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Analytical Methods

Resveratrols in *Vitis* berry skins and leaves: Their extraction and analysis by HPLC

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ABSTRACT

An orthogonal L_{36} $(6)^5$ test design was applied to select the optimum conditions for extracting resveratrols from grape berry skins and leaves. Solvent choice was the most important factor in the extraction of resveratrols, and mixed methanol and ethyl acetate [50:50 (v/v)] had much higher extraction efficiency than the other five solvents tested. For extracting resveratrols, 1 g of berry skins or leaf tissue extracted in 10 mL methanol and ethyl acetate [50:50 (v/v)] for 24 h at 25 °C in darkness was the optimized extraction condition. The optimized analytical method for HPLC was a multi-step gradient elution using acetonitrile and water. The optimized method was used to determine resveratrols among three different cultivars. The cultivar 'Zhi 168' had the highest total resveratrols in berry skins while 'Saint-Emilion' had the lowest resveratrols. The resveratrol content of 'Beta' was between that of 'Zhi 168' and 'Saint-Emilion'.

1. Introduction

Resveratrols are stilbenes belonging to a non-flavonoid class of phenolic compounds. They have two isomers (*trans*-resveratrol and *cis*-resveratrol), which can be transformed to piceids (*trans*-piceid and *cis*-piceid, respectively) in plant tissues (Cantos, Espín, & Tomás-Barberán, 2002; Donnez, Jeandet, Clément, & Courot, 2009). Resveratrols have been reported to be associated with resistance to fungal diseases, and can also occur in response to abiotic stresses, such as UV irradiation, ozone, heavy metal ions, injury and frost (Pezet, Gindro, Viret, & Spring, 2004; Jeandet et al., 2010).

Resveratrols have human-health benefits, such as antioxidant properties (Sanchez-Moreno, Larrauri, & Saura-Calixto, 1999), platelet aggregation inhibition (Bertelli et al., 1995), and anti-inflammatory (Martinez & Moreno, 2000), cardio-protection (Hung, Chen, Huang, Lee, & Su, 2000), cancer chemopreventive (Jang et al., 1997; Schneider et al., 2000) and anti-viral activities (Docherty et al., 1999). *Trans*-resveratrol is an important and leading stilbenic compound in some plants, and its biological activity for human-health benefits has been extensively studied for nearly 70 years (Aggarwal et al., 2004). The biological activities of the other isomers (*cis*-resveratrol, *trans*-piceid and *cis*-piceid) have not been thoroughly studied, but it seems that *cis*-resveratrol and the two piceids may also have beneficial effects on health (Chong, Poutaraud, & Hugueney, 2009).

Human sources of resveratrols include grape berries and red grape wine, Japanese knotweed (*Polgonum cuspidatum* Ziebold et Zucc.) roots, mulberry (*Morus rubra* L.) fruit, garden rhubarb (*Rheum rhaponticum* L.) roots, and peanuts (*Arachis hypogaea* L.) (Püssa, Floren, Kuldkepp, & Raal, 2006). However, resveratrols from grape berries and red wine are the most important sources due to their large production and consumption.

In grapes, *trans*-resveratrol is distributed mostly in the skin (Jeandet, Bessis, & Gautheron, 1991), and mainly in a glucosylated form (Roggero & Garcia-Parrilla, 1995). These compounds were also reported in lower abundance in grape seeds (Pezet & Cuenat, 1996) and stems (Bavaresco, Cantù, Fregoni, & Trevisan, 1997). The concentration of *trans*-resveratrol in grape skins and seeds varies considerably within *Vitis* germplasm (Li, Wu, Wang, & Li, 2006). Resveratrol, the major stilbene of grape, is present as two isomers; however, only *trans*-resveratrol has been studied extensively in grapes (Jeandet et al., 1991; Vrhovsek, Wendelin, & Eder, 1997). Besides *trans*-resveratrol, the other derivatives such as *trans*-piceid and *cis*-piceid distributed in varietal cultivars also play an important medicinal activities. It is valuable to know all resvertrol derivatives in grapes for their uses in the future. However, only one or two derivatives have been studied along with other phenolic compounds (Jeandet et al., 1991; Li et al., 2006; Vrhovsek et al., 1997; Okuda & Yokotsuka, 1996; Kalantari & Das, 2010), and little research can be found in the literature concerning extraction and analysis of different resveratrols in grapes. It is essential to

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optimize the extraction conditions and the measurement of resveratrols in grapes for future studies and uses.

The main analytical method for resveratrols is high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection. Several solvents, including methanol, ethyl acetate, acetone, and ethanol in water, have been used for extraction of resveratrols in grapes with extraction times varying from 10 min to 72 h (Li et al., 2006; Adrian et al., 2000b; Jerkovic, Callemien, & Collin, 2005). It is essential to know the efficiency of these solvents, and optimum extraction times for resveratrols. In addition, the temperature and the solvent volume per gram of sample may also affect extraction efficiency (Liang, 2008). Sun, Ribes, Leandro, Belchior, and Spranger (2006), Adrian et al. (2000b) and Jerkovic et al. (2005) used different chromatographic separation methods to analyze resveratrols quantification. However, these methods were not compared or optimized for analysis of resveratrols and their derivatives to obtain the highest peak resolution with the shortest analytical time.

The study presented here focused on optimizing the extraction conditions and on analytical methods for measuring resveratrols in grape berry skins and leaves. Moreover, resveratrols were investigated in representative *Vitis* germplasm to compare different resveratrol components for validating the method.

2. Materials and methods

2.1. Reagents and standards

All reagents used were of analytical or HPLC grade. Acetonitrile (HPLC grade) was purchased from Sigma Chemical Company (St. Louis, USA). Other analytical grade reagents were from Beijing Chemical Factory (Beijing, China). The *trans*-resveratrol standard was purchased from Sigma. *Trans*-piceid (analytical grade) was purchased from the Chinese Standards Research Institute (Beijing, China). Since *trans*-stilbenes can be partly converted to their respective *cis*-isomers by UV-C irradiation (Adrian et al., 2000b; Lamuela-Raventós, Romero-Pérez, Waterhouse, & Torre-Boronat, 1995), *cis*-resveratrol and *cis*-piceid were obtained by UV-C (254 nm) irradiation of the *trans*-isomers in methanol at 600 μW/cm^2 for 30 min. The *cis*-isomers formed were confirmed by their specific spectra using a photodiode array detector, and the amounts calculated on assumption that the decrease in *trans*-resveratrol and *trans*-piceid, respectively, was equal to the generation of the *cis*-isomers as described by Goldberg, Karumanchiri, Yan, Diamandis, and Soleas (1995). The content of each resveratrol was obtained according to the corresponding external standard solution calibration using HPLC peak areas.

2.2. Materials

Berries and leaves of 'Beiquan' ['Gros Colman' (*Vitis vinifera*) × 'Beichun' (*Vitis amurensis* × *V. vinifera*)] were used to optimize extraction and analytical conditions of resveratrols. Vines were planted in the spring of 1993 at the Institute of Botany, Chinese Academy of Sciences located in Beijing, China. Berries at the beginning of veraison, and fully-expanded leaves, were harvested in September of 2009, and individual berries were separated. A high content of resveratrols can increase the reliability of extraction and analysis, so the berries and abaxial surfaces of leaves were irradiated with UV-C (254 nm) at 600 μW/cm^2 for 10 min, based on previous research (Li, Zheng, Yan, & Li, 2008). This UV-C irradiation can induce rapid synthesis of resveratrols in berries and leaves (Adrian, Jeandet, Douillet-Breuil, Tesson, & Bessis, 2000a; Bonomelli et al., 2004). Moreover, the abaxial leaf surface was chosen because of its greater sensitivity to UV-C than the adaxial surface (Aggarwal et al., 2004; Adrian et al., 2000b; Lamuela-Raventós et al., 1995; Jean-Denis, Pezet, & Tabacchi, 2006). The irradiated berries and leaves were incubated in the laboratory in darkness for 48 h. Berry skins were separated from fruit flesh by hand, then skins and leaves were frozen respectively in liquid nitrogen, ground to powder and stored at −40 °C until analysis. Skin or leaf samples (1 g) were used for each extraction (n = 3).

Three cultivars, 'Saint-Emilion' (*V. vinifera*), 'Zhi 168' (*Vitis monticola* × *Vitis riparia*) and 'Beta' (*Vitis labrusca* × *V. riparia*) previously shown to have different contents of *trans*-resveratrol in berry skin were used to investigate resveratrols in berry skins and leaves for validating the method. 'Saint-Emilion' berry skin has low *trans*-resveratrol while a high content of *trans*-resveratrol was found in berry skin of 'Zhi 168' and 'Beta' (Li et al., 2006). Berries were sampled at maturity, and mature leaves of cultivars were harvested on one date in June. The grapevines were grown in the same vineyard as described above for 'Beiquan'. Leaves from three shoots on one vine were randomly chosen and combined, with three vines per cultivar sampled for three replicates per cultivar. Separated berry skins and leaves were ground to powder and stored at −40 °C until analysis.

2.3. Optimization of chromatographic separation of different resveratrols by HPLC

All samples were analyzed using a Dionex P680 HPLC system (Dionex Corporation, CA, USA) equipped with a reverse-phase C18 column of Atlantis® T3 (5-μm particle sizes, 4.6 × 250 mm I.D.; Waters, USA) and a C18 Nova Pack guard precolumn (Waters). Injection volume was 10 μL and column temperature was 30 °C. *Cis*-isomers were detected at 288 nm and *trans*-isomers at 306 nm, and photodiode array spectra were recorded from 240 to 600 nm.

Three analytical methods currently used for the separation of resveratrols with two mobile phases were compared:

(1) 'Sun' analytical method (Sun et al., 2006): at a flow rate of 1 mL/min, from 5% acetonitrile (A) in water (B) to 75% A in B within 75 min, followed by washing and re-equilibrating the column to initial conditions.
(2) 'Adrian' analytical method (Adrian et al., 2000b): at a flow rate of 1 mL/min, starting from 10% acetonitrile (A) and 90% water (B) to 85% A and 15% B within 0–18 min; at 85% A and 15% B for 5 min; then from 85% A and 15% B to 10% A and 90% B within 7 min; followed by re-equilibrating the column to initial conditions for 5 min.
(3) 'Jerkovic' analytical method (Jerkovic et al., 2005): at a flow rate of 0.2 mL/min, from 5% acetonitrile (A) and 95% water (containing 1% acetonitrile and 0.1% formic acid) (B) to 45% A and 55% B within 23 min; from 45% A and 55% B to 100% A within 7 min; followed by washing and re-equilibrating the column to initial conditions.

In these comparisons, irradiated skins or leaves of 'Beiquan' were used, with three replications for each analytical method where a single sample is one gram. The analytical method with the best result from the previous comparison was optimized for the separation of resveratrols, adjusting the multi-step gradients and time periods for each step, to obtain a high peak resolution with a relatively short analytical time.

2.4. Optimization of extraction for resveratrols

An orthogonal test design L_{36} $(6)^5$ was employed (Table 1) to determine suitable extraction conditions from a wide range of parameters with a minimum number of trials. Solvents, extraction times, ratios of sample to solvent, and extraction temperatures were considered to be the four major factors for effective extraction. There

Table 1
L_{36} $(6)^5$ orthogonal extraction design.

Test no.	Factors								
	(A) Extraction solvent		(B) Extraction time (h)		(C) Ratio of sample to solvent (g/mL)		(D) Temperature (°C)		Blank
Matrix 1	A1	100% MeOH	B1	24	C1	1:5	D1	25	
2	A1	100% MeOH	B2	24	C2	1:5	D2	25	
3	A1	100% MeOH	B3	48	C3	1:10	D3	25	
4	A1	100% MeOH	B4	48	C4	1:10	D4	4	
5	A1	100% MeOH	B5	72	C5	1:20	D5	4	
6	A1	100% MeOH	B6	72	C6	1:20	D6	4	
7	A2	100% EtOAC	B1	24	C2	1:5	D3	25	
8	A2	100% EtOAC	B2	24	C3	1:10	D4	4	
9	A2	100% EtOAC	B3	48	C4	1:10	D5	4	
10	A2	100% EtOAC	B4	48	C5	1:20	D6	4	
11	A2	100% EtOAC	B5	72	C6	1:20	D1	25	
12	A2	100% EtOAC	B6	72	C1	1:5	D2	25	
13	A3	75% Me2CO	B1	24	C3	1:10	D5	4	
14	A3	75% Me2CO	B2	24	C4	1:10	D6	4	
15	A3	75% Me2CO	B3	48	C5	1:20	D1	25	
16	A3	75% Me2CO	B4	48	C6	1:20	D2	25	
17	A3	75% Me2CO	B5	72	C1	1:5	D3	25	
18	A3	75% Me2CO	B6	72	C2	1:5	D4	4	
19	A4	MeOH:EtOAC (50:50)	B1	24	C4	1:10	D1	25	
20	A4	MeOH:EtOAC (50:50)	B2	24	C5	1:20	D2	25	
21	A4	MeOH:EtOAC (50:50)	B3	48	C6	1:20	D3	25	
22	A4	MeOH:EtOAC (50:50)	B4	48	C1	1:5	D4	4	
23	A4	MeOH:EtOAC (50:50)	B5	72	C2	1:5	D5	4	
24	A4	MeOH:EtOAC (50:50)	B6	72	C3	1:10	D6	4	
25	A5	MeOH:Me2CO (50:50)	B1	24	C5	1:20	D3	25	
26	A5	MeOH:Me2CO (50:50)	B2	24	C6	1:20	D4	4	
27	A5	MeOH:Me2CO (50:50)	B3	48	C1	1:5	D5	4	
28	A5	MeOH:Me2CO (50:50)	B4	48	C2	1:5	D6	4	
29	A5	MeOH:Me2CO (50:50)	B5	72	C3	1:10	D1	4	
30	A5	MeOH:Me2CO (50:50)	B6	72	C4	1:10	D2	4	
31	A6	EtOAC:Me2CO (50:50)	B1	24	C6	1:20	D5	25	
32	A6	EtOAC:Me2CO (50:50)	B2	24	C1	1:5	D6	25	
33	A6	EtOAC:Me2CO (50:50)	B3	48	C2	1:5	D1	25	
34	A6	EtOAC:Me2CO (50:50)	B4	48	C3	1:10	D2	25	
35	A6	EtOAC:Me2CO (50:50)	B5	72	C4	1:10	D3	25	
36	A6	EtOAC:Me2CO (50:50)	B6	72	C5	1:20	D4	4	

MeOH = methanol, EtOAC = ethyl acetate, Me_2CO = acetone.

were 36 separate extractions performed using six solvent solutions [methanol (100%), ethyl acetate (100%), acetone (70%) in water (v/v), and three combinations of two solvents from the three]; extraction times of 24, 48 and 72 h; three different ratios of sample to solvent volume (1:5, 1:10 and 1:20); and two temperatures (4 and 25 °C). In all scenarios (Table 1), samples were extracted in darkness, and then centrifuged at 10,000*g* for 10 min. The supernatants were evaporated to dryness by rotary vacuum evaporation at 40 °C. Dried residues were then dissolved in 2 mL methanol and stored at −40 °C before analysis by HPLC. The samples were filtered through a 0.45-μm PTFE membrane filter before analysis. Three replicate extractions were performed for each sample.

On the basis of the orthogonal test results, the most important factors, solvent solution, ratio of sample to solvent, and extraction temperature, were confirmed to significantly affect the extraction efficiency, so were further investigated in single-factor tests for their optimization. Lastly, the recovery experiment was conducted. The recovery was assessed by adding three concentrations of standard solutions (high, medium and low) to 'Beiquan' berry skin and leaf samples. The spiked samples were extracted and analyzed in triplicate. The percentage recoveries were calculated as (recovered amount − initial amount)/added amount × 100.

2.5. Statistical analysis

The results of the L_{36} $(6)^5$ test were weighted and statistically analyzed, and the evaluation indices *K*, *k* and *R* were calculated (Table 2). Capital letters A–D corresponded to the factors in the

Table 2(A)
Analysis of $L_{36}(6)^5$ test results of resveratrol contents (μg/g FW) in grape skins.

	Trans-piceid				*Trans*-resveratrol			
	A[a]	B	C	D	A	B	C	D
k1	7.16	3.48	2.87	3.19	300.10	281.63	172.81	242.10
k2	1.04	4.07	4.05	3.54	284.12	307.52	197.55	243.85
k3	2.02	3.90	3.89	3.57	122.52	243.99	279.85	239.04
k4	4.89	3.48	3.58	3.68	300.49	236.69	280.39	257.57
k5	5.65	3.40	4.27	4.01	293.38	211.54	302.22	256.02
k6	1.39	3.82	4.53	4.16	211.71	230.94	282.54	273.73
R	2.87	0.67	1.39	0.58	177.97	95.98	129.41	34.69
Optimal level	A1	B2	C6	D6	A4	B2	C5	D6
Importance order	A > C > B > D				A > C > B > D			

[a] Capital letters A–D correspond to the factors in the orthogonal test design in Table 1.

Table 2(B)
Analysis of $L_{36}(6)^5$ test results of stilbene contents (μg/g FW) in grape leaves.

	Trans-piceid				*Cis*-piceid				*Trans*-resveratrol			
	A[z]	B	C	D	A	B	C	D	A	B	C	D
k1	3.81	11.99	6.13	9.45	7.64	10.75	10.46	10.57	107.72	127.49	73.55	96.28
k2	2.33	9.44	6.00	9.86	8.04	14.65	11.43	12.62	97.88	128.83	85.77	91.22
k3	9.80	7.65	9.71	7.49	10.55	11.79	11.98	8.23	71.60	85.97	116.28	95.23
k4	13.95	9.39	10.90	11.34	15.68	13.87	11.03	10.87	110.63	100.95	116.41	114.32
k5	17.50	6.56	12.06	9.26	21.42	10.20	12.73	13.94	131.52	86.27	124.02	108.22
k6	9.25	11.61	10.70	9.24	7.70	9.76	13.55	14.79	96.92	86.77	110.09	111.02
R	15.17	5.43	6.06	3.84	13.78	4.89	3.09	6.56	59.92	41.51	50.47	23.10
Optimal level	A5	B1	C5	D4	A5	B2	C6	D6	A5	B2	C5	D4
Importance order	A > C > B > D				A > D > B > C				A > C > B > D			

[z] Capital letters A–D correspond to the factors in the orthogonal test design in Table 1.

orthogonal test design in Table 1. A: extraction solvent; B: extraction time; C: ratio of sample to solvent; D: temperature. Letter *i* corresponded to the levels of each factors. A_i corresponded to *i* level of factor *A*. K_i^A is the total amount of target compound at A_i, k_i^A is the mean amount of target compound at A_i, and R_i^A is the value of max $\{k_i^A\}$ subtracting min $\{k_i^A\}$. They were estimated as follows:

$$K_i^A = \sum \text{the amount of target compound at} A_i$$

$$K_i^A = K_i^A/6$$

$$R_i^A = \max(kiA) - \min(k_i)^A$$

The higher the value of k_i^A, the more effective is the '*i*' level of parameter *A*. The higher the value of R_i^A, the more important is parameter *A*. Similar considerations apply to the other factors.

All analyses were performed in triplicate. Analysis of variance and comparison of means ($P < 0.05$) were carried out using SPSS 13.0 (SPSS, USA). All figures were produced by SigmaPlot 10.0.

3. Results and discussion

3.1. HPLC analysis methods

The first step in the experiment was to obtain high separation efficiency and peak resolution of the target compounds. Of the three analytical methods, the 'Sun' method generated the best peak resolution for resveratrols. Then, the shortest possible period for chromatographic separation by the 'Sun' method (Sun et al., 2006) was developed: solvents acetonitrile (A) and water (B); at a flow rate of 1 mL/minute; 0 min of 10% A, 5 min of 17% A,

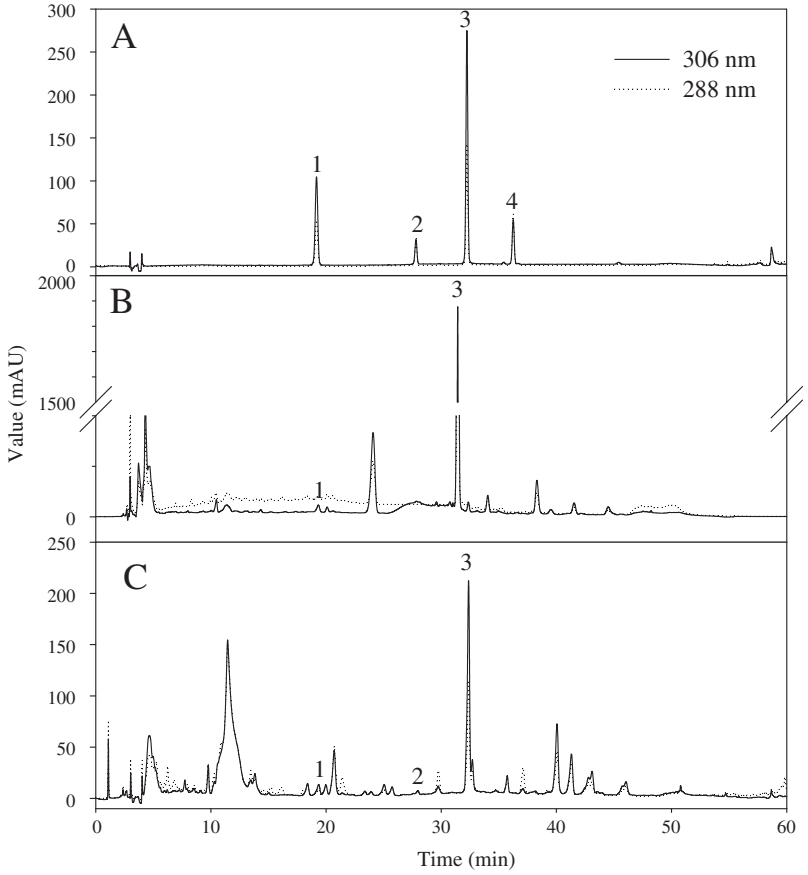

Fig. 1. HPLC chromatogram of standards (A), 'Beiquan' berry skins (B) and leaves (C) after UV-C irradiation at 306 nm (solid line) and 288 nm (dotted line). The numbers 1–4 in the chromatogram indicate *trans*-piceid, *cis*-piceid, *trans*-resveratrol and *cis*-resveratrol, respectively.

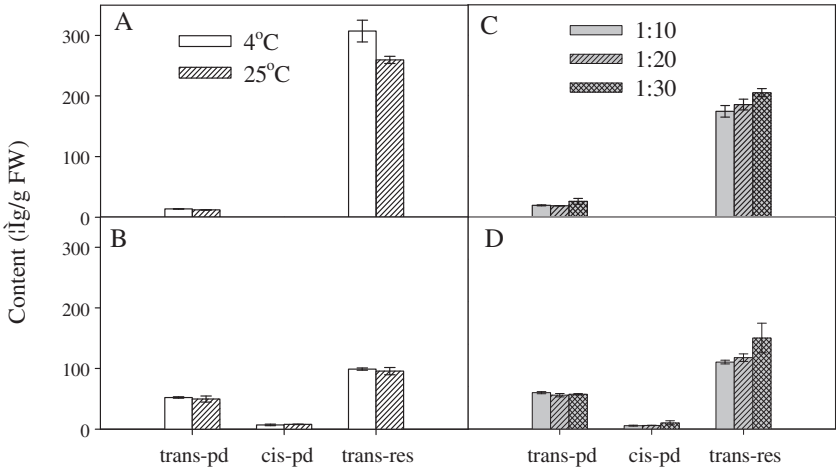

Fig. 2. Extraction efficiencies of temperatures (A and B) and ratios of sample to solvent (C and D) on resveratrols in 'Beiquan' berry skins (A and C) and leaves (B and D) after UV-C irradiation. The bars indicate standard errors.

12 min of 18% A, 22 min of 22% A, 30 min of 33% A, 45 min of 38% A, and 58 min of 100% A; followed by washing and re-equilibrating the column to initial conditions. The optimized HPLC analysis method described above gave a high separation efficiency of for four resveratrols of the standards, as well as for the extract of 'Beiquan' berry skins and leaves irradiated by UV-C detected at 306 and 288 nm (Fig. 1).

3.2. Optimization of methods for quantitative extraction of resveratrols

The results of the orthogonal test of extraction conditions are shown in Table 2. There were *trans*-resveratrol and *trans*-piceid in both berry skins and leaves. But, *cis*-resveratrol was not detected in either berry skins or leaves, and *cis*-piceid was only found in berry skins of 'Beiquan' despite being UV-C irradiated. The influence of the extraction factors on the mean yields of the three resveratrols in both skins and leaves was relatively consistent based on the *k* and *R* values. For *trans*-resveratrol and *trans*-piceid, the ranking of importance of extraction factors was extraction solvent > ratio of sample to solvent > extraction time > extraction temperature. For *cis*-piceid, extraction solvent > extraction temperature > extraction time > ratio of sample to solvent (Table 2). Solvent played the most important role in extracting resveratrols in grape berries and leaves. A1 (methanol) and A4 [methanol:ethyl acetate (50:50 (v/v))] gave the highest yield of *trans*-piceid and *trans*-resveratrol respectively in berry skin, however, A5 [methanol:acetone (50:50 (v/v))] was the best solvent solutions for extracting all the three resveratrols in leaves. As regards factors B and C, extraction for 24 h (B1 and B2) and ratio of sample to solvent of 1:20 (C5 and C6) had the highest yield of resveratrols among the three levels of the test (Table 2). Extraction temperature had the least effect on the extraction of resveratrols except the extraction of *cis*-piceid in leaves. For *cis*-piceid, extraction temperature was a more important factor than extraction time or the ratio of sample to solvent. Overall, extraction time was less important in the extraction of resveratrols than the other factors, and 24 h of extraction gave the best results of the three extraction times and was used for further study.

The effect of extraction at 4 °C versus 25 °C was compared. Methanol: ethyl acetate [50:50 (v/v)], and methanol: acetone [50:50 (v/v)] were used for extraction of skins and leaves, respectively, with the ratio of sample to solvent of 1:20 g/mL. The results showed no significant differences between the two temperatures in the extraction levels of all resveratrols for both skins and leaves (Fig. 2A and B). Temperatures of 4 and 25 °C have been used for extraction of resveratrols in previous studies (Larronde et al., 2003; Tassoni et al., 2005), but no one has compared their effect on extraction efficiency. Our result indicated it was not necessary to extract resveratrols at 4, and 25 °C was sufficient. Thus, 25 °C was used for subsequent extractions for convenience and saving energy.

Methanol: ethyl acetate [50:50 (v/v)] and methanol: acetone [50:50 (v/v)], were used as solutions for the extraction of skins and leaves, respectively for comparison of the ratio of sample to solvent. Three ratios of sample to solvent of 1:10, 1:20 and 1:30 g/mL were compared. There were no significant differences in resveratrol extraction (Fig. 2C and D), indicating that 10 mL of solution was sufficient for extracting 1 g of powder of berry skins or leaves.

Solvent solution had the greatest effect on resveratrols extraction as shown by the orthogonal test. Sun et al. (2006) reported

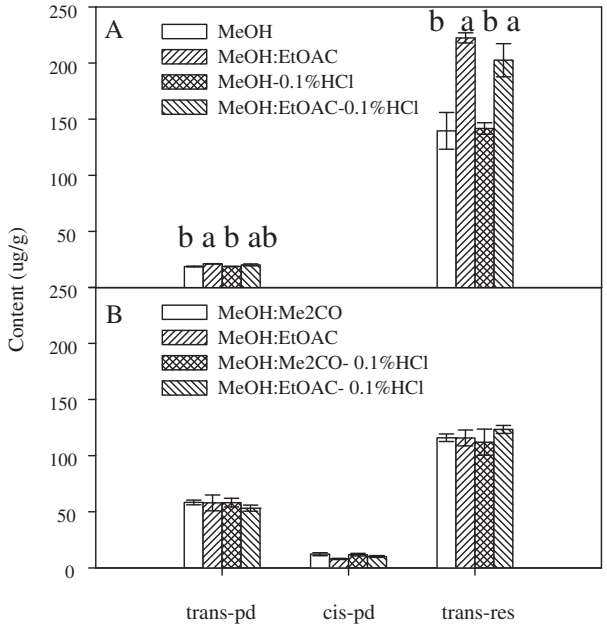

Fig. 3. Extraction efficiencies of various solvents on resveratrols in 'Beiquan' berry skins (A) and leaves (B) after UV-C irradiation. The bars indicate standard errors. Different letters above columns indicate significant differences among the solvents at $P < 0.05$ while no letters above columns indicate no significant differences were observed.

Table 3
Recovery of the resveratrol standards in the extraction of grape skins and leaves.

Tissue	Resveratrols	Added amount (μg)	Initial amount (μg)	Recovered amount (μg)	Recovery[a] (%)	Average recovery (%)
Berry skins	*Trans*-pieced	1.00	10.00 ± 0.08	11.08 ± 0.08	97.47	94.31 ± 2.79
		10.00	10.01 ± 0.08	19.68 ± 0.18	96.71	
		30.00	9.90 ± 0.14	36.53 ± 0.86	88.75	
	Trans-resveratrol	20.00	353.13 ± 5.94	371.75 ± 1.67	93.11	97.74 ± 2.41
		200.00	357.56 ± 1.02	555.36 ± 2.92	98.90	
		600.00	353.94 ± 8.16	961.24 ± 9.60	101.22	
Leaves	*Trans*-pieced	1.00	10.02 ± 0.05	10.97 ± 0.005	95.31	96.04 ± 0.39
		10.00	9.98 ± 0.04	19.59 ± 0.27	96.15	
		30.00	10.17 ± 0.36	39.17 + 0.98	96.66	
	Trans-resveratrol	20.00	24.37 ± 1.49	42.95 ± 0.91	92.91	101.08 ± 4.64
		200.00	25.63 ± 0.08	228.36 ± 0.08	101.36	
		600.00	26.34 ± 1.30	660.08 ± 3.97	105.96	

[a] Recovery = (recovered amount − initial amount)/added amount × 100.

Table 4
Resveratrol contents (μg/g FW) in berry skins and leaves of three representative grape cultivars.

Cultivars	Skins				Leaves			
	Trans-piceid	*Cis*-piceid	*Trans*-resveratrol	Total	*Trans*-piceid	*Cis*-piceid	*Trans*-resveratrol	Total
'Zhi 168'	2.21 ± 0.08[a]	336.25 ± 46.66[a]	6.21 ± 0.79[a]	344.68 ± 46.20[a]	4.54 ± 1.53[b]	8.23 ± 2.74[ab]	2.10 ± 0.51[ab]	14.87 ± 4.05[a]
Beta	2.30 ± 0.71[a]	42.12 ± 7.83[b]	1.59 ± 0.76[b]	46.02 ± 9.28[b]	9.28 ± 1.63[a]	12.29 ± 5.13[a]	4.13 ± 2.43[a]	25.70 ± 8.60[a]
Saint-Emilion	0.86 ± 0.23[b]	1.86 ± 0.21[b]	0.88 ± 0.10[b]	3.60 ± 0.24[b]	0.10 ± 0.02[c]	2.14 ± 0.70[b]	0.12 ± 0.40[b]	2.36 ± 0.76[b]

The data are presented as mean ± standard deviation and different letters within a column indicate significant difference of resveratrols contents among the three cultivars at $P < 0.05$.

that the extraction efficiency of MeOH–HCl was higher than that of MeOH although the difference was not significant, indicated that there was a possibility to further increase extraction efficiency of resveratrols through acidifying solvents. Thus, in this study, the two best solvent solutions for each tissue were: A1 (methanol) and A4 [methanol:ethyl acetate (50:50 (v/v))] for berry skins, and A4 and A5 [methanol:acetone (50:50 (v/v))] for leaves acidified to pH 2.1 using 0.1% HCl. These solutions were combined with the optimized conditions obtained in the previous experiments, i.e., an extraction time for 24 h at 25 °C, and a ratio of sample to solvent of 1 g /10 mL. Both methanol: ethyl acetate [50:50 (v/v)] and its acidified solution (0.1% HCl) yielded significantly higher resveratrols from both skin and leaf tissues than the two other solvents (Fig. 3A). But there was no significant difference on extraction efficiency between methanol:ethyl acetate [50:50 (v/v)] and its acidified solution (0.1% HCl). The result indicated that it is was not necessary to acidify solvents during extracting the resveratrols. Thus, for berry skin and leaf tissue, methanol:ethyl acetate [50:50 (v/v)] was quite adequate.

3.3. Accuracy of the method

Accuracy of the method was executed by measuring recovery. As shown in Table 3, resveratrols recoveries using the method ranged from 94.31% to 101.08 %. Vinas, Campillo, Martinez-Castillo, and Hernandez-Cordoba (2009) showed the recovery of resveratrols varied from 85% to 116% in wine and grapes by solid-phase microextraction on-fiber derivatization using gas chromatography–mass spectrometry. Paixao, Pereira, Marques, and Camara (2008) showed the recovery of *trans*-resveratrol was 98% in wine using reverse phase HPLC. These comparisons demonstrated that the optimized extraction method had a similar high or higher accuracy.

3.4. Content of resveratrols in representative cultivars

We used the above optimized extraction conditions and analytical method to compare the resveratrol content among three representative cultivars ('Zhi 168', 'Beta', and 'Saint Emilion'). One gram of berry skin or leaf tissue was extracted in 10 mL methanol:ethyl acetate [50:50 (v/v)] for 24 h at 25 °C in darkness. The result showed that the contents of resveratrols in grape skins and leaves varied considerably among cultivars (Table 4). *Trans*-resveratrol, *trans*-piceid and *cis*-piceid were found but *cis*-resveratrol was not detected in grape skins and leaves in all three cultivars. 'Zhi 168' had the highest total resveratrols content with 344.68 and 14.87 μg/g fresh weight (FW) in berry skins and leaves, while 'Saint-Emilion' had the lowest total resveratrol content at about 3.60 and 2.36 μg/g FW in berry skins and leaves, respectively. 'Beta' resveratrol contents were between 'Zhi 168' and 'Saint-Emilion'. Of the total resveratrols, *cis*-piceid was dominant in berry skins and leaves.

4. Conclusion

Solvent solution was the most important extraction factor for obtaining the highest yield of resveratrols from grape berry skins and leaves, followed by the ratio of solvent to sample and extracting temperature. The optimal conditions for extracting resveratrols were: 1 g of berry skin or leaf tissue was extracted in 10 mL methanol: ethyl acetate [50:50 (v/v)] for 24 h at 25 °C in darkness. The optimized HPLC separation conditions were: column temperature of 30 °C, with two mobile phases acetonitrile (A) and water (B), at a flow rate of 1 mL/min; a gradient elution of 0 min at 10% A, 5 min to 17% B, 12 min to 18% A, 22 min to 22% A, 30 min to 33% A, 45 min to 38% A, and 58 min to 100% A; followed by washing and re-equilibrating the column to initial conditions. Moreover, *trans*-resveratrol, *trans*-piceid and *cis*-piceid were only detected in grape skins and leaves and their contents varied among the cultivars.

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