

# Comparison of Standard Elution and Displacement Modes in Centrifugal Partition Chromatography for an Efficient Purification of Four Anthocyanins from *Hibiscus sabdariffa* L.

Nor Syaidatul Akmal Mohd Yousof<sup>1</sup>, Nikos Xynos<sup>3</sup>, Evanthia Dina<sup>2</sup>, Maizatul Hashima Omar<sup>1</sup>, Choong Yew Keong<sup>1</sup>, Mohd Isa Wasiman<sup>1</sup> and Nektarios Aligiannis<sup>2</sup>

1. Phytochemistry Unit, Herbal Medicine Research Centre, Institute for Medical Research, Jalan Pahang, Kuala Lumpur 50588, Malaysia

2. Laboratory of Pharmacognosy and Natural Products Chemistry, School of Pharmacy, University of Athens, Panepistimioupoli Zografou, Athens 15771, Greece

3. Rousselet Robatel Kromaton, 45 Avenue Rhin et Danube, Annonay 07104, France

**Abstract:** The increasing demand for natural food antioxidant has hastened research to extract the biologically active substances from a variety of raw materials. The consumption of anthocyanin-rich food promotes health by contributing to cardio protection, lowering high blood pressure and by helping to protect against cancer. In this study, centrifugal partition chromatography (CPC) was studied for anthocyanin separation of *Hibiscus sabdariffa* L. (HS). A simple elution and two displacement protocols were implemented. The displacement modes were strong ion-exchange (SIX) and pH-zone refining (pHZR) method. Among the three protocols, SIX CPC using trioctylmethylammonium chloride (Aliquat 336®) as a strong exchanger was the most successful in purifying four anthocyanins from an ethanolic extract of HS, identified as delphinidin-3-*O*-sambubioside (DS), cyanidin-3-*O*-sambubioside (CS), delphinidin-3-*O*-glucoside (DG) and cyanidin-3-*O*-glucoside (CG). The purification process was carried out on a gram scale using the ternary biphasic system ethyl acetate:*n*-butanol:water (mobile organic phase: 4.0/4.6/1.4; aqueous stationary phase: 0.5/0.5/9.0) in the ascending mode. Aliquat 336® was used as the anion extractant in the aqueous stationary phase and sodium iodide (NaI) as the cation displacer in the organic mobile phase. From 1 g of crude HS extract, 80 mg of DS, 40 mg of CS, 15 mg of DG, and 4 mg of CG were obtained after 75 minutes. High performance liquid chromatography (HPLC-UV and LC-MS) analysis revealed the identity and the purity of isolated compounds: 88% for DS, 92% for CS, 93% for DG and 70% for CG. This new methodology for the isolation of anthocyanin mixtures successfully increased the purity and efficiency especially for DG and CG, while maintaining excellent recovery rate and being relatively low cost. The availability of high purity anthocyanin mixtures will facilitate anthocyanin testing studies and will widen the use of anthocyanins in the food, supplements and pharmaceutical industries.

**Key words:** SIX, pHZR, CPC, *Hibiscus sabdariffa*, anthocyanins.

## 1. Introduction

HS (*Hibiscus sabdariffa* L.) known as Roselle is an important source of vitamin C, minerals and bioactive compounds such as polyphenols and anthocyanins

---

**Corresponding author:** Nikos Xynos, Pharmacist Ph.D., scientific affairs manager at Kromaton, research fields: natural products chemistry, botanical extraction and purification, formulation of drugs, cosmetics and Supplements.

[1-4]. In fruits and vegetable, anthocyanins health-promoting features are attributed to their antioxidant, anti-inflammatory, anti-cancer and anti-aging properties as shown by several *in vivo* studies [4-7]. Anthocyanins are a family of compounds that have aromatic rings containing polar substituent groups like hydroxyl, carboxyl and methoxyl groups [8, 9]. Due to their specific structure, anthocyanins are very

effective against ROS (reactive oxygen species) and they are consequently considered to be powerful natural antioxidants. The chemical structure of the main anthocyanins in HS has been identified and delphinidin-3-*O*-sambubioside (DS) and cyanidin-3-*O*-sambubioside (CS) are known as the major anthocyanins in HS aqueous, ethanol and methanol extracts [3, 10]. Isolation of anthocyanins from plants is a strenuous task. In the past, anthocyanins were successfully fractionated based on their polarities into the biphasic mixture of tert-butyl methyl ether/*n*-butanol/acetonitrile/water acidified with trifluoroacetic acid (TFA) [11].

The selection of an appropriate purification technique is essential in order to achieve high purity of the target compounds [11]. Centrifugal partition chromatography (CPC) has been developed as an effective technique for the fractionation of various classes of compounds ranging from very apolar to very polar [11-13]. CPC is mostly operated in two basic modes of separation called isocratic ascending and descending mode [14]. For the researcher who is dealing with ionisable compounds such as alkaloids or anthocyanins, pH-zone refining (pHZR) and ion-exchange modes are preferable [15]. The versatility of CPC makes it an ideal method for the separation and purification of bioactive natural products [16], providing high efficiencies, recoveries and purities. Various bioactive compounds, such as carotenoids [17], flavonoids [18], alkaloids [19], saponins [20], terpenes [21], coumarins [22], anthocyanins [10], as well as antibiotics [20] and lipids [23] have been separated and purified from plant extracts by CPC. To further extend the capabilities of CPC in isolating bioactive natural products, initial existing strategies of CPC have been modified and resulted improved complex CPC methodologies, such as displacement modes. pHZR and SIX (strong ion exchange) CPC are being used besides the usual isocratic elution in various applications. pHZR has been developed and applied by Ito and co-workers [16, 24] as a preparative

purification method for the separation of compounds whose electric charge depends on pH-values. On the other hand, SIX concerns analytes whose electric charge is invariant during the purification process. These techniques have also been investigated for their capacity to provide high throughputs and high recovery in the separations of highly polar molecules [25].

The objective of this study was to compare the performance between the standard elution and displacement modes in CPC for the purification of anthocyanins from HS extract. A separation study was conducted to provide a good pretext for the investigation of the analyte nature and the choice of cationic displacer-retainer that lead to the finetuning of efficient experimental conditions.

## **2. Methodology**

### *2.1 Chemical and Reagents*

Methanol, ethanol, *n*-butanol, ethyl acetate, TFA, acetic acid and formic acid were purchased from Merck (Germany). Methyltriethylammonium chloride (Aliquat 336®), sodium hydroxide (NaOH), and sodium iodide (NaI) were purchased from Sigma Aldrich (France). Water was purified by a Mili-Q-system.

### *2.2 Preparation of HS Crude Extract*

The powder form of HS calyx (250 g) obtained from Kepala Batas, Penang, was extracted three times consecutively with 500 mL acidified ethanol (with 0.1% TFA) for 30 min by sonication at room temperature (25 °C). The filtered extract was concentrated under vacuum at 30°C and then freeze-dried. The extract was stored in an amber vial at -20 °C until further processing.

### *2.3 CPC Apparatus*

CPC separations were performed on FCPC® (fast centrifugal partition chromatography) preparative 200 Kromaton apparatus (Rousselet Robatel Kromaton,

Annonay, France) using a rotor composed of 840 twin cells, with a total column capacity of 266 mL, of which 200 mL correspond to the cell volume. Rotation speed was set from 600 to 1,500 rpm, thus producing a centrifugal force field in the partition cells about  $120 \times g$  at 1,000 rpm and  $480 \times g$  at 2,000 rpm. Method development, pumping, detection, fraction collection and automated control of FCPC were ensured by the Kromaflash integrated CPC-peripheral system, using the Glider software. The sample was introduced into the column equipped with a 50 mL sample loop and effluent detection was monitored by UV detector at  $\lambda = 520$  nm.

## 2.4 Development of CPC Operating Conditions

### 2.4.1 Elution Mode

Anthocyanin separation was performed at a 200 mL hydrostatic column using the ternary biphasic system ethyl acetate:*n*-butanol:water (mobile organic phase: 4.0:4.6:1.4; aqueous stationary phase: 0.5:0.5:9.0) (Table 1). The column was loaded with the aqueous stationary phase at a flow rate of 30 mL/min with the rotation set at 600 rpm and was subsequently equilibrated with the organic mobile phase using ethyl acetate, *n*-butanol, water 4.0:4.6:1.6 and 0.5:0.5:9.0 acidified with 0.5% TFA. The rotation speed was set at 1,500 rpm and the mobile phase flow rate was 10 mL/min. Elution was performed in ascending mode under the same conditions as equilibration and then the whole content of the CPC column was extruded by pumping the aqueous phase at 30 mL/min in 600 rpm.

### 2.4.2 pHZR CPC Procedure

In order to implement the pHZR mode NaOH was used as a retainer and TFA was used as a displacer. The organic mobile phase was acidified with TFA at a concentration of 16 mM (pH~2) and the aqueous stationary phase was alkalized with NaOH (pH~10) at a concentration of 20 mM. The column was filled with the alkalized aqueous phase at a rotation of 600 rpm and it was subsequently equilibrated with the organic phase without the acidic displacer (TFA). After

injecting the sample through the injection loop, the organic mobile phase containing the acidic displacer was pumped at 1,500 rpm for 10 mL/min. And 50 fractions were collected in every 10 mL/tubes and the experiment was monitored at  $\lambda = 520$  nm.

### 2.4.3 Strong-Ion Exchange CPC Procedure

The elution-extrusion method with Strong-Ion Exchange application was also implemented for this application. A quaternary ammonium salt (Aliquat 336™) was added in the stationary phase as an extractant (retainer) and sodium iodide (NaI) was added to the mobile phase as a displacer. Elution of compounds was performed in ascending mode. A volume of 500 mL of the stationary phase (aqueous phase) containing retainer was pumped into the column with 600 rpm rotation speed at 30 mL/min. Displacer-free mobile phase (organic phase) was then pumped into the column with increasing of rotation speed to 1,500 rpm at 10 mL/min in order to achieve column equilibration. The sample was injected through the injection loop and subsequently the organic mobile phase containing the displacer was pumped at 10 mL/min. And 80 fractions were collected in every 10 mL/tubes and the experiment was monitored at  $\lambda = 520$  nm.

## 2.5 Fractions Analyses

All CPC fractions were screened by HPTLC on Merck 60 F<sub>254</sub> silica gel plates developed with ethyl acetate/formic acid/acetic acid/water [100:11:11:26 v/v/v/v] using natural product reagent. The purity of the isolated compounds was assessed by Dionex Ultimate 3000 UHPLC system. The optimized gradient elution was performed at a flow rate of 0.7 mL/min, using 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B) as follows: 0-2 min, 5% B; 2-18 min, 60% B; 18-20 min, 95% B. Kinetex C18 column (100 × 4.6 mm i.d., 2.6  $\mu$  particle size) was used and the temperature of column oven was set at 35 °C. The linear calibration curve was plotted at 520 nm from 1 mg/mL stock solution with six level of three point each

**Table 1** Experimental conditions of the separation of anthocyanin from *Hibiscus sabdariffa* by CPC.

Apparatus		FCPC® A200 preparative by Kromaton	
Biphasic solvent system		Ethyl acetate, <i>n</i> -butanol, water [4.0:4.6:1.4 and 0.5:0.5:9.0] v/v/v	
	Elution mode	pH zone refining	SIX CPC
Stationary phase	Aqueous lower phase + 0.5% TFA	Aqueous lower phase + 20 mM of NaOH	Aqueous lower phase 7 mM of AL336
	Organic upper phase + 0.5% TFA	Organic upper phase + 16 mM of TFA	Organic upper phase 7 mM of NaI
Sample mass	1 g	1 g	1 g
Injection volume	10 mL (organic mobile phase without TFA)	10 mL (organic mobile phase without TFA)	10 mL (organic mobile phase without NaI)
Rotation speed	1,500 rpm	1,500 rpm	1,500 rpm
Flow rate	10 mL/min	10 mL/min	10 mL/min

as a function of peak purity determination. All of the chromatographic data management was processed by the Chromaleon 7.2.2 software. All isolated compounds were characterized by Orbitrap mass spectrometers (MS) fitted with an electrospray interface (ESI) (Q Exactive, Thermo Fisher Scientific, Bremen, Germany).

### 3. Results and Discussion

In the present study, three techniques were applied and compared using CPC: (1) Standard elution; (2) pHZR and; (3) Strong-ion exchange; for the separation of four specific anthocyanins. Two aglycons: delphinidin and cyanidin with two sugars; glucoside and sambubioside were identified. The nature of the sugar moiety in the anthocyanin plays an important role in CPC. Glucoside group is expected to elute first in the CPC and as for the aglycons delphinidin, that has more phenolic hydroxyls than cyanidin, is expected to elute last [26]. In UHPLC, the nature of aglycons plays a major role in polarity. Therefore, there is a different elution pattern in UHPLC where delphinidin, which is more polar than cyanidin, elutes first with sambubioside sugar followed by the glucoside [26].

#### 3.1 Standard Elution CPC

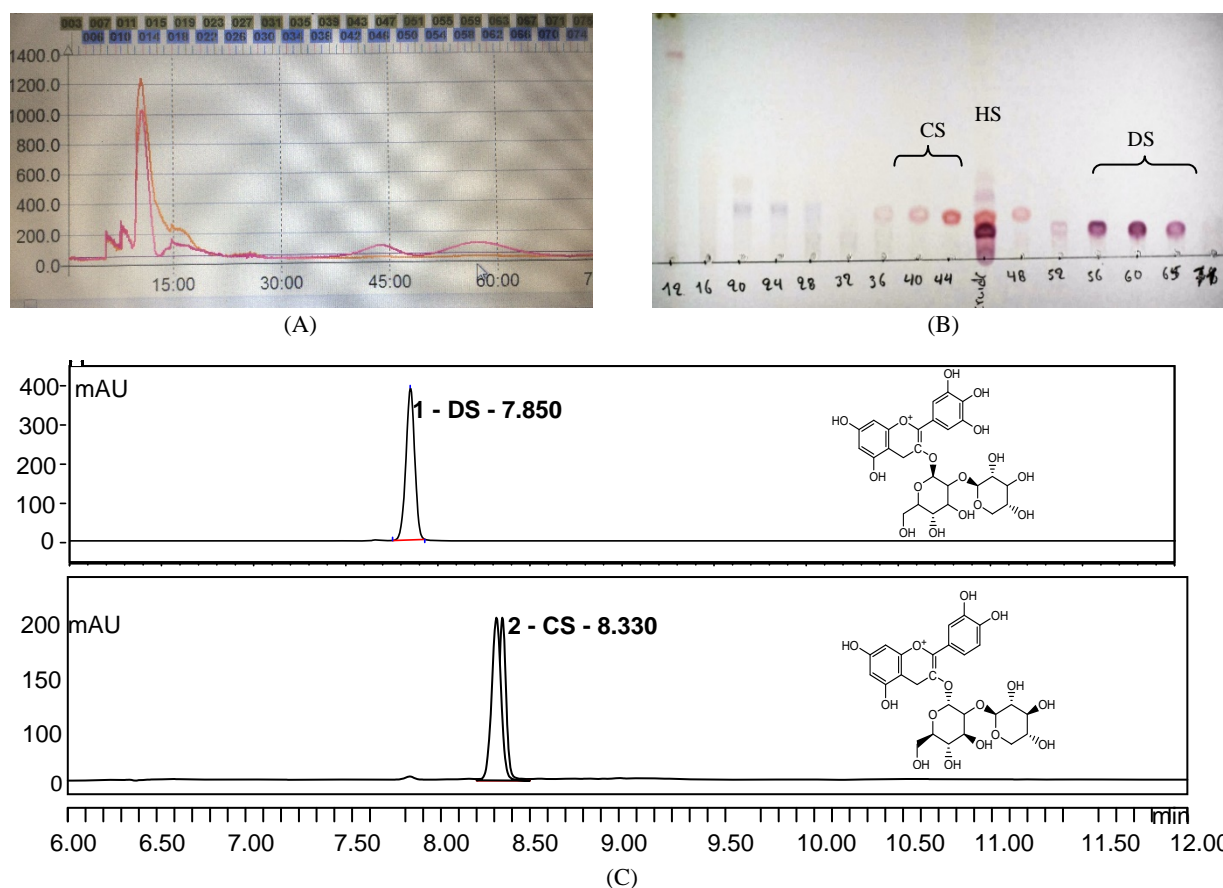
The biphasic solvent was prepared according to Kouakou et al. 2010 solvent ratio and the CPC conditions were modified accordingly [10]. An acid, TFA, was added in both phases in order to preserve

the pH-sensitive structure of the flavylum aglycon [27]. The analysed fractions demonstrate that CPC had the capability to separate DS and CS at a high purity (> 95%) but did not show good separation for cyanidin-3-*O*-glucoside (CG) and delphinidin-3-*O*-glucoside (DG) (Fig. 1, Table 2). This method was suitable for achieving large quantity of pure DS and CS with 16% and 14% recovery in a one-step purification.

#### 3.2 pHZR

The pHZR process was performed by adding aqueous phase with NaOH as the retainer (pH-10) and acidic displacer TFA (pH-2) to the organic phase. The role of the retainer is to ensure the deprotonation of the ionisable compounds so that they will remain in the stationary phase due to their significant partition [28]. Meanwhile, the acidic displacer TFA was added in the mobile phase in order to neutralize the retainer (OH<sup>-</sup>) and to displace the ionisable compounds from the stationary phase to mobile phase [28]. Thus, the aqueous stationary phase had a pH of approx. 2 and the organic mobile phase had a pH around 9. The pH of the eluent of the CPC column was measured as well. In the first fractions it was measured as pH = 6 and during the elution the acidity was constantly increasing, reaching up to pH = 2.

pHZR mode allows the separation of anthocyanins according to their hydrophobicity and their  $pK_a$  values [24, 29]. The elutropic sequence of pHZR was reported in Table 3. The separation effectiveness is poor



**Fig. 1** CPC chromatogram of (A) Standard elution separation corresponding (B) Thin layer chromatography (TLC) fractograms and corresponding (C) UHPLC (Ultra high performance liquid chromatography) spectrum at 520 nm for DS and CS fraction.

**Table 2** The elutropic sequence of UHPLC and CPC of the target compound.

Target compound	Elutropic sequence of CPC	Elutropic sequence of UHPLC	Purity by UHPLC relative area (%)
DS	2 (50.0 min, tubes 53-66)	1 (7.850 min)	95
CS	1 (39.0 min, tubes 38-48)	2 (8.330 min)	95
CS	NA	NA	NA
CG	NA	NA	NA

NA: Not applicable.

because DG, CS and CG are still co-eluting to each other (Fig. 2). It may be due to the difference between solute dissociation constants ( $pK_a$ ) which differ only 0.01 unit. The  $pK_a$  should be at least 0.2 units between each of the compound in order to achieve a good separation [15]. This technique also requires significant sample concentration, and it does not work well at low concentration as CG and DG are minor compound in HS [15].

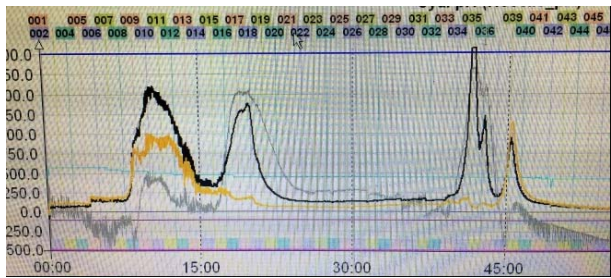
### 3.3 SIX CPC

Recently, researchers developed displacement mode strategies by ion exchange for the purification of anionic compounds including organic acids and anthocyanins by CPC [25, 30]. Depending on the nature of the targeted compounds, anthocyanins have significant polarity and ionisation behaviour in polar solution. The stability of anthocyanins could be limited,

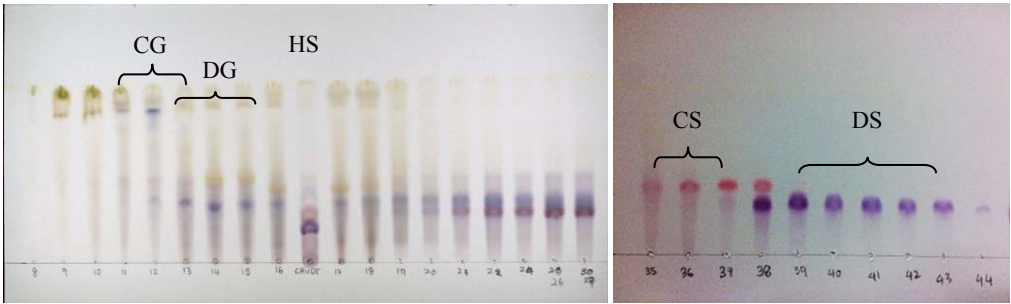
Table 3 The  $pK_a$  and the eluotropic sequence of UHPLC and pHZR CPC of the target compound.

Target compound	$pK_a$ values	Eluotropic sequence of CPC	Eluotropic sequence of UHPLC	Purity by UHPLC	Relative area (%)
DS	6.61	3 (45.0 min, tubes 39-43)	1 (7.850 min)	95	
DG	6.37	1 (12.0 min, tubes 13-16)	2 (8.040 min)	40	
CS	6.39	2 (39.0 min, tubes 35-37)	3 (8.337 min)	75	
CG	6.39	NA	NA	NA	

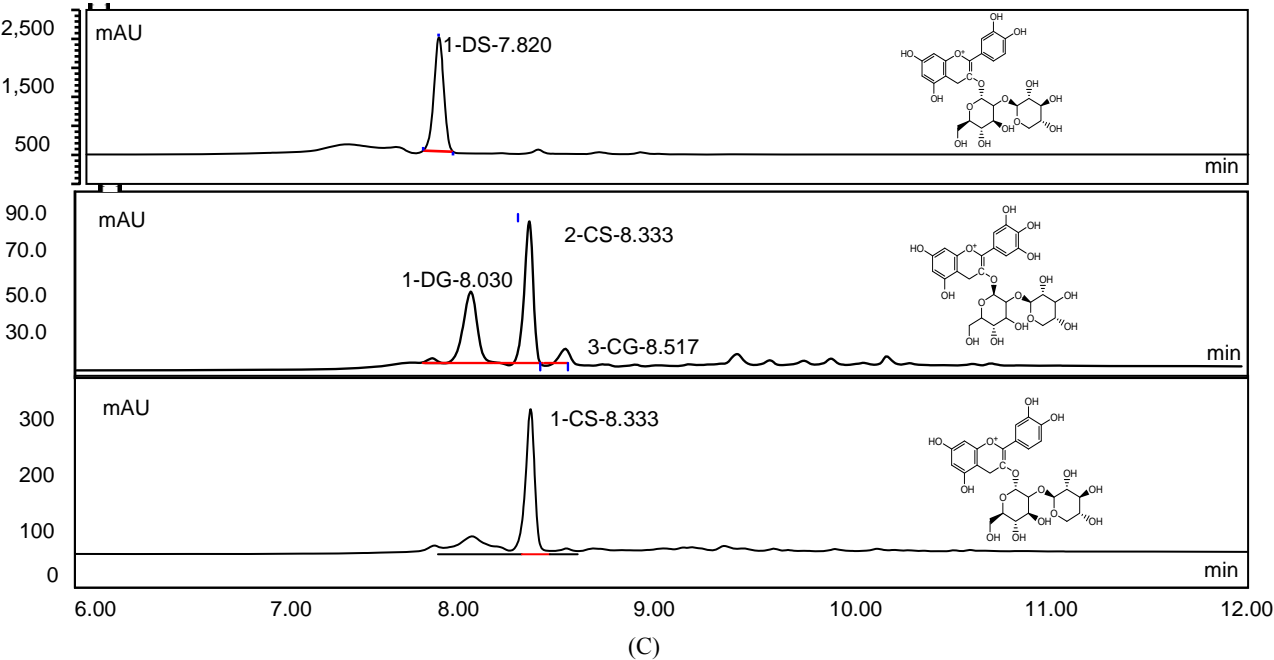
NA: Not applicable.



(A)



(B)



(C)

Fig. 2 CPC chromatogram of (A) pHZR corresponding (B) Thin layer chromatography (TLC) fractograms and corresponding (C) UHPLC spectrum at 520 nm for DS and CS fraction.

depending on pH values [30, 31]. Therefore, in this study, SIX CPC was applied for the isolation of four anthocyanins (CS, CG, DS & DG) from HS extract.

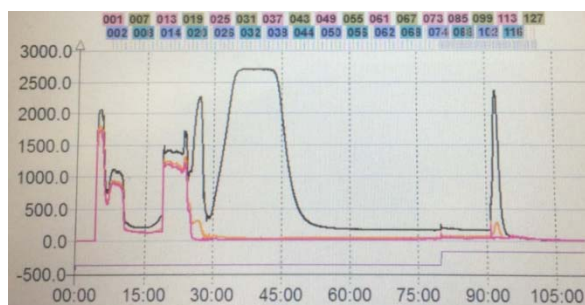
Aliquat 336® (Methyltrioctylammonium chloride) was used as a retainer in the stationary aqueous phase and NaI was used as a displacer in the mobile organic phase. During the equilibration step and after loading the stationary phase in the column, NaI-free mobile phase was pumped into the column in order to ensure that the anthocyanins will partition completely in the stationary phase after injecting the sample [25]. Anthocyanins in HS are present as anions in neutral solutions (pH = 7), and then interact with an anion-exchanger, Aliquat 336® in its protonated form (RNH<sub>4</sub><sup>+</sup>) thus forming ion-pairs that are well-soluble in the stationary phase. The organic mobile phase containing NaI was then pumped into the column with a flow rate of 10 mL/min, generating the displacement process by neutralizing the RNH<sub>4</sub><sup>+</sup>. The re-extraction of the hydrophilic analytes into the mobile phase occurs due to this and the analytes elute in highly concentrated rectangular peaks fused together with minimum overlapping, the so-called isotachic train [32, 33].

Fig. 3 shows the results of the displacement CPC run, monitored by UV absorbance at 520 nm. The separation and purity of anthocyanins using SIX CPC strategy was improved, by comparison with the corresponding pHZR and standard elution experiment (Table 4). The increment of purity is more obvious for

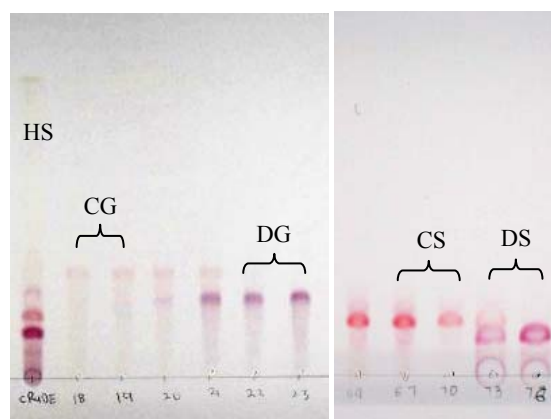
DG and CG as it was less contaminated by the major CS. This is due to the steep boundaries between each analyte zone in the SIX CPC mode [25]. However, CG required one more step of purification by preparative HPLC.

The identification of all four isolated compound was done with reference standards by UV-spectra and retention time. Moreover, Table 5 shows the pseudo-molecular ion and fragmentation ions of 4 purified compounds using full-scan ESI-MS in positive mode observed scanning from *m/z* 100 to 1,000. Compounds 1 and 2 were identified as delphinidin derivatives based on significant fragment masses of the aglycone at *m/z* 303. Compounds 3 and 4 were identified as cyanidin derivatives based on significant masses of the aglycone at *m/z* 287 [34-36].

Compound 1 exhibited [M-H]<sup>+</sup> at *m/z* 597 had similar neutral loss of 294 amu as compound 3; [M-H]<sup>+</sup> at *m/z* 581, which indicate that the molecule is being fragmented and confirmed the sambubioside unit [-3-*O*-(2"-xylosyl)glucoside] is linked to the anthocyanidins [35]. Thus, compound 1 is identified as DS and compound 3 is identified as CS. While, compound 2 exhibited [M-H]<sup>+</sup> at *m/z* 465, had similar neutral loss of 162 amu as compound 4; [M-H]<sup>+</sup> at *m/z* 449, which indicate that the molecule is being fragmented and confirmed the glucoside unit is link to the anthocyanidins [37, 38]. Thus, compound 2 is identified as DG and compound 3 is identified as CG.



(A)



(B)

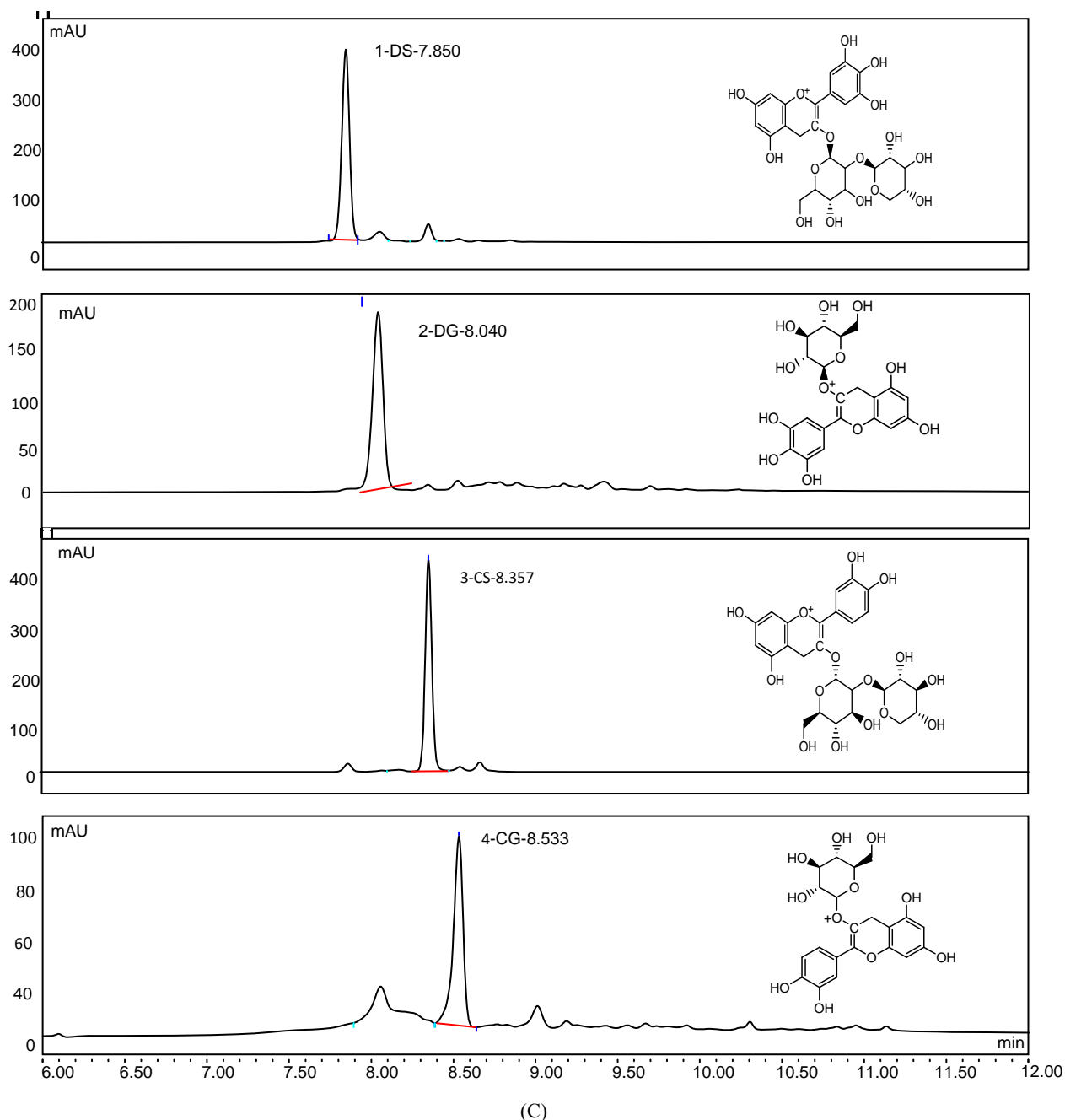


Fig. 3 CPC chromatogram of (A) Strong ion chromatography (SIX CPC) corresponding (B) Thin layer chromatography (TLC) fractograms and corresponding (C) UHPLC spectrum at 520 nm for DS, DG, CS ,CG fraction.

Table 4 The elutropic sequence of UHPLC and strong ion chromatography (SIX CPC) of the target compound.

Target compound	The elutropic sequence of SIX CPC	The elutropic sequence of UHPLC	Purity by UHPLC	Relative area (%)
DS	4 (80.0 min, tubes 75-80)	1 (7.850 min)	88	
DG	2 (29.0 min, tubes 22-23)	2 (8.040 min)	93	
CS	3 (75.0 min, tubes 68-71)	3 (8.357 min)	92	
CG	1 (26.0 min, tubes 18-19)	4 (8.533 min)	70	

**Table 5** Compound confirmation of isolated anthocyanins in HS extracts using HPLC-ESI-MS.

Compound	[M-H] <sup>+</sup> (m/z)	MS <sup>2</sup> (m/z)	Assignment
1	597.12393	303	Delphinidin-3-O-sambubioside
2	465.10153	303	Delphinidin-3-O-glucoside
3	581.15011	287	Cyanidin-3-O-sambubioside
4	449.12	287	Cyanidin-3-O-glucoside

## 4. Conclusions

A simple elution and two displacement protocols, SIX and pHZR, were compared in CPC for the purification of four anthocyanins from an ethanolic extract of HS, identified as DS, CS, DG and CG. Among the three protocols, SIX CPC using trioctylmethylammonium chloride (Aliquat 336®) as a strong exchanger was the most successful. This was achieved by an appropriate selection of the displacer and retainer and their concentrations in a selected solvent system without disturbing its biphasic nature [25]. The analytes were organized inside the column according to their solubility and polarity resulting in highly selective separations for DS: 88%, CS: 92%, DG: 93% and CG: 70%. An optimization scheme through experimental design regarding SIX CPC will be implemented in the future in order to develop a single protocol that maximizes the purities of all four target compounds.

## Acknowledgements

The authors express their deepest gratitude to the Director General of Health, Malaysia and the Director of Institute for Medical Research (IMR) for permission to publish this article. This work was financially supported by the NKEA AGRICULTURE (EPP#1), NKEA Research Grant Scheme (NRGS) (Grant No: NH064DO070). The authors also thank Laboratory of Pharmacognosy and Natural Products Chemistry (University of Athens) for laboratory assistance and technical support from Rousselet Robatel Kromaton (France). The support from Ministry of Health Malaysia for training in CPC under the LDP program is gratefully acknowledged.

## References

- [1] Tsai, P.-J., McIntosh, J., Pearce, P., Camden, B., and Jordan, B. R. 2002. "Anthocyanin and Antioxidant Capacity in Roselle (*Hibiscus sabdariffa* L.) Extract." *Food Research International* 35 (4): 351-6.
- [2] Ali, B. H., Wabel, N. A., and Blunden, G. 2005. "Phytochemical, Pharmacological and Toxicological Aspects of *Hibiscus sabdariffa* L.: A Review." *Phytotherapy Research* 19 (5): 369-75.
- [3] Du, C., and Francis, F. 1973. "Anthocyanins of Roselle (*Hibiscus sabdariffa* L.)." *Journal of Food Science* 38 (5): 810-2.
- [4] Rossi, A., Serraino, I., Dugo, P., Di Paola, R., Mondello, L., Genovese, T., et al. 2003. "Protective Effects of Anthocyanins from Blackberry in a Rat Model of Acute Lung Inflammation." *Free Radical Research* 37 (8): 891-900.
- [5] Bowen-Forbes, C. S., Zhang, Y., and Nair, M. G. 2010. "Anthocyanin Content, Antioxidant, Anti-inflammatory and Anticancer Properties of Blackberry and Raspberry Fruits." *Journal of Food Composition and Analysis* 23 (6): 554-60.
- [6] Wang, L.-S., and Stoner, G. D. 2008. "Anthocyanins and Their Role in Cancer Prevention." *Cancer Letters* 269 (2): 281-90.
- [7] Bagchi, D., Sen, C., Bagchi, M., and Atalay, M. 2004. "Anti-angiogenic, Antioxidant, and Anti-carcinogenic Properties of a Novel Anthocyanin-Rich Berry Extract Formula." *Biochemistry (Moscow)* 69 (1): 75-80.
- [8] Bueno, J. M., Ramos-Escudero, F., Sáez-Plaza, P., Muñoz A. M., Jose Navas, M., and Asuero, A. G. 2012. "Analysis and Antioxidant Capacity of Anthocyanin Pigments. Part I: General Considerations Concerning Polyphenols and Flavonoids." *Critical Reviews in Analytical Chemistry* 42 (2): 102-25.
- [9] Delgado-Vargas, F., Jiménez, A., and Paredes-López, O. 2000. "Natural Pigments: Carotenoids, Anthocyanins, and Betalains—Characteristics, Biosynthesis, Processing, and Stability." *Critical Reviews in Food Science and Nutrition* 40 (3): 173-289.
- [10] Kouakou, H. T. 2010. "Preparative Purification of Delphinidin 3-O-Sambubioside from Roselle (*Hibiscus sabdariffa* L.) Petals by Fast Centrifugation Partition Chromatography." *Journal of Advances in Chemistry* 6

- (2): 999-1004.
- [11] Dai, J., and Mumper, R. J. 2010. "Plant Phenolics: Extraction, Analysis and Their Antioxidant and Anticancer Properties." *Molecules* 15 (10): 7313-52.
- [12] Rostagno, M. A., and Prado, J. M. 2013. *Natural Product Extraction: Principles and Applications*. Royal Society of Chemistry.
- [13] Huie, C. W. 2002. "A Review of Modern Sample-Preparation Techniques for the Extraction and Analysis of Medicinal Plants." *Analytical and Bioanalytical Chemistry* 373 (1): 23-30.
- [14] Bojczuk, M., Żyżelewicz, D., and Hodurek, P. 2017. "Centrifugal Partition Chromatography—A Review of Recent Applications and Some Classic References." *Journal of Separation Science* 40 (7): 1597-609.
- [15] Yin, L., Li, Y., Lu, B., Jia, Y., and Peng, J. 2010. "Trends in Counter-Current Chromatography: Applications to Natural Products Purification." *Separation & Purification Reviews* 39 (1-2): 33-62.
- [16] Ito, Y. 2005. "Golden Rules and Pitfalls in Selecting Optimum Conditions for High-Speed Counter-Current Chromatography." *Journal of Chromatography A* 1065 (2): 145-68.
- [17] Chen, F., Li, H.-B., Wong, R. N.-S., Ji, B., and Jiang, Y. 2005. "Isolation and Purification of the Bioactive Carotenoid Zeaxanthin from the Microalga *Microcystis Aeruginosa* by High-Speed Counter-Current Chromatography." *Journal of Chromatography A* 1064 (2): 183-6.
- [18] Costa, Fd., and Leitão, G. G. 2010. "Strategies of Solvent System Selection for the Isolation of Flavonoids by Countercurrent Chromatography." *Journal of Separation Science* 33 (3): 336-47.
- [19] Renault, J.-H., Nuzillard, J.-M., Le Crouerour, G., Thepenier, P., Zeches-Hanrot, M., and Le Men-Olivier, L. 1999. "Isolation of Indole Alkaloids from *Catharanthus Roseus* by Centrifugal Partition Chromatography in the pH-Zone Refining Mode." *Journal of Chromatography A* 849 (2): 421-31.
- [20] Engelberth, A. S., Clausen, E. C., and Carrier, D. J. 2010. "Comparing Extraction Methods to Recover Ginseng Saponins from American Ginseng (*Panax Quinquefolium*), Followed by Purification Using Fast Centrifugal Partition Chromatography with HPLC Verification." *Separation and Purification Technology* 72 (1): 1-6.
- [21] Skalicka-Woźniak, K., and Garrard, I. 2014. "Counter-Current Chromatography for the Separation of Terpenoids: A Comprehensive Review with Respect to the Solvent Systems Employed." *Phytochemistry Reviews* 13 (2): 547-72.
- [22] Liu, R., Sun, Q., Sun, A., and Cui, J. 2005. "Isolation and Purification of Coumarin Compounds from Cortex *Fraxinus* by High-Speed Counter-Current Chromatography." *Journal of chromatography A* 1072 (2): 195-9.
- [23] Alvarez, J., Cazes, J., Touchstone, J., and Grob, R. 1990. "Isolation and Purification of Lipids by Centrifugal Partition Chromatography." *Journal of liquid chromatography* 13 (18): 3603-14.
- [24] Ito, Y. 2013. "pH-Zone-Refining Counter-Current Chromatography: Origin, Mechanism, Procedure and Applications." *Journal of Chromatography A* 1271 (1): 71-85.
- [25] Toribio, A., Delannay, E., Nuzillard, J.-M., Zèches, M., Richard, B., Waffo-Tégou, P., et al. 2006. "Large-Scale Isolation of Anthocyanins from Grapevine by Strong Ion-Exchange Centrifugal Partition Chromatography." *Macromolecules and Secondary Metabolites of Grapevine and Wine*, 247.
- [26] Renault, J.-H., Thépenier, P., Zèches-Hanrot, M., Le Men-Olivier, L., Durand, A., Foucault, A., et al. 1997. "Preparative Separation of Anthocyanins by Gradient Elution Centrifugal Partition Chromatography." *Journal of Chromatography A* 763 (1-2): 345-52.
- [27] Venter, A., Joubert, E., and de Beer, D. 2013. "Characterisation of Phenolic Compounds in South African Plum Fruits (*Prunus Salicina* Lindl.) Using HPLC Coupled with Diode-Array, Fluorescence, Mass Spectrometry and on-Line Antioxidant Detection." *Molecules* 18 (5): 5072-90.
- [28] Hamzaoui, M., Renault, J.-H., Reynaud, R., and Hubert, J. 2013. "Centrifugal Partition Extraction in the pH-Zone-Refining Displacement Mode: An Efficient Strategy for the Screening and Isolation of Biologically Active Phenolic Compounds." *Journal of Chromatography B* 937: 7-12.
- [29] Sun, C., Liu, F., Sun, J., Li, J., and Wang, X. 2016. "Optimisation and Establishment of Separation Conditions of Organic Acids from *Usnea Longissima* Ach. by pH-Zone-Refining Counter-Current Chromatography: Discussion of the Elutotropic Sequence." *Journal of Chromatography A* 1427: 96-101.
- [30] Hamzaoui, M., Hubert, J., Reynaud, R., Marchal, L., Foucault, A., and Renault, J.-H. 2012. "Strong Ion Exchange in Centrifugal Partition Extraction (SIX-CPE): Effect of Partition Cell Design and Dimensions on Purification Process Efficiency." *Journal of Chromatography A* 1247: 18-25.
- [31] Wybraniec, S., Stalica, P., Jerz, G., Klose, B., Gebers, N., Winterhalter, P., et al. 2009. "Separation of Polar Betalain Pigments from Cacti Fruits of *Hylocereus Polyrhizus* by Ion-Pair High-Speed Countercurrent Chromatography." *Journal of Chromatography A* 1216

- (41): 6890-9.
- [32] Maciuk, A., Renault, J.-H., Margraff, R., Trébuchet, P., Zèches-Hanrot, M., and Nuzillard, J.-M. 2004. "Anion-Exchange Displacement Centrifugal Partition Chromatography." *Analytical Chemistry* 76 (21): 6179-86.
- [33] Zhu, J., and Guiochon, G. 1995. "Production Rate of an Isotachic Train in Displacement Chromatography." *AIChE Journal* 41 (1): 45-57.
- [34] Oh, Y., Lee, J., Yoon, S., Oh, C., Choi, D. S., Choe, E., et al. 2008. "Characterization and Quantification of Anthocyanins in Grape Juices Obtained from the Grapes Cultivated in Korea by HPLC/DAD, HPLC/MS, and HPLC/MS/MS." *Journal of Food Science* 73 (5).
- [35] Grajeda-Iglesias, C., Figueroa-Espinoza, M. C., Barouh, N., Baréa, B., Fernandes, A., de Freitas, V., et al. 2016. "Isolation and Characterization of Anthocyanins from *Hibiscus sabdariffa* Flowers." *Journal of Natural Products* 79 (7): 1709-18.
- [36] Ezzat, S. M., Salama, M. M., Seif el-Din, S. H., Saleh, S., El-Lakkany, N. M., Hammam, O. A., et al. 2016. "Metabolic Profile and Hepatoprotective Activity of the Anthocyanin-Rich Extract of *Hibiscus sabdariffa* Calyces." *Pharmaceutical Biology* 54 (12): 3172-81.
- [37] Pikulski, M., and Brodbelt, J. S. 2003. "Differentiation of Flavonoid Glycoside Isomers by Using Metal Complexation and Electrospray Ionization Mass Spectrometry." *Journal of the American Society for Mass Spectrometry* 14 (12): 1437-53.
- [38] Tian, Q., Giusti, M. M., Stoner, G. D., and Schwartz, S. J. 2005. "Screening for Anthocyanins Using High-Performance Liquid Chromatography Coupled to Electrospray Ionization Tandem Mass Spectrometry with Precursor-Ion Analysis, Product-Ion Analysis, Common-Neutral-Loss Analysis, and Selected Reaction Monitoring." *Journal of chromatography A* 1091 (1-2): 72-82.