV) of 40 mL. Anthocyanins and flavonols were adsorbed onto the resin while sugars, organic acids, and other water-soluble compounds were removed by washing the column with 1 L of deionized water acidified with HCl (pH 2.0) at a flow rate of 1 BV/h. Elution of anthocyanins was performed using 40% aqueous ethanol acidified with HCl (pH 2.0) at 1 BV/h. Fractions were collected in 10-mL tubes and each tube was analyzed using a double-beam UV–vis spectrophotometer (TU-1900, Persee, Beijing, China).

The desired eluate was collected and concentrated at temperatures not exceeding 40 °C, and the resulting solution was freeze-dried. Freeze-dried anthocyanin powder (45.59 ± 3.61 mg) was dissolved in 10 mL of deionized water and loaded onto a YMC*Gel ODS-AQ-HG (50 μm) chromatographic column (50 × 1.7 cm) with a BV of 80 mL. The column was eluted with 500 mL of 20% aqueous ethanol acidified with HCl (pH 2.0) at 0.5 BV/h, and monomeric anthocyanins were collected based on clearly distinct color bands and freeze-drying to powder. Then the isolated monomers were weighed accurately and their purities were represented by percentage of their peak area to the total chromatogram area based on HPLC-PDA chromatogram (200–600 nm) [19,20].

2.6. Anthocyanins quantification

TACs in crude extracts were quantified by two different methods: pH differential and HPLC-PDA analysis. For the pH differential method [21], absorbance was measured at 520 and 700 nm, were expressed as cyanidin-3-glucoside (cyd-glu, molar extinction coefficient of 26,900 L/mol cm^{-1} and molecular weight of 449.2 g mol^{-1}). The TCA was expressed in terms of mg/g dry peanut skin. Analysis was carried out in triplicate. This method has been described in detail in our previous work [7].

HPLC-PDA analysis as described by Lee, et al. [22], using a Finnigan Spectra System HPLC (Thermo-Fisher Scientific, Inc., Waltham, MA, USA) equipped with a PDA detector and a Hypersil Gold C_{18} column (150 × 4.6 mm, 3 μm). The mobile phase and elution gradient kept consistent with the condition used in the following section 2.7. Absorbance was recorded at 520 nm. Quantification was done by the external standard method with cyanidin-3-glucoside.

The purity of TCAs in each fraction was calculated according to the method used by Yao, et al. [23]. Extracts obtained from each steps was placed into a weighing bottle of constant weight, which was then placed in an oven to dry to constant weight at 105 °C to completely remove the solvent. The purity of the extract was calculated using the following equation:

$$\text{purity} = \frac{C \cdot V}{W - W_0} \times 100\% \quad (1)$$

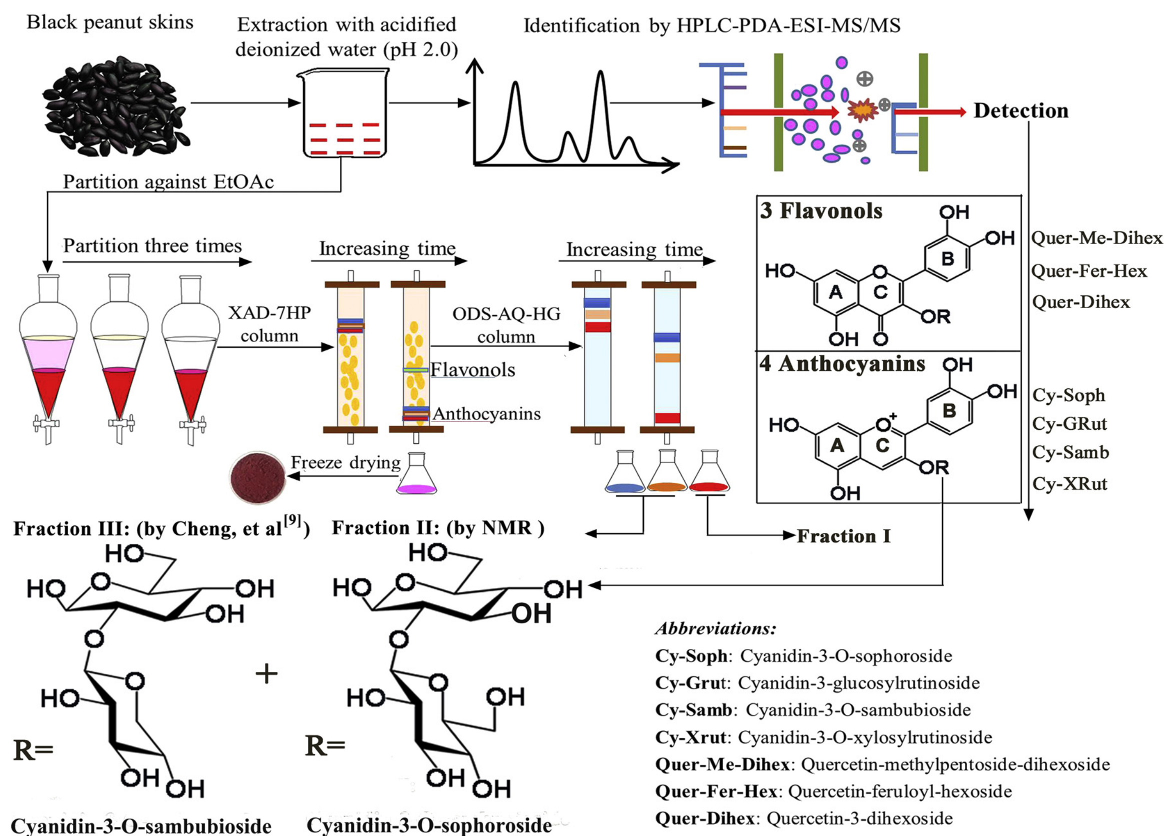

Fig. 1. Schematic representation for the preparation of high-purity anthocyanin mixtures and anthocyanin monomers from black peanut skins and structures of anthocyanin monomers and flavonols.

where C is the concentration of anthocyanins in various solution (mg/mL); V is the volume of the solution (mL); W is the total weight of the weighing bottle and residue solid after drying (g), W_0 is the weight of the weighing bottle (g).

Yields of different components (TAC, cyanidin-3-*O*-sophoroside, cyanidin-3-*O*-sambubioside) in each step were determined by quantities (mg) versus their corresponding quantities (mg) in the crude extracts.

2.7. Identification of anthocyanins by HPLC-PDA-ESI–MS/MS and NMR spectroscopy

HPLC-PDA-ESI–MS/MS was used to analyze the anthocyanin composition in black peanut skin. The samples obtained above were filtered through a 0.45-μm filter membrane (cellulose acetate and nitrocellulose) and then analyzed using a Finnigan Spectra System HPLC (Thermo-Fisher Scientific, Inc., Waltham, MA, USA) equipped with a PDA detector and a Hypersil Gold C_{18} column (150 × 4.6 mm, 3 μm). The mobile phase included aqueous 2% formic acid as solvent A (pH 2.0) and methanol containing 2% formic acid as solvent B (pH 2.1). The gradient was: 0 min, 15% B; 0–3 min, 15–20% B; 3–6 min, 20–25% B; 6–15 min, 25% B; 15–28 min, 25–70% B; 28–33 min, 70–90% B; 33–35 min, 90% B; 35–38 min, 90–15% B, followed by equilibration for 10 min at 15% B. The flow rate was 0.5 mL/min, the injection volume 10 μL, and the column temperature was maintained at 25 °C. HPLC chromatograms were recorded by using two different detecting models, total scan over the wavelength range 200–600 nm in steps of 2 nm and fixed wavelength at 525 nm, respectively.

The mass detector used was a Thermo-Finnigan mass spectrometer ion trap (Thermo-Fisher Scientific, Inc.) equipped with an electrospray ionization (ESI) source. The MS/MS parameters were: ESI source, positive ion mode; drying and nebulizing gas, nitrogen; nebulizing gas and nebulizer pressure 30 psi; dry gas flowing at 10 arbitrary units; temperature 300 °C; capillary voltage 5 kV; and scan range from m/z 100–1000.

Structure of the isolated monomeric anthocyanin was further identified by NMR spectrometry. The NMR sample was prepared by dissolving the purified fraction 2 (ca. 5 mg) in 0.5 mL of methanol-d4. NMR spectra were determined at 400 MHz for ^{1}H spectra and at 125 MHz for ^{13}C spectra using a UNITY INOVA 400 spectrometer (VARIAN Ltd., USA). Chemical shifts are reported relative to a tetra methyl silane (TMS) internal standard, and coupling constants are in hertz.

2.8. Statistical analysis

Data were collected from three preparative processes and measured in triplicate and reported as mean ± standard deviation. Significant differences between means were determined by t-tests with $P < 0.05$ considered statistically significant.

3. Results and discussion

3.1. TAC in black peanut skin

The shortest possible times for peanut peeling were: 15 min at 100 °C, 30 min at 70 °C, and 80 min at 40 °C. TACs in the black peanut skin peeled at the different temperatures, determined by the pH differential method, were: 8.18 ± 0.13 mg/g dry black peanut skin (70 °C, 30 min), 7.68 ± 0.10 mg/g (100 °C, 15 min), and 6.41 ± 0.16 mg/g (40 °C, 80 min). Thus, peeling temperature and time had a significant influence on the TAC ($P < 0.05$). Heating at 70 °C for 30 min was selected as the optimal condition for the peel-

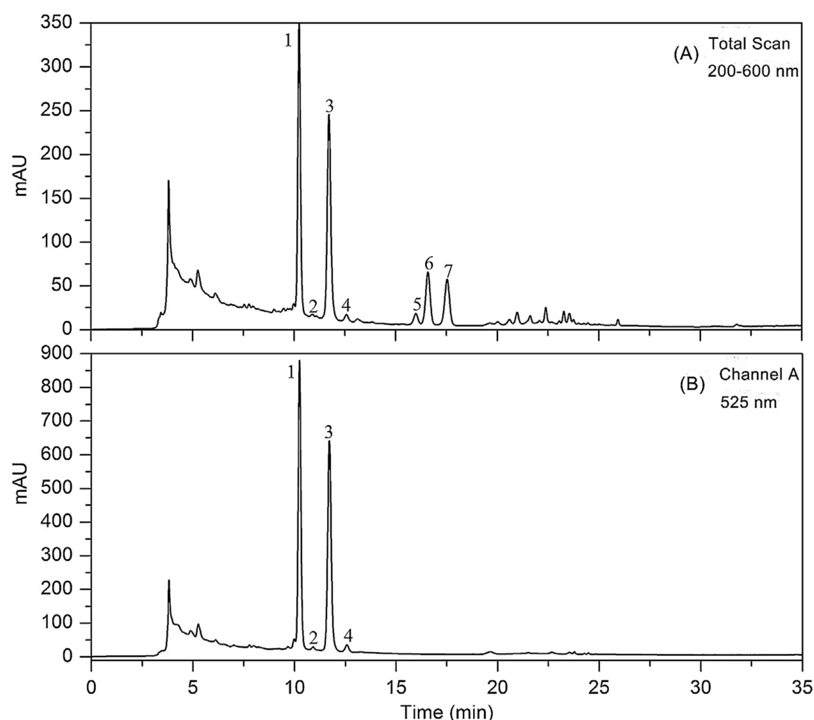

Fig. 2. HPLC-PDA chromatograms of crude black peanut skin extract: (A) total scan detection from wavelength 200–600 nm; (B) detection at 525 nm. The identities of the chromatographic peaks 1–6 represent Cyanidin-3-*O*-sophoroside, Cyanidin-3-*O*-glucosylrutinoside, Cyanidin-3-*O*-sambubioside, Cyanidin-3-*O*-xylosylrutinoside, Quercetin-methylpentoside-dihexoside, Quercetin-feruloyl-hexoside, and Quercetin-3-dihexoside, respectively.

ing of the black peanut skins. The average TAC (8.18 ± 0.13 mg/g) in black peanut skins peeled at 70 °C for 30 min in this research is much higher than the previous reported level of 4.93 mg/g [8]; the lower level in that work may be caused by use of improper peeling conditions (60 °C for 24 h) which led to thermal degradation of anthocyanins.

TAC in black peanut skins peeled at the optimal condition (70 °C for 30 min) was further determined by HPLC-PDA at wavelength of 520 nm using an authentic external standard cyanidin-3-glucoside, and the result was calculated as 8.61 ± 0.27 mg/g, which was similar with the value obtained by pH differential method. Correlation between the two values also corresponded to the regression equation that reported by Lee, et al. [22]. Quantitation of anthocyanins in following steps was all conducted by HPLC method.

3.2. Identification and composition of flavonoid

Fig. 2 shows a HPLC chromatogram of the anthocyanin profile obtained from black peanut skin. Seven major compounds, comprising four anthocyanins and three flavonols, were detected and identified.

All seven compounds could be detected by HPLC-PDA on scanning from 200 to 600 nm (Fig. 2A), while only the four anthocyanins could be detected at 525 nm (Fig. 2B). Therefore, to obtain comprehensive information about the compounds in black peanut skin, HPLC-PDA analyses should be conducted using a full spectral scan from 200 to 600 nm, while to evaluate the percentages of the monomeric anthocyanins account for the total anthocyanins amount, HPLC-PDA analyses at 525 nm should be used. The percentages of the two anthocyanin monomers gained from 525 nm have been shown in Table 2. Assigned peaks with area percentages < 0.6% were omitted from the chromatogram.

Peaks were identified (Table 1) by a combination of UV–vis and HPLC–MS spectra, comparison with the retention times of standards, the *m*/*z* of each anthocyanin molecule, and comparison of fragmentation patterns with available values in the literature. The aglycone ion at *m*/*z* 287 in positive ion mode suggests that compounds 1, 2, 3, 4 were all cyanidin glycosides. Generally, the glycosylation substitution position (3-position or 3- and 5-positions) can be determined by the $E_{440}/E_{\lambda_{max}}$ value [24]. The 3-hydroxyl group is glycosylated first [25]. The $E_{440}/E_{\lambda_{max}}$ ratios for the four anthocyanin compounds identified here ranged from 31.5% to 41.5% (Table 1), demonstrating a substitution only at position 3 [24,26].

Specifically, MS/MS analysis showed that compound 1 had its molecular ion $[M+H]^+$ at *m*/*z* 611 and a fragment ion at *m*/*z* 287. The *m*/*z* 287 fragment ion corresponds to the cyanidin aglycone, and the loss of 324 amu suggests a sophoroside. Taking into account these results and published data [26–29], peak 1 was assigned as cyanidin-3-*O*-sophoroside. Peak 2 had its molecular ion $[M+H]^+$ at *m*/*z* 757, an MS^2 = 611 (minor ion) after loss of 146 amu (a rhamnosyl group), and a major fragment at *m*/*z* 287 after loss of 324 amu (two glucosyl moieties), and was therefore assigned as cyanidin-3-*O*-glucosyl-rutinoside [27,28,30]. Peak 3 had its molecular ion $[M+H]^+$ at *m*/*z* 581 and an MS^2 major fragment at 287 after loss of 294 amu (corresponding to a sambubioside unit); it was identified as cyanidin-3-*O*-sambubioside [26,27,29,31]. Peak 4 with *m*/*z* 727, a major fragment ion at *m*/*z* 287, and a minor ion at *m*/*z* 581, corresponds to cyanidin-3-*O*-xylosyl-rutinoside [26,27,32]. The two most abundant monomeric anthocyanins in the crude black peanut skin extract were determined to be cyanidin-3-*O*-sophoroside (24.37%) and cyanidin-3-*O*-sambubioside (22.42%), and their structure could be further confirmed with the later NMR analysis and previous published research.

In addition, three flavonols were present in the black peanut skin extract. Unlike the anthocyanins, these compounds had obvious absorption peaks between 350 and 360 nm, which is characteristic of absorption by flavonols [33,34]. ESI–MS/MS spectra of compounds 5, 6 and 7 revealed molecular ions at *m*/*z* 773, 641 and

Table 1
The UV–vis and MS/MS data used for identification of anthocyanins and main flavonols from black peanut skin.

Peak number[a]	RT[b] (min)	vis λ_{max} (nm)	$E_{440}/E_{\lambda max}$ (%)	$[M+H]^+$ (m/z)	Fragments $[M+H]^+$ (m/z)	Compound
1	10.50	516	33.2	611	287	Cyanidin-3-*O*-sophoroside
2	11.14	519	41.5	757	611, 287	Cyanidin-3-*O*-glucosylrutinoside
3	12.01	517	31.5	581	287	Cyanidin-3-*O*-sambubioside
4	12.89	520	37.8	727	581, 287	Cyanidin-3-*O*-xylosylrutinoside
5	16.46	354	–	773	627, 465, 303	Quercetin-methylpentoside-dihexoside
6	17.23	353	–	641	479, 303	Quercetin-feruloyl-hexoside
7	18.07	354	–	627	465, 303	Quercetin-3-dihexoside

[a] Peak numbers refer to Fig. 1A.
[b] RT = retention time.

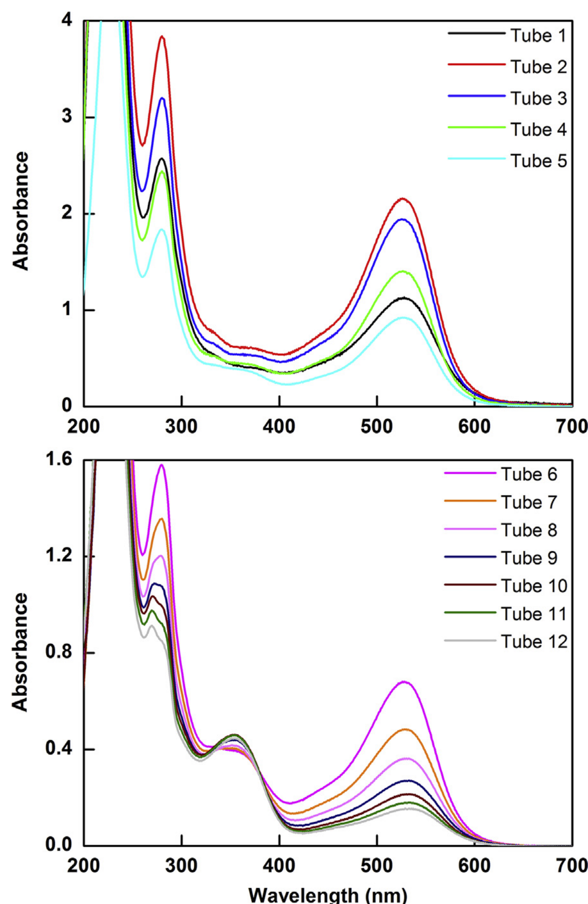

Fig. 3. UV–vis spectra of anthocyanin eluent in different tubes (Nos. 1–12) after elution from the XAD-7HP resin column using 40% aqueous ethanol solution acidified with HCl (pH 2.0).

627, which released unique MS² fragments at m/z 303/465/627, 303/479 and 303/465, respectively. Comparing with MS information in the literature [34–36], we conclude that these compounds were quercetin-methylpentoside-dihexoside, quercetin-feruloyl-hexoside, and quercetin-3-dihexoside, respectively. Thus, in this work, cyanidin-3-*O*-glucosylrutinoside, cyanidin-3-*O*-xylosylrutinoside and the three quercetin flavonols were tentatively identified for the first time in black peanut skin.

3.3. Pre-fractionation by EtOAc partitioning and Amberlite XAD-7HP resin column

The relatively high polarity of non-acylated anthocyanins makes them quite soluble in water but less soluble in nonpolar solvents such as EtOAc, n-hexane and petroleum ether [37]. EtOAc as safe and widely used solvent in the food industry, has been well known for its capability of removing not very polar phenolic compounds from crude anthocyanins extract [17]. After the crude extract solution had been extracted three times by EtOAc, the purity of TAC was obviously improved, from the initial 5.20% to 6.62%, but without loss of its total amount (Table 2). The two dominating monomeric anthocyanins (peak 1 and 3 in Fig. 2) were also improved from the initial 24.37% and 22.42% in crude extract to 32.30% and 30.21% (HPLC-PDA from 200 to 600 nm), respectively. In addition, the application of EtOAc partition could remove pectins, and other liposoluble compounds [17], which also ease the burden of the later XAD-7HP column in some degree.

The partitioned anthocyanin extracts were applied to an Amberlite XAD-7HP resin-containing column and then washed with deionized water acidified with HCl (pH 2.0). Afterwards, acidified aqueous ethanol (ethanol/H_2O = 40:60 v/v, pH 2.0, acidified with HCl) was introduced to elute the anthocyanins from the column. Amberlite XAD-7HP, as a weakly-polar, acrylic ester resin, has been widely used for the separation and purification of anthocyanins and other phenolic compounds [17,38,39]. In this study, the flavonols detected in the crude extracts were separated from the anthocyanins through non-polar and polar interactions in the XAD-7HP resin column. The separation was monitored using a UV–vis detector (Fig. 3).

The majority of anthocyanins eluted early from the column (Fig. 3, Tubes 1–5) because of their relatively high polarity and low adsorption affinity towards the acrylic ester polymer. As the elution continued, the absorption peak around 360 nm became increasingly obvious (Tubes 6–12), as non-anthocyanin impurities were eluted from the resin.

Quantitative analysis showed that 40% aqueous ethanol could elute the majority of the anthocyanins from the Amberlite XAD-7HP column with satisfactory yields— TAC 67.12%, cyanidin-3-*O*-sophoroside 92.43% and cyanidin-3-*O*-sambubioside 71.12% (Table 2). The purity of TAC increased to 25.40%, 5-folds of 5.20% in crude extracts. In addition, the content of the two major anthocyanins, cyanidin-3-*O*-sophoroside and cyanidin-3-*O*-sambubioside, was also increased to 51.12% and 38.22%, respectively, about twice their levels in the crude extract solution. In contrast, the three flavonols' contents present in the crude extracts (Fig. 2A, peaks 5–7) showed a noticeable decrease–peak 7 did not detected, peak 5 and 6 were respectively decreased from 1.63% and 7.52% to 0.59% and 3.16%–after the XAD-7HP resin column chromatography purification step. These results reveal that partitioning using EtOAc and succeeding Amberlite XAD-7HP column chromatography could be an efficient method for the preliminary purification of anthocyanins before loading onto a preparative ODS column.

3.4. Preparation of high-purity monomeric anthocyanins by ODS column chromatography

YMC*Gel ODS-AQ-HG is a C_{18} reversed phase silica-based chromatography packing material specifically designed for use in 100%

Table 2
Quantitative data for the anthocyanins (2 g dry black peanut skins) during the whole preparative process.

Steps	Times	TAC			Cyanidin-3-*O*-sophoroside			Cyanidin-3-*O*-sambubioside		
		Purity (%)	Yield (%)	Quantity (exp.) (mg)	Purity* (%)	Yield (%)	Quantity (cal.) (mg)	Purity* (%)	Yield (%)	Quantity (cal.) (mg)
Crude extracts	1	5.01 ± 0.21	–	17.24 ± 0.34	38.57 ± 0.23	–	6.65 ± 0.09	36.08 ± 0.17	–	6.22 ± 0.13
	2	5.37 ± 0.34	–	16.68 ± 0.23	38.37 ± 0.19	–	6.40 ± 0.11	36.33 ± 0.20	–	6.06 ± 0.08
	3	5.21 ± 0.28	–	17.74 ± 0.30	38.22 ± 0.26	–	6.78 ± 0.14	36.36 ± 0.24	–	6.45 ± 0.16
	AVG	5.20 ± 0.18	–	17.22 ± 0.53	38.39 ± 0.18	–	6.61 ± 0.19	36.26 ± 0.15	–	6.24 ± 0.20
EtOAc partition	1	6.72 ± 0.34	101.67 ± 1.37	17.53 ± 0.37	40.10 ± 0.17	105.71 ± 0.82	7.03 ± 0.16	37.14 ± 0.23	102.83 ± 1.94	6.51 ± 0.22
	2	7.02 ± 0.36	104.26 ± 2.01	17.39 ± 0.27	39.90 ± 0.29	108.44 ± 1.49	6.94 ± 0.10	36.96 ± 0.12	106.11 ± 1.35	6.43 ± 0.26
	3	6.11 ± 0.14	101.63 ± 2.54	18.03 ± 0.31	40.09 ± 0.23	106.64 ± 0.97	7.23 ± 0.25	37.02 ± 0.19	103.41 ± 2.62	6.67 ± 0.19
	AVG	6.62 ± 0.46	102.52 ± 1.51	17.65 ± 0.34	40.03 ± 0.11	106.93 ± 1.39	7.07 ± 0.15	37.04 ± 0.09	104.12 ± 1.75	6.54 ± 0.12
XAD-7HP	1	25.53 ± 0.32	67.40 ± 0.96	11.62 ± 0.28	53.36 ± 0.38	93.23 ± 2.74	6.20 ± 0.16	38.30 ± 0.14	71.54 ± 0.91	4.45 ± 0.10
	2	26.01 ± 0.39	65.53 ± 0.77	10.93 ± 0.22	52.24 ± 0.20	89.22 ± 3.18	5.71 ± 0.29	38.88 ± 0.36	70.13 ± 1.21	4.25 ± 0.17
	3	24.65 ± 0.46	68.43 ± 1.02	12.14 ± 0.30	52.97 ± 0.33	94.84 ± 1.56	6.43 ± 0.37	38.14 ± 0.31	71.70 ± 2.06	4.63 ± 0.26
	AVG	25.40 ± 0.69	67.12 ± 1.47	11.56 ± 0.61	52.86 ± 0.57	92.43 ± 2.89	6.11 ± 0.37	38.44 ± 0.39	71.12 ± 0.86	4.44 ± 0.19
ODS-AQ column	1	–	–	–	98.21 ± 0.28	89.62	5.9 (exp)	99.37 ± 0.13	68.17	4.2 (exp)
	2	–	–	–	98.46 ± 0.31	82.81	5.3 (exp)	99.19 ± 0.23	64.35	3.9 (exp)
	3	–	–	–	98.26 ± 0.22	89.97	6.1 (exp)	99.52 ± 0.21	65.12	4.2 (exp)
	AVG	–	–	–	98.31 ± 0.13	87.47 ± 4.04	5.77 ± 0.42	99.36 ± 0.17	64.27 ± 0.13	4.10 ± 0.17

Purity of TAC was obtained from Eq. (1), while purity* represented with the percentage of total peak area obtained from HPLC-PDA analysis at 525 nm. Quantity (exp.) of TAC was gained by experiment, while quantity (cal.) of cyanidin-3-*O*-sophoroside and cyanidin-3-*O*-sambubioside was calculated according the real quantity of TAC. Data shown indicate means from three independent experiments performed in triplicate.

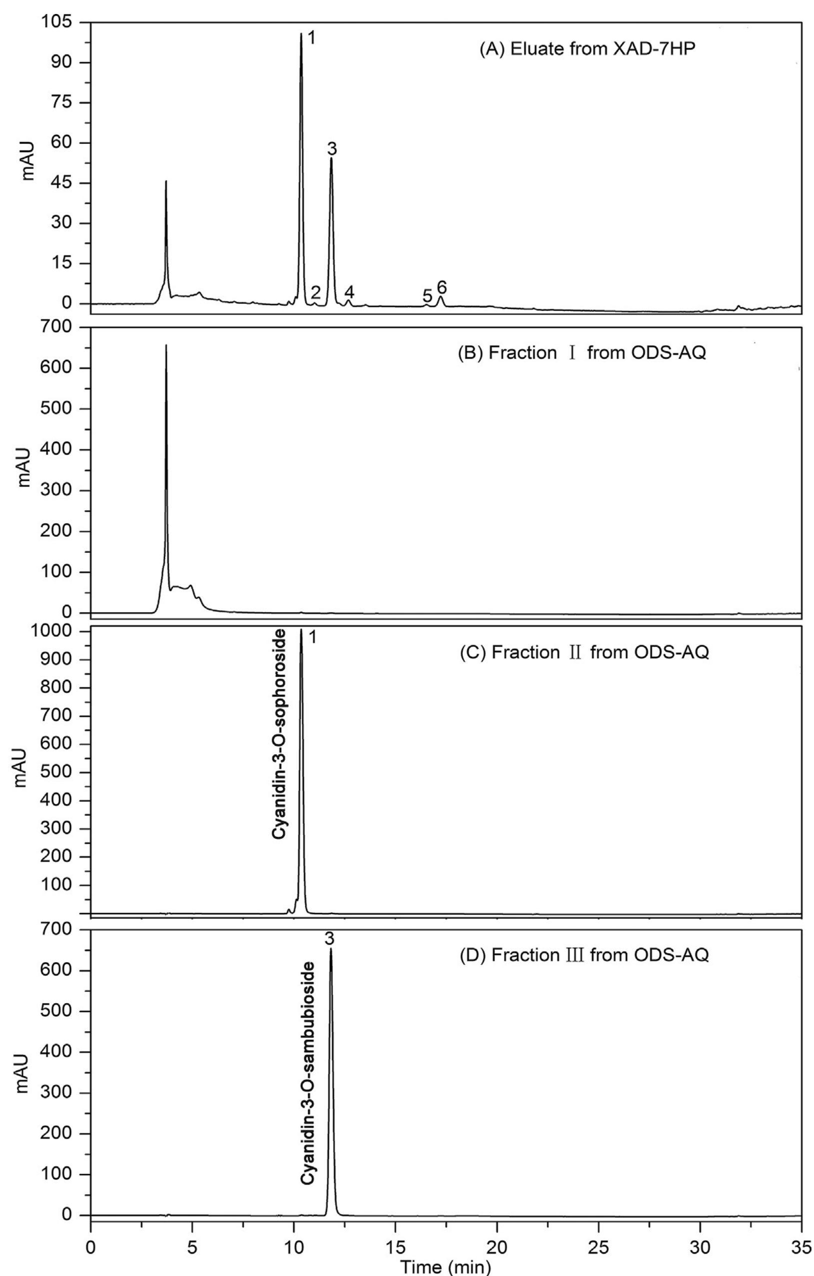

Fig. 4. HPLC-PDA chromatograms of purified anthocyanins observed from wavelength 200–600 nm. (A) Crude anthocyanin mixture eluted from the XAD-7HP resin column; (B) the first strong polar fraction obtained from ODS-AQ-HG column chromatography; (C) cyanidin-3-*O*-sophoroside obtained from ODS-AQ-HG column chromatography; (D) cyanidin-3-*O*-sambubioside obtained from ODS-AQ-HG column chromatography.

aqueous eluents which exhibits a different selectivity to that of traditional C_{18} stationary phases [40]. This difference substantially increases retention of polar compounds and, as a result, makes this chromatography material suitable for the isolation of relatively strong polar compounds. The material was packed in a high-pressure-resistant glass column (50 × 1.7 cm) with a simple pressure device. With a column length around 35 cm, the monomeric anthocyanins could be totally separated during the elution. In our study, three major fractions were selected and collected as distinct colored bands; the eluates of each fraction were concentrated with a rotary evaporator and freeze-dried.

The purities and compositions of each of the three fractions were determined using the HPLC-PDA-ESI–MS/MS technique (Fig. 4). From the HPLC profile of the crude extract solution (Fig. 2A) and the mass spectrum, the two main fractions (II and III) corresponded to cyanidin-3-*O*-sophoroside {retention time (RT) 10.50 min in Fig. 2A, $[M+H]^+$ (*m*/*z*) 611, MS^2 (*m*/*z*) 287} and cyanidin-3-*O*-sambubioside {RT 12.01 min in Fig. 2A, $[M+H]^+$ (*m*/*z*) 587, MS^2 (*m*/*z*) 287}. Fraction I (weighs 23.47 ± 1.19 mg) was not an anthocyanin but a mixture of strong polar compounds, presumably proanthocyanidins that are extensively present in peanut skins [41], we intend to purify and characterize this material specifically in future work for its outstanding capacity of oxidation resistance and other beneficial bioactivity [42]. The overall purity of each anthocyanin monomer was assayed by HPLC-PDA (200–600 nm) and was 97.29% for cyanidin-3-*O*-sophoroside (Fig. 4C) and 98.28% for cyanidin-3-*O*-sambubioside (Fig. 4D). The purities of the two monomeric anthocyanins were also measured by HPLC at 525 nm as 98.31% for cyanidin-3-*O*-sophoroside and 99.36% for cyanidin-3-*O*-sambubioside. The

yields based on their total amounts in crude extracts were 87.47% for cyanidin-3-*O*-sophoroside (weighs 5.77 ± 0.42 mg) and 64.27% for cyanidin-3-*O*-sambubioside (weighs 4.10 ± 0.17 mg). Therefore, the column chromatography combination of Amberlite XAD-7HP and ODS-AQ-HG used in this study could be an efficient technique for separating pure monomeric anthocyanins from black peanut skins.

3.5. Structure determination of the anthocyanin monomers

Although the combination of UV–vis spectra and HPLC-PDA-ESI–MS/MS methods has been widely used for the characterization and identification of anthocyanins from various plant resources [17,24,26], NMR experiment on the pure compounds should also be performed to exactly define the structure of the anthocyanin monomers. As fraction III has already been assumed for the well characterized cyanidin-3-*O*-sambubioside by Cheng, et al. [9] from black peanut skin, so the NMR experiment was only conducted on fraction II in this work.

The purified compound is a dark violet amorphous powder and the above MS/MS analysis gave molecular ion $[M+H]^+$ at *m*/*z* 611 and a fragment ion MS^2 at *m*/*z* 287 corresponded to the molecular weights calculated using the molecular formula: $C_{27}H_{31}O_{16}$ of cyaniding diglucoside. The ^{1}H NMR spectrum of this pigment gave as follows: δ 8.92 (1H, s, H-4), 6.64 (1H, s, H-6), 6.89 (1H, s, H-8), 8.03 (1H, d, J = 2.0 Hz, H-2′), 7.04 (1H, d, J = 8.8 Hz, H-5′), 8.23 (1H, dd, J = 8.8, 2.0 Hz, H-6′), 5.65 (1H, t, J = 6.4 Hz, H-1”), 4.74 (1H, d, J = 7.2 Hz, H-2”), 4.87 (1H, t, J = 7.6 Hz, H-1”’); ^{13}C NMR δ 164.7 (C-2), 146.2 (C-3), 137.2 (C-4), 159.6 (C-5), 103.7 (C-6), 170.6 (C-7), 95.3 (C-8), 157.2 (C-9), 113.6 (C-10), 121.5 (C-1′), 118.8 (C-2′), 148.0 (C-3′), 155.4 (C-4′), 117.6 (C-5′), 127.9 (C-6′), 102.7 (C-1”), 82.5 (C-2”), 78.2 (C-3”), 70.8 (C-4”), 78.4 (C-5”), 64.3 (C-6”), 105.2 (C-1”’), 76.6 (C-2”’), 77.9 (C-3”’), 70.6 (C-4”’), 75.8 (C-5”’), 62.5 (C-6”’). Despite the result of HPLC-PDA-ESI–MS/MS, the presence of cyanidin was once again verified by ^{1}H NMR with three 1H singlets at δ 6.64, 6.89, and 8.92 and ABX-type signals at δ 8.03, 7.04, and 8.23 [9]. In addition, the C-1 signals of the two sugar moieties were 102.7 and 105.2 (δ > 100 ppm), while the coupling constants J of anomeric carbon C_1—H and C_2—H in the glc-1 and glc-2 were all between 6 and 8 Hz, which undoubtedly prove the sugar moiesties were β-type. Besides, the ^{13}C signal of the C-2 carbon in the glc-1 (δ = 82.5) shifted to a lower magnetic field. Fraction II was identified as cyanidin-3-*O*-(2”- *O*-β-D-glucosyl)-β-D-glucoside, cyanidin-3-*O*-sophoroside, by both those NMR spectra data analysis and previous LC–MS/MS results. Chemical structure of cyanidin-3-*O*-sophoroside presented in Fig. 5.

4. Conclusion

In this work, four anthocyanins and three major flavonols from black peanut skins were detected and identified by HPLC-PDA-ESI–MS/MS. The four anthocyanins were non-acylated cyanidin derivatives, while the three identified flavonols were all quercetin glycosides. The two main anthocyanin compounds in black peanut skin, cyanidin-3-*O*-sophoroside and cyanidin-3-*O*-sambubioside, were ultimately isolated and purified by the combined column chromatography technique with purities of 97.29% and 98.28%, at the yields of 87.47% and 64.27%, respectively. The pre-fractionation of the crude anthocyanins extract using EtOAc extraction and succeeding Amberlite XAD-7HP column chromatography to eliminate flavonols, improves fractionation on ODS-AQ-HG. The new fractionation and isolation procedure is easy to operate and gives high yields of anthocyanins products due to the lesser number of chromatographic steps. The proposed method is suitable laboratory research and potentially applicable in industry for a large-scale production.

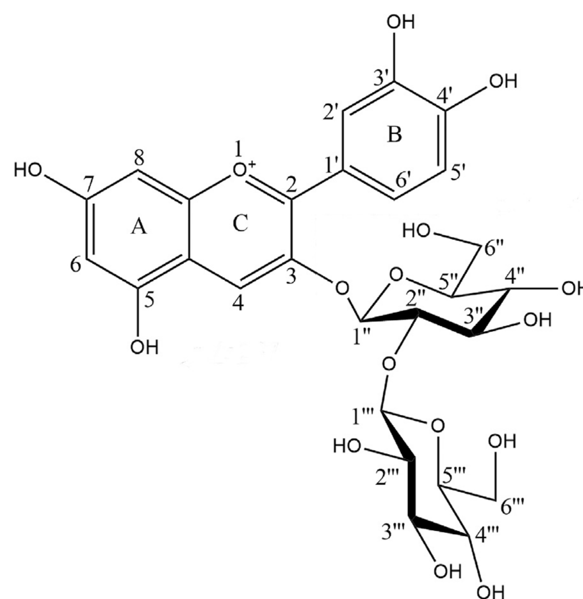

Fig. 5. Chemical structure of cyanidin-3-*O*-sophoroside (cyanidin-3-*O*-(2”- *O*-β-D-glucosyl)-β-D-glucoside).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2017.08.078.

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