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Extraction optimization and identification of anthocyanins from *Nitraria tangutorun* Bobr. seed meal and establishment of a green analytical method of anthocyanins

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ABSTRACT

This study aimed to extract and identify anthocyanins from *Nitraria tangutorun* Bobr. seed meal and establish a green analytical method of anthocyanins. Ultrasound-assisted extraction of anthocyanins from *N. tangutorun* seed meal was optimized using response surface methodology. Extraction at 70 °C for 32.73 min using 51.15% ethanol rendered an extract with 65.04 mg/100 g of anthocyanins and 947.39 mg/100 g of polyphenols. An *in vitro* antioxidant assay showed that the extract exhibited a potent DPPH radical-scavenging capacity. Eight anthocyanins in *N. tangutorun* seed meal were identified by HPLC-MS, and the main anthocyanin was cyanidin-3-*O*-(*trans*-*p*-coumaroyl)-diglucoside (18.17 mg/100 g). A green HPLC-DAD method was developed to analyse anthocyanins. A mixtures of ethanol and a 5% (v/v) formic acid aqueous solution at a 20:80 (v/v) ratio was used as the optimized mobile phase. The method was accurate, stable and reliable and could be used to investigate anthocyanins from *N. tangutorun* seed meal.

1. Introduction

Nitraria tangutorun Bobr, belonging to Nitrariaceae, is endemic to China, especially in the desert areas of Qinghai-Tibetan plateau (Pan, Shen, & Peng, 1999). *N. tangutorun* Bobr. has been developed for use in many products, such as juice (Chen et al., 2011), red dry wine (Wang, Chen, Li, Gao, & Zhang, 2012) and compound seed oil soft capsules (Ding et al., 2008).

N. tangutorun fruit contained abundant anthocyanins that showed antioxidant activities *in vitro* and *in vivo* (Ma et al., 2016; Zheng et al., 2011). *N. tangutorun* seed contained flavonoids, such as quercetin, isorhamnetin-7-*O*-α-l-rhamnoside, and kaempferol-7-*O*-α-l-rhamnoside (Jia, Zhu, & Wang, 1989; Wang, Li, Li, & Suo, 2007). According to our preliminary experiment, the ethanolic extract of *N. tangutorun* seed meal was red coloured; the extract was analysed according to previous methods (Zheng et al., 2011) and some peaks were detected using an HPLC chromatogram (Fig. S1). As a result, we conjectured that *N. tangutorun* seed meal contains anthocyanins. To date, no detailed studies have been published in the literature about the anthocyanins in *N. tangutorun* seed and the parameters used to extract anthocyanins from *N. tangutorun* seed meal even though this by-product was substantially generated from seed oil factories. Anthocyanins have many pharmacological activities and possible health benefits. Additionally, anthocyanins extracted from plants might be suitable substitutes for synthetic dyes because of their attractive bright colours, water solubility and absence of adverse health effects (Giusti & Wrolstad, 2003). Therefore, it is worthy extracting anthocyanins from *N. tangutorun* seed meal. The seed meal, as a raw material, was applied to solve the resource waste problem and improve the resource value. However, traditional extraction techniques often require relatively high consumption of organic solvents, cooling water and electric energy. In addition, long extraction times often result in degradation of phenolic compounds due to the oxidation, ionization and hydrolysis during the process (Ivanovic et al., 2014).

Ultrasound-assisted processes have dramatically improved many sample-preparation approaches (e.g., digestion, dissolution and extraction) in the analytical laboratory and have become popular in many areas, including those for food, industry and the

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environment (Bendicho et al., 2012). In addition, an ultrasound-assisted extraction (UAE) technique has been extensively used to extract antioxidant compounds from plant materials (Roselló-Soto et al., 2015) and many reports have demonstrated that UAE is better than conventional extraction methods to recover phytochemicals from various seeds, such as flax seeds (Corbin et al., 20155), annatto seeds (Yolmeh, Habibi Najafi, & Farhoosh, 2014) and papaya seeds (Samaram, Mirhosseini, Tan, & Ghazali, 2014). UAE parameters, such as the extraction solvent, temperature and time, have a significant influence on the extraction process. The determination of optimal conditions is important to obtain a maximum yield of anthocyanins. The response surface methodology is an important method for developing and optimizing processes and products, and it can be used to determine the best conditions for extracting compounds from plant materials (Eren & Kaymak-Ertekin, 2007; Tabaraki, Heidarizadi, & Benvidi, 2012).

Green Chemistry seeks to develop chemistry techniques and methodologies that reduce or eliminate the use or generation of some substances that are hazardous to human health or environment, and the same philosophy and ideas on Green Chemistry have been developed in analytical laboratories (Anastas, 1999; Armenta, Garrigues, & de la Guardia, 2008). RP-HPLC with C18 columns has been the usual method of choice for separating anthocyanins from different sources (Valls, Millán, Martí, Borràs, & Arola, 2009). It should be noted that the wide application of liquid chromatography in analytical laboratories and industrial processes has generated substantial toxic waste. Consequently, decreasing the levels of solvents used for liquid chromatography is highly desirable (Plotka et al., 2013). Acetonitrile is by far the preferred organic solvent for use in the mobile phase of RP-HPLC; however, because it is hazardous to humans and the environment, greener alternatives like ethanol are promising for use in analytical processes (Welch, Brkovic, Schafer, & Gong, 2009). The colour and structure of anthocyanins depend on the acidity of the environment; these compounds were in the flavylium cation state and a red colour could be observed at low pH levels (Barnes, Nguyen, Shen, & Schug, 2009). Acidic mobile phases were frequently used to analyse anthocyanins because low pH levels allowed for complete displacement of the equilibria to the flavylium cation, resulting in a better resolution and great characteristic absorbance between 515 and 540 nm (Valls et al., 2009). Various formic acid and trifluoroacetic acid concentrations are usually chosen to adjust the mobile phase acidity; however, few detailed studies report on the effect of the acid concentration on the RP-HPLC analysis of anthocyanins in the ethanol-based mobile phase.

In the present study, the UAE of total anthocyanins and polyphenols from *N. tangutorun* seed meal was optimized using the response surface methodology. The anthocyanins extracted from *N. tangutorun* seed meal were identified using HPLC-MS and a green HPLC-DAD method was developed to analyse these anthocyanins.

2. Materials and methods

2.1. Chemicals and reagents

Cyanidin-3-*O*-glucoside (purity ⩾ 98%) and gallic acid (purity ⩾ 98%) were purchased from Chengdu Must Bio-Technology Co., Ltd. (Chengdu, China). Folin-Ciocalteu reagent was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich (Milwaukee, WI, USA). The methanol, ethanol and acetonitrile (Fisher Chemicals Co., Ltd., USA) used for HPLC-MS analysis were HPLC grade. Formic acid and trifluoroacetic acid (HPLC grade) were purchased from Tianjin Kermel Chemical Reagent Co., Ltd. (Tianjin, China). Ascorbic acid (Vc) was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). 2,6-Di-tert-butyl-4-methylphenol (BHT) was purchased from Chengdu Ai Keda Chemical Technology Co., Ltd. (Chengdu, China). All other chemicals and reagents were of analytical grade, and deionized water was used in all experiments.

2.2. Sample preparation

N. tangutorun fruits were purchased from the local market, Qing Hai. These fruits were juiced and seed pieces were separated and rinsed with clean water. The seed pieces were dried at 40 °C and then ground into powder using a grinder (DFT-50, Wenlinglinda Machine Co., Ltd, Zhejiang, China). The seed powder was sieved through a calibrated granulometric sieve (60 mesh). The seed oil was extracted with a Soxhlet apparatus. Briefly, 100 g of seed powder was refluxed with 500 mL of petroleum ether (b.p., 60–90 °C) until the extract was colourless. The seed meal was obtained after it was dried at 40 °C for 1 h to remove residual petroleum ether and moisture.

2.3. Extraction of anthocyanins

A Box-Behnken design was used to evaluate the effects of three independent variables (extraction temperature, ethanol concentration and extraction time) on the extraction yield of total anthocyanins and polyphenols. The values for the temperature were 30 °C, 50 °C and 70 °C, while extraction times of 15, 30 and 45 min and ethanol concentrations of 30%, 50%, and 70%, were studied. These values were established from earlier experiments and studies (data not shown). The experimental design presented 18 combinations (Table 1), including 12 factorial and 6 centre point experiments. The experimental results were fitted with a second-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii} X_i^2 + \sum_{i=1}^{2}\sum_{j=i+1}^{3}\beta_{ij} X_i X_j \qquad (1)$$

where Y is the predicted response and β_0, β_i, β_{ii} and β_{ij} are the regression coefficients for the intercept, linearity, square and interaction of the model, respectively. X_i and X_j represent the independent variables.

The ultrasonic equipment used in our study was an ultrasonic bath (TCX-600 *S*, Jiningtianyu Ultrasonic Instrument Co. Ltd., Shandong, China) that worked at a fixed frequency (30 kHz) and 300 W input power. A 50-mL beaker flask containing 1.0 g of seed meal and 15 mL of aqueous ethanol (0.1% HCl, v/v) was put in the middle of the ultrasonic bath (internal dimension: 500 mm × 300 mm × 150 mm) to ensure constant ultrasonic waves for every piece of experiment. The surface of the water in the ultrasonic bath was 5 cm higher than the level of the mixture in the beaker flask to ensure that there was a similar temperature between the mixture and water bath. The extraction temperature was controlled using a thermometer in the process of extraction. The extract was filtered and stored at −20 °C for later analysis. All experiments were performed randomly and in triplicate.

2.4. Analysis of total anthocyanins and polyphenols

The total anthocyanin content was measured according to a previous report (Lu et al., 2015) and quantified by cyanidin-3-*O*-glucoside with a final concentration, ranging from 0.002 to 0.016 mg/mL, and the calibration curve was Y (absorbance) = 54.994 × X (C3G equivalent content) + 0.0087 (R^2 = 0.9998). The absorbance of each sample were measured using a spectrophotometer (UV-2450, Shimadzu, Kyoto, Japan). The results were