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Quantitative Analysis of Anthocyanins in Grapes by UPLC-Q-TOF MS Combined with QAMS

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Abstract: A method for quantifying the anthocyanins in grapes was firstly developed by ultra-high performance liquid chromatography-quadrupole-time of flight mass spectrometry (UPLC-Q-TOFMS) combined with quantitative analysis of multi-components by single marker (QAMS). A total of 10 main anthocyanins were analyzed by using peonidin 3-*O*-glucoside as the reference standard. The accuracy of this method was evaluated by an established and validated external standard quantification method with 10 reference compounds. The standard method difference (SMDs) of the quantification results between QAMS and the external standard methodwasless than 15%. Furthermore, the QAMS method was used to analyzefour batches of grapes and the data was compared with those obtained using the external standard method. No significant difference wasobtained in the results obtained by both methods. These results indicated that the QAMS method could accurately determine the anthocyanins in grapes. This method can provide a basis to address the absence of reference standards for analyzing anthocyanins in other foods.

Keywords: anthocyanins; grapes; UPLC-Q-TOFMS; QAMS

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1. Introduction

Grapes are recognized as one of the most important commercial fruits worldwide [1]. Besides having adelightful flavor, they possess abundant nutrients and bioactive compounds [2]. In particular, a high content of anthocyanins, reaching up to 2300 mg/kg (fresh weight), isfound in red grapes [3]. Anthocyanins belong to water-soluble flavonoid-type polyphenols, which mainly exist in vacuoles of grape skin cells as free and acylated 3-*O*-glycosides derivatives [4]. Mostanthocyanin aglycones are based on anthocyanidins including cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin, which share a 2-phenylbenzopyrilium skeleton hydroxylated in the 4′, 5′, and 7 positions [5]. The presence of anthocyanins is responsible for the sensory attributes of grapes, including aroma, taste, mouthfeel, and color [4]. Apart from their organoleptic properties, anthocyanins also have a variety of unique biological features that can promote human health. Some studies have proved that anthocyanins exhibited antioxidant [6], anti-tumor [7], anti-inflammatory [8], anti-diabetic [9], anti-obesity [10], anti-cardiovascular [11], and neuroprotective [12] properties. Accordingly, these compounds have aroused appreciable attention and have been the focus of many studies [2,13]. Therefore, a reliable method to quantify multiple bioactive anthocyanins in grapes is essential for their comprehensive quality control and improved utilization. However, despite the quantitative analyses of anthocyanins in grapes thathavebeen reported [14–17], most of these studies used the area

normalization method to determine the chemical composition, which isa semi-quantitative procedure based on the chromatographic separationof analytes and a consistent detector response. The more commonly used external standard methods needstandards for each chemical. It may be difficult to obtain due to a limited number and the high cost of commercially available anthocyanin standards. Considering these reasons, an accurate and sensitive method is necessary to solve the major bottleneck of reference compounds absence in the quantitative analysis of anthocyanins in grapes.

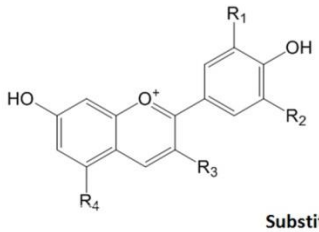
In the absence of reference standards, the quantitative analysis of complex components in foods is difficult. To tackle this urgency, a quantification method, quantitative analysis of multi-components by single marker (QAMS), has been first proposed in 2006 [18]. QAMS method is a relatively mature method to simultaneously detect the contents of multi-components in the sample, in which only one reference standard would be needed [19]. In QAMS method, each compoundwas quantified directly or calculated by a relative correction factor (RCF). QAMS can not only improve environmental friendliness, but decrease the operational complexity and experiment cost. This method has been widely used to analyze the components of Chinese herbal medicines [20,21], metabolites of aflatoxin [22], walnut leaves [23], and green tea extracts [24], and has been adopted by some national pharmacopoeia such as China, United States, and Europe [22]. Usually, QAMS was combined with high-performance liquid chromatography (HPLC) coupled with ultraviolet detection (UV) and/or mass spectrometry (MS) [24,25]. HPLC-UV has been proven to be an effective method for the multi-component analysis in plant-derived foods based on its good separation ability. However, its application can be limited because of low sensitivity, complex matrix interference, and the characterization of compounds only by the relative retention time [23]. Comparatively, HPLC-MS has advantages of high accuracy, sensitivity, and high separation efficiencyto demonstrate trace detection ability [22], and provides molecular weight, characteristic fragment ions and retention time to ensure compound specificity [19]. Therefore, QAMS combined with HPLC-MS could be a high-effective method to quantify anthocyanins in grapes.

Due to complex anthocyanins existing in grapes, it is challenging to accurately and comprehensively analyze the anthocyanins of grapes. In this study, UPLC-Q-TOF MS was used to qualitatively screen the target anthocyanins in grapes through the molecular mass and retention time. The RCFs of QAMS were then determined by different calculation formulas and reference standards. Moreover, QAMS was used to carry out the quantitative analysis and the results were compared with those obtained by the external standard method. Finally, a sensitive and reliable QAMS coupled with the UPLC-Q-TOFMS technique was developed to quantify 10 main anthocyanins in grapes. To the best of our knowledge, using UPLC-Q-TOFMS coupled with the QAMS method for analysis of the anthocyanins in grapes has not been reported yet.

2. Materials and Methods

2.1. Chemicals and Reagents

Peonidin 3-*O*-glucoside chloride (Pn-G), malvidin 3-*O*-glucoside chloride (Mv-G), delphinidin 3-*O*-glucoside chloride (Dp-G), cyanidin 3-*O*-glucoside chloride (Cy-G), pelargonidin 3-*O*-glucoside chloride (Pe-G), petunidin 3-*O*-glucoside chloride (Pt-G), malvidin-3,5-*O*-diglucoside chloride (Mv-DG), peonidin-3,5-*O*-diglucoside chloride (Pn-DG), pelargonidin-3,5-*O*-diglucoside chloride (Pe-DG), and cyanidin-3,5-*O*-diglucoside chloride (Cy-DG) were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). The purities of the 10 reference standards were over 98% and their structures were shown in Figure 1. Acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany). Methanol (analytical grade) was obtained from Lingfeng Chemical Reagent Co., Ltd. (Shanghai, China). Ultrapure water (18 MΩ cm) was prepared using a purification system from Fulham Technology Co., Ltd. (Qingdao, China).



Anthocyanins	Substitutions			
	R_1	R_2	R_3	R_4
Peonidin 3-*O*-glucoside	OCH_3	H	Glc	OH
Malvidin 3-*O*-glucoside	OCH_3	OCH_3	Glc	OH
Delphinidin 3-*O*-glucoside	OH	OH	Glc	OH
Cyanidin 3-*O*-glucoside	OH	H	Glc	OH
Pelargonidin 3-*O*-glucoside	H	H	Glc	OH
Petunidin 3-*O*-glucoside	OCH_3	OH	Glc	OH
Malvidin-3,5-*O*-diglucoside	OCH_3	OCH_3	Glc	Glc
Peonidin-3,5-*O*-diglucoside	OCH_3	H	Glc	Glc
Pelargonidin-3,5-*O*-diglucoside	H	H	Glc	Glc
Cyanidin-3,5-*O*-diglucoside	OH	H	Glc	Glc

Figure 1. Structures of 10 anthocyanins (Glc: glucosyl).

Each solid analyte was weighed accurately and dissolved in methanol to prepare the stock solution (1 mg/mL). The mixed working solution was prepared by further dilution using 50% aqueous methanol daily for the optimization of extraction conditions. All the solutions were stored at 4 °C before analysis.

The samples named as "Meiselan", "Xila", "Chixiazhu1", and "Chixiazhu2" were obtained from Yantai City, Shandong Province of China.

2.2. Sample Preparation

The grape samples were prepared by a literature procedure [26]. Grape samples were pre-frozen withliquid nitrogen, and then ground to powder by a mortar and pestle. The accurately weighed grape powder (0.5 g) was dark extracted with 10 mL of 2% formic acid-methanol (*v/v*) in an ultrasonic bath at room temperature for 10 min. The extracts were on a shaker at 25 °C, 140 rpm/min, and centrifuged at 8000× *g* rpm for 10 min. The process was then repeated two times. Then the extracts were combined and evaporated on a rotary evaporator at 35 °C until dry. The residue was redissolved in 5 mL of methanol. The samples were filtered through a 0.22 μm millipore filter before analysis.

2.3. The Instrument Conditions

Waters Acquity UPLC system (Waters, Milford, MA, USA) was used toperform-Chromatographic analysis of the target anthocyanins. A Waters Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm)was used in this study. The column temperature was set at 35 °C.The injection volume was 5 μL. The mobile phases were comprised of 1% formic acid aqueous solution (solvent A) and acetonitrile (solvent B) at a flow rate of 0.3 mL/min. An elution gradient was performed according to the following conditions: 0–1 min, 95.0–90.0% A; 1–6 min, 90.0–90.0% A, 6–9 min, 90.0–75.0% A; 9–11 min, 75.0–5.0% A; 11–13 min, 5.0–5.0% A; 13–13.5 min, 5.0–95.0% A; and 13.5–18 min, 95.0–95.0% A.

Mass spectrometry was performed on a Waters Q-TOFSynapt G2S high definition mass spectrometer (Waters, MA, USA). The quantification of the components was achieved on anelectrospray ionization source (ESI) in positive mode. The ion source working parameters were set as follows: source temperature, 80 °C; desolvation gas temperature, 300 °C; the flow rates of cone and desolvation gas, 50 L/h and 600 L/h, respectively; the voltages of capillary, cone and extraction cone, 3.0 kV, 35 V and 5.0 V, respectively. Full-scan mass range was 100–1000 Da.

2.4. QuantitativeAnalysis of Anthocyanins

In the present study, the anthocyanins were quantified bythe QAMS method. The appropriate calculation method and reference analyte were selected to establish the relative correction factor (RCF) of each substance. RCF was applied to calculate the contents of the components. The external standard method (ESM) was used to verify the results of QAMS. The result with a lower standard method difference (SMD) compared with those from ESM was selected.

In the current study, RCF was calculated by the multipoint method (MP) and slope method (SP), respectively [23]. The formulas of RCF were as follow:

$$\mathrm{RCF} = \frac{1}{\mathrm{n}} \times \sum_{\mathrm{i}=1}^{\mathrm{n}} \frac{\mathrm{Asi}}{\mathrm{Csi}} \times \frac{\mathrm{Cxi}}{\mathrm{Axi}} \quad (1)$$

$$\mathrm{RCF} = \frac{\mathrm{Ks}}{\mathrm{Kx}} \quad (2)$$

Formula (1) was used to calculate the RCF via the MP method. Where As and Ax represented the peak areas of the reference analyte and analytes, respectively; Cs and Cx were the concentrations of the reference analyte and analytes, respectively; n represented the concentration numbers of the reference analyte and analytes; i represented the sum variables. Formula (2) was used to calculate the RCF the via SP method. Ks and Kx were the slopes of the standard curves.

$$\mathrm{Cx} = \mathrm{RCF} \times \frac{\mathrm{Ax} \times \mathrm{Cs}}{\mathrm{As}} \quad (3)$$

Cs was calculated via ESM. Cx was determined based on Formula (3).

$$\mathrm{SMD}(\%) = \frac{|\mathrm{C_{ESM}} - \mathrm{C_{QAMS}}|}{\mathrm{C_{ESM}}} \times 100\% \quad (4)$$

SMD was calculated based on Formula (4), which could be used to indicate the difference between QAMS and ESM, and to verify the accuracy of QAMS. $\mathrm{C_{ESM}}$ and $\mathrm{C_{QAMS}}$ represented the concentrations of analytes calculated by ESM and QAMS methods, respectively.

2.5. Data Analysis

In this study, the data were represented by the mean and relative standard deviation (RSD) of three repeated experiments. GraphPad prism(v.5, GraphPad Software®, San Diego, CA, USA) was used for statistical analysis. The linear regression was performed using SPSS (v. 18.0, IBM®, Chicago, IL, USA). Statistical significance was set at the 95% confidence level ($p < 0.05$).

3. Resultsand Discussion

3.1. Optimization of the Instrumental Conditions

It is a challenging task to make chromatographic separation of anthocyanins due to their high diversity and structural similarity. However, MS detection allows simultaneous determination of chromatographically unresolved compounds by extraction of different m/z signals [25]. In this study, several parameters were optimized, including different concentrations of formic acid aqueous solution (water with formic acid—0%, 0.5%, and 1%), the organic phases (methanol and acetonitrile), gradient elution program, and different chromatographic columns (Waters ACQUITY UPLC BEH C18 (100 mm × 2.1 mm, 1.7 μm), Waters ACQUITY UPLC RP18 (100 mm × 2.1 mm, 1.7 μm)). The results showed that the best separation degree and peak shape were obtained using Waters ACQUITY UPLC BEH C18 (100 mm × 2.1 mm, 1.7 μm) column and a gradient elution program with acetonitrile and water containing 1% formic acid. Typical extract ion chromatograms of the anthocyanin standards and grape extract were shown in Figure 2.

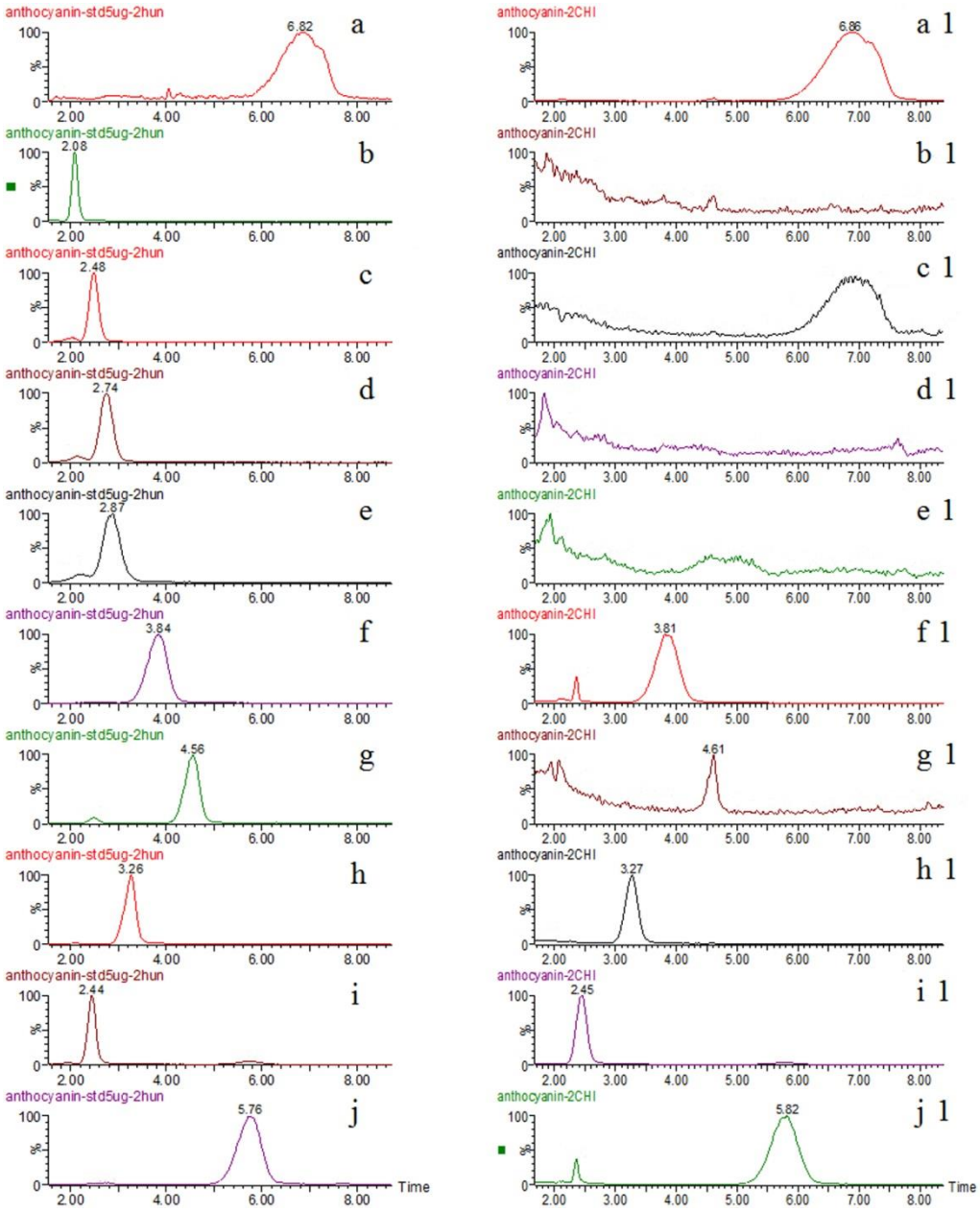


Figure 2. Typical extract ion chromatograms of mixed reference standards (**a**–**j**) and the grape extract (**a1**–**j1**) ((**a**,**a1**), Mv-G; (**b**,**b1**), Cy-DG; (**c**,**c1**), Pe-DG; (**d**,**d1**), Pn-DG; (**e**,**e1**), Mv-DG; (**f**,**f1**), Pt-G; (**g**,**g1**), Pe-G; (**h**,**h1**), Cy-G; (**i**,**i1**), Dp-G; (**j**,**j1**), Pn-G).

3.2. Validation of the Method

The retention time and MS^1 mass were gotunder the optimized instrumental conditions. In order to verify the applicability of the method, the linear ranges, regression coefficients, the limit of detection (LOD), the limit of quantification (LOQ), precisions, and repeatability were performed. The linearity was established with the peak areas of sixdifferent concentrations for each anthocyanin. As shown in Table 1, good linear relationships were obtained with satisfactory correlation coefficients (R^2) greater than 0.99. The LOD and LOQ were calculated as the signal to noise ratios (S/N) of 3 and 10, respectively. The instrument precision was evaluated by the relative standard deviation (RSD) calculated for the intra-day and inter-day variations. Both RSD values of the intra-day (0.58–4.09%) and inter-day (0.61–4.23%) variations were within the acceptable range. The precision of this method was evaluated by repeatability. Six repeated samples from the

same batch were measured by the developed method, and the results (RSD)were in the range of 1.22–2.43%. The above results indicated that the method was considered to be effective and reliable. Therefore, this method could be used for the quantitative analysis of anthocyanins in grapes.

Table 1. The results of method validation for the 10 analytes.

No.	Compound	Regression Equation	Linearity Range (μg/mL)	Correlation Coefficient (R^2)	LOD (μg/mL)	LOQ (μg/mL)	Precision		Repeatability
							Intra-Day RSD (%)	Inter-Day RSD (%)	RSD (%)
1	Pn-G	Y = 9558X + 1598.3	0.25–5.00	0.9992	0.05	0.20	1.33	0.79	1.22
2	Mv-G	Y = 7824.3X + 740.48	0.24–4.88	0.9999	0.05	0.20	2.62	1.65	1.76
3	Dp-G	Y = 2387X + 2139.5	0.50–9.90	0.9943	0.10	0.40	3.08	1.71	2.16
4	Cy-G	Y = 5336.4X + 3894.4	0.75–15.00	0.9988	0.15	0.50	3.99	0.61	2.22
5	Pe-G	Y = 8655.4X + 1850.4	0.25–4.99	0.9963	0.05	0.20	3.45	2.35	2.43
6	Pt-G	Y = 5021X + 2382.5	0.50–9.90	0.9949	0.10	0.40	4.00	2.28	1.59
7	Mv-DG	Y = 547.08X + 1619.8	1.75–34.95	0.9958	0.50	1.50	3.31	1.87	1.95
8	Pn-DG	Y = 3951.6X + 2697.6	0.37–7.42	0.9932	0.10	0.30	0.58	0.80	2.01
9	Pe-DG	Y = 9817X + 6488.5	0.75–7.5	0.9951	0.15	0.50	4.45	4.23	2.15
10	Cy-DG	Y = 2130.2X + 3240.5	1.25–12.5	0.9922	0.30	1.00	4.09	3.72	2.05

3.3. QAMS Method Development

At present, the QAMS approach coupled with HPLC-UV or HPLC-QqQ-MS was widely used in the quantitative analysis [19,24]. However, these methods could not be used for the qualitative and quantitative analysis of complex matrix samples simultaneously. Therefore, for better authentication and convenience forquantitative determination of compounds, the position of target compounds needs to be further corrected by the relative retention time of these compounds with reference substancesin different columns and instruments [20]. UPLC-Q-TOF MS is an instrument based on the quadrupole-time of flight technique. The method based on the instrument could not only reduce the matrix interference, but also quantify the analytes with low and high contentssimultaneously and preliminarily identify the analytes by the mass spectrometric data without reference compounds. Thus, it could be a powerful tool for the qualitative and quantitative analysis of complex matrix samples at the same time. In the present research, UPLC-Q-TOF MS was combined with QAMS to determine the anthocyanins in grapes. The obtained chromatographic peaks were identified by comparing the retention times, molecular weight, and fragment ions with chemical reference substances (Table 2).

Table 2. Parameters for the 10 analytes in MS mode.

No.	Compound	PrecursorIon (*m*/*z*)	Fragment Ion (*m*/*z*)	Rt (min)
1	Pn-G	463.2524	301	5.76
2	Mv-G	493.2640	331	6.82
3	Dp-G	465.1743	303	2.44
4	Cy-G	449.2361	287	3.26
5	Pe-G	433.2406	271	4.56
6	Pt-G	479.2479	317	3.84
7	Mv-DG	655.2778	331	2.87
8	Pn-DG	625.2689	301	2.74
9	Pe-DG	595.2590	271	2.48
10	Cy-DG	611.2512	287	2.08

QAMS method was designed according to the principle of linear relationship between the component amount and the detector response within a certain range [22]. Forthe QAMS method, RCFs between the components werea critical parameter because its ruggedness and robustness heavily influence the accuracy of the QAMS method [21]. The value of RCF was affected by many factors. Usually, the factors including the instrument, mobile phase, pH, flow rate, chromatographic column, and the column temperature, were the

most often considered [22,24], while the previous results indicated that these factors had little effect on the RCF. Up to now, the RCF of the QAMS method could be divided into two types based on their calculation methods. One was calculated by the ratio of slopes of the analytes (SP); the other was calculated using the average of several RCFs from the referring standard and the analyte detected under multiple concentration levels (MP) [21]. However, few studies have compared the results of the two methods. Moreover, the selection of a reference standard significantly affects the accuracy of RCF. However, very few studies have focused on the choice of a single marker. Usually, the reference substance was a cheap and readily available typical component and was used to determine the RCF between the analytes and the reference component. Anthocyanins in grapes are complex and diverse, while the structure of the reference analyte might influence the RCF. Therefore, we focused on the selection of reference analyte and the calculation method of RCF in this study.

The RCF values of analytes were calculated with different reference standards according to Formulas (1) and (2). The concentration contents of 10 anthocyanins in the test sample were determined by QAMS and ESM methods. In ESM, the contents of all the analytes were calculated based on linear regression equations listed in Table 1. In QAMS, the contents of the target analytes were calculated by Equation (3) with the different RCFs. To select the proper formula of RCF, the values of the SMDs were determined with the help of Equation (4) as the evaluation principle. The boxplots of the SMDs were shown in Figure 3, and the MP and SP methods were applied to calculatethe RCF values, respectively. According to the results, the SMDs calculated from the RCF values obtained by MP were all higher than those obtained by SP. It indicated that the quantification of the analytes with the RCF values calculated by SP was more accurate than that obtained by MP. It may be due to the fluctuations at different concentration levels. As described previously, SP was ultimately selected to calculate RCF values. On the other hand, the selection of the reference standard is very important for the QASM method. Figure 3b shows that Pn-G could be regarded as a reference standard for calculatingthe RCF values of the other compounds because the SMDs of all components with Pn-G as a reference standard were lower than those of the othersevenreference standards. In addition, Pn-Ghas the advantages of simple structure, low price, and easily obtained. Moreover, the SMDs of the other nineanthocyanins calculated with Pn-G as reference standard were between 0.15% and 13.66%. Therefore, it should be noted that the SMDs of the analytes with different structures calculated by using the same reference standard wasdifferent. If Mv-G, Dp-G, Cy-G, Pt-G, Mv-DG, Pn-DG, and Cy-DG were used as reference standards, the SMDs of the 10 anthocyanins were 4.93–27.94%, 4.23–23.05%, 3.14–30.12%, 0.61–22.12%, 1.60–23.37%, 6.21–40.07%, and 2.36–25.68%, respectively. The results indicated that the structure of reference standard and analytes could influence the values of RCF and the accuracy of the QAMS method. This result was consistent with that of the literature [21]. Therefore, the established QAMS method was more appropriate for the quantitative analysis of multiple compounds with similar structures in foods. Finally, to compare the difference between ESM and QAMS, the *t*-test was used for statistical analysis, and the *p* values were all greater than 0.05. The results are shown in Table 3. Therefore, the QAMS, which used Pn-G as the reference standard and adopted the SP method to calculate the RCF values, can simultaneously determine the 10 anthocyaninsinstead of the ESM.

Table 3. The contents of the 10 anthocyanins by ESM and QAMS (μg/mL).

Compounds	Quantitative Method	Mean	RSD%	*p*
Pn-G	ESM	2.07	2.50	/
Mv-G	ESM	1.96	1.79	0.63
	QAMS	1.98	3.77	
Dp-G	ESM	4.94	0.54	0.63
	QAMS	4.96	1.04	
Cy-G	ESM	6.39	1.18	0.56
	QAMS	6.35	1.40	

Table 3. *Cont.*

Compounds	Quantitative Method	Mean	RSD%	*p*
Pe-G	ESM	1.92	4.90	0.56
	QAMS	1.96	4.15	
Pt-G	ESM	4.19	3.41	0.42
	QAMS	4.09	3.07	
Mv-DG	ESM	18.64	1.31	0.08
	QAMS	18.28	0.66	
Pn-DG	ESM	4.07	0.74	0.06
	QAMS	3.86	3.43	
Pe-DG	ESM	5.60	1.19	0.78
	QAMS	5.62	1.07	
Cy-DG	ESM	10.44	0.97	0.15
	QAMS	10.20	2.14	

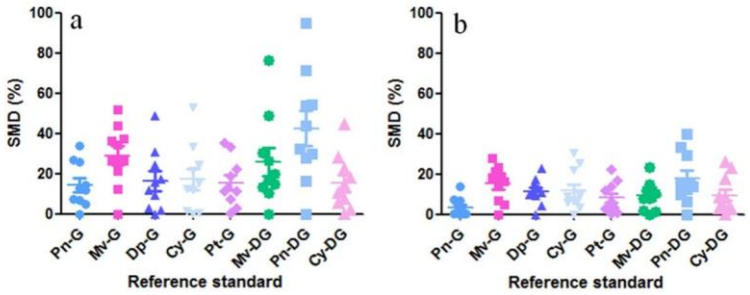


Figure 3. Boxplotsof the differences in quantification results between ESM and QAMS: (**a**) the SMDs of all anthocyanins with different anthocyanins as reference standards, where F is calculated by MP; (**b**) the SMDs of all anthocyanins with different anthocyanins as reference standards, where F is calculated by SP.

3.4. Application of Proposed Method to Grape Samples

To validate the feasibility of QAMS to determinemulti-compounds in grapes, the 10 anthocyanins contentswere determined by ESM and QAMS in four batches, respectively. Six anthocyanins were detected in these grapes including Pn-G, Dp-G, Cy-G, Pe-G, Pt-G and Mv-G and the contents of Pe-G were all below the limit of quantitation. The linear regression model was built between the two variables to measure the deviation between QAMS and ESM (Table 4). The independent and dependent variables were determined by QAMS and ESM, respectively. In the regression model, allstatistically significant coefficientswere 0.000, and the R^2 valuesof Dp-G, Cy-G, Pt-G, and Mv-G were 1.000, 1.000, 0.999, and 0.999, respectively. The results indicated that there was a good statistically significant correlation between the two variables, and no significant difference in the contents obtained by QAMS and ESM. Therefore, QAMS could be used to determine anthocyanins in grapes.

Table 4. The summary of the linear regression model and ANOVA.

	Dp-G	Cy-G	Pt-G	Mv-G
R	1.000	1.000	1.000	1.000
R^2	1.000	0.999	0.999	0.999
F	7326.6	2435.9	2165.0	2085.4
Sig.	0.000	0.000	0.000	0.000

4. Conclusions

In this study, a method for quantifyinganthocyanins in grapes was established by UPLC-Q-TOF MS combined with QAMS, and the different calculation formulas of RCF and the reference standards were compared. SP was ultimately selected to calculate RCF values and Pn-G was chosen as a reference standard to determinethe 10 compounds'

contents. The SMDs between QAMS and ESM were below 15%. No significant deviations of anthocyanin contents between QAMS and ESM were obtained in the four batches of grapes. The results showed that the established QAMS method could replace the ESM method under the condition ofa lack of a reference standard. The established QAMS method displayed the advantages of simplicity, accuracy, and low price. It may provide new ideas for the quantitative study of anthocyanin in other foods like colored grain, fruits, and vegetables, etc.

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