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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.

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:

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