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# Optimisation of High Hydrostatic Pressure Assisted Extraction of Anthocyanins from Rabbiteye Blueberry Pomace

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**Abstract**

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The purpose of this study was to evaluate the influence of high hydrostatic pressure assisted extraction (HHPE) on the anthocyanins from blueberry (*Vaccinium ashei)* pomace. From the Plackett-Burman Experimental Design (PBD), only the liquid-solid ratio, ethanol concentration, and extraction pressure were found to significantly affect the extraction yield of anthocyanin content. Hence, the outcome of Box-Behnken Design suggested that the optimal operating conditions of the HHPE for the yield of anthocyanin content were liquid-solid ratio 41 ml/g, ethanol concentration 63%, and extraction pressure 443 MPa. At these conditions, 107.9 mg/100 g anthocyanins was obtained, which was more than by the control extraction (67.63 mg/100 g). 10 anthocyanins were identified by HPLC-ESI-MS, malvidin-3-galactoside and malvidin-3-glucoside were the major anthocyanins.

**Keywords**: extraction conditions; *Vaccinium ashei*; extraction/separation; response surface methodology (RSM); HPLC-MS

Rabbiteye blueberry (*Vaccinium ashei*) is a fruit widely cultivated in Nanjing in China, which is a rich source of biological compounds, chiefly anthocyanins that are well-known for their health related properties (Li *et al*. 2013). Indeed, various studies have been devoted to the health benefits of blueberry to its antioxidant (Su & Silva 2006), anti-inflammatory (Huang *et al*. 2014), anticancer (Faria *et al*. 2010), and atherosclerosis protection (Del Bo' *et al*. 2015) properties.

However, due to its limited shelf-life, blueberry is usually processed into juice, wine, and vinegar (Johnson & Gonzalez de Mejia 2012; Kim *et al*. 2012). In fact, fermented anthocyanin-rich beverages have been demonstrated to exhibit a good biological activity by inhibiting inflammation (Johnson *et al*. 2013). Although anthocyanins are generally extracted during the fermentation process, the largest part of anthocyanins remains in pomace (Lee *et al*. 2002; Farrukh *et al*. 2006; Khanal *et al*. 2010). Therefore, the recovery of polyphenols, especially anthocyanins, in pomace is an imperative owing to their potential use in the food industry as natural colorants and nutraceuticals. Hence, among the processes employed in the food industry, high hydrostatic pressure extraction (HHPE) has been proposed as a suitable technique for the polyphenol extraction (Corrales *et al*. 2009). Based on its ability to enhance the mass transfer, damage the cell membrane, and increase permeability (Jun 2013), HHPE has been successfully used for anthocyanins and flavonols from berry fruits and berry juices (Altuner & Tokuşoğlu 2013). Moreover, HHPE as a non-thermal process has been reported to increase the production efficiency such as reduction of processing time or improvement in operating conditions. Thereby, HHPE is considered as a non-thermal and environment-friendly technology (Shouqin *et al*. 2004).

Therefore, the objectives of this study were to assess, in the first approach, the effect of HHPE conditions

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on anthocyanin extraction efficiency using a Plackett-Burman design (PBD). Furthermore, the study sought to identify the optimal operative conditions of HHPE using response surface methodology (RSM). Thereafter, the anthocyanin profile of blueberry pomace extract was determined using HPLC-MS.

## MATERIAL AND METHODS

***Chemicals***. Gallic acid (99% of purity) was purchased from J&K Technology Co. Ltd (China). Folin-Ciocalteu phenol reagent was purchased from Shanghai Lida Biotechnology Co. Ltd (China). Other chemicals, as analytical grade, were purchased from Sinopharm Chemical Reagent (China).

***Plant material***. Blueberry pomace was obtained after blueberry wine fermentation, fresh rabbiteye blueberries (*Vaccinium ashei*) were picked from a plantation in Lishui District (China). Blueberries were broken using a presser, after enzymolysis 1.5 h at 35°C blueberries were fermented at 21°C for 7 days. The wine pomace was pressed in a small laboratory extruder, the obtained wastes were dried to a constant weight at 40°C in a hot-air drying oven. The dried pomace was powdered using a grinder. The powders were frozen immediately and stored at –20°C until used.

***High hydrostatic pressure assisted extraction (HHPE)***. The extraction processing was carried out with high hydrostatic pressure equipment (Intelligent Super High Pressure Food Processing Device, Jiangsu University, China). The machine had an operational volume of 3 l. Dioctyl sebacate was used as the pressurising fluid, the maximum operational pressure of 600 MPa was reached in about 95 s and the depressurisation time was approximately 10 seconds.

The packaged sample that contained blueberry pomace powder and extraction solvent was extracted under different pressures and holding times (according to the experimental design) at ambient temperature. After treatment, the extraction solution was centrifuged using a TGL-20M tabletop high-speed refrigerated centrifuge (Changsha Xiangyi Centrifuge Instrument Co. Ltd, China) at 5000 rpm for 10 min at 10°C. Afterward, the supernatant (100 ml) was concentrated to dryness at 40°C for 20 min using vacuum rotary evaporation (Ya Rong Biochemical Instrument Factory, China). Then, the dry extract was diluted to 100 ml with phosphate buffer (pH 3.0) prior to the total anthocyanin analysis.

Table 1. Variables and levels encoded for PBD

| Input variables | Levels | |
|---|---|---|
| | –1 | 1 |
| Liquid-solid ratio (ml/g) ($X_1$) | 10 | 50 |
| Ethanol concentration (%) ($X_2$) | 20 | 80 |
| Hydrochloric acid concentration (%) ($X_3$) | 0.185 | 0.74 |
| Extraction pressure (MPa) ($X_4$) | 100 | 600 |
| Holding time (min) ($X_5$) | 5 | 30 |
| Extraction cycles ($X_6$) | 1 | 3 |

***Control extraction***. 5 g blueberry pomace in a 150 ml extraction solvent which contained 60% ethanol and 12 M HCl (99 : 1, v/v), extraction conditions were carried out in a water bath incubated at a temperature of 60°C for 1 hour.

***PBD experiment***. There are many parameters, including pressure, extraction time, solid-to-solvent ratio, solvent concentration, solvent constituents and extraction cycles that may influence the extraction efficiency of anthocyanins.

The PB design was employed to determine the effect of liquid-to-solid ratio ($X_1$), ethanol concentration ($X_2$), hydrochloric acid concentration ($X_3$), extraction pressure ($X_4$), holding time ($X_5$), and extraction cycles ($X_6$) on the total anthocyanin content. The PB two levels (+1) and (–1) (Table 1) were used to screen the significant variables during HHPE. From the outcome of the PB design, 15 runs with 3 centre point experiments (Table 2), the interactive effect of

Table 2. Anthocyanin extraction yield obtained from PBD

| Runs | $X_1$ | $X_2$ | $X_3$ | $X_4$ | $X_5$ | $X_6$ | Anthocyanin content (mg/g) |
|---|---|---|---|---|---|---|---|
| 1 | 1 | 1 | –1 | 1 | 1 | 1 | 0.91 |
| 2 | –1 | 1 | 1 | –1 | 1 | 1 | 0.67 |
| 3 | 1 | –1 | 1 | 1 | –1 | 1 | 0.84 |
| 4 | –1 | 1 | –1 | 1 | 1 | –1 | 0.79 |
| 5 | –1 | –1 | 1 | –1 | 1 | 1 | 0.51 |
| 6 | –1 | –1 | –1 | 1 | –1 | 1 | 0.61 |
| 7 | 1 | –1 | –1 | –1 | 1 | –1 | 0.54 |
| 8 | 1 | 1 | –1 | –1 | –1 | 1 | 0.72 |
| 9 | 1 | 1 | 1 | –1 | –1 | –1 | 0.62 |
| 10 | –1 | 1 | 1 | 1 | –1 | –1 | 0.76 |
| 11 | 1 | –1 | 1 | 1 | 1 | –1 | 0.86 |
| 12 | –1 | –1 | –1 | –1 | –1 | –1 | 0.41 |
| 13 | 0 | 0 | 0 | 0 | 0 | 0 | 0.8 |
| 14 | 0 | 0 | 0 | 0 | 0 | 0 | 0.83 |
| 15 | 0 | 0 | 0 | 0 | 0 | 0 | 0.81 |

doi: 10.17221/189/2016-CJFS

Table 3. Variables and levels encoded for BBD

| Input variables | Levels | | |
|---|---|---|---|
| | –1 | 0 | 1 |
| Liquid-solid ratio (ml/g) ($X_1$) | 25 | 35 | 45 |
| Ethanol concentration (%) ($X_2$) | 45 | 60 | 75 |
| Extraction pressure (MPa) ($X_4$) | 300 | 400 | 500 |

the variables was assessed based on the first order model:

$$Y = \beta_0 + \sum \beta_i x_i \quad (1)$$

where: $Y$ – response (total anthocyanin content); $\beta_0$ – constant; $\beta_i$ – linear regression coefficient; $x_i$ – level of the independent variable

***Box-Behnken Design (BBD)***. BBD was used in this study to determine the optimal conditions of the HHPE of anthocyanins in blueberry pomace. Based on the results obtained from the PBD liquid-solid ratio ($X_1$), ethanol concentration ($X_2$), and pressure ($X_4$) were selected as experimental variables. Therefore, 3-level 3 factors (Table 3) were employed requiring 15 experiments with 3 centre point experiments (Table 4) for the optimisation of HHPE.

To evaluate the interactions among the factors, the experimental data was analysed by multiple regression equation to fit the second order polynomial model:

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

where: $Y$ – predicted response, here it is total anthocyanin content; $\beta_0$ – constant; $\beta_i$, $\beta_{ii}$, $\beta_{ij}$ – linear coefficient, quadratic coefficient, and interaction coefficient, respectively; $X_i$, $X_j$ – independent variables

***Total anthocyanin content***. The total anthocyanin content (TCA) of blueberry pomace extracts was determined according to Giusti and Wrolstad (2001).

***HPLC-ESI-MS analysis***. The extraction sample obtained at the optimal conditions was purified as described by Rodriguez-Saona and Wrolstad (2001) using the solid phase extraction method with Sep-Pak C18 cartridge (Waters Co., USA). Thereafter, the purified sample was filtered through a 0.45 µm Millipore filter before anthocyanin determination using HPLC-DAD (HP Agilent 1100 Series) and ESI-MS Bruker Esquire LC-MS ion trap multiple-stage mass spectrometer (Germany) in positive ionisation mode analysing ions from *m/z* 100 to *m/z* 1200.

The HPLC characteristics and working conditions were: DAD detector (G1315A): 200–700 nm full scan, quaternary pump system (G1311A), autosampler (G1313A). The column used was Agilent 20RBAX-SB C18 4.6 mm × 250 mm, 5 µm (Agilent, USA). Solvent A (methanol) and solvent B (3% formic acid in deionised water) were membrane-filtered (0.45 µm) and de-aerated by sonication at 25°C for 40 minutes. The linear gradient elution was carried out as follows: 0~10 min A – increased from 20% to 25%; 10~25 min A – increased from 25% to 30%; 25~35 min A – increased from 30% to 35%; 35~45 min A – increased from 35% to 50%; 45~50 min A – decreased from 50% to 20%; 50~60 min A – kept 20%. The flow rate was 1.0 ml/min and injection volume was 30 µl. The column temperature was 25°C.

The conditions of ESI-MS were as follows: ESI source voltage 4.5 kV, capillary voltage 30 V, sheath gas flow rate 30 arbitrary units, tube lens voltage 120 V, and capillary temperature 300°C.

The Xcalibur 2.0.7 SP1 software (Thermo Fisher Scientific Inc., USA) was used to create and edit the mass spectrometry data for the precursor and fragment ions.

***Statistical analysis***. Design Expert 8.0.6.1 software (Stat-Ease Inc., USA) was used for the PB and BBD. All the experiments were conducted in triplicate and results are expressed as means. The model adequacy was evaluated using: the *F*-test obtained from the analysis of variance represented at 0.05 and 0.01 level of significance, the coefficient of variation (*CV*), the predicted versus actual plots, the lack of fit test, the coefficient of determination ($R^2$), the three-dimensional (3D) surface plots of model interaction terms.

## RESULTS AND DISCUSSION

***Screening of significant variables using PBD***. PBD as a useful methodology which allows a rapid screening of significant factors from a multivariable system (Cao *et al*. 2012) was employed to investigate the effect of six processing variables as well as their interactive effects on the extraction yield of anthocyanins from blueberry pomace during HHPE.

Only three processing variables such as extraction pressure ($X_4$), liquid-solid ratio ($X_1$), and ethanol concentration ($X_2$) were found to significantly affect the extraction yield of anthocyanins (Figure 1). The finding is similar with Jun (2006), who also found that extraction pressure, liquid-solid ratio, and ethanol concentration had significant effects on lycopene

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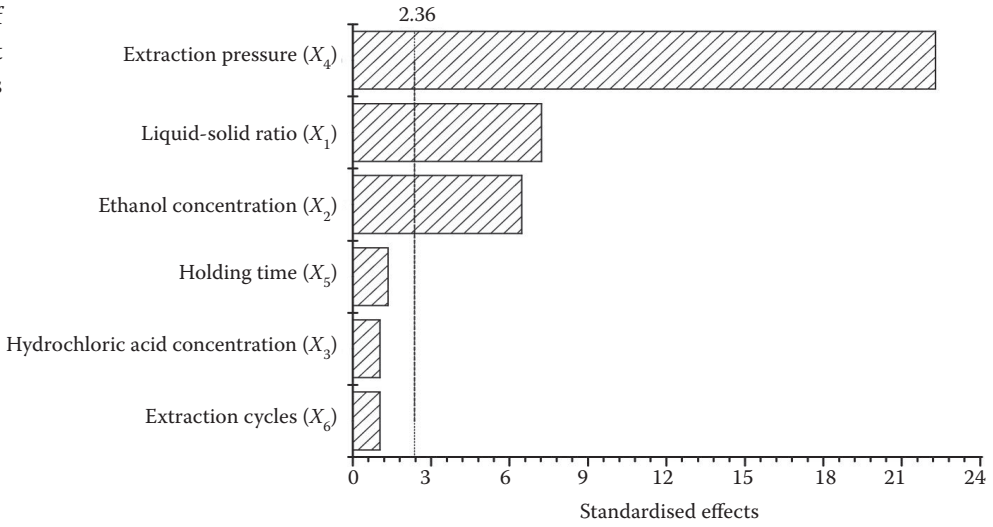


Figure 1. Pareto chart of the standardised effect of independent variables (α = 0.05)

extraction during HHPE. Indeed, some researchers reported that the pressure had significant effects on extraction active compounds (Prasad *et al.* 2009; Briones-Labarca *et al.* 2013; Altuner *et al.* 2014). As explained by the mass transfer theory, the pressure accelerated mass transfer; the higher the pressure, the more solvent could enter into cells and the more anthocyanins dissolved out (Corrales *et al.* 2009).

Therefore, the non-significant processing variables such as hydrochloric acid concentration ($X_3$), holding time ($X_5$), and extraction cycles ($X_6$) were fixed at 0.185%, 5 min, and 1 cycle, respectively. Other three significant variables (extraction pressure, liquid-solid ratio, and ethanol concentration) were selected for further optimisation studies.

Table 4. The extraction yield of anthocyanins using BBD response surface methodology

| Runs | $X_1$ | $X_2$ | $X_4$ | Anthocyanin content (mg/100 g) |
|---|---|---|---|---|
| 1 | –1 | –1 | 0 | 85.14 |
| 2 | 1 | –1 | 0 | 93.65 |
| 3 | –1 | 1 | 0 | 92.34 |
| 4 | 1 | 1 | 0 | 103.5 |
| 5 | –1 | 0 | –1 | 89.32 |
| 6 | 1 | 0 | –1 | 99.25 |
| 7 | –1 | 0 | 1 | 92.74 |
| 8 | 1 | 0 | 1 | 103.72 |
| 9 | 0 | –1 | –1 | 89.09 |
| 10 | 0 | 1 | –1 | 91.56 |
| 11 | 0 | –1 | 1 | 89.01 |
| 12 | 0 | 1 | 1 | 104.46 |
| 13 | 0 | 0 | 0 | 108.76 |
| 14 | 0 | 0 | 0 | 101.92 |
| 15 | 0 | 0 | 0 | 106.32 |

***Optimisation of significant variables using RSM.*** RSM as a powerful tool successfully employed for the optimisation of anthocyanin extraction in food processing (Fan *et al.* 2008; Borges *et al.* 2011; Meng *et al.* 2014) was used to optimise the significant variables of the HHPE.

Hence, the extraction conditions at liquid-solid ratio 35 : 1/ethanol concentration 60% per extraction pressure 400 MPa (run 13) resulted in the highest extraction of total anthocyanin content (Table 4). While at liquid-solid ratio 25 : 1/ethanol concentration 45% per extraction pressure 400 MPa (run 1), the extraction yield of total anthocyanins was found to be minimal. These results indicated that the liquid-solid ratio, ethanol concentration, and extraction pressure had a significant effect on the total anthocyanin content depending upon the experimental conditions.

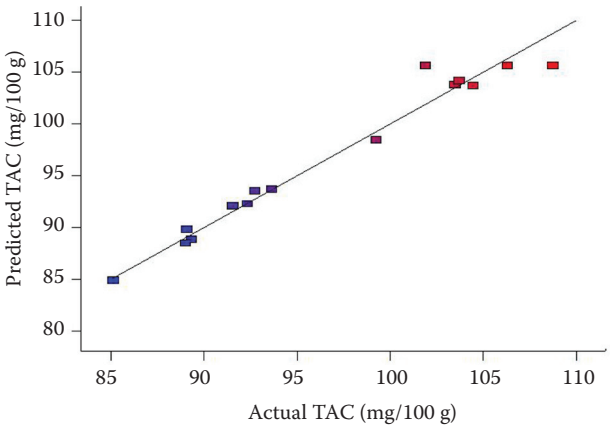


Figure 2. Comparison between predicted and actual values of total anthocyanin content (TAC) extracted by HHPE

doi: 10.17221/189/2016-CJFS

Table 5. The ANOVA for the regression equation

| Source | DF | SS | MS | *F*-value | *P*-value | |
|---|---|---|---|---|---|---|
| Model | 9 | 775.67 | 8619 | 15.75 | 0.0037 | ** |
| Liquid-solid ratio $X_1$ | 1 | 205.84 | 205.84 | 37.62 | 0.0017 | ** |
| Ethanol concentration $X_2$ | 1 | 152.86 | 152.86 | 27.94 | 0.0032 | ** |
| Extraction pressure $X_4$ | 1 | 53.61 | 53.61 | 9.80 | 0.026 | * |
| $X_1X_2$ | 1 | 1.76 | 1.76 | 0.32 | 0.5956 | |
| $X_1X_4$ | 1 | 0.28 | 0.28 | 0.05 | 0.8313 | |
| $X_2X_4$ | 1 | 42.12 | 42.12 | 7.70 | 0.0392 | * |
| $X_1X_1$ | 1 | 79.52 | 79.52 | 14.53 | 0.0125 | * |
| $X_2X_2$ | 1 | 200.46 | 200.46 | 36.64 | 0.0018 | ** |
| $X_4X_4$ | 1 | 83.95 | 83.95 | 15.34 | 0.0112 | * |
| Lack of fit | 3 | 3.32 | 1.11 | 0.092 | 0.9576 | ns |
| Residual | 5 | 5.47 | | | | |
| Pure error | 2 | 12.02 | | | | |
| Total | 14 | 803.03 | | | | |
| $R^2$ = 0.9659 | Adj-$R^2$ = 0.9046 | | | | | |
| Pred $R^2$ = 0.8664 | *CV*% = 2.42 | | | | | |

DF – degrees of freedom; SS – sum of squares; MS – mean square; $R^2$ – coefficient of determination; *CV* – coefficient of variation; * and ** indicate 5% and 1% significant levels, respectively; ns – non-significant

Therefore, to investigate the effect of independent variables, their interactions, and their quadratic effect on anthocyanin extraction, the data obtained from Table 4 was fitted to the quadratic model by the regression analysis and analysis of variance (ANOVA).

The regression model was highly significant ($P < 0.01$) and the lack of fit was significant ($P > 0.05$) (Table 5). Moreover, $R^2$ was higher than 0.965 and *CV*% was lower than 2.41%. Furthermore, the diagnostic plots such as the predicted versus experimental values (Figure 2) showed no significant difference between predicted and experimental values. Hence, these results indicated that the polynomial regression model was an accurate and reliable result. Therefore the linear effects of $X_1$, $X_2$, and $X_4$, the interactive effects term in $X_2X_4$, and quadratic effects of $X_1X_1$, $X_2X_2$, and $X_4X_4$ were demonstrated to significantly affect the TAC extraction (Table 5). Hence, the importance of independent variables on the TAC rank is in the following order: $X_1 > X_2 > X_4$.

According to Cao *et al.* (2012), circular contour plots suggest that the interaction between the interactive variables could be negligible while elliptical contour plots indicate the significant influence of the interactive effect between the corresponding variables. Therefore the interactive effect of ethanol concentration and extraction pressure resulted in an increment of the extraction of TAC up to a threshold level of 55% and 400 MPa (Figure 3C). This finding is consistent with that of Corrales *et al.* (2009), who

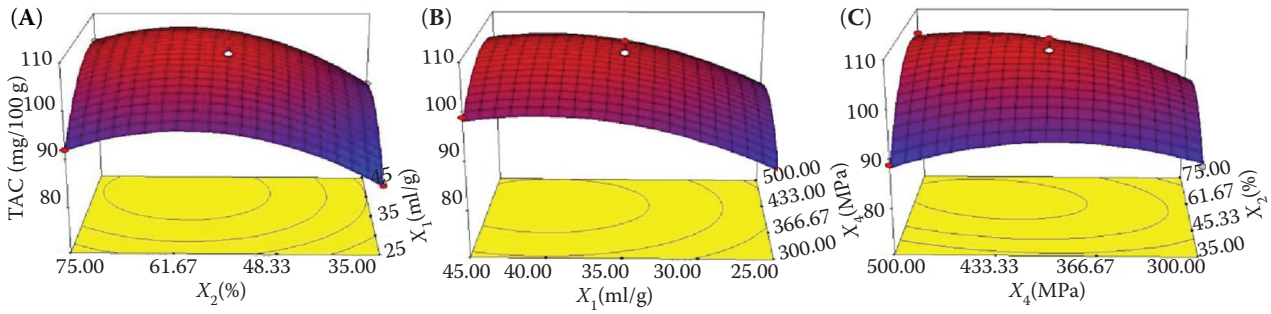


Figure 3. Response surface plots and contour plots for the effect of three variables on total anthocyanin content (TAC): (**A**) ethanol concentration ($X_2$); (**B**) liquid-solid ratio ($X_1$); (**C**) extraction pressure ($X_4$)

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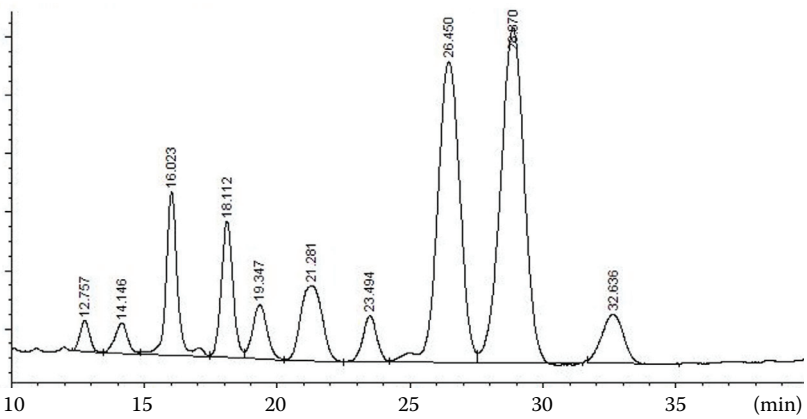


Figure 4. Anthocyanin chromatogram of blueberry pomace

reported an increase of the yield of anthocyanins from grape skins with an increase in pressure and ethanol concentration. This positive effect may be due to the fact that HHPE could change the solvent properties by decreasing the dielectric constant, while increasing the $H^+$ ionisation (San Martin *et al.* 2002). Besides, a high pressure has also been reported to increase the polarity of some anthocyanin compounds (Garcia *et al.* 2001).

***Optimisation***. The optimal HHPE conditions were selected to obtain the maximum extraction of anthocyanins from blueberry pomace. Based on this criterion, the liquid-solid ratio of 40.89 ml/g, ethanol concentration of 63.34%, and extraction pressure of 442.96 MPa, the anthocyanin content of extraction was predicted to be 108.628 mg/100 g. In order to verify the accuracy of the model to predict the optimal extraction content of anthocyanins, three experiments were carried out at optimised conditions with a slight modification as follows: liquid-solid ratio of 41 ml/g, ethanol concentration of 63%, and extraction pressure of 443 MPa. The experimental value of anthocyanin extraction was found to be 107.9 mg/100 g, which was higher than the control extraction (67.63 mg/100 g). The predicted results matched well with experimental results obtained at optimal HHPE conditions, which confirmed that the BBD model is with good correlation ($R^2 > 0.95$). As a result, the quadratic model Eq. (3) obtained from the BBD was considered to be precise and reliable for predicting the extraction of anthocyanins from blueberry pomace during the HHPE.

$$Y_{TCA} = 105.67 + 5.07X_1 + 4.37X_2 + 2.59X_4 + 0.66X_1X_2 + \\ + 0.26X_1X_4 + 3.24X_2X_4 - 4.64X_1^2 - 4.77X_4^2 \quad (3)$$

***HPLC-ESI-MS analysis***. The individual anthocyanins were tentatively identified according to retention times, λ max of UV/vis, mass spectral data ($M^+$, $MS^2$, $MS^3$), and those obtained from literature (Figure 4). The results are shown in Table 6.

Table 6. Anthocyanin profile of blueberry pomace

| Peak number | $t_R$ (min) | $\Lambda_{max}$ (nm) | $[M^+]$ ($m/z$) | $MS^2$ ($m/z$) | Anthocyanins[a] | Relative amount (%) |
|---|---|---|---|---|---|---|
| 1 | 12.757 | 277/523 | 465 | 303 | delphinidin-3-galactoside | 1.31 |
| 2 | 14.146 | 277/524 | 465 | 303 | delphinidin-3-glucoside | 1.67 |
| 3 | 16.023 | 279/515 | 449 | 287 | cyanidin-3-galactoside | 8.06 |
| 4 | 18.112 | 279/515 | 449 | 287 | cyanidin-3-glucoside | 6.95 |
| 5 | 19.347 | 277/525 | 479 | 317 | petunidin-3-galactoside | 3.42 |
| 6 | 21.281 | 277/523 | 479 | 317 | petunidin-3-glucoside | 7.04 |
| 7 | 23.494 | 279/517 | 463 | 301 | peonidin-3-galactoside | 2.89 |
| 8 | 26.450 | 275/520 | 493 | 331 | malvidin-3-galactoside | 29.48 |
| 9 | 28.870 | 274/525 | 493 | 331 | malvidin-3-glucoside | 34.53 |
| 10 | 32.636 | 277/526 | 463 | 331 | malvidin-3-arabinoside | 4.65 |

[a]identified from the literature: cyanidin 287, peonidin 301, delphinidin 303, petunidin 317, and malvidin 331 (Giusti & Wrolstad 2001); the order of the elution of glycosides on the $C_{18}$ column is galactoside before glucoside, which is before arabinoside (Prior *et al.* 2001)

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Among the 10 anthocyanins identified in the extract, malvidin-3-galactoside, and malvidin-3-glucoside were found to be the major individual anthocyanins (relative amount 64%). While delphinidin-3-galactoside, delphinidin-3-glucoside, cyanidin-3-galactoside, cyanidin-3-glucoside, petunidin-3-galactoside, petunidin-3-glucoside, peonidin 3-galactoside, and malvidin-3-arabinoside were considered as the minor individual anthocyanins (relative amount 36%). This result is in line with that of Li *et al*. (2013), who found 9 anthocyanins in rabbiteye blueberry (Brightwell) from Nanjing with malvidin-3-galactoside and malvidin-3-glucoside as the major individual anthocyanins. While Wang *et al*. (2012) reported 11 anthocyanins in rabbiteye blueberry (Garden Blue) from the USA, malvidin-3-galactoside was the major anthocyanin. However, conversely to these studies, a new anthocyanin such as cyanidin-3-glucoside was identified in the extract obtained at the optimised HHPE.

## CONCLUSION

High hydrostatic pressure assisted extraction (HHPE) was demonstrated to be an effective method for anthocyanin extraction from blueberry pomace. From the six processing variables investigated, only liquid-solid ratio, ethanol concentration, and extraction pressure were revealed to have a significant effect on anthocyanins during the HHPE. Therefore, at optimal HHPE conditions: liquid-solid ratio of 41 ml/g, ethanol concentration of 63%, and extraction pressure of 443 MPa, 107.9 mg/100 g anthocyanins were obtained, then individual anthocyanins were identified in the extract. 10 anthocyanins were identified by HPLC-ESI-MS, malvidin-3-galactoside and malvidin-3-glucoside were the major anthocyanins.

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