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High-Performance liquid chromatography-tandem mass spectrometry with polar C18 for rapid quantification of anthocyanin and flavonoid in black soybean extracts

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ABSTRACT

Black soybean (*Glycine max* L.) is one of the most popular crops with various application in the world. Its seed coat contains various natural pigments compounds such as anthocyanin and flavonoid. In this study, we optimize the extraction conditions of eight polyphenols from whole of black soybean and seed coat, evaluate their extraction effect, and separation resolution at different pH-range. The multiple reaction monitoring (MRM) method was analyzed by using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) with simultaneous positive and negative electrospray ionization in a single analytical run. Eight polyphenols were separated within 9 minutes of run time with LC-MS/MS operated mode. The calibration graphs were linear over the concentration ranges of 0.01 to 1 mg/L for all polyphenol. Regression coefficients were in the range > 0.99. The limit of detection value, calculated from the blank tests based on 3σ, was 1 μg/L for all polyphenol. The RSD values corresponding to the intra-day and inter-day repeatability were lower than 11% and accuracies between 85-115%. It is shown that the modified solvent system that applied in LC-MS/MS detection could be developed as a useful tool for the study of water-soluble phenolic compounds.

Keywords: Black soybean; LC-MS/MS; MRM; Ultrasound-assisted; Anthocyanin; Flavonoid.

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1. INTRODUCTION

Black soybean (*Glycine max* L.) has been used as a common agricultural product in the world because of its seed coat contains various natural pigments compounds such as anthocyanin and flavonol (Ganesan and Xu, 2017; Kim et al., 2011). In Taiwan, black soybean is widely cultivated and utilized as grains, tofu, and soy milk for one of the dietary sources for hundreds of years (Jeng et al., 2013). In previous studies, black soybean is an excellent dietary source for disease prevention and health promotion (Ganesan and Xu, 2017), including cyanidin-3-glucoside (Matsukawa et al., 2015) and petunidin-3-glucoside (Rossetto et al., 2002) for anti-cancer activity (Yun et al., 2010; Shin et al., 2009) and anti-oxidative (Nakajima et al., 2004), delphinidin-3-pyranoside for against cardiovascular diseases (Yang et al., 2012), peonidin-3-glucoside for inhibit tumor cell growth (Chen et al., 2005), myricetin for anti-obesity (Akindehin et al., 2018), quercetin for anti-toxic activities (Nathiya et al., 2014) and rutin for anti-Alzheimer activity (Bahadori et al., 2017). In plants, the accumulation of phytochemicals can protect from UV radiation, disease, oxidative stress, and severe climatic conditions (Tanaka et al., 2008; Ezekiel et al., 2013). The soybean contains various colors of seed

coat including black, yellow, green, and brown (Baek et al., 2020). It is due to the presence of anthocyanin and other pigments, such as anthocyanin, myricetin, quercetin and rutin are water-soluble natural pigments that belong to basic structure of flavonoids, and a widely distributed in black soybean and the structure of polyphenol-rich black soybean (Tripoli et al., 2007; Kumari et al., 2015) .

In the last two decades, liquid chromatography coupled with a tandem mass spectrometry (LC-MS/MS) has been the most widely used analytical system for the qualification and quantification of black soybean (Rue et al., 2018). Although various methods using LC-MS/MS have been developed for the simultaneous quantitation of polyphenol in black soybean (Koh et al., 2014; Lang et al., 2019), the analysis time of the HPLC method required for these methods are still longer than in UPLC system (Wang et al., 2019). However, the chemical analysis regarding anthocyanin or flavonoid present in black soybean was generally performed using HPLC-PDA-MS system (Green et al., 2007). In this study, our separation method was achieved using high-performance liquid chromatography (HPLC) with a Kinetex polar C18 core shell columns operated in the gradient mode. Furthermore, it coupled with triple quadrupole tandem mass spectrometry (QqQ) by the multiple reaction monitoring (MRM) modes also simultaneously detect analytes in positive and negative electrospray ionization in a single analytical run. Anthocyanins are very unstable and would be degraded under pH value above 7 (Petersson et al., 2015). Its color of the anthocyanins is affected by both pH and the storage temperature. However, at pH 2.5 and stored under temperatures of 4°C, the half-life of anthocyanins can be extended to 277 days (Mojica et al., 2017). In this research, it is found that with more acidic conditions as both in black soybean extraction solvent system and the mobile phase in LC-MS/MS technique could enhance the efficiency of the qualification and quantification of the 8 bioactive water soluble constituents in black soybean. The stability and resolution of the separation and identification in the experiment are also improved. Therefore, a simple and rapid methods were developed in the analysis of the polyphenol components in black soybean and seed coat extract.

2. EXPERIMENTAL SECTION

2.1 Plant Materials

All black soybean (*Glycine max* L.) seeds were provided by Wu, C. H. from Tainan District Agricultural Research and Extension Station (Muchang Hsinhua District, Tainan, Taiwan, ROC). The intact black soybeans were washed and air-dried, after which they were stored at 4 °C before analysis.

2.2 Materials and Reagents

Cyanidin-3-glucoside, delphinidin-3-galactosid, delphinidin-3-glucoside, peonidin-3-glucoside, petunidin-3-glucoside, myricetin, quercetin, rutin were purchased from Sigma-Aldrich Company (St. Louis, MO, USA), acetonitrile (ACN) and formic acid were the HPLC-grade reagents and were purchased from Merck Company (Darmstadt, Germany). Distilled water was purified using a Sartorius arium pro (Göttingen, Germany).

2.3 Sample Extraction and Preparation

First, weighed 2 g of the whole black soybean extract (WBSE) and 0.5 g of black soybean seed coat extract (BSSCE) and transferred into polypropylene tubes, respectively. Then, add 20 mL extraction buffer (5% ACN with 0.1 to 2.5% formic acid), and the sample extractions were accelerated with the ultrasonicator (Branson Ultrasonic Corp., Danbury, CT, USA) for 5 minutes (Lin et al., 2019). After sonication, 1 mL of supernatant of each sample was transferred to an Eppendorf centrifuge tube. Subsequently, the extracts were centrifuged at 3,000 rpm for 5 minutes, and the supernatants were filtered through a 0.22 μm membrane (PTFE Millex-GV, 13 mm, Millipore). After that, all filtered samples were stored at -20°C prior to injection for the LC-MS/MS analysis.

2.4 Instruments and HPLC–MS/MS Conditions

HPLC-MS/MS analysis was carried out using an Agilent 1260 infinity binary LC system coupled with an Agilent 6470 Triple Quadrupole MS system and equipped by an ESI ionization source with Agilent Jet Stream (Waters, Milford, MA, USA). Chromatographic separation was performed on a UHPLC Kinetex polar C18 column (2.1 x 100 mm, 2.6 μm, Phenomenex). All compounds were separated by a gradient elution program with 2.5% formic acid in DI water used as the mobile phase A, and acetonitrile used as the mobile phase B. The gradient elution program was performed as follows: 0.0–7.0 minutes, 5 to 15% B; 7.1–10.5 minutes, 15 to 90% B; 10.6–11 minutes, 90 to 5% B; 11.1–15.0 minutes, 5% B, and the total run time was 15 minutes. The flow rate was set at 0.3 mL/minutes, the column chamber and sample chamber were kept at constant temperatures of 40°C and 10°C, respectively. The injection volume of the sample was 1.0 μL. In terms of the MS parameters, ESI was operated simultaneously in the positive (4.0 kV) and negative (3.5 kV) electrospray ionization modes with the detection of multiple reaction monitoring (MRM). The drying gas temperature and the sheath gas temperature were 350°C, also the flow rate was 10 and 15 L/minutes, respectively. Experimental data acquisition and processed were executed by MassHunter software (Agilent Corp., Milford, MA, USA). The MRM parameters in analyzing the eight compounds, including the quantitative and qualitative ions, collision energy, dwell time, and retention time are shown in Table 1.

2.5 Preparation of Stock Standard Solutions and Quality Control (QC) Samples

All polyphenol standards were dissolved in water/ACN

Table 1. MRM parameters and transitions of eight bioactive polyphenol

Compound Group	Compound Name	Formula	Precursor Ion	Product Ion	Fragmentor	Collision Energy	Cell Accelerator Voltage	Polarity
Anthocyanin	Cyanidin-3-glucoside	$C_{12}H_{21}O_{11}^+$	449	287	130	24	5	Positive
				213*	130	60		
Anthocyanin	Delphinidin-3-galactoside	$C_{21}H_{21}O_{12}^+$	465	303	120	20	5	Positive
				229*	120	60		
Anthocyanin	Delphinidin-3-glucoside	$C_{21}H_{21}O_{12}^+$	465	303	120	24	5	Positive
				229*	120	60		
Anthocyanin	Peonidin-3-glucoside	$C_{22}H_{23}O_{11}^+$	463	301	120	24	5	Positive
				286*	120	60		
Anthocyanin	Petunidin-3-glucoside	$C_{22}H_{23}O_{12}^+$	479	317	120	24	5	Positive
				302*	120	60		
Flavonoid	Myricetin	$C_{15}H_{10}O_8$	317	151	135	25	5	Negative
				137*	135	32		
Flavonoid	Quercetin	$C_{15}H_{10}O_7$	301	151	135	24	5	Negative
				179*	135	32		
Flavonoid	Rutin	$C_{27}H_{30}O_{16}$	609	300	160	36	5	Negative
				271*	160	36		

*Qualitative ion of analyte.

(95:5 *v/v*) as primary stock standard solutions of 1 mg/mL. Serial dilution of each stock standard solution with water/ACN (95:5 *v/v*) was carried out to allow them to serve as working stock standards solutions and quality control (QC) samples at the required concentrations. QC samples were prepared for low (10 ng/mL), medium (400 ng/mL), and high (1,000 ng/mL) concentrations to evaluate the recovery efficiency and matrix effect. The stock solutions and QC samples were stored at −20°C in amber glass vials prior to use.

2.6 Method Validation

To verify the method, calibration curves were constructed from the concentration ranges of 10 to 1,000 ng/mL for the eight anthocyanin and flavonoid compounds and analyzed in six consecutive runs. The calibration curves established by the peak area ratio against the response concentration were studied by the weighted ($1/x^2$) least-squares linear regression method.

The sensitivity of the method was determined by both the limit of detection (LOD) and limit of quantification (LOQ). The samples were prepared in the same manner as the calibration standards. The signal-to-noise ratio (S/N) for the lower limit of quantification (LLOQ) should be higher than 10 and the relative error (RE) should be within ± 20% for accuracy. While RE of other QC points on the standard calibration curve should be within ± 15% for accuracy. To evaluate the precision and accuracy of the linearity over intra-day and inter-day periods, six repeats of QC samples (10 ng/mL, 400 ng/mL and 1,000 ng/mL) were evaluated on the same day, and repeated analysis at each concentration of QC was conducted once per day for six consecutive validation days.

Spiked at medium QC levels were applied in the investigation of the matrix effect and extraction efficiency. The matrix effect was evaluated by comparing the peak area response of the post-extracted spiked sample with the corresponding standard containing equivalent concentrations of the analytes in blank solvents. The matrix effect test was calculated as follows: matrix effect (%) = [(peak area of post-extracted spiked sample) – (peak area of endogenous sample)] / [peak area of spiked standard solution] x 100. The extraction efficiency was calculated by comparing the peak areas of the analytes extracted using the filter membrane procedure with those acquired from analytes spiked directly into post 0.22 μm PTFE membrane eluent. That undergo analyte spiking before and after the extraction in four replicates. The equation was calculated as follows: extraction efficiency (%) = [medium QC spiked pre-extraction-blank / medium QC spiked post-extraction] x 100. The RSD (%) of the mean recovery values must be within 15% at medium QC level.

2.7 Statistical Analysis

Statistical analysis was processed by Microsoft Office Excel 2016(Microsoft Crop., USA). The values of mean ± SD, RSD %, recovery % and relative error % were determined from QCs precision, accuracy, extraction recovery, and matrix effect were applied to evaluate the reliability and reproducibility of the approach developed.

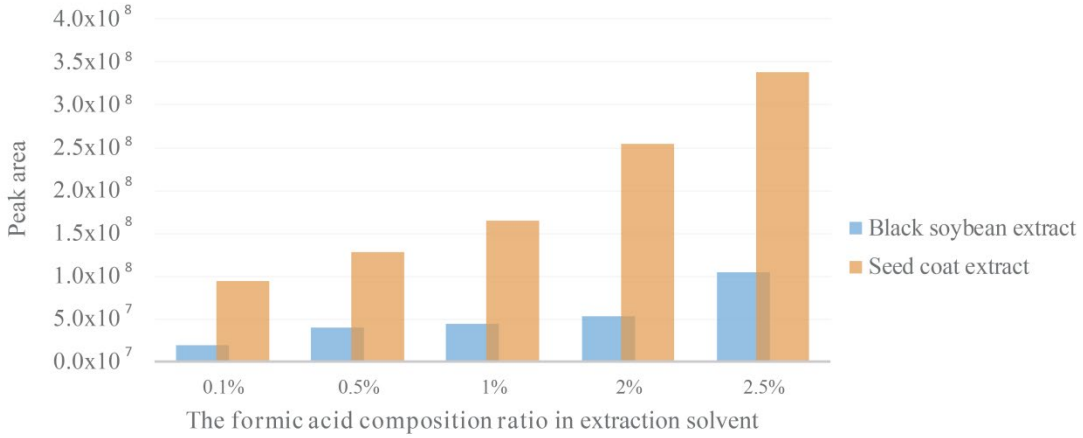

Fig. 1. Total polyphenol contents from different organs of black soybean and seed coat with different proportions of formic acid

3. RESULTS AND DISCUSSION

3.1 Sample Extraction on pH Dependent Based

The color changes of anthocyanin with different pH values, regarding the effect of pH on the stability of anthocyanin-based crude extracts from whole of black soybean and the seed coat, we selected the sample of Tainan 11 sample and investigated using five different extraction buffer solutions of formic acid percent (0.1, 0.5, 1.0, 2.0 and 2.5%, respectively). Thus, the extraction efficiency of the extraction solvent with different formic acid percent was compared in this study. The sum of peak areas in an HPLC-MS/MS chromatogram of the anthocyanin and flavonoid from the whole of black soybean and seed coat were calculated Fig. 1. The highest sum of peak area from extraction buffer by 5% acetonitrile with 2.5% formic acid. On the other hand, the lowest sum of peak area by 5% acetonitrile with 0.1% formic acid. These results indicated that a lower range of pH values can extract the highest amount of anthocyanin from whole of black soybean and seed coat sample. Therefore, 5% acetonitrile with 2.5% formic acid was chosen as the extraction buffer. As previously reported, under strongly acidic conditions, anthocyanin exist as flavylium cation which showed lower degradation, with the increasing of pH, the structural form becomes colorless by the rapid proton loss. These results agree with those of most studies regarding the stability of anthocyanin extracted from different plants (Xie et al., 2018; Tang et al., 2019).

3.2 Optimization of the Chromatographic Separation Conditions and MRM Method

A major challenge in the mass spectrometry analysis of anthocyanin components is the separation of the delphinidin-3-galactoside and delphinidin-3-glucoside homologs differ in structure merely by the position of the pyranoside group on the basic structure. Despite the fact that mass spectrometry detection offers structural characterization and identification of anthocyanin, but is difficult to separate the components with same molecule weight ion by MRM mode. Therefore, liquid chromatography as the premier separation and purification method for delphinidin-3-pyranoside could improve this problem. The HPLC system was chosen for method development and optimization to achieve higher efficiency of anthocyanin separation. Polar C18 stationary phase could be suitable for the separation of desired anthocyanin and flavonoid in this study. This stationary phase combines C18 ligands with a polar-modified surface to enable superior retention of polar and nonpolar compounds while ensuring 100 percent aqueous stability. Because its polar-modified surface can provide flexibility in the solvent, and gradient system selection needed to achieve the desired analyte separation (Desmarchelier et al., 2020). The particle surface of the polar C18 contains a positive charge that facilitates greater acidic compound retention through ionic interaction. In the earlier published methods (Willemse et al., 2013; Chorfa et al., 2016), acidic mobile phases containing diluted formic acid (2–5%) were used. In acidic solutions (pH < 3), the anthocyanins are in their flavylium-cationic forms that show a red color and absorption maximum at around 520 nm. For comparison of the analytical method, we choose delphinidin-3-pyranoside to prove better peak resolution under acidic conditions, as a separation shown in Fig. 2. The primary condition for resonable anthocyanin and flavonoid sensitivity could be achieved in the MRM method, based on triple quadrupole technology. Hence, in this study, a high-throughput quantitative method were developed for the simultaneous determination of all 8 target components in whole of black soybean and in seed coat samples. Before the optimization of MRM parameters (i.e., transitions, collision energies, fragmentor voltage, and electrospray ionization mode), the ion mode was determinutesed first. If the analytes are ionized in the correction mode, it can increase the signal intensity, thereby improve the ESI efficiency and sensitivities. Anthocyanins were more sensitive in the positive mode than the negative mode; on

the other hand, flavonoids in negative mode better (data not shown). Therefore, all compounds were ionized in the positive/negative mode in the following experiment. Then, 1000 ng/mL of all polyphenol standards were optimized with Agilent MassHunter Optimizer Software to determinutese the MRM parameters for quantification, and two transitions were selected for each analyte (Table 1). More specifically, the LC gradient method was designed to include two segments, the first being for the anthocyanin group and the second being for the flavonoid group. The gradient conditions of the LC and its flow rate were optimized during the experiment to achieve optimal chromatographic separation. The RTs of these compounds were determinutesed as follows: Delphinidin-3-galactoside at 5.1 minutes, Delphinidin-3-glucoside at 5.5 minutes, Cyanidin-3-glucoside at 6.4 minutes, Petunidin-3-glucoside at 7.1 minutes, Peonidin-3-glucoside at 7.9 minutes, Myricetin at 9.1 minutes, Quercetin 9.1 minutes, and Rutin at 9.2 minutes. All of the analytes were separated within the first 9.2 minutes in the analytical run. The MRM chromatograms are shown in Fig. 3(A).

3.3 Calibration Curves, Linearity, LOD and LOQ

The calibration curves of all polyphenols were obtained over the concentration ranges of 10 to 1000 ng/mL for six consecutive runs. For each curve, the peak area of the analyte was calculated, and plotted against the nominal analyte concentration. Excellent linearity for all polyphenols of analytes was achieved with a good correlation and regression coefficients were in the range > 0.99 (Table 2). The LOD and LOQ at the corresponding peak-area for each QC sample were calculated at ratios of signal to noise of 3 and 10, respectively. The LODs and LOQs for all polyphenols were 1 ng/mL and 4 ng/mL, respectively (Table 2). These results showed that the method developed in this study had good sensitivity and a good linear range for quantification.

3.4 Precision and Accuracy

The intra-day and inter-day precision and accuracy were determined by measuring the RSD% and the RE% at three different concentrations levels (QC samples: 10 ng/mL, 400 ng/mL and 1,000 ng/mL) were found to acceptable limits, the intra-day and inter-day RSD% values ranged from 0.4% to 10.4 %; with respect to accuracy, the RE% values ranged from − 5 to 13% (Table 2). These results proved that the method had excellent precision, accuracy, and stability, as whether the measurements were taken on an intra- or inter-day basis method still exhibited good performance.

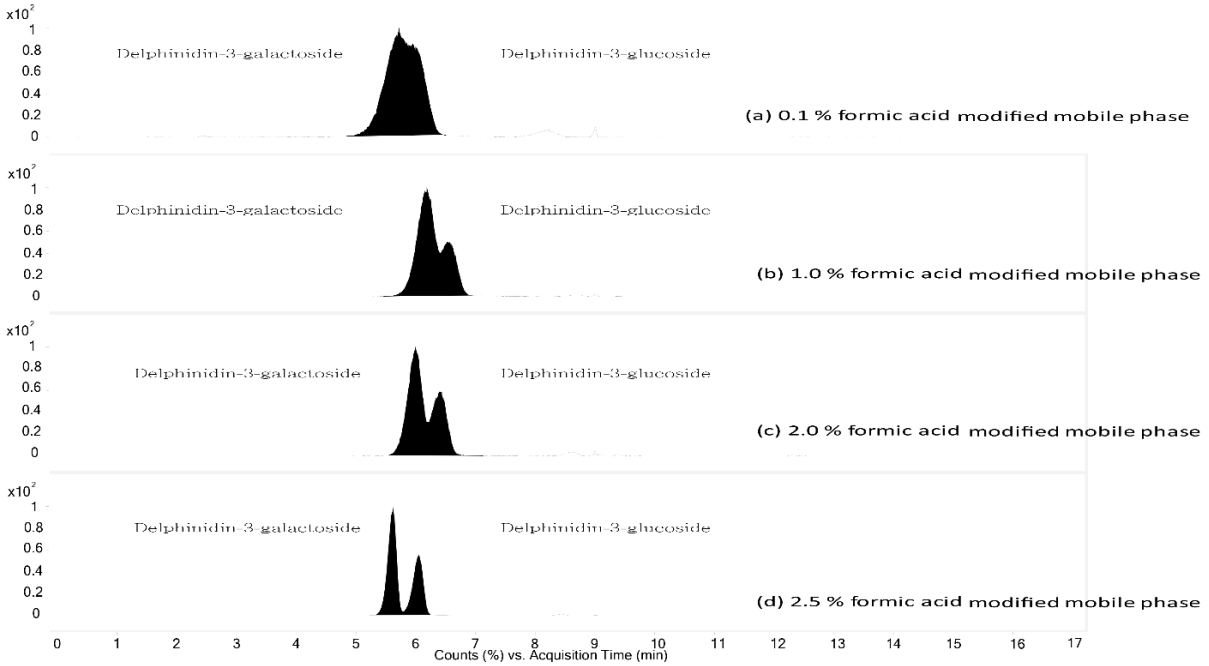

Fig. 2. Compare different resolutions in different formic acid conditions

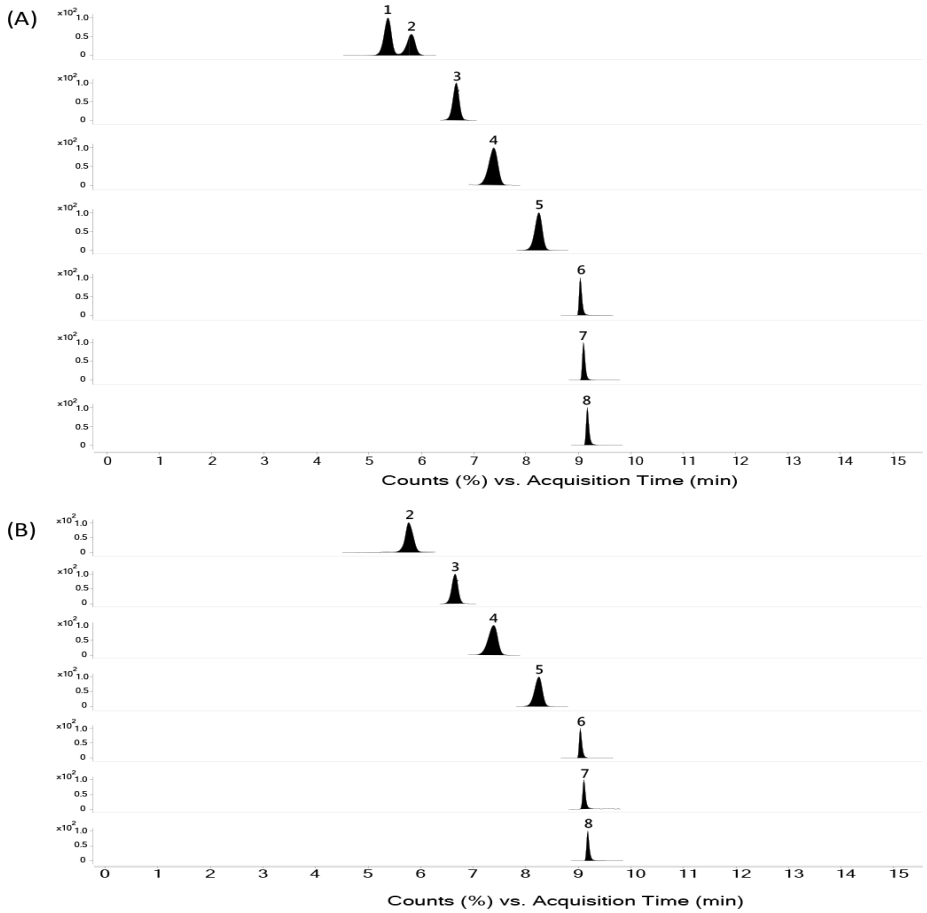

Fig. 3. LC-MS/MS chromatogram in MRM mode of (A) 8 standard compounds and (B) black soybean seed coat extract. (1) Delphinidin-3-galactoside, (2) Delphinidin-3-glucoside, (3) Cyanidin-3- glucoside, (4) Petunidin-3-glucoside, (5) Peonidin-3-glucoside, (6) Myricetin, (7) Quercetin, (8) Rutin

3.5 Extraction Recovery and Matrix Effect

The extraction recovery of delphinidin-3-galactoside and myricetin with medium QC (400 ng/mL) extracted from black soybean and seed coat ranged from 78% to 105%, and all the RSD values were below 15% (Table 3). The matrix effects of the ESI method in the positive and negative modes were also evaluated. Table 3 shows that the matrix effect of delphinidin-3-galactoside in the medium QC samples of the black soybean and seed coat were investigated in the positive mode, while that of myricetin was investigated in the negative mode. In evaluating the matrix effects in different samples of black soybean and seed coat, it was found that the matrix effects for delphinidin-3-galactoside in the positive ion mode at the medium QC (400 ng/mL) were between -21 and 16%, while those for myricetin in the negative ion mode at the same concentrations were between -2% and 44 %.

3.6 Real Samples Analysis

The method described above was applied to determine the anthocyanin and flavonoid in samples of 5 black soybean cultivars. Fig. 3(B) shows, as an example, the MRM chromatograms of the polyphenol, obtained by HPLC–MS/MS, for the analysis of black soybean seed coat extract. The sums of the anthocyanin and flavonoid in the different organs are shown in Table 4. Delphinidin-3-galactose was not quantified from all black soybean cultivars, while the myricetin and quercetin were not quantified in the WBSC samples. Black soybean seed coat extract has higher abundant levels of anthocyanin and flavonoid than black soybean extract. The main reason is that these natural color pigments are concentrated on the seed coat with antioxidant function that can protect the growth of seed from external damage (UV radiation, disease, oxidative stress). The strong acidic conditions can increase the extraction efficiency of anthocyanin and separation of delphinidin-3-pyranoside from black soybean and the seed coat. In addition, the advantages of this method is the simple process in sample preparation and is suitable for the routine analysis of the target bioactive compounds. However, according to the literature, the pH stability range of analytical column polar C18 are 1.5 to 8.5. The hydrolysis of the Si-O-Si bond occurs faster at low pH, that the loss of the bonded phase occurs slowly by running time.

Table 2. Linearity, correlation coefficient (r^2), LOD, LOQ of eight bioactive polyphenol and intra-, inter-day precision and accuracy tests of 3 concentrations of QCs

Analyte	Linear range (ng/mL)	Correlation coefficient (r^2)	LOD (ng/mL)	LOQ (ng/mL)	Conc. (ng/mL) of analyte in QCs	Intra-day (n = 6)		Inter-day (n = 6)	
						RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
Cyanidin-3-glucoside	10~1000	0.9984	1.0	4.0	10	4.3	108.3	7.3	101.7
					400	1.8	105.6	2.1	101.4
					1000	0.6	101.6	4.7	101.1
Delphinidin-3-galactoside	10~1000	0.9930	1.0	4.0	10	6.5	106.7	9.7	99.8
					400	5.4	102.1	1.5	101.2
					1000	2.4	100.5	0.9	99.9
Delphinidin-3-glucoside	10~1000	0.9910	1.0	4.0	10	6.5	100.9	8.3	98.1
					400	10.2	102.7	2.9	99.8
					1000	5.3	99.5	1.9	99.7
Peonidin-3-glucoside	10~1000	0.9934	1.0	4.0	10	10.4	101.3	0.4	102.5
					400	8.0	95.2	1.9	101.5
					1000	3.7	102.2	2.3	98.4
Petunidin-3-glucoside	10~1000	0.9993	1.0	4.0	10	3.7	108.2	9.1	98.0
					400	1.0	113.2	4.1	100.2
					1000	0.7	100.3	4.4	102.1
Myricetin	10~1000	0.9996	1.0	4.0	10	1.9	103.2	6.0	102.4
					400	7.9	114.0	2.0	99.5
					1000	8.5	97.5	1.8	99.5
Quercetin	10~1000	0.9912	1.0	4.0	10	6.4	102.2	10.1	99.2
					400	3.0	100.6	3.2	99.7
					1000	1.6	99.4	4.1	100.0
Rutin	10~1000	0.9961	1.0	4.0	10	2.6	111.2	3.3	97.8
					400	1.1	102.4	3.9	101.6
					1000	0.6	102.2	4.6	99.1

Table 3. Matrix effect and extraction efficiency of 2 bioactive compounds (n = 4)

Compunds	Ionization mode	Matrix effect(mean ± SD)	Conc. of analyte in QCs	Efficiency (%)	RSD (%)
Delphinidin-3-galactoside[a]	Positive	16 (±1.4)	400 ng/mL	93	7
Myricetin[a]	Negative	44 (±2.2)	400 ng/mL	105	4
Delphinidin-3-galactoside[b]	Positive	-21 (±3.9)	400 ng/mL	78	14
Myricetin[b]	Negative	-2 (±0.8)	400 ng/mL	95	4

[a]post-extracted spiked black soybean sample; [b]post-extracted spiked seed coat sample

Table 4. Comparison of anthocyanin and flavonoid contents in different extraction organs of five black soybean cultivars

No. of cultivar	Kaohsiung 7 (mg/Kg)		Tainan 11 (mg/Kg)		Hengchun (mg/Kg)		Tainan 5 (mg/Kg)		Tainan 3 (mg/Kg)	
	WBSE	BSSCE	WBSE	BSSCE	WBSE	BSSCE	WBSE	BSSCE	WBSE	BSSCE
Compunds										
Cyanidin-3-glucoside	2187.7	3876.9	867.8	4472.7	5891.8	12788.3	1191.2	2479.7	1080.9	10244.2
Delphinidin-3-galactoside	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Delphinidin-3-glucoside	48.4	110.9	47.6	361.0	90.7	110.1	30.3	65.5	38.3	578.0
Peonidin-3-glucoside	4.8	6.4	0.6	4.9	23.4	75.4	2.2	4.9	1.1	13.8
Petunidin-3-glucoside	1.1	1.9	0.5	4.9	2.6	5.2	0.6	1.4	0.8	12.3
Quercetin	< LOD	0.2	< LOD	0.1	< LOD	0.2	< LOD	0.8	< LOD	1.1
Rutin	0.4	1.6	0.2	3.0	0.3	1.6	0.1	1.4	0.2	4.3
Myricetin	< LOD	0.2	< LOD	0.1	< LOD	0.4	< LOD	0.1	< LOD	0.3

WBSE: whole of black soybean extract

BSSCE: black soybean seed coat extract

Meanwhile, after using high acidic mobile phases in the HPLC instrument should wash the system by DI water to prevent piston seals and stainless steel damage.

4. CONCLUSION

In this study, a rapid sample extraction and simple analytical method was developed and validated for the simultaneous determinutesation of eight target compounds in samples of 5 different cultivars of black soybean. The results of this study demonstrate that convenient sample throughput can be realized by employing HPLC technologies and appropriate reversed-phase Polar C18 column separation coupled triple quadrupole tandem mass spectrometry, together with simplified sample preparation under strongly acidic conditions. The uses of high-pressure chromatography look forward to reducing the cost of ultra-performance chromatography provided effectiveness in terms of separation and the low pressure of total runtime. The strong acidic conditions for both the solvent system extraction and the mobile phase for HPLC-MS/MS assay might facilitate the separation and identification of the polyphenols in black soybean. It could be applied as a routine condition in the study of crops with abundant polyphenols on the purpose of nutritional research and agricultural breeding development as well.

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