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An effective sample preparation approach for screening the anticancer compound piceatannol using HPLC coupled with UV and fluorescence detection

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Abstract

Piceatannol, compared with the renowned resveratrol, is a better anticancer agent and a superior agent with other biological activities. However, as there are only few plants reported to contain minute quantity of piceatannol, the scarcity of sources greatly impedes the piceatannol-related researches. To explore new sources of piceatannol, we established a sample preparation approach for screening the piceatannol in plants using HPLC-UV-fluorescence detection. When the HPLC is coupled with UV and fluorescence detectors, the decrease of signals in interferences and increase of signal in piceatannol in the fluorescence chromatogram mark clearly the position of the piceatannol peak; ultimately, it allows identification without standards. In this study, we systematically evaluated the factors affecting the extraction efficiency of piceatannol in sample preparation. Of the sample preparation strategies studied, direct solvent extraction and liquid nitrogen treatment followed by solvent extraction gave satisfactory recoveries for both piceatannol and resveratrol. These approaches avoided time-consuming lyophilization procedure. In addition, all procedures must be done in the dark to avoid negative impact of irradiation from fluorescence light on the recoveries of piceatannol and resveratrol. With the present method, we re-examined the plants previously claimed to contain only resveratrol for their piceatannol contents. The species examined included *Polygonum cuspidatum*, *Arachis hypogaea*, *Vitis thunbergii*, and *Ampelopsis brevipedunculaata*. The results showed, for the first time, all these plants contain piceatannol. The finding implies that the resveratrol-containing plants may also contain piceatannol. The results demonstrate the feasibility of these sample preparation approaches and may further the understanding for the distribution of piceatannol in plants.

Keywords: Anticancer agent; Piceatannol; Resveratrol; Sample preparation; HPLC; Fluorescence detection

1. Introduction

Piceatannol and resveratrol, two of the phytoalexins produced by plants in response to fungal infection, mechanical damage, or ultra-violet irradiation [1–9], are very important compounds for health due to their antioxidative activities and other biological functions. Compared with resveratrol, piceatannol has similar chemical structure (Fig. 1), but much potent biological activities. For instance, Clement et al. suggested that piceatannol, instead of resveratrol, is a more efficient inducer of apoptosis [10]. Other reports suggested that both piceatannol and resveratrol are able to induce apoptosis in many cancer cell lines, but to different extent [11–14]. In addition, Potter and colleagues found that the cytochrome p450 enzyme CYP1B1 that is generally present in human tumors converts the chemopreventive compound resveratrol to the anticancer compound piceatannol [13]. This observation provides a novel explanation for the cancer preventive property of resveratrol and elucidates clearly the important role that piceatannol can play in cancer treatment. Getting the anticancer compound piceatannol rather than the chemopreventive resveratrol from foods may be a more effective and direct way in cancer prevention. Thus, considering the anticancer activity and the ability to trigger apoptosis, piceatannol becomes a promising natural compound in cancer treatment.

In general, the amount of piceatannol in plants was much lower than that of resveratrol. For detecting piceatannol in plants, efficiency and sensitive methods should be necessary. However, the current analysis methods developed for polyhydroxystilbenes are mainly for resveratrol [15–25], and most of which are HPLC coupled with DAD or mass spectrometer. With these

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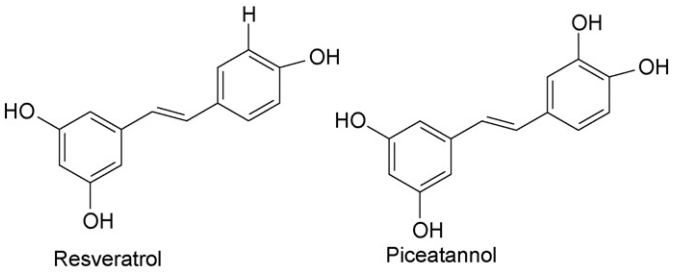

Fig. 1. The chemical structure of *trans*-resveratrol and *trans*-piceatannol.

already-developed analytical methods, there are 72 plant species reported to contain resveratrol [26], but only a handful of species contain both piceatannol and resveratrol, despite their structural similarities. This phenomenon might be due to inadequate method for piceatannol analysis in plants.

To investigate the cancer therapeutic and disease preventing effects of piceatannol, a large quantity of piceatannol is required. Hence, it is well worth to search for new sources of piceatannol and establish its distribution profiles in plants. Observing changes in the distribution profile is the basic requirement to investigate the underlying mechanism of piceatannol biosynthesis, which ultimately will help the agriculture society to induce plants with higher levels of piceatannol. To the best of our knowledge, there is still lack of analytical methods specific for piceatannol in plants. Thus, an effective analytical method, which includes adequate sample preparation with minimum loss of analytes and a suitable tool with low detection limit for piceatannol, will definitely alleviate the difficulties existed in the piceatannol-related research.

The main challenge of the sample preparation encountered in this study was the labile nature and low concentration of the analytes. Near-thorough and rapid extractions of analytes from matrix to avoid loss or decomposition are necessary to ensure a correct analysis of piceatannol in plant matrix. Like other botanical samples, the common practices used in stilbenoids sample pretreatment include lyophilization [27,28] and solvent extraction [29,30]. Since lyophilization is considered a better procedure than other drying methods, compounds assume to be able to sustain the lyophilization condition. Recently, researches revealed lyophilization treatment may reduce some bioactive compounds in plants [31] and has unanticipated effects in the constituent profiles of medicinal plants [32]. Therefore, the idea that piceatannol is stable throughout these procedures is in fact an unverified assumption. Thus, for proper preparation of the samples, the influences of solvent extraction and lyophilization on the stabilities of resveratrol and piceatannol should be evaluated systematically.

The goal of this study was to provide an effective analytical method with proper sample preparation to facilitate the screening of piceatannol and to re-evaluate the distribution profiles of piceatannol in plants that were previous reported to have resveratrol only. The species studied included *A. hypogaea*, *P. cuspidatum*, *V. thunbergii*, and *A. brevipedunculaata*. Beside the plant bodies, peanut calli were used as experimental materials as well, since they are excellent material for piceatannol induction and production [30]. We hope this method should be expanded to screen piceatannol induced in variety of calli. To overcome the difficulty associated with the low abundance in plants, a fluorescence detector was incorporated in the HPLC method which was developed in our laboratory for stilbenoid analysis using a UV-vis detector. The recoveries of spiked piceatannol and resveratrol in calli and *V. thunbergii* samples were determined to evaluate the impacts of several sample preparation procedures.

2. Experimental

2.1. Materials and reagents

HPLC-grade acetonitrile was purchased from TEDIA (Fairfield, Ohio, USA). *trans*-Resveratrol and *trans*-piceatannol standards were purchased from Merck (Darmstadt, Germany) and used as authentic compound throughout the present study. Fresh samples of *V. thunbergii* and *A. brevipedunculaata* used to evaluate their piceatannol and resveratrol contents were collected on the university campus. Both the plants are similar to the morphology of grapevine and used as herbal medicine in Taiwan. The radixes of the herbal plants: *P. cuspidatum*, *V. thunbergii*, and *A. brevipedunculaata* were also examined for their piceatannol and resveratrol contents in this study. The dry radix samples were commercially available. Peanut (*A. hypogaea*) calli and *V. thunbergii* were used to evaluate the sample preparation methods. *V. thunbergii* samples were collected on campus, while calli were cultured in our own laboratory.

2.2. Apparatus

An L-7100 HPLC pump coupled with an L-7420 UV-vis detector and an L-7485 fluorescence detector (Hitachi Co. Ltd., Tokyo, Japan) was used in this study for piceatannol and resveratrol analysis. The separation was carried out on a 250 mm × 4.6 mm i.d. Mightysil reverse-phased C-18 column (Kanto Chemical Co. Inc., Kanagawa, Japan). Both absorption and fluorescence signals were recorded simultaneously for each analysis. The wavelength of the UV-vis detector was set at 306 nm, the excitation and emission wavelengths of the fluorescence detector were set at 343 and 395 nm, respectively. The mobile phase used was the combination of acetonitrile and deionized water. The pH of deionized water was adjusted to 2.1 with formic acid. In each analysis, the acetonitrile composition was initially set at 20%, linearly increased to 32% in 20 min, increased to 90% in 10 min, and then held at 90% for 5 min. The flow rate of mobile phase was constant and kept at 1.0 mL/min. A volume of 20 μL sample was manually injected in each analysis.

2.3. Investigation of sample preparation efficiencies

Leaves of *V. thunbergii* and peanut calli were used to investigate the efficiency of different sample preparation procedures. Methods of sample preparation included direct solvent extraction, liquid nitrogen treated solvent extraction, and lyophilization treated solvent extraction. In a typical solvent extraction procedure for chlorophyll containing samples, such as

V. thunbergii, 0.5 g of the leaves were ground and then extracted with 2.5 mL of 100% methanol. The resulting methanol extract was purified by solid-phase extraction (SPE) procedure to remove the interference, such as chlorophyll prior to HPLC analysis. Briefly, the methanol extract was passed through a C-18 SPE cartridge (C18-E, 500 mg/3 mL) (Phenomenex, Torrance, CA). The cartridge was then eluted with 1 mL of a methanol/water mixture (60:40, v/v). All eluates were collected and combined. Finally, the combined eluent was diluted with 60% aqueous methanol to a volume of 10 mL for HPLC analysis.

For samples, such as calli, that do not have chlorophyll, SPE purification was not necessary prior to HPLC analysis. Peanut calli were grown and induced to produce resveratrol and piceatannol based on the procedures reported previously [30]. In each analysis, 1 g of the UV-treated calli was dispersed and ground in 1 mL of HPLC-grade methanol. This homogenized mixture was filtered to collect the methanol extract. The callus residues were then extracted two more times with additional 2 mL methanol. A total of approximately 3 mL combined methanol extract was then transferred to a 5 mL volumetric flask and diluted to the mark with methanol. This diluted methanol extract was then ready for HPLC analysis.

To examine the effect of liquid nitrogen treatment on the recoveries of piceatannol and resveratrol, leaves were dipped in liquid nitrogen, ground to fineness, and then subjected to solvent extraction. Similarly, to examine the effect of lyophilization treatment on the recoveries of piceatannol and resveratrol, leaves were first dipped in liquid nitrogen and then water was removed in a freeze-dryer (FD-Series, Panchum Scientific Corp., Taiwan). The resulting dry powder was then subjected to solvent extraction. Meanwhile, the effects of light on the stability of piceatannol and resveratrol during sample preparation were also investigated. Fluorescent lamps emitted white light for regular illuminations in laboratory were used as light source. The percent recoveries of piceatannol and resveratrol from samples handled under the exposure of light were compared to those handled under the protection against light. The recoveries of piceatannol and resveratrol were determined using authentic compounds that were spiked into the samples.

2.4. Validation of analytical method

To construct a calibration curve for quantization, a series of standards were made by diluting aliquots of either resveratrol or piceatannol standard solutions with HPLC-grade acetonitrile followed by HPLC analysis. The final standard concentrations were 5, 10, 25, 50, 100, and 500 ng/mL for piceatannol. Similarly, the final standard concentrations were 10, 25, 50, 100, 125, 250, 500, and 1000 ng/mL for resveratrol. For each concentration, three injections were made to get the averaged peak area to construct a calibration curve. The data points were fitted by the least-squared method. The corresponding standard deviation (STD) of slope and intercept were then determined.

To determine the theoretical limit of detection (LOD) concentration and the theoretical limit of quantitation (LOQ) concentration, the blank signals were measured to derive a mean and its standard deviation. The theoretical LOD calculated was based on the mean and three times of the standard deviation. Similarly, the theoretical LOQ calculated was based on the mean and 10 times of the standard deviation.

The analytical method was validated by spiking the piceatannol and resveratrol standards into the *V. thunbergii* or the peanut callus samples. The spiked samples then followed the same sample preparation and analysis procedures described in Section 2.3 to calculate the percent recovery of piceatannol and resveratrol. Briefly, the intraday precision was determined by analyzing three spiked callus samples over 24 h. Similarly, the interday precision was determined by analyzing three spiked callus samples over three consecutive days. The final standard concentrations of piceatannol and resveratrol added were 50 ng/mL.

The influence of sample matrix on the LOQ was also examined by spiking different amounts of piceatannol and resveratrol standards into the leaves of *V. thunbergii*. The recoveries of piceatannol and resveratrol from leaves were examined at six different concentrations. Various quantities of piceatannol and resveratrol standards were added to the leaves of *V. thunbergii* collected from outdoors. The final concentrations of piceatannol and resveratrol standards spiking into the samples were 10, 25, 50, 100, 1000, and 2000 ppb, respectively.

3. Results and discussion

3.1. Elution profiles, calibration curve, linearity, limit of detection, and limit of quantitation of piceatannol and resveratrol

The representative fluorescence and UV-vis chromatograms are shown in Fig. 2. The *trans*-piceatannol and *trans*-resveratrol were eluted at 11.4 and 17.1 min, respectively, in the condition of analysis. For convenience, *trans*-piceatannol and

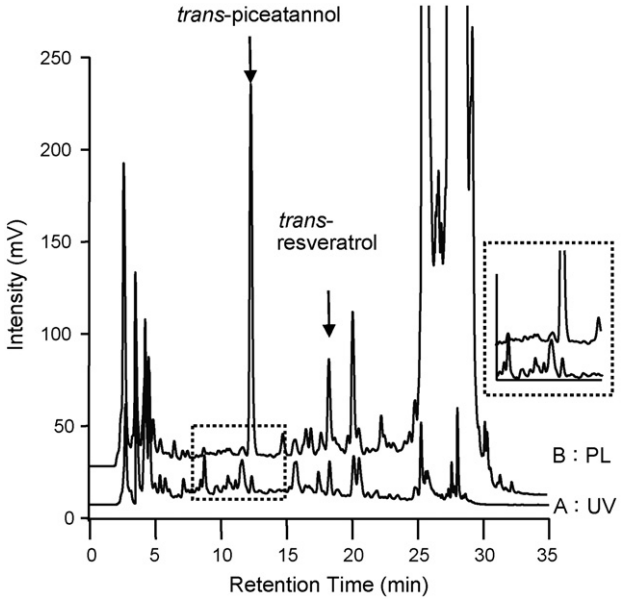

Fig. 2. Representative UV and fluorescence chromatograms of methanol extract obtained from the callus of *Arachis hypogea* (A) UV signals (B) fluorescence signals.

Table 1
The linear range, LOD, and LOQ for quantitative analyses of piceatannol and resveratrol

Compound	Calibration range (ng/mL)[a]	Detector	Calibration equation[b]	Slope standard deviation ($n=3$)	Intercept standard deviation ($n=3$)	Square of correlation coefficient, R^2	LOD (ng/mL)	LOQ (ng/mL)
trans-Piceatannol	5–500	Fluorescence	$y=1514.10x-710.98$	5.66	190.43	0.9998	0.82	1.63
	25–500	UV	$y=66.52x-400.62$	3.02	163.49	1.0000	9.81	13.65
trans-Resveratrol	10–1000	Fluorescence	$y=633.77x+24.07$	4.41	175.80	1.0000	0.81	2.76
		UV	$y=191.13x+117.77$	2.67	94.58	0.9999	6.08	6.65

[a] To construct a calibration curve, a standard solution with the concentrations of 5, 10, 25, 50, 100, 125, 250, 500, and 1000 ppb were prepared.
[b] To construct a calibration curve, the averaged peak area of fluorescence signals were plotted against the standard concentrations. The resulting data points were then fitted by linear regression to get the calibration curve.

trans-resveratrol are simply expressed as piceatannol and resveratrol throughout this report. The presence of the piceatannol and resveratrol was confirmed by spiking authentic compounds and MS spectra as described in the previous study [30]. The selectivity of this method was illustrated in the dashed box in Fig. 2. As shown, the UV signal magnitude of piceatannol is smaller than the one ahead of it. When shift to fluorescence detection, the piceatannol signal increased, while all the other signals appeared around the retention time of piceatannol decreased. These decreases of signals in interferences and increase of signal in piceatannol clearly demonstrate the advantages of this method. Of plants analyzed in this study, if a sample contains piceatannol, this phenomenon is always present. Therefore, utilizing this hyphenated UV and fluorescence mode, a piceatannol-containing plant can be easily identified, even at a concentration lower then 10 ng/mL. Since the UV chromatogram in the boxed region are noisy in real samples, spiking to identify the piceatannol peak was necessary even at a concentration as high as microgram level. Therefore, if only one detector is permitted, fluorescence detector is preferred.

Table 1 shows the calibration curves of the piceatannol and the resveratrol constructed by different concentrations of authentic solutions and the fluorescence and UV signals. The fluorescence detector was convinced to be more sensitive by the slopes of the curves and the corresponding LOD and LOQ. The sensitivity of fluorescence detection was 22.8 times higher than that of UV detection (1514.10:66.52) in the case of piceatannol. Likewise, the sensitivity was 3.5 times more (633.77:191.13) for resveratrol when using the fluorescence detection. Another benefit associated with the fluorescence detection is the LOD. As shown in the Table 1, the LOD determined by the fluorescence for piceatannol was much lower than the LOD determined by the UV signals.

Since methods specifically developed for piceatannol analysis are limited, not many data are available for comparison. The LOD and LOQ values determined by Rimando et al. are 21 and 69 ng/mL, respectively [27]. These numbers are higher than the LOD and LOQ determined in this study. Table 2 lists the LOD and LOQ values of methods developed for resveratrol analysis in literatures for comparison. In the case of resveratrol, the LOD determined by fluorescence in this study is at least one order of magnitude lower than the reported values except in one case, in which Zhou et al. reported an LOD of 0.166 ng/mL with chemiluminescent detection [33]. The limit of detection of chemiluminescent method is low, but post-column derivatization of analytes is required. Similarly, the LOQ determined in the present for resveratrol is also at least one order of magnitude lower than the values reported.

Table 2
The LOD and LOQ reported in literatures for resveratrol analysis

	Detection method	LOD (ng/mL)	LOQ (ng/mL)
Rudolf et al. (2005)[a]	DAD	10	40
Rimando et al. (2004)[b]	LC–MS	1	23
Zhou et al. (2004)[c]	Chemiluminescence	0.166	
Vitrac et al. (2002)[d]	UV-vis/fluorescence	10	30
Gamoh and Nakashima (1999)[e]	LC–MS	20	
Soleas et al. (1997)[f]	GC–MS	84	
Sobolev and Cole (1999)[g]	DAD		10000
Lamuela-Raventos et al. (1995)[h]	DAD	3000	10000

[a] Ref. [25].
[b] Ref. [27].
[c] Ref. [33].
[d] Ref. [22].
[e] Ref. [17].
[f] Ref. [34].
[g] Ref. [35].
[h] Ref. [36].

3.2. Comparison of sample preparation methods used for piceatannol and resveratrol extraction

To analyze piceatannol and resveratrol in plants or related samples, extraction or other steps to remove interferences is usually required. However, too many sample preparation steps will cause serious loss and even decomposition of the analytes. Thus, sample treatment used in this study was limited to grinding, solvent extraction, and removing some interference, such as chlorophyll with solid-phase extraction in some cases; otherwise, the chromatograms would be too complex to resolve piceatannol (data not shown). Additionally, the effects of liquid nitrogen treatment and lyophilization on the recoveries of piceatannol and resveratrol were investigated and compared. In the case of liquid nitrogen treatment, leaf samples of *V. thunbergii* were frozen by liquid nitrogen, ground, and then extracted with solvent. In the case of lyophilization, samples were frozen, ground, freeze-dried, and then extracted with solvent. All procedures were done in the dark to avoid the impact of fluorescence light. The influence of fluorescenc light on the recoveries of piceatannol and resveratrol were then examined in another experiments with the same sample preparation procedures. The recoveries associated with different treatments under darkness and fluorescence light are shown in Tables 3 and 4, respectively. As shown in the Table 3, direct solvent extraction gives a quantitative recovery. The averaged recovery for samples treated with liquid nitrogen was around 95%, close to quantitative recovery. Beside the analytes are stabilized at low temperature, one of the advantages of liquid nitrogen treatment is the samples will become fragile; which makes the grinding easier even for sample as hard as roots. On the contrary, lyophilization has a great impact on the recovery. Only approximately 1.7% of piceatannol and 12% of resveratrol was recovered after the treatment. Sample preparation carried out under light using the same protocols also revealed a similar trend in percent recovery. The recoveries of the analytes were higher for direct solvent extraction and lower for lyophilization. However, only 65% of piceatannol and 50% of resveratrol were recovered from the matrix when extracted by solvent directly under the exposure of fluorescence light, whereas almost all piceatannol lost after liquid nitrogen treatment or lyophilization. Thus, exposure of light during analysis has definitely a great impact on the stability of resveratrol and piceatannol. Irradiation of UV light to *trans*-resveratrol induces its isomerization to the *cis*-form and greatly alters its UV spectrum [37]. The λ_{max} shifts from 320 nm for *trans*-piceatannol to 286 nm for *cis*-piceatannol, and therefore the absorbance at 320 nm will diminish if the *cis*-form appears to be the dominated compound. Even the UV-induced isomerization between *cis*- and *trans*-form has not been reported for piceatannol, similar analogy may be applied. Hence, it is highly recommended to prepare and analyze the piceatannol and resveratrol-containing samples in a dark environment.

The piceatannol and resveratrol contents of samples treated with lyophilization prior to solvent extraction confirmed the negative effect of lyophilization. The amounts of piceatannol and resveratrol determined in *V. thunbergii* samples treated with lyophilization under darkness are listed in Table 5 and are compared with the results obtained by treating with liquid nitrogen. In general, lyophilization prior to solvent extraction yields lower levels of both piceatannol and resveratrol as compared with the liquid nitrogen treatment. In the first sample, the piceatannol content was 183 ng per gram of material when treated with liquid nitrogen, but no piceatannol was detected when the same sample was lyophilized prior to extraction. In the case of resveratrol, the quantity extracted after liquid nitrogen treatment was four times higher than that after lyophilization. We also found prolong lyophilization may greatly reduce the recovery of piceatannol and resveratrol. The reason for the reduction in the contents and recoveries of piceatannol and resveratrol might be simply due to the molecules sublimate during lyophilization pro-

Table 3
Percent recoveries of piceatannol and resveratrol standards from leaf samples of *V. thunbergii* in different sample preparation conditions[a]

	Piceatannol[b] (%)	Resveratrol[b] (%)
Direct solvent extraction	100.0 ± 1.5	100.0 ± 1.7
Liquid nitrogen treatment[c]	96.5 ± 1.1	95.0 ± 13.7
Lyophilization[c]	1.7 ± 2.5	12.7 ± 11.0

[a] Sample preparation and analysis were done in dark to exclude the influence of light on piceatannol and resveratrol. The data were acquired by fluorescence detector.
[b] Averaged recovery of three spiked samples. Two injections were made for each sample.
[c] After liquid nitrogen treatment or lyophilization, samples were subjected to solvent extraction following the same procedures described in the experimental section.

Table 4
Influence of light on the recoveries of piceatannol and resveratrol standards from leaf samples of *V. thunbergii* in different sample preparation conditions[a]

	Piceatannol[b] (%)	Resveratrol[b] (%)
Direct solvent extraction	64.8 ± 3.4	49.2 ± 19.3
Liquid nitrogen treatment[c]	0.5 ± 0.1	7.4 ± 4.9
Lyophilization[c]	0.5 ± 0.2	3.3 ± 1.8

[a] Sample preparation and analysis were done under regular fluorescence lamps and the data were acquired by fluorescence detector.
[b] Averaged recovery of three spiked samples. Two injections were made for each sample.
[c] After liquid nitrogen treatment or lyophilization, samples were subjected to solvent extraction.

Table 5
The effect of lyophilization on the amounts of piceatannol and resveratrol extracted from *V. thunbergii*

	Lyophilization		Liquid nitrogen treatment	
	Piceatannol (ng/g)[a]	Resveratrol (ng/g)[a]	Piceatannol (ng/g)	Resveratrol (ng/g)
Sample 1[b]	ND[c]	238 ± 64	183 ± 5	589 ± 174
Sample 2	ND	110 ± 99	ND	447 ± 230

[a] The concentrations were normalized based on 1 g of fresh material.
[b] Samples 1 and 2 were from different plants collected from the outdoors at two separate days. For each sample, leaves of plants were pooled and homogenized. The homogenized material was then used in three experiments.
[c] ND: not detected.

cess. According to a recent reports, lyophilization may reduce bioactive compounds in matrix [31,32]. Here, we observed the same phenomenon; therefore, lyophilization may not be an appropriate sample preparation method for piceatannol or resveratrol-containing materials. Depending on the nature of plant tissues, it is better to extract tender tissues, such as calli and leaves, with solvent directly or to freeze hard samples, like roots and stems, quickly with liquid nitrogen followed by solvent extraction.

3.3. Method validation

V. *thunbergii* spiked with piceatannol and resveratrol standards was used to examine the accuracy and precision of the developed method. Since calli can be a good source for mass production of piceatannol, the analytical method was also validated using the peanut calli. The recoveries and relative standard deviation (RSD) of piceatannol and resveratrol from the leaves of V. *thunbergii* are summarized in Table 6. As shown in the table, when the spiked piceatannol was higher than 25 ng/mL, the recoveries were almost quantitative, higher then 90%. The RSD range was from 0.8 to 5.7%. Similarly, the recoveries of resveratrol were between 96.8 and 100.0% in the range from 50 to 1000 ng/mL and the RSD were below 3.5%. The low recovery of 66.6% and high RSD of 10.4% observed at the low standard concentration of 10 ng/mL for piceatannol were probably due to the complicated sample matrix of *V. thunbergii* as compared with that of calli. The low recoveries and high RSD observed at the low standard concentrations (25 and 10 ng/mL) in the case of resveratrol could be attributed to the matrix effect as well. Since the theoretical LOD and LOQ listed in Table 1 were calculated using the piceatannol and resveratrol standards, the influence of matrix on quantitative analysis was not as complicated as real samples. The LOQ and LOD values shown in Table 1 are therefore lower than the numbers shown in Table 6. Considering the matrix effect, the real LOQ may be a little higher than the theoretical values. When analyzing the plant samples, such as *V. thunbergii*, minor components appeared on the chromatogram may interfere with the quantitative analysis, resulting in poor recovery and high RSD at the concentration lower than 10 ng/mL.

To assess the reproducibility and accuracy of this method, the interday and intraday recoveries determined using peanut calli are listed in Table 7. Among samples analyzed, piceatannol and resveratrol standards were almost quantitatively recovered in all cases. The recovery yields range from 92% in the case of resveratrol to 101% in the case of piceatannol. The quantitative recovery of spiked standards suggests that the sample preparation steps used are able to extract analytes completely from the matrix. Thus, this analytical method should be able to give good accuracy for resveratrol and piceatannol analysis. The relative standard deviations for intraday and interday precisions were 3 and 1%, respectively, in the case of resveratrol. Similarly, the RSD for intraday and interday precisions were 6 and 3% for piceatannol, respectively. All these values are less than 6%, which reveals excellent precision of this analytical method.

3.4. Screening the piceatannol-containing plants

One of the purposes of the present study was to find new piceatannol-containing plants using this proposed method. We selected some plants, which reported to contain resveratrol only. The free form of piceatannol has never been detected in these plant species. In addition, we collected some local folk medicine plants, which are lack of information about stilbenoids. The samples included dry herbal materials that are commonly used in Chinese medicine and plants that were collected from our university campus. The plants analyzed included *P. cuspidatum*, a Chinese herbal medicine; and *A. brevipedunculaata* and *V. thunbergii*. The major plant parts used as Chinese herbal materials are their roots. For plants collected from the outdoors roots, stems, and leaves were analyzed. Peanut plants (*A. hypogaea*) collected from field were also analyzed for their piceatannol and resveratrol contents. The tissues analyzed included roots, stems, and leaves. The quantities of piceatannol and resveratrol in plant samples are thus listed in Tables 8 and 9. Interestingly, all plants analyzed contained piceatannol. For example, the root of peanut was found to have high levels of piceatannol. This observation of piceatannol in peanut plants differs from the literature report that no piceatannol is present in the tissues of peanut except for calli [30]. In the fresh plants collected outdoors, *V. thunbergii* have the highest contents of piceatannol and resveratrol. In the case of roots, the piceatannol and resveratrol contents in *V. thunbergii* were approximately 5 and 314 μg per gram of fresh material, respectively. When comparing the quantities of piceatannol and resveratrol in different tissues of

Table 6
Recovery and reproducibility of piceatannol and resveratrol from the spiked leaves of *V. thunbergii*

Spiked concentration (ng/mL)	Piceatannol		Resveratrol	
	Recovery (%)[a]	RSD (%)[b]	Recovery (%)[a]	RSD (%)
2000	101.0	5.7	92.3	3.5
1000	100.0	1.5	100.0	1.7
100	98.8	1.0	97.9	2.1
50	97.4	0.8	96.8	1.2
25	90.4	2.3	79.4	12.3
10	66.6	10.4	44.7	35.0

[a] The percent recovery was the average of three spiked samples.
[b] Relative standard deviation.

Table 7
Recovery and reproducibility of piceatannol and resveratrol from spiked peanut calli

Compounds	Recovery (%) (Interday)[a]	RSD (%) (Interday)	Recovery (%) (Intraday)[b]	RSD (%) (Intraday)
Piceatannol	101	3	98	6
Resveratrol	93	1	92	3

[a] The interday precision was determined by ananlzing three spiked samples over three consecutive days. The concentrations of resveratrol or piceatannol standards spiked in samples were 50 ng/mL.
[b] The intraday precision was determined by analyzing three samples in 24 h. The concentrations of resveratrol or piceatannol standards spiked in samples were 50 ng/mL.

Table 8
Piceatannol and resveratrol contents in roots, stems, and leaves of *A. brevipedunculaata*, *V. thunbergii*, and *A. hypogaea*

	Piceatannol[a,b] (ng/g)[c]	Resveratrol[a,b] (ng/g)[c]
A. brevipedunculaata		
Roots	412 ± 20	673 ± 164
Stems	ND[d]	ND
Leaves	288 ± 137	326 ± 133
V. thunbergii		
Roots	4970 ± 130	314570 ± 9680
Stems	540 ± 10	17870 ± 2050
Leaves	184 ± 6	589 ± 175
A. hypogaea		
Roots	2945 ± 4126	6336 ± 8825
Stems	8 ± 2	ND
Leaves	57 ± 14	18 ± 31
UV-treated calli	5310 ± 1	11940 ± 640

[a] The quantities of piceatannol and resveratrol in roots, stems, and leaves were the averaged values of three individual samples.
[b] To determine the piceatannol and resveratrol contents, samples collected from fields were prepared by the procedures described in the experimental sections. The calculation was done based on the fluorescence using the calibration curved listed in Table 1.
[c] The concentrations were normalized based on 1 g of fresh material.
[d] ND: not detected.

the same plant, roots were found to have the highest levels. Surprisingly, no piceatannol and resveratrol were detected in the stems of *A. brevipedunculaata*. The higher levels of piceatannol and resveratrol detected in roots are probably due to their consistent exposure to the microorganisms in soil. As a result, roots can produce higher levels of phytoalexins in response to environmental stimulations.

The high levels of resveratrol found in *V. thunbergii* and *A. hypogaeae* agree with the observation reported in literature. On the contrary, the presence of piceatannol in *V. thunbergii* [38] and *A. hypogaeae* has never been reported. Thus, the finding of piceatannol in *V. thunbergii* and *A. hypogaeae* is of particular important. It not only proves the applicability of this analytical method for screening piceatannol, but also provides a new source for large production of piceatannol. In fact, to the best of our knowledge, this is the first time piceatannol was found in these plant species. In the case of *A. hypogaeae*, data showed large standard deviations. These high standard deviations came from variances between plants collected. Three different peanut plants were analyzed to get the averaged numbers. Visual inspection prior to analysis revealed that the leaves of one plant had clear insect bites caused by the insect pests, thrips, which might pose as one kind of stress to induce peanut plants to produce the unusual amounts of resveratrol and piceatannol. This particular sample had higher piceatannol and resveratrol contents than the other two peanut plants (data not shown), which resulted in high standard deviations of the data.

Table 9
Piceatannol and resveratrol contents in the radixes of Chinese herbal medicine, *P. cuspidatum*, *A. brevipedunculaata*, and *V. thunbergii*[a]

	Piceatannol[b,c] (μg/g)[d]	Resveratrol[b,c] (μg/g)[d]
P. cuspidatum	66 ± 36	1083 ± 666
A. brevipedunculaata	17 ± 5	556 ± 36
V. thunbergii	17 ± 7	409 ± 13

[a] The materials were commercially available. The major parts of these materials are roots.
[b] The quantities of piceatannol and resveratrol in each Chinese herbal medicine were the averaged of three analyses.
[c] Each sample was ground to fineness followed by solvent extraction using the same sample preparation procedures described in the experimental section.
[d] The concentrations were normalized based on 1 g of dry material.

Shown in Table 9 are the piceatannol and resveratrol contents in some dried Chinese herbal materials. All three species analyzed contained exceptionally high quantities of piceatannol and resveratrol. In the case of *P. cuspidatum*, the free form of piceatannol was detected for the first time in this plant. The amount of piceatannol ranged from 66 μg per gram of dry material in *P. cuspidatum* to 17 μg in *A. brevipedunculaata* and *V. thunbergii*. The resveratrol contents ranged from over 1000 μg for 1 g of dry material in *P. cuspidatum* to approximately 500 μg in *A. brevipedunculaata* and *V. thunbergii*. Since *P. cuspidatum* is a resveratrol-producing plant, it is not surprising to identify resveratrol in the dry *P. cuspidatum*. However, the quantity was much higher than expected. Since the material analyzed was mainly the roots, high levels of phytoalexins may be found. Interestingly, the piceatannol found in this particular *P. cuspidatum* sample was the free form instead of the piceatannol glycoside and its level was also much higher than other plants.

4. Conclusions

Many scientific evidences have shown that the piceatannol is a more important anticancer compound than resveratrol. A large amount of piceatannol is required to investigate its potential as an anticancer drug and other possible health beneficial effects. As a result, continuous discovery of plants that can provide large quantity of piceatannol is very important. To screen for the piceatannol-containing plants, a sensitive analytical method with proper sample preparation is necessary. In this study, we provided a sensitive analytical method that was specifically designed for piceatannol analysis. More importantly, the presence of piceatannol in plants can be easily identified by using this method. When the HPLC is coupled with UV and fluorescence detectors, the decrease of signals in interferences and increase of signal in piceatannol in the fluorescence chromatogram mark clearly the position of the piceatannol peak; ultimately, it allows identification without standards. This study found inappropriate sample preparation would cause dramatic loss of piceatannol and resveratrol. We believe this inappropriate sample preparation may be the key factor that interferes the observation of piceatannol in plants. Thus, lyophilization is not recommended. All procedures should be done in the dark to avoid the negative impact of light.

The piceatannol and resveratrol profiles in some plant species were re-evaluated by this method. Literatures show that *A. brevipedunculaata*, *V. thunbergii*, *A. hypogaea*, and *P. cuspidatum* have resveratrol only, however, by the method, we found differ-

ent amounts of piceatannol in all test species for the first time. This result implies that piceatnnol may be present along with resveratrol in all other resveratrol-containing plants. Simply due to the improper sample preparation, piceatannol was not detected in most cases. Thus, the findings of this study have successfully demonstrated the applicability of this analytical method and its importance. With this method, we hope that more people can be promoted and start the piceatannol research in the future.

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