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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:

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

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

Table 1
Box-Behnken design and the response values of total anthocyanins and total polyphenols.

Run	Independent variables			Response values [a]			
	X_1: Temperature (°C)	X_2: Time (min)	X_3: Ethanol concentration (%)	Total anthocyanins (mg/100 g)		Total polyphenols (mg/100 g)	
				Experimental	Predicted	Experimental	Predicted
1	70 (1)	30 (0)	70 (1)	56.68	58.16	876.71	891.69
2	70 (1)	15 (−1)	50 (0)	65.23	64.74	828.65	843.49
3	30 (−1)	45 (1)	50 (0)	56.27	56.76	711.05	696.20
4	50 (0)	30 (0)	50 (0)	60.68	59.44	880.67	850.13
5	30 (−1)	30 (0)	30 (−1)	55.50	54.03	636.98	622.00
6	50 (0)	30 (0)	50 (0)	59.91	59.44	839.96	850.13
7	50 (0)	30 (0)	50 (0)	59.82	59.44	810.56	850.13
8	30 (−1)	30 (0)	70 (1)	48.14	48.12	615.49	633.87
9	50 (0)	15 (−1)	30 (−1)	53.96	54.43	644.33	647.86
10	70 (1)	30 (0)	30 (−1)	60.82	60.84	813.38	795.01
11	30 (−1)	15 (−1)	50 (0)	53.14	54.12	638.67	650.12
12	50 (0)	30 (0)	50 (0)	58.55	59.44	865.40	850.13
13	50 (0)	15 (−1)	70 (1)	50.36	49.38	724.62	694.79
14	70 (1)	45 (1)	50 (0)	64.00	63.00	945.12	933.67
15	50 (0)	45 (1)	70 (1)	51.05	50.57	773.81	770.27
16	50 (0)	30 (0)	50 (0)	59.77	59.44	855.22	850.13
17	50 (0)	30 (0)	50 (0)	57.91	59.44	849.00	850.13
18	50 (0)	45 (1)	30 (−1)	53.14	54.12	678.82	708.64

[a] Each value is the mean of triplicate measurements.

expressed as mg cyanidin-3-*O*-glucoside equivalent/100 g dry seed meal.

The total polyphenol content was measured by following a published procedure (Borges, Vieira, Copetti, Gonzaga, & Fett, 2011) with slight modifications. Briefly, 300 μL of extract was poured into a 10-mL volumetric flask and then mixed with 4 mL of deionized water, 500 μL of Folin-Ciocalteu reagent and 1500 μL of Na_2CO_3 solution (20%, w/v). Deionized water was added to bring the volume of the mixture to 10 mL. The mixture was kept for 2 h at room temperature. The absorbance of the mixture was determined at 765 nm using a spectrophotometer (UV-2450, Shimadzu, Kyoto, Japan). Gallic acid was standard, and the calibration curve was Y (absorbance) = 9.8259 × X (gallic acid equivalent content) + 0.0428 (R^2 = 0.9997). The results were expressed as the mg gallic acid equivalent/100 g dry seed meal.

2.5. HPLC-MS analysis of anthocyanins

HPLC-MS analysis was performed with a LCMS-8040 (Shimadzu Co., Ltd., Kyoto, Japan) consisting of a LC-20AD solvent delivery pump, SIL-20AC autosampler, CTO-20A Oven, SPD-20A detector, CBM-20A controller and electrospray ionization source. The mobile phase composition included 80% aqueous trifluoroacetic acid (0.1%, v/v) and 20% acetonitrile. The analytical column was a Benetnach™ C_{18} column (250 mm × 4.6 mm, 5 μm particle, Hanbon Sci & Tech. Co., Ltd., Jiangsu, China), flow rate was 0.4 mL/min, column temperature was 35 °C, injection volume was 10 μL and detection wavelength was 520 nm. The MS conditions were as follows: positive mode; curved desolvation line temperature, 300 °C; heat block temperature, 400 °C; dry gas (N_2) flow rate, 15 L/min; cone gas (N_2) flow, 3 L/min; ESI source voltage, 4.5 kV; detector voltage, 1.96 kV; and scan range, 100–1000 *m/z*.

2.6. HPLC-DAD analysis of anthocyanins

The anthocyanins of *N. tangutorun* seed meal were analysed with an Agilent HPLC series 1260 (Agilent Technologies Co., Ltd., USA) equipped with a quaternary gradient pump, auto-sampler, diode array detector, thermostatically controlled column oven and Agilent Chemstation software. The analytical column was a Benetnach™ C_{18} column (250 mm × 4.6 mm, 5 μm particle, Hanbon Sci & Tech. Co., Ltd., Jiangsu, China). The mobile phase consisted of various types and concentrations of acid aqueous solution and ethanol in a 80:20 (v/v) ratio for *N. tangutorun* seed meal extract and a 85:15 (v/v) ratio for cyanidin-3-*O*-glucoside. The injection volume was 5 μL and detection wavelength was 520 nm. To keep a lower column pressure, the flow rate was set at 0.6 mL/min and column temperature was set at 35 °C.

2.7. Determination of free-radical scavenging activity

The optimum extract of *N. tangutorun* seed meal that was recovered under optimal UAE conditions was freeze-dried after it was concentrated using a rotary evaporator at 50 °C. The antioxidant activity was measured as previously described (Liu et al., 2009) with slight modifications. Briefly, 100 μL of various concentrations of the sample solutions were mixed with 3.0 mL of DPPH solution (100 μmol/L in 95% ethanol). The absorbance of the mixtures was measured at 517 nm using an ELIASA (M4, Molecular Devices Co., ShangHai, China) after mixing and incubation in the dark for 30 min. A lower absorbance of the reaction mixture indicates a higher antioxidant capacity. The blank control consisted of 95% ethanol; BHT and Vc were the positive controls. The antioxidant capacity was calculated according to Eq. (2). The concentration of the optimum extract, BHT and Vc producing 50% DPPH free radical-scavenging activity (IC_{50}) was used as an index to compare their antioxidant activity.

$$\textit{Antioxidant capacity}\ (\%) = \frac{A_0 - A_i}{A_0} \times 100 \qquad (2)$$

where A_0 is the absorbance of the DPPH solution (100 μmol/L in 95% ethanol) and A_i is the absorbance of the reaction mixture after incubation for 30 min in the dark.

2.8. Statistical analysis

All experiments were performed in triplicate. Response surface methodology was performed using Design-Expert.V8.0.6.1 software and other statistical analysis was performed using GraphPad.Prism.v.5.0. Analysis of variance (ANOVA) was employed to determine the statistical significance; $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Fitting the model for predicting total anthocyanins and polyphenols

The experimental and model-predicted values are shown in Table 1. The ANOVA for the experimental results of the Box-Behnken design (Table 2) showed that the coefficients of determination (R^2) of the models were 96% for both total anthocyanins and polyphenols, suggesting that 96% of the actual levels can be matched with the model-predicted levels. The adj R^2 values showed that the insignificant terms were not included in the models of total anthocyanins (adj R^2 = 0.9181) and total polyphenols (adj R^2 = 0.9217). The analysed results (Table 2) also showed that the p-values for lack of fit were 0.1361 and 0.2171 at confidence levels for total anthocyanins and total polyphenols, implying that the two models were well fitted. In addition, the p-values ($p < 0.0001$ for total anthocyanins and polyphenols) indicated that the model predictions were significant. Moreover, the values of the coefficient of variation were 2.35% and 3.67% for total anthocyanins and polyphenols, respectively. These results indicated that the models were reproducible. Therefore, the two mathematical models were adequate for predicting the total anthocyanins and polyphenols and were fitted to the following second-order polynomial formula (Eq. (3) and (4)) for total anthocyanins and polyphenols, respectively:

$$Y_1 = 59.44 + 4.21X_1 + 0.22X_2 - 2.15X_3 - 1.09X_1X_2 + 0.81X_1X_3 + 0.38X_2X_3 + 1.69X_1X_1 - 1.47X_2X_2 - 5.85X_3X_3 \quad (3)$$

$$Y_2 = 850.13 + 107.71X_1 + 34.07X_2 + 27.14X_3 + 11.03X_1X_2 + 21.20X_1X_3 + 3.68X_2X_3 - 19.51X_1X_1 - 49.76X_2X_2 - 94.99X_3X_3 \quad (4)$$

3.2. Response surface optimization of extraction

3.2.1. Response surface optimization of extraction for total anthocyanins

Table 2 shows the variables with significant effects on the total anthocyanin extraction. Three-dimensional response surface plots and two-dimensional contour plots (Fig. 1) were constructed according to Eq. (3), which were the graphical representations of the regression equation and made it easy to analyse the relationship between independent and dependent variables (Dahmoune et al., 2014). The extraction temperature was a significant parameter ($p < 0.0001$); rising extraction temperature correlated to a higher extraction yield. Mild heating might soften the plant tissue, weaken the cell wall integrity and enhance the phytochemical solubility, allowing for more compounds to distribute to the solvent (Tabaraki & Nateghi, 2011). However, prolonged ultrasonic treatment at a higher temperature may induce anthocyanin degradation. In accordance with this, the extraction yield of total anthocyanins was first increased with increasing extraction time, which was then followed by a decrease, as shown in a previous study (Ramić et al., 2015). The ethanol concentration was also a significant parameter ($p = 0.0019$) and with increasing ethanol concentration, the extraction yield of total anthocyanins increased rapidly up to a maximum and then decreased. This effect may

Table 2
Estimated regression coefficients for the quadratic polynomial model and the analysis of variance for the experimental results.

Source	Sum of Squares	DF[a]	Mean square	*F*-value	*p*-value
Total anthocyanins					
Model	357.87	9	39.76	22.17	<0.0001[b]
X_1	141.84	1	141.84	79.08	<0.0001[b]
X_2	0.39	1	0.39	0.22	0.6522
X_3	36.91	1	36.91	20.58	0.0019[b]
X_1X_2	4.76	1	4.76	2.65	0.1419
X_1X_3	2.60	1	2.60	1.45	0.2626
X_2X_3	0.56	1	0.56	0.31	0.5908
X_1X_1	12.46	1	12.46	6.94	0.0299[b]
X_2X_2	9.43	1	9.43	5.26	0.0511
X_3X_3	149.10	1	149.10	83.12	<0.0001[b]
Lack of Fit	9.19	3	3.06	2.97	0.1361[c]
Pure Error	5.16	5	1.03		
Cor Total	372.22	17			
R^2 = 0.9614					
Adj R^2 = 0.9181					
Coefficient of variation (%) = 2.35					
Total polyphenols					
Model	169800	9	18869.75	23.25	<0.0001[b]
X_1	92809.02	1	92809.02	114.34	<0.0001[b]
X_2	9283.55	1	9283.55	11.44	0.0096[b]
X_3	5892.27	1	5892.27	7.26	0.0273[b]
X_1X_2	486.23	1	486.23	0.60	0.4612
X_1X_3	1798.18	1	1798.18	2.22	0.1750
X_2X_3	54.03	1	54.03	0.067	0.8029
X_1X_1	1660.34	1	1660.34	2.05	0.1905
X_2X_2	10802.50	1	10802.50	13.31	0.0065[b]
X_3X_3	39371.08	1	39371.08	48.51	0.0001[b]
Lack of Fit	3631.04	3	1210.35	2.11	0.2171[c]
Pure Error	2862.38	5	572.48		
Cor Total	176300	17			
R^2 = 0.9632					
Adj R^2 = 0.9217					
Coefficient of variation (%) = 3.67					

[a] DF, degrees of freedom.
[b] Means significance ($p < 0.05$).
[c] Means not significance ($p > 0.05$).

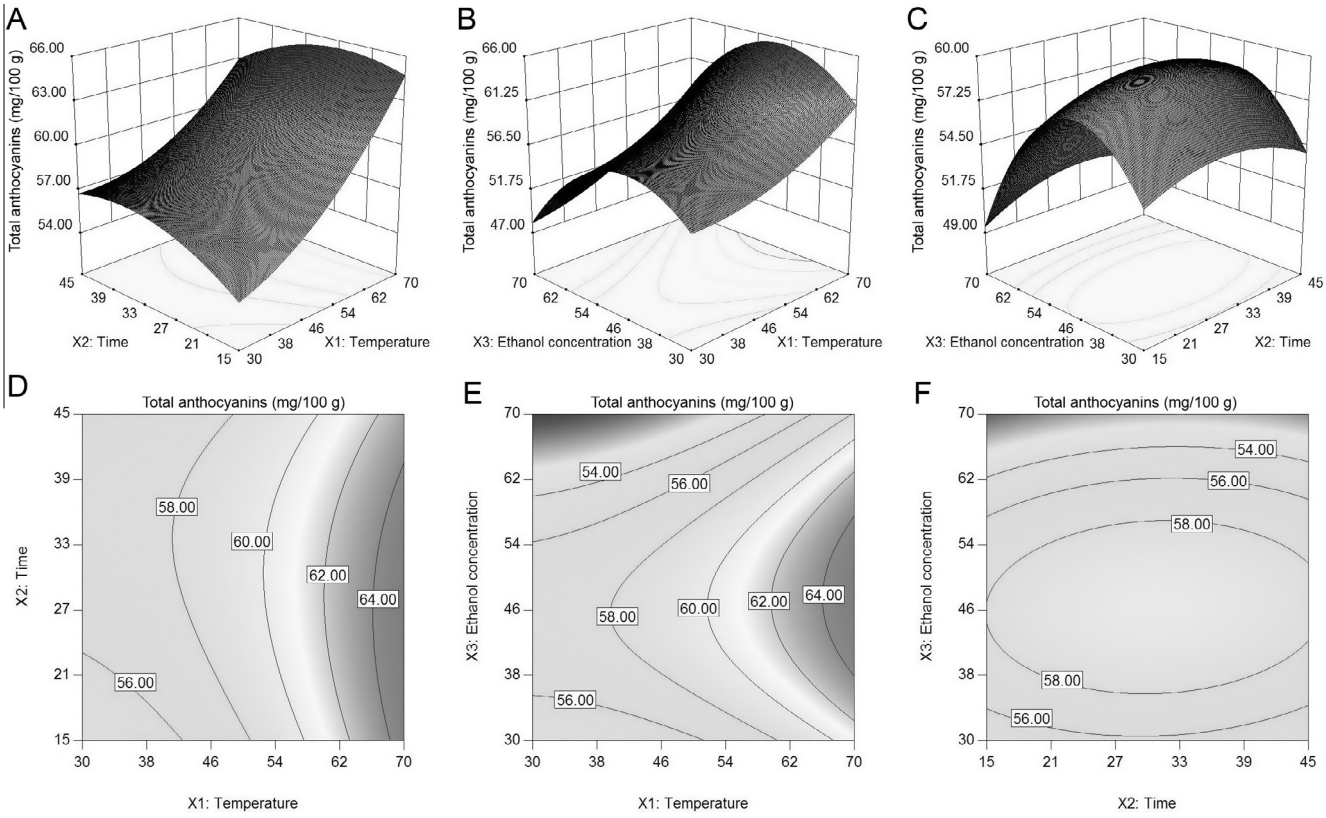

Fig. 1. Response surface plots (A, B and C) and contour plots (D, E and F) showing the combined effect of extraction temperature (X1), extraction time (X2) and ethanol concentration (X3) on extraction yield of total anthocyanins from *N. tangutorun* seed meal.

be attributed to the change in the solvent polarity, and more compounds could distribute to the solvent according to the "like dissolves like" principle (Tabaraki et al., 2012). Therefore, the optimum procedure for maximizing the extraction yield of total anthocyanins was predicted as follows: extraction temperature of 70 °C, extraction time of 25.30 min and ethanol concentration of 47.49%. Under optimal ultrasonic conditions, the modelling predicted a total anthocyanin yield of 65.56 mg cyanidin-3-*O*-glucoside equivalents/100 g dry seed meal. To verify this prediction, *N. tangutorun* seed meal was extracted under optimal conditions. The results revealed an average experimental yield of 65.37 ± 0.91 mg cyanidin-3-*O*-glucoside equivalents/100 g dry seed meal, which was within the experimental tolerance of the predicted value (RSD = 1.39%). Therefore, the model was useful to optimize the conditions for total anthocyanins.

3.2.2. Response surface optimization of extraction for total polyphenols

The variables with a significant effect on total polyphenol extraction are summarized in Table 2. Three-dimensional response surface plots and two-dimensional contour plots (Fig. 2) were constructed according to Eq. (4). The extraction temperature had a significant effect ($p < 0.0001$) on the extraction of total polyphenols, and the maximum yield could be achieved at 70 °C. A high system temperature could facilitate the extraction of more phytochemicals from plant material by softening the tissues, increasing the solubility and diffusion coefficient of the substances. The extraction time was another critical variable ($p = 0.0096$). Because the largest yield for total polyphenols was achieved at a moderate time of ultrasonic treatment, it seems that increasing the extraction temperature was more efficient than prolonging the extraction time to obtain the maximum yield of total polyphenols. This observation is in good agreement with a previous study (Xie et al., 2014). In addition, the ethanol concentration had a remarkable impact ($p = 0.0096$) on the response, playing a dominant role in the total polyphenol recovery. The extraction yield of total polyphenols increased with increasing ethanol concentration at a fixed extraction time and extraction temperature. However, the yield decreased after reaching a peak value. This finding was similar to a previous conclusion on a process for extracting bioactive compounds from grape seeds (Ghafoor, Choi, Jeon, & Jo, 2009). The optimum extract conditions for total polyphenols were predicted as follows: extraction temperature of 70 °C, extraction time of 36.94 min and ethanol concentration of 55.27% with a maximum yield of total polyphenols (955.14 mg gallic acid equivalents/100 g dry seed meal). *N. tangutorun* seed meal was extracted under optimal conditions and achieved an average yield 951.67 ± 9.24 mg gallic acid equivalents/100 g dry seed meal, which is similar to the predicted value (RSD = 0.97%). Therefore, the model was useful for optimizing the extract conditions of total polyphenols.

The mathematical models of total anthocyanins and polyphenols were established, and the optimal UAE conditions of these compounds were obtained using response surface methodology. To maximize the yield of these bioactive compounds from *N. tangutorun* seed meal, simultaneous optimization was performed using Design-Expert.V8.0.6.1 software. The final results suggested that the optimal UAE conditions were an extraction temperature of 70 °C, extraction time of 32.73 min and ethanol concentration of 51.15%. Additionally, 65.04 mg/100 g of total anthocyanins and 947.39 mg/100 g of total polyphenols could be recovered.

3.3. Anthocyanin composition analysis

Anthocyanins of *N. tangutorun* seed meal were identified based on the UV/Vis spectrum, HPLC-MS (Fig. S2) and previous reports.

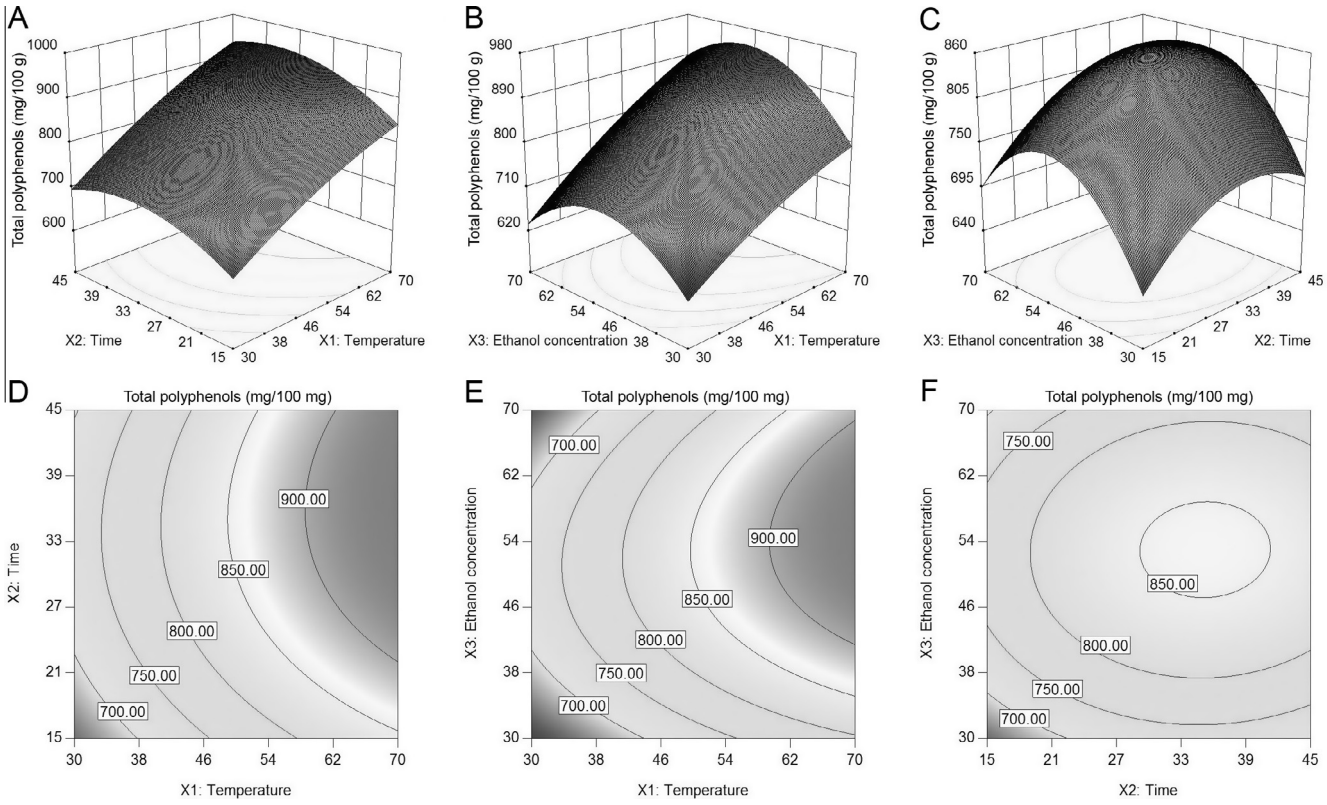

Fig. 2. Response surface plots (A, B and C) and contour plots (D, E and F) showing the combined effect of extraction temperature (X1), extraction time (X2) and ethanol concentration (X3) on extraction yield of total polyphenols from *N. tangutorun* seed meal.

The absorbance band approximately 315 nm presented in the UV–vis spectrum (200–600 nm) implied that the anthocyanin may be acylated; as a result, peaks 1 and 2 were not acylated-anthocyanins and peaks 3–8 were acylated-anthocyanins. Four nonacylated anthocyanins were found in *N. tangutorun* fruit, including cyanidin-3-*O*-diglucoside ($M^+ = m/z$ 611), pelargonidin-3-*O*-diglucoside ($M^+ = m/z$ 595), delphinidin-3-rutinoside ($M^+ = m/z$ 611) and peonidin-3-*O*-diglucoside ($M^+ = m/z$ 625) (Zheng et al., 2011). Cyanidin-3-*O*-diglucoside had a shorter retention time than pelargonidin-3-*O*-diglucoside; as a result, peak 1 (retention time = 8.769 min, $M^+ = m/z$ 611.20) was identified as cyanidin-3-*O*-diglucoside and peak 2 (retention time = 9.186 min, $M^+ = m/z$ 595.25) was identified as pelargonidin-3-*O*-diglucoside. Peak 3 (retention time = 11.108 min) and peak 4 (retention time = 11.820 min) had the same molecular weights ($M^+ = m/z$ 773.35). Zheng et al. (2011) did not separate delphinidin-3-*O*-(caffeoyl)-diglucoside ($M^+ = m/z$ 773) from cyanidin-3-*O*-(caffeoyl)-diglucoside ($M^+ = m/z$ 773) in a HPLC chromatogram. However, delphinidin had a shorter retention time than that of cyanidin (Barnes et al., 2009) and delphinidin-3-*O*-(caffeoyl)-diglucoside could first be eluted from a reverse phase column and cyanidin-3-*O*-(caffeoyl)-diglucoside afterwards. As a result, peaks 3 and 4 were identified as delphinidin-3-*O*-(caffeoyl)-diglucoside and cyanidin-3-*O*-(caffeoyl)-diglucoside, respectively. Cyanidin-3-*O*-(*trans*-*p*-coumaroyl)-diglucoside ($M^+ = m/z$ 757) was the main anthocyanin in *N. tangutorun* fruit and its *cis* configuration exhibited a shorter retention time in RP-HPLC (Zheng et al., 2011). Therefore, peak 5 (retention time = 14.620 min, $M^+ = m/z$ 757.30) was identified as cyanidin-3-*O*-(*cis*-*p*-coumaroyl)-diglucoside and peak 6 (retention time = 15.997 min, $M^+ = m/z$ 757.30), the principal chromatographic peak, was identified as cyanidin-3-*O*-(*trans*-*p*-coumaroyl)-diglucoside. Peak 7 (retention time = 21.996 min, $M^+ = m/z$ 741.35) was identified as pelargonidin-3-*O*-(*p*-coumaroyl)-diglucoside. Peak 8 ($M^+ = m/z$ 611.30, MS/MS = m/z 465.20, 303.15) could not be correlated with a previous report of *N. tangutorun* fruit. The fragment signal at m/z 303.20 suggested peak 8 might be a derivative of delphinidin. Because of the absorbance band at 317 nm in UV–vis spectrum and the neutral loss of 146 Da, the compound might be *p*-coumaroyl-acylated anthocyanin. Neutral loss of 162 Da may be a hexose residue; however, MS could not distinguish the glycosidic isomers, such as -glucoside and -galactoside (Willemse, Stander, & de Villiers, 2013). As a result, peak 8 was tentatively identified as delphinidin-3-*O*-(*p*-coumaroyl)-hexose. Eight anthocyanins were identified from *N. tangutorun* seed meal; cyanidin-3-*O*-diglucoside (0.34%) and pelargonidin-3-*O*-diglucoside (0.21%) were less than other acylated-anthocyanins and cyanidin-3-*O*-(*trans*-*p*-coumaroyl)-diglucoside accounted for almost 80.64% of the total peak area. *N. tangutorun* seed meal could be a source of anthocyanins, and the utilization of these by-products was of significance.

3.4. Optimization of HPLC-DAD conditions

3.4.1. Effect of acids on the peak area

The effects of formic acid (0–10%, v/v), trifluoroacetic acid (0–0.5%, v/v) and HCl (0–0.25%) on the HPLC analysis of anthocyanins were studied. The peak area of cyanidin-3-*O*-(*trans*-*p*-coumaroyl)-diglucoside was used to evaluate the influences of the acids. When the acid concentration was 0%, the peak area of cyanidin-3-*O*-(*trans*-*p*-coumaroyl)-diglucoside was a minimum (453.87 ± 6.45). With increasing formic acid concentration, a rising tendency of the peak area of cyanidin-3-*O*-(*trans*-*p*-coumaroyl)-diglucoside was observed although a downtrend was found (from 3% to 7.5%), and the maximum value of the peak area was found at 10% formic acid (977.50 ± 3.12), which was 2.15-fold higher than

that of 0% formic acid (Fig. 3A). A higher acid concentration and lower the pH level could raise the percentage of flavylium cation, increasing the peak area (Valls et al., 2009). As shown in Fig. 3C and E, rising trends in the peak area were also found when trifluoroacetic acid and HCl were used, albeit an insignificant increase, and the highest values were 906.33 ± 2.10 for 0.5% trifluoroacetic acid and 881.50 ± 5.74 for 0.25% HCl. In addition, cyanidin-3-*O*-glucoside was used to perform the same experiments and significant increases in the peak area were observed with increasing concentrations of formic acid, trifluoroacetic acid and HCl (Fig. 3). To improve the HPLC-DAD analysis of anthocyanins, a higher acid concentration in the mobile phase should be used to increase the peak area and resolution of target compounds. However, the acidic conditions of HPLC-DAD for anthocyanins in plant extract must be evaluated because of the complicated composition of crude extract and interaction between anthocyanins and other compounds.

3.4.2. Effect of acids on the retention time

As shown in Fig. 4, the retention times of cyanidin-3-*O*-(*trans*-*p*-coumaroyl)-diglucoside and cyanidin-3-*O*-glucoside strongly depended on the acid concentration. The retention time of the two anthocyanins was increased at first and then decreased with increasing formic acid and trifluoroacetic acid concentrations (Fig. 4A–D); however, sustained growth in the retention time of both cyanidin-3-*O*-(*trans*-*p*-coumaroyl)-diglucoside and cyanidin-3-*O*-glucoside was found when various HCl levels were used (Fig. 4E and F). First, the increase could be explained as the addition

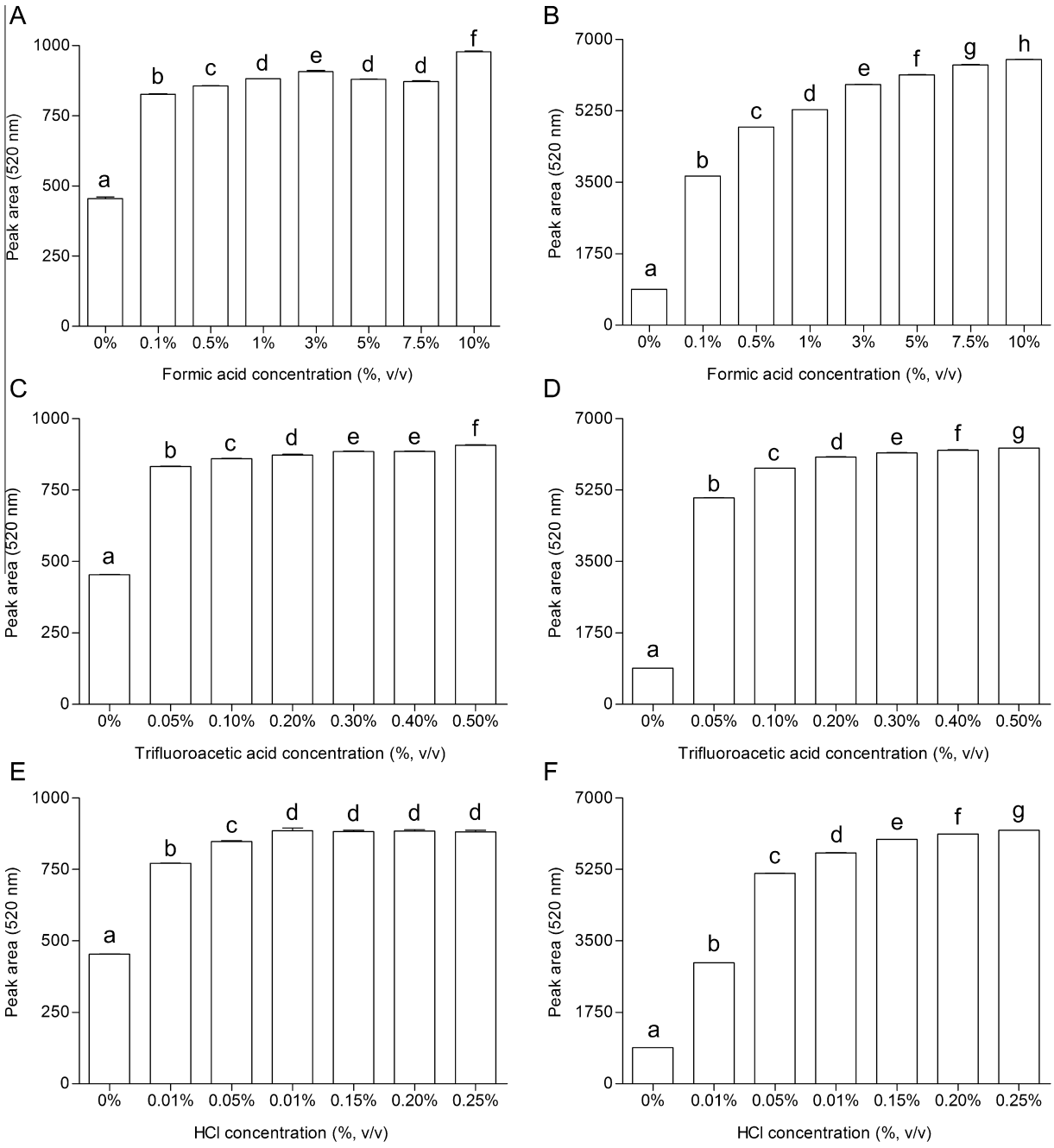

Fig. 3. Effect of various concentrations (%, v/v) of formic acid, trifluoroacetic acid and HCl on the peak area of cyanidin-3-*O*-(*trans*-*p*-coumaroyl)-diglucoside (A, C and E) and cyanidin-3-*O*-glucoside (B, D and F) in HPLC using ethanol-based mobile phase. Means with different letters were significantly different at the level of $p < 0.05$.

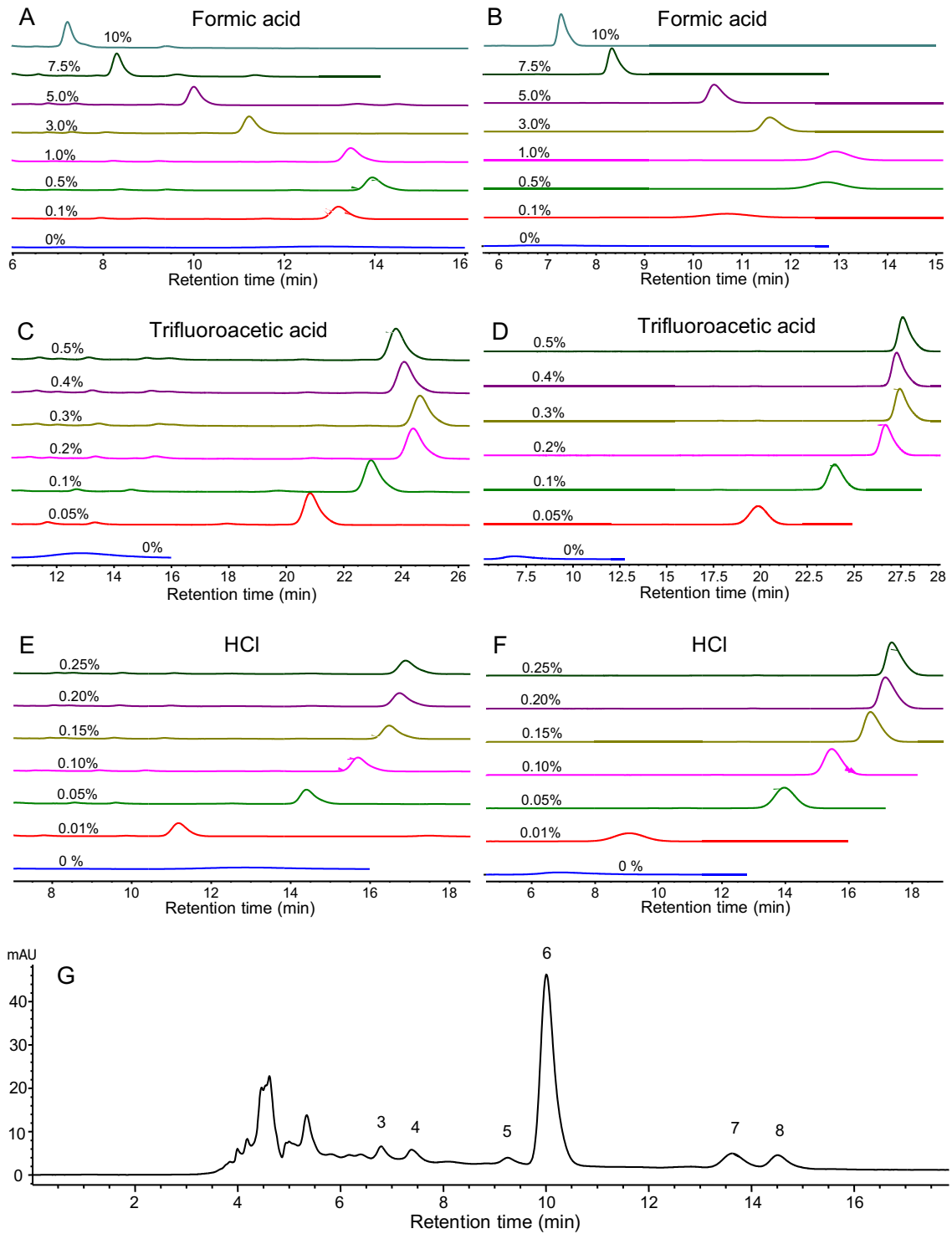

Fig. 4. Chromatographic behavior of cyanidin-3-*O*-(*trans*-*p*-coumaroyl)-diglucoside (A, C and E) and cyanidin-3-*O*-glucoside (B, D and F) when various concentration (%, v/v) of acids (formic acid, trifluoroacetic acid and HCl) were used as mobile phase additive, and the optimal HPLC-DAD chromatogram (G) of anthocyanins from *N. tangutorun* seed meal.

of formic acid or trifluoroacetic acid could increase the polarity of the mobile phase, resulting in weaker power of elution and thus a longer retention time. Subsequent reduction in the retention time could be attributed to a competitive interaction between HCOOH or CF_3COOH molecules and the polar groups of anthocyanins for the silanol group on the alkylsilica surface when excessive organic acid was added. A similar phenomenon was observed when ionic liquids and deep eutectic solvents were used as mobile phase additives (He, Zhang, Zhao, Liu, & Jiang, 2003; Tan, Zhang, Wan, & Qiu, 2016). When HCl was added into the mobile phase, the competitive interaction was not obvious in our experiments. Acids added in the mobile phase could improve the peak area and resolution of target anthocyanins; however, the effects on the retention time should be evaluated. Considering the analysis time and pH range of the

analytical column and apparatus, suitable acid concentrations should be determined.

3.5. Validation of the HPLC-DAD method

The optimal HPLC-DAD method conditions were determined as follows: the composition of the mobile phase was 5% (v/v) formic acid aqueous solution with absolute ethanol in an 80:20 (v/v) ratio, flow rate of 0.6 mL/min, column temperature of 35 °C and detection wavelength of 520 nm (Fig. 4G). Cyanidin-3-*O*-(*trans*-*p*-coumaroyl)-diglucoside was used to verify and evaluate the green HPLC-DAD method of anthocyanins in *N. tangutorun* seed meal extract.

3.5.1. Linearity

Cyanidin-3-*O*-glucoside was used as a standard with concentrations at ten levels in the 0.01–0.25 mg/mL range, and the results are expressed in milligrams of cyanidin-3-*O*-glucoside equivalent/mL sample solution. The calibration curve was given by Y (peak area) = 33637 × X (cyanidin-3-*O*-glucoside equivalent concentration) + 26.897 (R^2 = 0.9999).

3.5.2. Limits of detection and quantitation

The limits of detection and quantitation of cyanidin-3-*O*-(*trans*-*p*-coumaroyl)-diglucoside, calculated as signal-to-noise ratios of 3 and 10, were 0.0636 mg/L and 0.2119 mg/L, respectively, indicating the method was sufficiently sensitive to measure cyanidin-3-*O*-(*trans*-*p*-coumaroyl)-diglucoside in *N. tangutorun* seed meal.

3.5.3. Precision

The precision was evaluated using six replicate determinations of a sample, and the RSD was used to evaluate the precision of the analysis method. The RSD values of intra-day and inter-day analyses were 0.53% (n = 6) and 0.66% (n = 6), respectively.

3.5.4. Repeatability

The repeatability was examined in six replicate samples and the RSD value (2.07%) was below the recommended value of 5%.

3.5.5. Stability

To evaluate stability, the sample was measured at different times after preparation (0, 30, 60, 90, 120, 150 and 180 min). The concentration of cyanidin-3-*O*-(*trans*-*p*-coumaroyl)-diglucoside did not change significantly (RSD = 0.19%) within 180 min at room temperature.

These results for method validation suggested that the proposed green HPLC-DAD method that was established in our study was reliable for evaluating the anthocyanins of *N. tangutorun* seed meal. For the first time, the HPLC-DAD method with the ethanol-based mobile phase was used to analyse anthocyanins, which could effectively reduce the utilization of a toxic solvent and generation of harmful waste. Additionally, six anthocyanins (peak 3–8) of *N. tangutorun* seed meal were determined within 16 min, and an isocratic elution program and lower backpressure (⩽160 bar) could reduce the cost of the analysis apparatus. This reduced cost could promote the wide use of green analytical methods. A previous report of *N. tangutorun* fruit (Zheng et al., 2011) analysed anthocyanins using HPLC with a longer analytical process and higher percentage of the acid and toxic mobile phases. As a result, substantial harmful waste was generated. A rapid HPLC method (less than 20 min) was used to determined anthocyanins from haskap berries; however, toxic solvent (methanol) was used (Celli, Ghanem, & Brooks, 2015). He et al. (2016) used UPLC to analyse anthocyanins from blueberry wine pomace within 14 min, and the investment in UPLC apparatus was much higher. Therefore, environmentally friendly, rapid and cost-effective methods should be established.

3.6. Antioxidant capacity of the extract

An *in vitro* antioxidant assay showed that the DPPH free radical-scavenging activity of the optimal *N. tangutorun* seed meal extract was increased from 27.24 ± 0.89% to 60.71 ± 0.06% when the extract concentration increased from 0.1 to 0.6 mg/mL (Fig. S3). However, the DPPH free radical-scavenging activity saturated (p > 0.05) when the extract concentration was over 0.6 mg/mL. The antioxidant capacity of the optimum extract (IC_{50} = 0.3438 mg/mL) was lower than that of Vc (IC_{50} = 0.1473 mg/mL) and higher than that of BHT (IC_{50} = 0.5484 mg/mL), a synthetic antioxidant. Therefore, *N. tangutorun* seed meal was a potential raw material for recovering natural antioxidant and could be used as a substitute synthetic antioxidant with possible side effects. The optimal *N. tangutorun* seed meal extract could be developed as a product with health benefits.

4. Conclusions

Response surface methodology was used to evaluate the effects of the extraction temperature, extraction time and ethanol concentration on the UAE of anthocyanins from *N. tangutorun* seed meal. An extraction temperature of 70 °C, extraction time of 32.73 min and aqueous ethanol concentration of 51.15% were optimal conditions for recovering total anthocyanins and polyphenols. The optimal extract exhibited potent antioxidant capacity *in vitro*. Eight anthocyanins identified from *N. tangutorun* seed meal extract were derivatives of cyanidin, pelargonidin and delphinidin, and cyanidin-3-*O*-(*trans*-*p*-coumaroyl)-diglucoside was the principal anthocyanin (18.17 ± 0.16 mg cyanidin-3-*O*-glucoside equivalents/100 g dry seed meal). *N. tangutorun* seed meal utilization was noteworthy because this by-product could be used as a natural raw material to extract bioactive and pigmented compounds.

An environmentally friendly HPLC-DAD method with the ethanol-based mobile phase was established to analyse the anthocyanins of *N. tangutorun* seed meal. Satisfactory accuracy and precision of the green method were demonstrated. The proposed HPLC-DAD method could simultaneously analyse six anthocyanins (peak 3–8) within 16 min, which was achieved by isocratic elution (5% formic acid aqueous solution and absolute ethanol in a 80:20 (v/v) ratio). A low-cost HPLC apparatus could be used to perform the entire analytic process because of the application of a single pump and lower backpressure (⩽160 bar). Our study demonstrated the potential of an environmentally friendly analytic method and might promote practical applications of green analytical chemistry.

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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.foodchem.2016.09.093.

References

Anastas, P. T. (1999). Green chemistry and the role of analytical methodology development. *Critical reviews in analytical chemistry, 29*, 167–175.

Armenta, S., Garrigues, S., & de la Guardia, M. (2008). Green analytical chemistry. *Trends in Analytical Chemistry, 27*, 497–511.

Barnes, J. S., Nguyen, H. P., Shen, S., & Schug, K. A. (2009). General method for extraction of blueberry anthocyanins and identification using high performance liquid chromatography-electrospray ionization-ion trap-time of flight-mass spectrometry. *Journal of Chromatography A, 1216*, 4728–4735.

Bendicho, C., De La Calle, I., Pena, F., Costas, M., Cabaleiro, N., & Lavilla, I. (2012). Ultrasound-assisted pretreatment of solid samples in the context of green analytical chemistry. *Trends in Analytical Chemistry, 31*, 50–60.

Borges, G. D. S. C., Vieira, F. G. K., Copetti, C., Gonzaga, L. V., & Fett, R. (2011). Optimization of the extraction of flavanols and anthocyanins from the fruit pulp of *Euterpe edulis* using the response surface methodology. *Food Research International, 44*, 708–715.

Celli, G. B., Ghanem, A., & Brooks, M. S. (2015). Optimization of ultrasound-assisted extraction of anthocyanins from haskap berries (*Lonicera caerulea* L.) using Response Surface Methodology. *Ultrasonics Sonochemistry, 27*, 449–455.

Chen, C., Zhang, L., Liu, Z., Tao, Y., Shao, Y., & Mei, L. (2011). Processing technology of *Nitraria tangutorun* Bobr. juice. *Science and Technology of Food Industry, 32*, 289–291.

Corbin, C., Fidel, T., Leclerc, E. A., Barakzoy, E., Sagot, N., Falguiéres, A., Renouard, S., et al. (2015). Development and validation of an efficient ultrasound assisted extraction of phenolic compounds from flax (*Linum usitatissimum* L.) seeds. *Ultrasonics Sonochemistry, 26*, 176–185.

Dahmoune, F., Spigno, G., Moussi, K., Remini, H., Cherbal, A., & Madani, K. (2014). *Pistacia lentiscus* leaves as a source of phenolic compounds: Microwave-assisted extraction optimized and compared with ultrasound-assisted and conventional solvent extraction. *Industrial Crops and Products, 61*, 31–40.

Ding, C., Suo, Y., Li, Y., Wang, H., Wang, L., & Wang, X. (2008). Immune regulation research of soft capsule of compound *Nitraria tangutorun* Bobr. seed oil. *Chinese Traditional Patent Medicine, 30*, 125–128.

Eren, İ., & Kaymak-Ertekin, F. (2007). Optimization of osmotic dehydration of potato using response surface methodology. *Journal of Food Engineering, 79*, 344–352.

Ghafoor, K., Choi, Y. H., Jeon, J. Y., & Jo, I. H. (2009). Optimization of ultrasound-assisted extraction of phenolic compounds, antioxidants, and anthocyanins from grape (*Vitis vinifera*) seeds. *Journal of Agricultural and Food Chemistry, 57*, 4988–4994.

He, B., Zhang, L., Yue, X., Liang, J., Jiang, J., Gao, X., & Yue, P. (2016). Optimization of Ultrasound-Assisted Extraction of phenolic compounds and anthocyanins from blueberry (*Vaccinium ashei*) wine pomace. *Food Chemistry, 204*, 70–76.

He, L., Zhang, W., Zhao, L., Liu, X., & Jiang, S. (2003). Effect of 1-alkyl-3-methylimidazolium-based ionic liquids as the eluent on the separation of ephedrines by liquid chromatography. *Journal of Chromatography A, 1007*, 39–45.

Ivanovic, J., Tadic, V., Dimitrijevic, S., Stamenic, M., Petrovic, S., & Zizovic, I. (2014). Antioxidant properties of the anthocyanin-containing ultrasonic extract from blackberry cultivar "Čačanska Bestrna". *Industrial Crops and Products, 53*, 274–281.

Jia, Z., Zhu, G., & Wang, J. (1989). Flavonoid constituents from the seeds of *Nitraria tangutorum* Bolor. *Journal of Integrative plant biology, 31*, 241–243.

Liu, L., Sun, Y., Laura, T., Liang, X., Ye, H., & Zeng, X. (2009). Determination of polyphenolic content and antioxidant activity of kudingcha made from *Ilex kudingcha* C.J. Tseng. *Food Chemistry, 112*, 35–41.

Lu, M., Li, Z., Liang, H., Shi, M., Zhao, L., Li, W., ... Li, Y. (2015). Controlled release of anthocyanins from oxidized konjac glucomannan microspheres stabilized by chitosan oligosaccharides. *Food Hydrocolloids, 51*, 476–485.

Giusti, M. M., & Wrolstad, R. E. (2003). Acylated anthocyanins from edible sources and their applications in food systems. *Biochemical Engineering Journal, 14*, 217–225.

Ma, T., Hu, N., Ding, C., Zhang, Q., Li, W., Suo, Y., ... Ding, C. (2016). *In vitro* and *in vivo* biological activities of anthocyanins from *Nitraria tangutorun* Bobr. fruits. *Food Chemistry, 194*, 296–303.

Pan, X., Shen, G., & Peng, C. (1999). A preliminary research on taxonomy and systematic of genus *Nitraria*. *Acta Botanica Yunnanica, 21*, 287–295.

Plotka, J., Tobiszewski, M., Sulej, A. M., Kupska, M., Górecki, T., & Namiesnik, J. (2013). Green chromatography. *Journal of Chromatography A, 1307*, 1–20.

Ramić, M., Vidović, S., Zeković, Z., Vladić, J., Cvejin, A., & Pavlić, B. (2015). Modeling and optimization of ultrasound-assisted extraction of polyphenolic compounds from *Aronia melanocarpa* by-products from filter-tea factory. *Ultrasonics Sonochemistry, 23*, 360–368.

Roselló-Soto, E., Galanakis, C. M., Brnčić, M., Orlien, V., Trujillo, F. J., Mawson, R., ... Barba, F. J. (2015). Clean recovery of antioxidant compounds from plant foods, by-products and algae assisted by ultrasounds processing. Modeling approaches to optimize processing conditions. *Trends in Food Science & Technology, 42*, 134–149.

Samaram, S., Mirhosseini, H., Tan, C. P., & Ghazali, H. M. (2014). Ultrasound-assisted extraction and solvent extraction of papaya seed oil: Crystallization and thermal behavior, saturation degree, color and oxidative stability. *Industrial Crops and Products, 52*, 702–708.

Tabaraki, R., Heidarizadi, E., & Benvidi, A. (2012). Optimization of ultrasonic-assisted extraction of pomegranate (*Punica granatum* L.) peel antioxidants by response surface methodology. *Separation and Purification Technology, 98*, 16–23.

Tabaraki, R., & Nateghi, A. (2011). Optimization of ultrasonic-assisted extraction of natural antioxidants from rice bran using response surface methodology. *Ultrasonics Sonochemistry, 18*, 1279–1286.

Tan, T., Zhang, M., Wan, Y., & Qiu, H. (2016). Utilization of deep eutectic solvents as novel mobile phase additives for improving the separation of bioactive quaternary alkaloids. *Talanta, 149*, 85–90.

Valls, J., Millán, S., Martí, M. P., Borràs, E., & Arola, L. (2009). Advanced separation methods of food anthocyanins, isoflavones and flavanols. *Journal of Chromatography A, 1216*, 7143–7172.

Wang, H., Li, Y., Li, X., & Suo, Y. (2007). Chemical constitutions of *Nitraira tangutorun* seed from Qaidam Basin. *Natural Product Research and Development, 19*, 614–616.

Wang, Y., Chen, N., Li, C., Gao, H., & Zhang, Y. (2012). Production technology of red dry wine from *Nitraria tangutorun* Bobr. fruits. *Food Science and Technology, 37*, 111–116.

Welch, C. J., Brkovic, T., Schafer, W., & Gong, X. (2009). Performance to burn? Re-evaluating the choice of acetonitrile as the platform solvent for analytical HPLC. *Green Chemistry, 11*, 1232.

Willemse, C. M., Stander, M. A., & de Villiers, A. (2013). Hydrophilic interaction chromatographic analysis of anthocyanins. *Journal of Chromatography A, 1319*, 127–140.

Xie, J., Dong, C., Nie, S., Li, F., Wang, Z., Shen, M., & Xie, M. (2014). Extraction, chemical composition and antioxidant activity of flavonoids from *Cyclocarya paliurus* (Batal.) Iljinskaja leaves. *Food Chemistry, 186*, 97–105.

Yolmeh, M., Habibi Najafi, M. B., & Farhoosh, R. (2014). Optimisation of ultrasound-assisted extraction of natural pigment from annatto seeds by response surface methodology (RSM). *Food Chemistry, 155*, 319–324.

Zheng, J., Li, H., Ding, C., Suo, Y., Wang, L., & Wang, H. (2011). Anthocyanins composition and antioxidant activity of two major wild *Nitraria tangutorun* Bobr. variations from Qinghai-Tibet Plateau. *Food Research International, 44*, 2041–2046.