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Quantification of anthocyanosides in grapes by QuEChERS and biphenyl-UHPLC tandem mass spectrometry

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Abstract

In this study, the QuEChERS and UHPLC-MS/MS method using a biphenyl stationary phase was developed and applied for the rapid separation of 12 anthocyanosides in grapes. Efficient separation was achieved for a wide range of mono- and un-glycosylated anthocyanosides. The linear ranges of the target anthocyanosides were 0.5–500 ng mL⁻¹. The intra- and inter-day precisions were both below 11.8%. Anthocyanosides in grapes from different sources were successfully quantified. Thus, the developed QuEChERS and UHPLC-MS/MS method provide an attractive alternative for the quantification of anthocyanosides in grapes.

Keywords: Anthocyanosides, Biphenyl stationary phase, LC-MS/MS, QuEChERS, Quantification

1. Introduction

Anthocyanosides (AnCS) are widely distributed in plant tissues where they contribute to the red, blue, and purple colors in plants and are mainly distributed in flowers or fruits, but also in leaves, stems, and roots. AnCS are classified as having biological activity since they are known to show anti-oxidative and anti-inflammatory properties [1]. They were considered to contribute to the prevention of various diseases, such as diseases of the neuronal system as well as cardiovascular diseases [2]. AnCS are mainly derived from natural products, the most common structure being a mono-glucoside derivative which is quite abundant in plants. These structures are derived from the glycosidation of anthocyanins (AnC) or anthocyanidins (AnCD) by adding sugar groups to the basic framework through glycosidic bonds. They differ from each other in terms of the degree of hydroxylation and methoxylation. Therefore, the structural differences in AnCS derivatives are due to differences in the degree of glycosylation and acylation, which result in a diverse array of glycosylation patterns and structures. Although the AnCS configurations in many natural fruits and vegetables are relatively simple, the characteristics of AnCS in grapes are complicated. There are many types of AnCS in nature, and there are no reliable reference standards for most of these derivatives. As a result, accurately quantifying the AnCS content in grapes remains a challenge.

Several analytical techniques have been developed to assess AnCS levels in plant tissue samples. The popular approaches are based on liquid-liquid extraction (LLE) [3] and solid-phase extraction (SPE) [4]. Nevertheless, recent analytical procedures with the intent of obtaining a high extraction specificity and minimizing the co-extraction of matrix substances have been proposed. Thus, supercritical fluid extraction (SFE) [5], ultrasound-assisted extraction (UAE) [6], and microwave-assisted extraction (MAE) [7] can be remarked. Despite their advantages, these procedures usually require prolonged operating times and specific devices. In recent years, the quick, easy, cheap, effective, robust and safe method (QuEChERS) [8,9] has been widely used for the analysis of multiple residual pesticides in fruits and vegetables [10], veterinary drugs [11], or plant growth regulators [12]. The QuEChERS methodology
presents some advantages, such as its simplicity, minimum steps, and effectiveness for cleaning up complex samples. It involves two steps: the first one is an extraction step based on partitioning via salting-out extraction involving the equilibrium between an aqueous and an organic layer, and the second one is a dispersive SPE (d-SPE) step that involves further clean-up using a combination of different sorbents, such as C18 or enhanced matrix removal (EMR-lipid), to remove interfering substances. QuEChERS based methods have been recently reported for the extraction of different analytes in seafood [13], meat matrices [14], cereal products [15], and in the multi-residue extraction of different contaminants.

Liquid chromatography coupled with tandem mass spectrometry detection is the most frequently used method for the determination of AnCS [16]. The octadecyl-silica (ODS, C18) column is widely used for the separation of individual AnCS derivatives for various fruits [17]. However, despite the well-documented benefits of the C18 stationary phase for the determination of some types of AnCS [18], the similar structures of the glycosylated AnCS derivatives combined with their structural diversity complicate their analysis, and the complete separation of natural mixtures of complex AnCS is not always possible. In the recent development of separation targeted for liquid chromatography, the use of a pentfluorophenyl (F5) stationary phase has received considerable attention. It has now been applied for B6 metabolites in human plasma [19], or vitamin metabolite [20]. The biphenyl stationary phase is often used in the analysis of steroid hormones [21]. For food composition, Ferro et al. [22] previously published articles about phenolic compounds in virgin olive oil and their separation using a biphenyl stationary phase. In this study, the QuEChERS for extracting AnCS was developed for the first time. The 12 types of AnCS derivatives were detected and quantified in grapes using modified QuEChERS and biphenyl ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). Based on our knowledge, no previous studies have been reported in which a biphenyl column was applied for the separation and identification of AnCS. The method was validated and then applied to determining target AnCS derivatives in commercial grape samples from various sources.

2. Materials and methods

2.1. Chemicals and reagents

Standards of AnCS: cyanidin chloride (Cy, ≥ 96%), delphinidin chloride (Dp, ≥ 97%), pelargonidin-3-O-glucoside chloride (Pe, ≥ 97%), cyanidin-3-O-glucoside chloride (Cy-3-glu, ≥ 96%), delphinidin-3-O-glucoside chloride (Dp-3-glu, ≥ 95%), malvidine-3-O-glucoside chloride (Mv-3-glu, ≥ 95%), pelargonidin-3-O-glucoside chloride (Pl-3-glu, ≥ 95%), peonidin-3-O-glucoside chloride (Pe-3-glu, ≥ 95%) and petunidin-3-O-glucoside chloride (Pt-3-glu, ≥ 95%) were purchased from EXTRASYNTHÈSE (Genay, France). Malvidine chloride (Mv, ≥ 98%) and petunidin chloride (Pt, ≥ 98%) both were obtained from Cayman Chemical (Ann Arbor, MI, USA). Pelargonidin chloride (Pl, ≥ 95%) was obtained from Sigma–Aldrich (Isère, France).

HPLC grade acetonitrile (ACN), methanol (MeOH), and ACS reagent grade formic acid (FA) were purchased from Merck (Darmstadt, Germany). Ultra-pure water was purified using a Merck Millipore Direct-Q® 3 system (Darmstadt, Germany). Analytical reagent grade ethanol (EtOH), anhydrous magnesium sulfate (MgSO4), and sodium chloride (NaCl) were obtained from J. T. Baker (Phillipsburg, NJ, USA). Acetic acid (Acet. acid), citric acid (Cit. acid), hydrochloric acid (HCl), isopropyl alcohol (IPA), and anhydrous calcium chloride (CaCl2) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Sorbents required for clean-up, primary and secondary amines (PSA) were purchased from Agilent Technologies (Santa Clara, CA, USA), SiliaBond C18 (octadecysilane) was obtained from Silicycle Inc. (Quebec, Canada) and graphitized carbon black (GCB) was obtained from Waters (Dublin, Ireland).

2.2. Sample collection

The developed method was applied to determine the AnCS composition of some commercially available fresh grapes. The grapes (including Beauty, Autumn Royal, Marroo, Black Globe, Red Globe, Moon Drops, and Kyoho in Table S1) were obtained from local markets and supermarkets (Taipei, Taiwan). The samples were ground with a WONDER.tw homogenizer (New Taipei City, Taiwan) and the extracts were filtered through 0.20 μm PTFE syringe filters (Merck, Cork, Ireland) before the analysis.

2.3. Extraction processes for AnCS analysis

2.3.1. QuEChERS procedure

An accurately weighed 5 g grape was placed into a 50 mL transparent centrifuge tube. Extraction was carried out by adding 5 mL of ethanol (including 0.2% HCL (v/v)) followed by vortexing at room temperature. For salting-out, 3 g of anhydrous MgSO4 and 1.5 g NaCl were added and the sample was then centrifuged for 4 °C at 7000 rpm for 5 min.
(Hettich universal 320R centrifuge, Westfalen, Germany). An aliquot of the organic layer was collected, 300 mg of PSA sorbent and 150 mg of anhydrous CaCl2 were added for d-SPE in the clean-up step. The tubes were then stirred by vortexing and centrifuged. Finally, the collected supernatant was filtered through a PTFE syringe filter before injecting it into the UHPLC-MS/MS system.

2.3.2. The ultrasound-assisted extraction

Extractions were carried out in an open rectangular DENTAL DC150H ultrasonic cleaner bath (40 kHz, 150 W, Delta Ultrasonic Co., Ltd., New Taipei City, Taiwan) with a useful volume of 7.2 L (internal dimensions: 300 × 160 × 150 mm) and controlled a 50 ± 1 °C by a thermostat. The 5.0 g grape sample was placed in a 50 mL centrifuge tube, using ethanol that was acidic with 0.2% HCl (v/v). After the extraction, the mixture was centrifuged for 5 min at 4 °C at 7000 rpm and filtered through a PTFE syringe filter before subjecting it to UHPLC-MS/MS.

2.4. Liquid chromatography and mass spectrometry analysis

The chromatographic analyses for AnCS were carried out on an ExionLC™ AD Series UHPLC system (SCIEX Pte. Ltd., Framingham, MA, USA) incorporating a binary pump, vacuum degasser, auto-sampler, column oven, and coupled to the mass spectrometer. Reversed-phase separation was performed using a Kinetex biphenyl column (2.1 × 100 mm, 2.6 μm particle size, Phenomenex, Torrance, CA, USA). The flow rate was 500 μL min⁻¹ and the oven temperature was thermostatted at 40 °C. The auto-sampler temperature was set at 10 °C and the injection volume was 5 μL. The mobile phase components were 2% formic acid containing ultrapure water (A) and 2% formic acid containing acetonitrile (B) in a gradient started from 5% B to 20% B in the initial to 8 min time. The mobile phase B was then increased to 80% for 2 min and equilibrated back to the initial gradient conditions for 5% B. The UHPLC system was coupled to a SCIEX Triple Quad 5500 plus QTrap Ready mass spectrometer (SCIEX Pte. Ltd., Woodlands, Singapore) equipped with electrospray ionization (ESI) using a Turbo V™ ion source. ESI-MS/MS spectra were acquired in the positive ion mode and the acquisition method was multiple reaction monitoring (MRM). The conditions for the MS analysis were as follows: the electrospray voltage was set at 5500 V, ion source temperature of 600 °C for desolvation, the curtain gas was 20 psi and the collision gas was 9 psi, the nebulizer gas (gas 1) and the heater gas (gas 2) were both held at 55 psi. Precursor, product ions, and the mass spectrometric parameters settings for the MRM transitions of AnCS are given in Table 1. The target analytes was confirmed using the guidance of COMMISSION IMPLEMENTING REGULATION (EU) 2021/808 [23]. The entire system, including data acquisition analysis, was controlled by the Analyst® software version 1.7.1 (Framingham, MA, USA) and SCIEX OS-Q software version 1.7 (Ontario, Canada).

2.5. Method validation

Method validation was conducted by investigating the linearity, limits of detection (LOD) and quantification (LOQ), accuracy (recovery, R%), matrix effect (ME%), and intra-inter-day precision [24]. Selectivity of analytes was optimized and confirmed with their retention time, quantitative and qualitative ion transitions using a fortified sample spiked with anthocyanoside standards. Linearity was evaluated by triplicate analyses of six grape samples spiked between 0.5 and 500 ng mL⁻¹. The LOD and LOQ were determined based on signal-to-noise ratios of 3 and 10, respectively. The results are expressed as the percentage recovery and calculated as follows:

$$\text{Recovery (\%)} = \frac{C_s - C_n}{C_t} \times 100$$  

Eq.1

The recovery was calculated as the difference between the concentrations of AnCS measured in spiked (C_s) and non-spiked in samples (C_n) divided by the theoretical spiked concentration (C_t) of the sample and multiplied by 100. The matrix effect was calculated as the slope of matrix-matched (S_m) divided by slope for a standard solution (S_s), then minus one and multiplied by 100. The matrix effects were calculated as follows:

$$\text{Matrix effect (\%)} = \left( \frac{S_m}{S_s} - 1 \right) \times 100$$  

Eq.2

If the value of the matrix effect was negative, this indicates signal suppression while a positive value indicates an enhancement of the signal.

The intra- and inter-day precisions were evaluated for each compound in three replicates on three days (n = 9) and are expressed as the relative standard deviation (%RSD).

2.6. Statistical analysis

For statistical data analyses, results are present as mean values ± standard deviation (SD). Data were submitted for analysis of variance using Duncan’s new multiple range test (MRT) or independent...
sample t-test (IBM SPSS Statistics 25, Armonk, NY, USA). Differences are considered significant at \( p < 0.05 \). All experiments were carried out in triplicate \((n = 3)\).

3. Results and discussion

3.1. Evaluation of QuEChERS extraction

3.1.1. Optimization of extraction conditions

Conventionally, AnCS extraction has been performed using polar solvents (e.g., methanol, ethanol, acetone, and even water acidified with organic or inorganic acids). To optimize the QuEChERS method for AnCS extraction from grapes, various extraction solvents were evaluated including ethanol, isopropanol, methanol, and their mixture (all acidified with 0.1% HCl (v/v)). Although acetonitrile is a popular choice for analyte extraction [8], this study found that solvents with hydroxyl groups gave better responses, and ethanol was selected for subsequent analyses (Fig. 1(A)). The acidified solvent increased the extraction efficiency, and the extent to which it affected extraction efficiency depended on the type of acid being used. Different types of acids can have different effects on extraction efficiency, even when the same solvent is used. Based on previous studies, hydrochloric, tartaric, or trifluoroacetic acid were used as acidifiers [25]. Four acidifiers including acetic, citric, formic, and concentrated HCl were compared in this study. Among them, HCl gave a better result than others (Fig. 1(B)).

Although the addition of acid can enhance the extraction efficiency, excess acid may cause partial hydrolysis and lead to glycosidic bond cleavage between AnCS and their sugar moieties. To prevent the hydrolysis of acylating groups in AnC [26], it was suggested that extraction should be performed using a solvent containing less than 0.12 M HCl. In this study, a series of HCl concentrations (0.01–0.3%, v/v) were evaluated (Fig. 1(C)). It was found that the content of AnCS increased as the HCl concentration increased. With 0.3% HCl (v/v), trace amounts of AnCD (e.g. Cy, Dp, and Pe) were detected in the extract because the high concentration of acid caused hydrolysis or glycosidic bond breakage. However, as it is preferable to stabilize AnCS for extraction and subsequent quantitative analysis, 0.2% HCl (v/v) was chosen as the acidifier for the extraction solvent. The sample-to-solvent ratio was assessed by determining the highest peak area that could be obtained with the minimum amount of solvent. Parallel experiments were carried out using 5 g of grapes with different ethanol volumes (5, 10, 15, and 20 mL). For all the AnCS compounds, the best results were obtained when

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**Table 1. Basic structures of AnCS and their UHPLC-MS/MS results.**

| Peak no. | t_R (min) | Compound | Precursor ion \((m/z)\) | Product ion \((m/z)\) | R_1 | R_2 | R_3 |
|---------|-----------|----------|------------------------|-----------------------|-----|-----|-----|
| 1       | 1.98      | Dp-3-glu | 465.2                  | 303.2, 257.1          | OH  | OH  | Glu |
| 2       | 2.85      | Cy-3-glu | 449.1                  | 287.2, 137.0          | OH  | H   | Glu |
| 3       | 3.18      | Pt-3-glu | 479.2                  | 317.1, 302.2          | OH  | OCH_3| Glu |
| 4       | 3.20      | Dp       | 303.1                  | 229.0, 201.0          | OH  | OCH_3| Glu |
| 5       | 3.63      | Pt-3-glu | 433.1                  | 271.2, 121.0          | H   | H   | Glu |
| 6       | 4.15      | Pe-3-glu | 463.0                  | 301.2, 286.2          | OCH_3| H   | Glu |
| 7       | 4.38      | Mv-3-glu | 493.2                  | 331.1, 315.2          | OCH_3| OCH_3| Glu |
| 8       | 4.51      | Cy       | 287.0                  | 137.1, 185.0          | OH  | H   | H   |
| 9       | 5.00      | Pt       | 317.0                  | 274.0, 245.0          | OH  | OCH_3| H   |
| 10      | 5.82      | Pl       | 271.2                  | 121.2, 093.0          | H   | H   | H   |
| 11      | 6.60      | Pe       | 301.2                  | 201.0, 230.2          | OCH_3| H   | H   |
| 12      | 6.85      | Mv       | 331.2                  | 315.0, 242.0          | OCH_3| OCH_3| H   |

*Indicators were confirmed using reference [23].

* Retention time.

* Glucose.
5 mL of ethanol containing 0.2% HCl (v/v) was used. Thus, a sample-to-solvent ratio of 1:1 (5 g of grapes/5 mL of acidiﬁed ethanol) was selected for subsequent experiments.

3.1.2. Optimization of d-SPE clean-up

The addition of anhydrous CaCl₂ or MgSO₄ was evaluated for use in the clean-up step. Anhydrous CaCl₂ was found to be more effective for all AnCS. The CaCl₂ retains the remaining water after the salting-out/partition step and increases the ionic strength of the medium. In addition, lower amounts of water and a high ionic strength favor the partition of neutral AnCS into the organic phase [27]. In this work, as a consequence, a combination of salts that were amenable for use in conjunction with CaCl₂ was used instead of a conventional salt combination (MgSO₄) for the removal of various types of interfering matrix components in the organic extracts. To optimize the purification efficiency for the clean-up step, combinations of different sorbents were evaluated. For all the analytes, significant differences were observed when sorbent mixtures containing GCB were used for the d-SPE step. This effect was especially high for all analytes. This can be explained by the fact that GCB has a mesh-like structure, and interacts easily with compounds that contain ring structures [28]. It is suitable for removing pigments and planar compounds (e.g. sterols), resulting in a decreased AnCS content. Concerning the PSA sorbent, it is a weak anion exchanger that is usually used to eliminate polar organic acids, sugars, or lipids from samples. In comparing the adsorbent combinations listed in Fig. 2 (A), the results indicate that a single PSA adsorbent has superior adsorption capacity compared to a mixed adsorbent of C18 and PSA. Therefore, PSA was chosen as the optimal sorbent and was used in subsequent experiments. To preserve the AnCS content, adding different amounts of sorbent to the extracts was evaluated. The amounts of PSA sorbents in d-SPE were...
examined within the range of 150–600 mg, and with the amount of CaCl₂ fixed at 150 mg. It was observed that by increasing the amount of PSA from 150 mg to 300 mg, the responses for most of the analytes increased (except for Dp-3-glu). This can be attributed to the presence of other nutrients, pesticides, or plant growth regulators in the extract. Therefore, a combination of 150 mg of anhydrous CaCl₂ and 150 mg of PSA was used in subsequent experiments (Fig. 2(B)).

3.2. Assessment of the UAE method

Alternatively, UAE method has been used to isolate AnCS. For grape samples, the UAE conditions were optimized to improve the AnCS extraction efficiency. In particular, the extraction time (5, 20, 35, and 50 min) and temperature (room temperature, 30 °C, 50 °C, and 70 °C) were investigated in this study. The results indicated that 5 min was the optimal extraction time for the target analytes using the UAE method (Fig. S1 (A)). During extraction processes, enhanced extraction yields are typically obtained at higher temperatures. However, increasing the temperature may also induce sample degradation, thus resulting in the loss of some AnCS. Thus, 50 °C was selected as a suitable temperature for the UAE method (Fig. S1 (B)).

3.3. Liquid chromatographic conditions

3.3.1. Stationary phase and column selection
Reverse-phase liquid chromatography is widely used for the determination of AnCS. Several mechanisms play important roles in column selectivity and include hydrophobicity, hydrogen bonding, dipole-dipole, and ion exchange. Three different stationary phases were investigated and compared in the present study, including biphenyl, F5, and C18. The specifications for the columns (2.1 mm i.d × 100 mm L, particle size 2.6 μm) were obtained from Phenomenex (Torrance, CA, USA). The use of C18 and F5 columns for the separation, identification, and quantification of AnCS has been extensively reported in the literature, but much less information is available concerning the use of biphenyl phases for the analysis of AnCS. In the past, the separation of AnCS using conventional C18 stationary phases had some shortcomings, such as unsatisfactory chromatographic efficiency and long elution runtimes [29]. The F5 stationary phases are known for their high selectivity for several classes of compounds, including tocotrienols and trimethylamine N-oxide. Unlike conventional C18 or F5, the main biphenyl structure contains two benzene rings with rotational characteristics, which is highly prone to electrophilic interactions due to the delocalized electrons in the p-orbitals above and below the planar ring. The π–π interactions caused by the overlapping p-orbitals of both rings create an attraction that may allow the solute to arrange itself over the stationary phase group via discriminating interactions, which would greatly improve the efficiency of separation for compounds such as estrogen, antibiotics, and aromatic compounds [30].

3.3.2. Mobile phase composition and the acid additive used for the separation
The analysis of AnCS in extracts that are rich in phenolic compounds is rather laborious due to their great diversity and the number of interfering
compounds. In the present study, an LC method based on biphenyl as the stationary phase for the analysis of AnCS is proposed. The developed biphenyl-LC-MS/MS method permitted the 12 AnCS derivatives to be separated within 8 min. Generally, 0.1% FA was found to be the preferred additive for the LC-MS/MS analysis. The use of a 0.1% FA gradient was used for the separation of the major AnCS component. Except for Dp, other non-polar AnCD derivatives showed distinct characteristic peaks. Nevertheless, for most of the analytes in this separation scheme, there were no prominent differences among the three types of stationary phases mentioned in Fig. S2. However, when the concentration of formic acid was increased to 1%, differences began to appear. This phenomenon could be explained based on the fact that the addition of formic acid increased the polarity of the mobile phase, resulting in a weaker elution strength and resulted in a longer retention time. There subsequent reduction in the retention time can be attributed to a competitive interaction between formic acid and the polar functional groups (e.g. −OH) of AnCS for the silanol groups attached to the stationary phase surface when excessive acid was added. The pH of the elution system was normally maintained below 2 by the addition of a small amount of formic acid. It was found that at pH below 2, the AnCS is mainly present in the flavilyum cationic form, but the stability of this form decreased with increasing pH [31]. For the chromatographic separation in this study, at pH values above 2, severe peak broadening was observed because of the slow interconversion between these species, leading to poor resolution and reduced detection limits, the above observation is illustrated in Table S5. AS shown in Fig. 3, it was found that the use of the biphenyl, Dp, resulted in a different order of retention compared to the other two columns. This is because Dp contains multiple hydroxy groups, which allow the analytes to the more easily retained on C18 and F5 compared to the biphenyl column. The different selectivity and specificity of biphenyl permits a sufficient separation to be obtained.

Many studies have recently appeared on the use of acetic acid as a mobile phase modifier [32,33]. Acetic acid was also investigated for use in biphenyl LC separation, but the results were not satisfactory, even with 3% acetic acid (Fig. S3). The reason for this is because the pKa of acetic acid (4.75) is higher than that of formic acid (pKa 3.75). Because of this, acetic acid cannot allow the AnCS to be maintained in the flavilyum form and it would not be fully retained on the column. Finally, 2% FA as a mobile phase modifier on the biphenyl column was used for AnCS separation in this study. It was found that retention times of the majority of the AnCS derivatives were between 6.5 and 9.5 min with methanol as the mobile phase in all three columns. Poor separation (low peak capacity) was also observed for some AnC derivatives and the less polar AnCD. On the other

Fig. 3. Chromatograms of 12 AnCS using biphenyl, F5, and C18 stationary phase columns with different FA concentrations.
hand, acetonitrile interrupted the retention between AnCS for the 3 stationary phases. Acetonitrile offered satisfactory results and peak capacity was increased dramatically. As such, the less polar AnCD and relatively polar AnC could be efficiently separated using acetonitrile as the mobile phase.

### 3.4. Mass spectrometry analysis

For electrospray ionization, the positive ion mode produced higher precursor ion signal intensities than the negative ion mode for all of the AnCS compounds. Some fragmentation patterns by collisional induced dissociation (CID) were observed for the 12 types AnCS, they were illustrated in supplementary data (Fig. S4-Fig. S15). All of the target AnCS structures are relatively rigid, and bond cleavage requires high collisional energy; especially for the closed-shell cation fragments obtained by hydrogen rearrangements and fragments induced by charge dissociation. Moreover, for some AnCS derivatives, resonance acylium ions are produced. In the case of the flavium form of AnCS, these fragmentation processes generally predominate over fragmentations related to the rupture of the B or C-ring bonds, and the majority occur on the C-ring (Table 1). In addition to the indicated fragmentation pathways (loss of functional group), the product ion spectra also displayed fragments corresponding to the loss of CH₃, CH₂OH, C=O, a hydrogen atom, or radicals from the respective AnCS derivative by CID. The product ion spectra for hydroxylated AnCD (Cy, Dp, and Pl) showed the molecular ion as the characteristic peak, whilst the spectra for the methoxylated AnCD (Mv, Pe, and Pt) forms also provided relatively intense peaks for the molecular ions [34]. Glycosylated AnC derivatives are known to fragment via the loss of an anhydrous glycoside (m/z 162) moiety, a characteristic signal produced from Cy-3-glu (m/z 449 > 287), Dp-3-glu (m/z 465 > 303), or Mv-3-glu (m/z 493 > 331). However, in comparing the mass spectra of the anhydro form of AnC with AnCD, both showed similar fragmentation patterns but the intensities of the fragment ions were different.

### 3.5. Analytical method validation

The validation results for the developed QuEChERS are summarized in Table 2a (a) and Table S6. Linearity was in the range from 0.5 to 500 ng mL⁻¹ with the R² value around 0.9963 to 0.9992 for all of the analytes. The LOD and LOQ were 0.125 ng mL⁻¹ and 0.5 ng mL⁻¹, respectively. Trueness and precision were evaluated in recovery studies by triplicate analysis on three successive days. The AnCS was spiked at levels of 0.5 (low), 1 (medium), and 5 (high) μg mL⁻¹, and the recoveries were then evaluated. The recoveries of the 12 AnCS derivatives were 88.5–113.7% in the case of the QuEChERS. The intra- and inter-day precision was less than 4.42% and 11.8% for all of the investigated compounds. The slopes of the calibration graph obtained with matrix-matched standards were compared with those obtained with solvent-based standards, the matrix to solvent slope ratios was then calculated, and the ME% was obtained for each of the analytes. For QuEChERS, the ME% values for only four types of AnCS (Cy, Dp, Mv, and Pl-3-glu) exceeded ±20%, and the others showed only minor matrix effects. The analytical performance of UAE is shown in Table 2b and Table 57. For linearity was in the range from 5–500 ng mL⁻¹ with R² values from 0.9937–0.9987, and the LOD and LOQ were 1 ng mL⁻¹ and 5 ng mL⁻¹, respectively. Recoveries

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### Table 2a. Analytical performance of the QuEChERS method for the quantification of AnCS.

| Compound | Linear equation | Linear range (ng mL⁻¹) | LOD (ng mL⁻¹) | LOQ (ng mL⁻¹) | ME% |
|----------|-----------------|------------------------|---------------|---------------|-----|
| Cy       | \( y = 0.0163x - 0.0039 \) | 5–500                  | 1             | 5             | -29.1% |
| Dp       | \( y = 0.0131x - 0.0127 \) | 5–500                  | 1             | 5             | -24.7% |
| Mv       | \( y = 0.0228x + 0.0159 \) | 0.5–500                | 0.25          | 0.5           | +32.5% |
| Pe       | \( y = 0.0167x - 0.0036 \) | 0.5–500                | 0.25          | 0.5           | -10.2% |
| Pl       | \( y = 0.0109x + 0.0108 \) | 1–500                  | 0.5           | 1             | -14.2% |
| Pt       | \( y = 0.0122x - 0.0153 \) | 5–500                  | 1             | 5             | -83.3% |
| Cy-3-glu | \( y = 0.0014x + 0.0166 \) | 0.5–500                | 0.25          | 0.5           | -17.6% |
| Dp-3-glu | \( y = 0.0098x + 0.0011 \) | 0.5–500                | 0.25          | 0.5           | -18.3% |
| Mv-3-glu | \( y = 0.0203x + 0.0147 \) | 0.5–500                | 0.125         | 0.5           | +18.7% |
| Pe-3-glu | \( y = 0.0098x + 0.0054 \) | 0.5–500                | 0.125         | 0.5           | -10.1% |
| Pl-3-glu | \( y = 0.0114x - 0.0136 \) | 0.5–500                | 0.25          | 0.5           | -21.4% |
| Pt-3-glu | \( y = 0.0046x + 0.3244 \) | 0.5–500                | 0.25          | 0.5           | -11.5% |

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**a** Coefficient of determination.  
**b** Limit of detection (S/N ≥ 3).  
**c** Limit of quantification (S/N ≥ 10).  
**d** Matrix effect.
and precisions were also evaluated using an experimental setup identical to that for QuEChERS. The recoveries were 82.4—117.6%, and the intra- and interday precision was less than 4.98% and 14.3%, respectively. Only Pl, Dp-3-glu, and Pe-3-glu displayed minor matrix effects in the case of the UAE method but significant matrix effects were found for the other compounds. The validation results indicate that the developed QuEChERS method was superior compared to the conventional UAE, and provided an efficient pretreatment method for the grape samples examined in this study.

3.6. Application for samples analysis

The validated QuEChERS method was applied to the determination of AnCS in commercial grape samples from different varieties and origins. The data for the analyzed samples are summarized in Table 3. The total contents of AnCS in these grapes ranged from 3.31—23.9 mg/100g. Among them, detected contents of AnCS in Red Globe and Moon Drops were 4.66 ± 1.39 and 20.0 ± 3.58 mg/100g, respectively. For all samples, the results showed that Mv-3-glu was the most abundant component, followed by Pe-3-glu, values that are in agreement with data reported in previous studies. Some interesting findings were reported by Wang et al. [35] and HE et al. [36], who proposed that the trace existence of Pl-3-glu was confirmed in some grapes and grape products. In this study, trace amounts of AnCS were also quantified using the proposed QuEChERS treatment coupled with the developed biphenyl-LC-MS/MS method, which confirms that

Table 2b. Analytical performance of the UAE for determination of AnCS.

| Compound | Linear equation | Linear range (ng mL⁻¹) | LODb | LOQc | ME%d |
|----------|-----------------|-------------------------|-----|-----|-----|
| Cy       | y = 0.0155 x − 0.0067 | 0.9945 | 10—500 | 5 | 10 | −32.6% |
| Dp       | y = 0.0125 x + 0.0021  | 0.9937 | 50—500 | 10 | 50 | −28.1% |
| Mv       | y = 0.02145 x − 0.0065 | 0.9946 | 5—500 | 1 | 5 | +24.9% |
| Pe       | y = 0.0148 x + 0.0099  | 0.9979 | 10—500 | 5 | 10 | −20.4% |
| Pl       | y = 0.0102 x − 0.0183  | 0.9965 | 10—500 | 5 | 10 | −19.7% |
| Pt       | y = 0.018 x − 0.0247   | 0.9961 | 10—500 | 1 | 10 | +35.3% |
| Cy-3-glu | y = 0.0022 x + 0.0109  | 0.9978 | 10—500 | 5 | 10 | −29.4% |
| Dp-3-glu | y = 0.0103 x − 0.0052  | 0.9983 | 10—500 | 5 | 10 | −14.2% |
| Mv-3-glu | y = 0.021 x − 0.0076   | 0.9987 | 5—500 | 1 | 5 | +22.8% |
| Pe-3-glu | y = 0.0127 x + 0.0028  | 0.9953 | 10—500 | 5 | 10 | +16.5% |
| Pl-3-glu | y = 0.0108 x − 0.0252  | 0.9962 | 50—500 | 10 | 50 | −25.5% |
| Pt-3-glu | y = 0.0041 x + 0.0016  | 0.9974 | 5—500 | 5 | 10 | −21.1% |

* Coefficient of determination.

a Limit of detection (S/N ≥ 3).

b Limit of quantification (S/N ≥ 10).

c Matrix effect.

d

Table 3. Quantification of AnCS (mg/100 g) in commercial grapes by the developed QuEChERS and UHPLC-MS/MS.

| Code | Mv  | Pe  | Pt  | Cy-3-glu | Dp-3-glu | Mv-3-glu | Pe-3-glu | Pl-3-glu | Pt-3-glu | Total (mg/100g) |
|------|-----|-----|-----|---------|---------|---------|---------|---------|---------|-----------------|
| G1   | 0.35 ± 0.07 | 0.04 ± 0.02 | < LOQa | 0.11 ± 0.08 | 0.05 ± 0.02 | 3.96 ± 0.11 | 0.85 ± 0.04 | < LODb | 0.69 ± 0.14 | 6.05 ± 0.48 |
| G2   | 0.07 ± 0.03 | 0.04 ± 0.01 | 0.05 ± 0.01 | 0.62 ± 0.11 | 0.02 ± 0.01 | 3.95 ± 0.13 | 1.82 ± 0.05 | < LODc | 0.42 ± 0.07 | 6.97 ± 0.42 |
| G3   | 0.18 ± 0.02 | 0.05 ± 0.01 | < LODd | 0.96 ± 0.13 | 0.02 ± 0.01 | 3.07 ± 0.04 | 2.03 ± 0.03 | < LODe | 0.06 ± 0.02 | 3.67 ± 0.26 |
| G4   | 0.16 ± 0.03 | 0.03 ± 0.02 | < LOFe | 0.08 ± 0.02 | 0.03 ± 0.01 | 4.88 ± 0.09 | 1.74 ± 0.11 | < LODf | 0.23 ± 0.05 | 7.15 ± 0.33 |
| G5   | 1.17 ± 0.11 | 0.06 ± 0.03 | < LOFg | 0.11 ± 0.04 | 0.04 ± 0.02 | 2.34 ± 0.12 | 1.39 ± 0.12 | < LODh | 0.88 ± 0.09 | 5.99 ± 0.53 |
| G6   | < LODi | < LODj | < LOKe | 0.16 ± 0.07 | < LODl | 2.06 ± 0.13 | 0.75 ± 0.08 | < LODm | 0.34 ± 0.01 | 3.31 ± 0.29 |
| G7   | 0.83 ± 0.05 | 0.02 ± 0.01 | 0.04 ± 0.02 | 0.19 ± 0.11 | 0.06 ± 0.03 | 2.64 ± 0.07 | 1.26 ± 0.12 | < LODn | 0.72 ± 0.07 | 5.72 ± 0.47 |
| G8   | < LOQo | 0.09 ± 0.18 | < LODp | 0.12 ± 0.05 | 0.03 ± 0.01 | 1.85 ± 0.15 | 1.04 ± 0.04 | < LODq | 0.49 ± 0.11 | 3.62 ± 0.54 |
| G9   | 0.36 ± 0.05 | 0.05 ± 0.02 | 0.03 ± 0.02 | 0.97 ± 0.12 | 0.05 ± 0.03 | 9.08 ± 0.09 | 4.02 ± 0.07 | 4.75 ± 10⁻³ | 2.42 ± 0.16 | 16.9 ± 0.56 |
| G10  | 0.39 ± 0.14 | 0.08 ± 0.02 | 0.16 ± 0.05 | 0.82 ± 0.19 | 0.03 ± 0.01 | 9.70 ± 0.06 | 5.21 ± 0.06 | 1.70 ± 10⁻³ | 2.76 ± 0.08 | 19.1 ± 0.61 |
| G11  | 0.53 ± 0.12 | 0.12 ± 0.09 | 0.14 ± 0.07 | 1.04 ± 0.15 | 0.08 ± 0.02 | 9.91 ± 0.07 | 8.59 ± 0.11 | 2.16 ± 10⁻³ | 3.56 ± 0.13 | 23.9 ± 0.76 |
| G12  | 0.22 ± 0.05 | 0.06 ± 0.01 | < LOQr | 0.58 ± 0.11 | 0.04 ± 0.02 | 3.31 ± 0.02 | 1.46 ± 0.09 | < LODs | 0.67 ± 0.14 | 6.34 ± 0.44 |

a Below limit of quantification.

b Limit of detection. The grapes species (sample name, original source) was follow: G1 (Beauty, Chile), G2 (Autumn Royal, Peru), G3 (Marroo, South Africa), G4 (Black Globe, USA), G5 (Red Globe, Chile), G6 (Red Globe, Peru), G7 (Red Globe, South Africa), G8 (Red Globe, USA), G9 (Moon Drops, Peru), G10 (Moon Drops, South Africa), G11 (Moon Drops, USA), G12 (Kyoho, Taiwan).
this method provides an effective method for the detection and analysis of trace substances in grapes.

4. Conclusion

In the present study, a QuEChERS extraction method was used for the first time for the quantification of anthocyanosides. Compared with ultrasound-assisted extraction, the developed QuEChERS method showed superior sensitivity for the determination of anthocyanosides in grape samples. The extraction and clean-up procedures were simple and efficient, allowing the grape extracts to be sufficiently purified. Furthermore, a novel liquid chromatography method was developed for the separation of anthocyanosides in grape samples. For chromatographic separation, a biphenyl stationary phase was found to be an attractive alternative to conventional C18 reversed phases. The biphenyl column enabled the separation of 12 type mono- and un-glycosylation anthocyanosides. Finally, with the fast elution and short analysis time, the developed method is reliable for the routine analyses of grapes extracts.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Lun-Chi Yang: Conceptualization, Methodology, Validation, Investigation, Data curation, Writing - original draft, Writing - review & editing.

Sung-Fang Chen: Resources, Supervision, Conceptualization, Writing - Review & Editing, Project administration.

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Appendix.

Table S1. Sample information for commercially available fresh grapes.

| Code | Sample name | Varieties | Source |
|------|-------------|-----------|--------|
| G1   | Beauty      | Sugrathirteen variety | Chile  |
| G2   | Autumn Royal| Vitis vinifera | Peru   |
| G3   | Marroo      | Vitis vinifera | South Africa |
| G4   | Black Globe | Vitis vinifera 'Black Globe' | USA |
| G5   | Red Globe   | Vitis vinifera 'Red Globe' | Chile |
| G6   | Red Globe   | Vitis vinifera 'Red Globe' | Peru |
| G7   | Red Globe   | Vitis vinifera 'Red Globe' | South Africa |
| G8   | Red Globe   | Vitis vinifera 'Red Globe' | USA |
| G9   | Moon Drops  | Vitis vinifera | Peru |
| G10  | Moon Drops  | Vitis vinifera | South Africa |
| G11  | Moon Drops  | Vitis vinifera | USA |
| G12  | Kyoho       | Vitis vinifera x Vitis labrusca | Taiwan |

Table S2. Retention time (min) of AnCS in different material of stationary phases in 0.1% FA.

| Peak no. | Compound name | Biphenyl (1% FA) | F5 (1% FA) | C18 (1% FA) | Biphenyl (2% FA) | F5 (2% FA) | C18 (2% FA) | Biphenyl (3% FA) | F5 (3% FA) | C18 (3% FA) |
|----------|---------------|------------------|------------|-------------|------------------|------------|-------------|------------------|------------|-------------|
| 1        | Dp-3-glu      | 2.13             | 2.80       | 2.19        | 1.98             | 2.64       | 2.13        | 1.92             | 2.55       | 1.99        |
| 2        | Cy-3-glu      | 3.01             | 3.33       | 2.67        | 2.85             | 3.18       | 2.63        | 2.75             | 3.09       | 2.52        |
| 3        | Pt-3-glu      | 3.34             | 3.86       | 3.15        | 3.18             | 3.76       | 3.14        | 3.06             | 3.68       | 3.03        |
| 4        | Dp            | 3.42             | 4.50       | 3.49        | 3.20             | 4.29       | 3.41        | 3.12             | 4.17       | 3.28        |
| 5        | Pl-3-glu      | 3.78             | 3.90       | 3.18        | 3.63             | 3.75       | 3.17        | 3.54             | 3.67       | 3.09        |
| 6        | Pe-3-glu      | 4.27             | 4.42       | 3.65        | 4.15             | 4.32       | 3.66        | 4.08             | 4.25       | 3.61        |
| 7        | Mv-3-glu      | 4.46             | 4.82       | 4.01        | 4.38             | 4.76       | 4.06        | 4.32             | 4.71       | 4.02        |
| 8        | Cy            | 4.73             | 5.49       | 4.35        | 4.51             | 5.29       | 4.33        | 4.40             | 5.17       | 4.25        |
| 9        | Pt            | 5.20             | 6.04       | 4.92        | 5.00             | 5.88       | 4.93        | 4.89             | 5.78       | 4.84        |
| 10       | Pl            | 6.01             | 6.48       | 5.22        | 5.82             | 6.31       | 5.26        | 5.74             | 6.19       | 5.23        |
| 11       | Pe            | 6.76             | 7.10       | 5.81        | 6.60             | 6.97       | 5.86        | 6.52             | 6.89       | 5.86        |
| 12       | Mv            | 7.00             | 7.51       | 6.23        | 6.85             | 7.40       | 6.30        | 6.77             | 7.34       | 6.28        |

* Take retention time of biphenyl stationary phase as representation.

Table S3. Retention time (min) of AnCS in 3 different stationary phases and percentage of FA used as mobile phase modifier.

| Peak no. | Compound name | Biphenyl (1% FA) | F5 (1% FA) | C18 (1% FA) | Biphenyl (2% FA) | F5 (2% FA) | C18 (2% FA) | Biphenyl (3% FA) | F5 (3% FA) | C18 (3% FA) |
|----------|---------------|------------------|------------|-------------|------------------|------------|-------------|------------------|------------|-------------|
| 1        | Dp-3-glu      | 2.13             | 2.80       | 2.19        | 1.98             | 2.64       | 2.13        | 1.92             | 2.55       | 1.99        |
| 2        | Cy-3-glu      | 3.01             | 3.33       | 2.67        | 2.85             | 3.18       | 2.63        | 2.75             | 3.09       | 2.52        |
| 3        | Pt-3-glu      | 3.34             | 3.86       | 3.15        | 3.18             | 3.76       | 3.14        | 3.06             | 3.68       | 3.03        |
| 4        | Dp            | 3.42             | 4.50       | 3.49        | 3.20             | 4.29       | 3.41        | 3.12             | 4.17       | 3.28        |
| 5        | Pl-3-glu      | 3.78             | 3.90       | 3.18        | 3.63             | 3.75       | 3.17        | 3.54             | 3.67       | 3.09        |
| 6        | Pe-3-glu      | 4.27             | 4.42       | 3.65        | 4.15             | 4.32       | 3.66        | 4.08             | 4.25       | 3.61        |
| 7        | Mv-3-glu      | 4.46             | 4.82       | 4.01        | 4.38             | 4.76       | 4.06        | 4.32             | 4.71       | 4.02        |
| 8        | Cy            | 4.73             | 5.49       | 4.35        | 4.51             | 5.29       | 4.33        | 4.40             | 5.17       | 4.25        |
| 9        | Pt            | 5.20             | 6.04       | 4.92        | 5.00             | 5.88       | 4.93        | 4.89             | 5.78       | 4.84        |
| 10       | Pl            | 6.01             | 6.48       | 5.22        | 5.82             | 6.31       | 5.26        | 5.74             | 6.19       | 5.23        |
| 11       | Pe            | 6.76             | 7.10       | 5.81        | 6.60             | 6.97       | 5.86        | 6.52             | 6.89       | 5.86        |
| 12       | Mv            | 7.00             | 7.51       | 6.23        | 6.85             | 7.40       | 6.30        | 6.77             | 7.34       | 6.28        |

* Take retention time of biphenyl stationary phase as representation.
Table S4. Retention time (min) of AnCS in 3 different stationary phases in 3% Acet. used as mobile phase modifier.

| Peak no. | Compound name | Biphenyl | F5 | C18 |
|----------|---------------|----------|----|-----|
| 1        | Dp-3-glu      | 1.39     | 1.80 | 1.16 |
| 2        | Cy-3-glu      | 2.11     | 2.33 | 1.45 |
| 3        | Pt-3-glu      | 2.33     | 2.77 | 1.78 |
| 4        | Dp            | 2.56     | 3.58 | 2.18 |
| 5        | Pt-3-glu      | 2.85     | 2.90 | 1.86 |
| 6        | Pe-3-glu      | 3.28     | 3.36 | 2.24 |
| 7        | Mv-3-glu      | 3.38     | 3.69 | 2.52 |
| 8        | Cy            | 3.84     | 4.58 | 2.93 |
| 9        | Pt            | 4.29     | 5.17 | 3.53 |
| 10       | Pe            | 5.15     | 5.59 | 3.73 |
| 11       | Mv            | 5.89     | 6.24 | 4.35 |
| 12       |               | 6.12     | 6.69 | 4.79 |

*a Take retention time of biphenyl stationary phase as representation.

Table S5. The peak capacity comparison of three stationary phase.

| Biphenyl | F5 | C18 |
|----------|----|-----|
| 0.1% FA (pH ≈ 2.6) | 37.2 | 39.5 | 28.1 |
| 1% FA (pH ≈ 2.2)   | 48.1 | 51.9 | 39.2 |
| 2% FA (pH = 1.9)    | 54.6 | 58.5 | 45.8 |
| 3% FA (pH ≈ 1.7)    | 63.7 | 67.3 | 53.6 |
| 3% AcOH (pH ≈ 2.5)  | 41.3 | 43.1 | 27.4 |

*The specification was 2.1 × 100 mm, 2.6 μm particle size.

Table S6. Recoveries and precisions (intra- and inter-day) of the QuEChERS for 12 AnCS.

| Compound Spiked level (μg mL⁻¹) | R%a | Precision (%RSD)b |
|--------------------------------|-----|------------------|
|                                |     | Intra-day (n = 3) | Inter-day (n = 9) |
| Cy                              | 0.5 | 94.6 | 2.34 | 8.81 |
|                                | 1   | 97.9 | 2.28 | 5.62 |
|                                | 5   | 93.7 | 1.27 | 6.13 |
| Dp                              | 0.5 | 104.8 | 3.52 | 11.2 |
|                                | 1   | 103.5 | 2.73 | 10.4 |
|                                | 5   | 94.2 | 1.39 | 9.26 |
| Mv                              | 0.5 | 91.4 | 3.14 | 10.1 |
|                                | 1   | 105.3 | 2.32 | 7.53 |
|                                | 5   | 109.2 | 1.86 | 6.91 |
| Pe                              | 0.5 | 93.2 | 4.24 | 9.82 |
|                                | 1   | 102.9 | 3.52 | 10.9 |
|                                | 5   | 108.5 | 0.784 | 6.33 |
| Pt                              | 0.5 | 88.5 | 2.73 | 11.7 |
|                                | 1   | 98.8 | 1.91 | 9.44 |
|                                | 5   | 98.3 | 0.942 | 8.17 |
| Cy-3-glu                       | 0.5 | 106.4 | 2.6 | 7.45 |
|                                | 1   | 93.2 | 4.1 | 6.82 |
|                                | 5   | 103.5 | 3.5 | 5.66 |
| Dp-3-glu                       | 0.5 | 96.2 | 4.3 | 10.3 |
|                                | 1   | 97.8 | 0.841 | 7.47 |
|                                | 5   | 105.3 | 0.623 | 5.24 |
| Mv-3-glu                       | 0.5 | 96.8 | 4.18 | 8.15 |
|                                | 1   | 113.7 | 3.44 | 6.32 |
|                                | 5   | 102.4 | 2.86 | 6.78 |

*a Recoveries.  
*b Relative standard deviation of precision values.

Table S6. (continued)

| Compound Spiked level (μg mL⁻¹) | R%a | Precision (%RSD)b |
|--------------------------------|-----|------------------|
|                                |     | Intra-day (n = 3) | Inter-day (n = 9) |
| Mv-3-glu                       | 0.5 | 90.6 | 3.15 | 6.68 |
|                                | 1   | 105.3 | 3.22 | 7.59 |
|                                | 5   | 106.7 | 1.27 | 5.86 |
| Pe-3-glu                       | 0.5 | 106.5 | 3.39 | 7.91 |
|                                | 1   | 92.4 | 3.48 | 10.4 |
|                                | 5   | 90.5 | 2.46 | 8.32 |
| Pl-3-glu                       | 0.5 | 94.6 | 4.41 | 8.26 |
|                                | 1   | 104.9 | 2.35 | 9.55 |
|                                | 5   | 108.4 | 3.72 | 5.37 |
| Pt-3-glu                       | 0.5 | 98.3 | 3.53 | 7.14 |
|                                | 1   | 106.8 | 2.67 | 7.36 |
|                                | 5   | 104.2 | 0.912 | 6.82 |

*a Recoveries.  
*b Relative standard deviation of precision values.

Table S7. Recoveries and precision (intra- and inter-day) of the UAE for 12 AnCS.

| Compound Spiked level (μg mL⁻¹) | R%a | Precision (%RSD)b |
|--------------------------------|-----|------------------|
|                                |     | Intra-day (n = 3) | Inter-day (n = 9) |
| Cy                             | 0.5 | 88.2 | 2.14 | 14.2 |
|                                | 1   | 104.4 | 2.73 | 8.94 |
|                                | 5   | 114.8 | 0.637 | 7.18 |
| Dp                             | 0.5 | 89.6 | 3.95 | 8.45 |
|                                | 1   | 105.4 | 1.82 | 9.72 |
|                                | 5   | 104.3 | 1.43 | 6.83 |
| Mv                             | 0.5 | 94.8 | 4.28 | 9.54 |
|                                | 1   | 98.3 | 4.15 | 10.2 |
|                                | 5   | 109.7 | 1.71 | 8.69 |
| Pe                             | 0.5 | 111.4 | 3.13 | 8.26 |
|                                | 1   | 95.1 | 3.93 | 8.52 |
|                                | 5   | 97.4 | 0.671 | 6.77 |
| Pt                             | 0.5 | 108.7 | 3.89 | 15.6 |
|                                | 1   | 109.6 | 3.46 | 9.41 |
|                                | 5   | 113.5 | 2.54 | 10.8 |
| Cy-3-glu                       | 0.5 | 95.6 | 3.22 | 10.1 |
|                                | 1   | 90.3 | 3.64 | 9.42 |
|                                | 5   | 109.5 | 1.13 | 7.48 |
| Dp-3-glu                       | 0.5 | 110.9 | 4.97 | 8.53 |
|                                | 1   | 98.1 | 3.42 | 6.94 |
|                                | 5   | 92.6 | 2.93 | 5.56 |
| Mv-3-glu                       | 0.5 | 109.3 | 2.35 | 11.1 |
|                                | 1   | 117.6 | 2.43 | 7.85 |
|                                | 5   | 98.4 | 1.28 | 7.24 |
| Pe-3-glu                       | 0.5 | 101.9 | 3.42 | 9.33 |
|                                | 1   | 98.9 | 3.61 | 5.11 |
|                                | 5   | 96.3 | 1.76 | 12.5 |
| Pl-3-glu                       | 0.5 | 94.1 | 2.83 | 9.66 |
|                                | 1   | 105.8 | 2.72 | 7.29 |
|                                | 5   | 82.4 | 4.19 | 4.86 |
| Pt-3-glu                       | 0.5 | 107.4 | 4.84 | 10.3 |
|                                | 1   | 92.5 | 3.15 | 7.91 |
|                                | 5   | 109.2 | 2.73 | 6.64 |

*a Recoveries.  
*b Relative standard deviation of precision values.
Fig. S1. Assessed of extraction time (A), extraction temperatures (B) for UAE.

Fig. S2. The chromatograms of 12 AnCS in different stationary phases with 0.1% FA as mobile phase modifier.

Fig. S3. The chromatogram of 12 AnCS in different material of stationary phases in 3% Acet.
Fig. S4. Fragmentation for cyanidin (Cy).

Fig. S5. Fragmentation for delphinidin (Dp).
Fig. S6. Fragmentation for malvidin (Mv).

| m/z  | Product Ion                  |
|------|------------------------------|
| 299  | [M-CH₃OH]⁺                   |
| 287  | [M-CH₂-CO-H]⁺                |

Fig. S7. Fragmentation for peonidin (Pe).

| m/z  | Product Ion                  |
|------|------------------------------|
| 286  | [M-CH₂]⁺                     |
| 258  | [M-CH₃-CO]⁺                  |
| 230  | [M-CH₂-2CO]⁺                 |
| 213  | [M-CH₂OH-2CO]⁺               |
| 201  | [M-CH₂-3COH]⁺                |
Fig. S8. Fragmentation for pelargonidin (Pl).

Fig. S9. Fragmentation for petunidin (Pt).
Fig. S10. Fragmentation for cyanidin-3-O-glucoside (Cy-3-glu).

| m/z   | Product Ion                |
|-------|----------------------------|
| 213   | [M-Glu+H₂O-2CO]⁺           |
| 185   | [M-Glu+H₂O-3CO]⁺           |

Fig. S11. Fragmentation for delphinidin-3-O-glucoside (Dp-3-glu).

| m/z   | Product Ion                |
|-------|----------------------------|
| 303   | [M-Glu]⁺                   |
| 257   | [M-Glu+H₂O-CO]⁺            |
| 229   | [M-Glu+H₂O-2CO]⁺           |
| 465.2 |                            |
Fig. S12. Fragmentation for malvidin-3-O-glucoside (Mv-3-glu).

Fig. S13. Fragmentation for peonidin-3-O-glucoside (Pe-3-glu).
Fig. S14. Fragmentation for pelargonidin-3-O-glucoside (Pl-3-glu).

Fig. S15. Fragmentation for petunidin-3-O-glucoside (Pt-3-glu).

Fig. S16. The chromatogram of Pl-3-glu in the sample by developed QuEChERS.
References

[1] Alam MA, Islam P, Subhan N, Rahman MM, Khan F, Burrows GE, et al. Potential health benefits of anthocyanins in oxidative stress related disorders. Phytochemistry Rev 2021;20:705–49.

[2] Wallace TC. Anthocyanins in cardiovascular disease. Adv Nutr 2011;2:1–7.

[3] Oliveira J, Alinhno da Silva M, Teixeira Nr, De Freitas V, Salas E. Screening of anthocyanins and anthocyanin-derived pigments in red wine grape pomace using LC-DAD/MS and MALDI-TOF techniques. J Agric Food Chem 2015;63: 7636–44.

[4] Denev P, Ciz M, Ambrozova G, Lojek A, Yanakieva I, Stajnbaher D, Schenck F. Fast dispersive solid-phase extraction/partitioning and easy multiresidue method employing acetonitrile for residue analysis of 122 pesticides, 20 mycotoxins in cereals. J Chromatogr A 2015;1382.

[5] Jiao G. Extraction of anthocyanins from haskab berry pulp using supercritical carbon dioxide: influence of co-solvent. J Food Drug Anal 2018:98:237–44.

[6] Ryu D, Koh E. Optimization of ultrasound-assisted extraction of anthocyanins and phenolic compounds from black soybeans (Glycine max L.). Food Anal Methods 2019;12:1055–61.

[7] Jafari SM, Mahdavee Khazaei K, Assadpour E. Production of a natural color through microwave-assisted extraction of saffron tepal’s anthocyanins. Food Sci Nutr 2019;7:1438–45.

[8] Anastassiades M, Lehotay S, Jafari SM, Mahdavee Khazaei K, Assadpour E. Production of a natural color through microwave-assisted extraction of saffron tepal’s anthocyanins. Food Sci Nutr 2019;7:1438–45.

[9] Matsyšk S, Liebisch G. Quantification of steroid hormones in human serum by liquid chromatography-high resolution tandem mass spectrometry. J Chromatogr A 2017;1526: 112–8.

[10] Montiel-León JM, Duy SV, Munoz G, Verner M-A, Hendawi MY, Moya H, et al. Occurrence of pesticides in fruits and vegetables from organic and conventional agriculture by QuEChERS extraction liquid chromatography-tandem mass spectrometry. Food Control 2019;109:15–25.

[11] Burrows GE, et al. Potential health benefits of anthocyanins and evaluation of their antioxidative properties. Food Food Compos Anal 2019;84:103314.

[12] Khoo HE, Azlan A, Tang ST, Lim SM. Anthocyanidins and anthocyanins: colored pigments as food, pharmaceutical and nutraceutical ingredients. J Agric Food Chem 2020;68:4657–69.
ingredients, and the potential health benefits. Food Nutr Res 2017;61:1361779.

[32] Brand MH, Connolly BA, Levine LH, Richards JT, Shine SM, Spencer LE. Anthocyanins, total phenolics, ORAC and moisture content of wild and cultivated dark-fruited Aronia species. Sci Hortic 2017;224:332–42.

[33] Peanparkdee M, Patrawart J, Iwamoto S. Effect of extraction conditions on phenolic content, anthocyanin content and antioxidant activity of bran extracts from Thai rice cultivars. J Cereal Sci 2019;86:86–91.

[34] Alberts P, Stander MA, de Villiers A. Advanced ultra high pressure liquid chromatography–tandem mass spectrometric methods for the screening of red wine anthocyanins and derived pigments. J Chromatogr A 2012;1235:92–102.

[35] Wang H, Race EJ, Shrikhande AJ. Characterization of anthocyanins in grape juices by ion trap liquid Chromatography–Mass spectrometry. J Agric Food Chem 2003;51:1839–44.

[36] He F, He JJ, Pan QH, Duan CQ. Mass-spectrometry evidence confirming the presence of pelargonidin-3-O-glucoside in the berry skins of Cabernet Sauvignon and Pinot Noir (Vitis vinifera L.). Aust J Grape Wine Res 2010;16:464–8.