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Isolation and Development of Quantification Method for Cyanidin-3-Glucoside and Cyanidin-3-Rutinoside in Mulberry Fruit by High-Performance Countercurrent Chromatography and High-Performance Liquid Chromatography

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Abstract – Cyanidin-3-glucoside (C3G) and cyanidin-3-rutinoside (C3R) were isolated by high-performance countercurrent chromatography (HPCCC) using a two-phase solvent system composed of *tert*-butyl methyl ether/*n*-butanol/acetonitrile/water/trifluoroacetic acid (1 : 3 : 1 : 5 : 0.01, v/v) to give pure C3G (34.1 mg) and C3R (14.3 mg) from 1.5 g crude mulberry fruit extract. Using the pure C3G and C3R, a reliable high-performance liquid chromatography (HPLC) method was developed and validated to determine the C3G and C3R contents in mulberry fruit. C3G and C3R were separated simultaneously using an Eclipse XDB-C18 column (4.6 × 250 mm I.D., 5 μm) coupled with a photodiode array detector (PDA). The gradient elution of the mobile phase consisting of acetonitrile (0.5% formic acid) and water (0.5% formic acid) was applied (1.0 mL/min), and the detection wavelength was 520 nm. The calibration curves of C3G and C3R showed good linearity (both with $r^2 = 0.9996$) in the concentration range 15.625 - 500 μg/mL, and the relative standard deviations (RSD%) of intra- and inter-day variability were in the ranges 2.1 - 8.2% and 4.1 - 17.1%, respectively. The accuracies were ranged 96.5 - 102.6% for C3G and C3R, respectively. The developed HPLC method was used to determine the contents of C3G and C3R in newly harvested mulberry from eight different provinces of Korea.
Keywords – Cyanidin-3-glucoside, Cyanidin-3-rutinoside, Mulberry fruit, High-performance countercurrent chromatography, HPLC-PDA method

Introduction

Mulberry (*Morus alba* L.) is a tree belonging to the Moraceae family, and its root bark and leaves have been used as traditional oriental medicines for antidiabetic, diuretic, anti-inflammatory, and antipyretic purposes.[1] Besides the root bark and leaves, the mulberry fruit has long been used as a seasonal food and regarded as a good material for healthcare products in food industries due to its high content of anthocyanins. Many studies have revealed that cyanidin-3-glucoside (C3G) and cyanidin-3-rutinoside (C3R) (Fig. 1) are the typical anthocyanins of mulberry fruit,[2-3] and possess diverse pharmacological effects such as neuroprotective,[4] antidiabetic[5] and antioxidant[6] activities. Because of these diverse biological activities, pure C3G and C3R are increasingly required for quantitative analysis and biological screenings such as

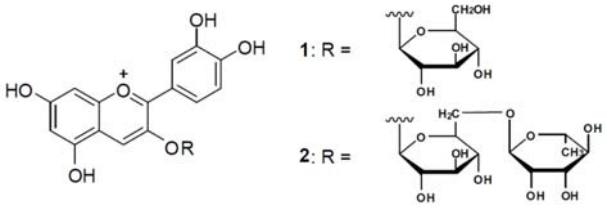

Fig. 1. Chemical structures of cyanidin 3-glucoside (C3G, **1**) and cyanidin 3-rutinoside (C3R, **2**).

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in vitro and *in vivo* experiments, but commercial standards of C3G and C3R are provided at a high price in small amounts. Therefore, easy and rapid methods are required to obtain pure C3G and C3R.

The countercurrent separation (CS) technique is a type of liquid-liquid chromatography that does not employ solid adsorbents, thus avoiding chemical degradation by and irreversible adsorption onto solid adsorbents such as silica gel. Because of the aforementioned advantages, many natural products have been separated by the CS technique through methods such as high-speed countercurrent chromatography (HSCCC), high-performance countercurrent chromatography (HPCCC), and centrifugal partition chromatography (CPC).[7] Up to now, many CS methods have been applied to separate anthocyanin derivatives from natural resources such as mulberry, black rice, and sweet purple potato.[8-10] In the present study, the quick and easy HPCCC method was applied to isolate C3G and C3R in high purities from mulberry fruit extract, and a reliable HPLC method was developed to determine the C3G and C3R contents in mulberry fruit by using the pure C3G and C3R isolated by HPCCC.

Experimental

General experimental procedures – HPLC analyses were carried out on a Waters Alliance HPLC (Waters, Milford, MA, USA) composed of a binary pump, an online degasser, and an autosampler. The semipreparative HPCCC (Spectrum, Dynamic Extractions, UK) possessed two sets of two bobbins. One bobbin was equipped with an analytical coil (13 mL, 0.8 mm I.D.), and the other with a preparative coil (70.5 ml, 3.2 mm I.D.). Organic solvents for HPCCC and HPLC were purchased from Daejung Chemical and Metals Co. Ltd. (Kyunggi-Do, Korea), and deionized water was produced with a Millipore Milli-Q water purification system (Millipore, USA).

Plant material – Fresh mulberry fruits from different localities were purchased from a Gyeungdong local market in Seoul, Korea. Each mulberry was lyophilized and ground into fine powder. The mulberry powder was kept in a deep freezer (–80 °C) until HPCCC separation and HPLC analysis.

Isolation of standards of C3G and C3R – Fresh mulberry fruit (100 g) was extracted with 60% methanol acidified with 0.1% TFA (2 L, 90 min, three times) and evaporated under vacuum to give 8 g of crude mulberry extract (CME). The CME (300 mg) was dissolved in a 1 : 1 mixture of the upper and lower phases of *tert*-butyl methyl ether/*n*-butanol/acetonitrile/water/trifluoroacetic acid (TBME/B/A/W/TFA, 1 : 3 : 1 : 5 : 0.01, v/v) and subjected to HPCCC (stationary and mobile phase: upper phase and lower phase of TBME/B/A/W/TFA (1 : 3 : 1 : 5 : 0.01, v/v), respectively; flow rate: 4 mL/min; rotational speed: 1600 rpm; detection wavelength: 520 nm).

Preparation of standard stock solutions of C3G and C3R – The standards of C3G (10 mg) and C3R (10 mg) were dissolved separately in 10 mL methanol (0.5% HCl) to give a concentration of 1 mg/mL. The stock solutions of C3G and C3R were pooled and diluted sequentially to six concentration levels from 15.625 to 500 μg/mL, to establish the calibration curves.

Sample preparation – Each mulberry powder (100 mg) was weighed accurately and transferred to a 20 mL glass bottle. Methanol (10 mL, acidified with 0.5% HCl) was added in a weight-to-volume ratio (w/v) of 1 : 100, and extracted by ultrasonic extraction for 2 h. After centrifugation, the supernatant was filtered using a syringe membrane filter (0.22 μm), and then injected to the HPLC.

HPLC condition – HPLC analysis was performed using an Eclipse XDB-C18 column (4.6 × 250 mm I.D., 5 μm, Agilent Technologies, CA, USA). The flow rate was 1.0 mL/min and the detection wavelength was 520 nm. The sample temperature was maintained at 5 °C. The organic mobile phase (A) was a methanol/formic acid (95 : 5, v/v) mixture, and the aqueous mobile phase (B) was a water/formic acid (95 : 5, v/v) mixture. The mobile phase gradient was 10% A (0 - 5 min) and 10 - 60% A (5 - 30 min). The mulberry samples and C3G and C3R standard mixtures were injected with the volume of 10.0 μL.

Linearity – The calibration curves for C3G and C3R were generated using six concentration levels under the HPLC method described above. The calibration curves were plotted according to linear regression analysis of the peak area (y) versus concentration (x), and the regression equation was expressed as $y = ax + b$. The slope (a), intercept (b), and regression coefficient (r^2) were evaluated as regression parameters. The limit of detection (LOD) and limit of quantification (LOQ) of C3G and C3R were estimated form the signal-to-noise (S/N) ratio. C3G and C3R were prepared separately in methanol (0.5% HCl) at 1 mg/mL, and diluted until the smallest detectable peaks were achieved. The LOD and LOQ were evaluated when the S/N ratios reached 3 and 10, respectively.

Precision – The precision was evaluated using intra- and inter-day variability. Four different concentration levels of working standard solution [15.625 (lowest), 50 (low), 200 (medium), and 400 μg/mL (high)] of C3G and C3R mixtures were prepared in methanol (0.5% HCl). For the intra-day variability, five replicas of each concent-

ration level were analyzed in a day, and the intra-day variability was evaluated by analyzing each concentration level of standard solutions in duplicate for five consecutive days. The amounts of C3G and C3R were calculated from the corresponding calibration curves, and the relative standard deviation (RSD%) was taken as a measure of precision.

Recovery – The accuracy was determined through the following recovery tests. Three concentration levels of working standard solutions of C3G and C3R mixtures [50 (low), 200 (medium), and 400 μg/mL (high)] were added to the known amounts of mulberry sample solution. The spiked samples were filtered and analyzed five times by using the described HPLC method. The amounts of C3G and C3R of each spiked solution were calculated from the corresponding calibration curves. The recoveries of C3G and C3R were obtained from the following formula: Recovery % = [(added amount + original amount) – original amount] / added amount × 100%.

Stability – The stability test was carried out using triplicate injection of a working standard solution of C3G and C3R mixtures (200 μg/mL) under a variety of storage time and temperature conditions. At room temperature (25 °C), the standard solution was kept for 6, 24, 48, and 72 h, and then analyzed by HPLC. The standard solution was also stored for 72 h and 12 days in a refrigerator (4 °C) and subjected to HPLC. The peak areas of the stored standard solutions obtained from HPLC were compared with those of freshly prepared standard solutions.

Results and Discussion

Isolation of C3G and C3R standards from crude mulberry fruit extract by HPCCC – Many reports have revealed that C3G and C3R are the major anthocyanins found in mulberry extracts. Accordingly, the HPLC chromatogram of crude mulberry extract used in this study showed C3R and C3G as primary components (Fig. 2A). The one-step HPCCC method was applied to isolate C3G and C3R. Crude mulberry extract (300 mg) was subjected to HPCCC to give 7.2 mg and 3.4 mg of C3G and C3R, respectively (Fig. 2B). The purities of C3G and C3R were evaluated to be over 98% by the HPLC method (Fig. 2C and 2D). Consequently, five repeated HPCCC runs yielded 34.1 mg of C3G and 14.3 mg of C3R. Generally, large portion of mulberry extract contain primary metabolites such as proteins and fatty acids,[11] while anthocyanin contents were relatively low as secondary metaboliets. In order to fractionate and isolate anthocyanins, time and labor consuming multi-step solid based column

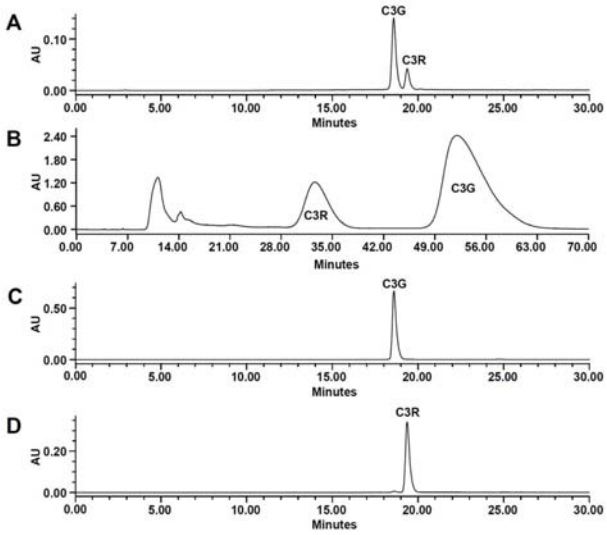

Fig. 2. HPLC chromatogram of crude mulberry fruit extract (A), HPCCC separation (B), and HPLC chromatogram of isolated C3G (C) and C3R (D). HPLC and HPCCC conditions: see Materials and Methods.

chromatography methods have been used so far. In the present study, C3G and C3R were isolated by only one-step HPCCC method, demonstrating countercurrent separation method is very powerful and efficient for the fractionation and isolation of mulberry anthocyanins.

Linearity, limit of detection (LOD), and limit of quantification (LOQ) – The stock standard solutions of C3G and C3R (1 mg/mL each) were diluted with methanol (0.5% HCl) to cover the concentration range 1.25 - 500 μg/mL and injected in the HPLC to evaluate the LOD and LOQ. The LOD and LOQ for the two analytes are listed in Table 1A. The calibration curves showed good linearity in the range 15.625 - 500 μg/mL for both C3G and C3R. The linear regression equations of C3G and C3R were $y = 17238x + 6412$ ($r^2 = 0.9996$) and $y = 5808x + 189$ ($r^2 = 0.9996$), respectively (Table 1A).

Precision and accuracy – The RSD% values of the intra-day and inter-day variabilities for C3G were 2.1 - 8.9% and 4.1 - 11.8%, respectively. For C3R, the intra- and inter-day precisions were 4.1 - 8.2% and 8.2 - 17.1%, respectively (Table 1B). It was found that the recoveries of C3G and C3R were in the ranges 95.6 - 102.6% and 96.5 - 98.9%, respectively. Thus, the results demonstrated that the developed HPLC method is sufficiently reproducible and accurate to quantify C3G and C3R simultaneously.

Stability – As shown in table 1C, the range of relative error percentages between the peak areas of the stored standard solutions and freshly prepared standard solution were within 5%, which demonstrated that methanol acidified

Table 1. Method validation data for cyanidin-3-glucoside (C3G) and cyanidin-3-rutinoside (C3R).

A. Regression equation, limit of detection (LOD) and quantification (LOQ) for C3G and C3R

Compounds	Regression equation	r^2	Linear range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
C3G	$y = 17238x + 6412$	0.9996	15.625 - 500	0.96	2.9
C3R	$y = 5808x + 189$	0.9996	15.625 - 500	1.90	6.0

B. Inter-, intra-day precision and accuracy test for C3G and C3R

Compounds	Conc. (μg/mL)	Intra-day variability (n = 5)		Inter-day variability (n = 10)		Recovery% (RSD%) (n = 5)
		Mean ± SD (μg/mL)	RSD %	Mean ± SD (μg/mL)	RSD %	
C3G	15.625 (lowest)	16.2 ± 0.7	4.4	15.2 ± 0.7	4.1	
	50 (low)	49.6 ± 2.1	2.1	48.1 ± 5.7	11.8	102.6 (2.2)
	200 (medium)	200.7 ± 16.3	8.1	199.2 ± 19	9.5	101.0 (2.8)
	400 (high)	449.4 ± 22.0	4.9	404.6 ± 30.7	7.6	95.6 (4.2)
C3R	15.625 (lowest)	16.0 ± 0.7	4.1	14.9 ± 2.6	17.1	
	50 (low)	47.0 ± 1.9	4.1	48.2 ± 6.4	13.3	98.9 (2.1)
	200 (medium)	190.3 ± 8.2	8.2	199.4 ± 21.1	10.6	96.6 (2.7)
	400 (high)	428.3 ± 21.3	5.0	404.2 ± 33.2	8.2	96.5 (4.4)

C. Stability test for C3G and C3R

Compounds	Room temperature (25 °C) RE%				Refrigerator (4 °C) RE%	
	6 h	24 h	48 h	72 h	72 h	12 day
C3G	–0.33	–0.68	–1.20	–0.64	–0.54	–2.04
C3R	0.14	–0.76	–1.35	–0.79	–0.57	–2.03

Table 2. C3G and C3R contents of mulberry fruits from different localities.

Locality	Anthocyanin contents (mg/g dry weight)		Locality	Anthocyanin contents (mg/g dry weight)	
	C3G	C3R		C3G	C3R
#1 (Jangseong)	30.03 ± 0.07	17.50 ± 0.04	#6 (Geochang)	28.89 ± 0.58	11.45 ± 0.23
#2 (Gochang I)	36.79 ± 0.36	14.74 ± 0.16	#7 (Sangju)	12.28 ± 0.20	4.34 ± 0.07
#3 (Gochang II)	52.88 ± 0.30	22.92 ± 0.13	#8 (Yangpyeong)	4.11 ± 0.03	1.51 ± 0.02
#4 (Jinju I)	31.55 ± 0.23	16.62 ± 0.11	#9 (Pocheon)	5.84 ± 0.07	1.12 ± 0.02
#5 (Jinju II)	38.04 ± 0.19	15.22 ± 0.05	#10 (Inje)	34.07 ± 0.43	12.73 ± 0.12

with 0.5% HCl was a good solvent for maintaining the stability of C3G and C3R during HPLC analysis.

Application of HPLC method for the evaluation C3G and C3R contents – The developed HPLC method was applied to determine the C3G and C3R contents in newly harvested mulberry from eight different provinces of Korea (Table 2). The quantities of C3R and C3G were determined directly from the corresponding calibration curves. The contents of C3G and C3R varied in different localities. Mulberry from Gochang (#3) contained the highest amounts of C3G and C3R (52.88 ± 0.3 and 22.92 ± 0.13 mg/g dry weight, respectively), while that from Yangpyeong (#8) showed the lowest C3G and C3R contents (4.11 ± 0.03 and 1.51 ± 0.02 mg/g dry weight, respectively). It seems that the storage conditions of mulberry fruits, such as light exposure, storage temperature, and time, may affect the contents of C3G and C3R. The results demonstrated that the developed HPLC method can be used to determine C3G and C3R not only in mulberry but also in diverse healthcare foods containing C3G and C3R as major anthocyanins.

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