Research Article

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The assay of pterostilbene in spiked matrices by liquid chromatography tandem mass spectrometry and isotope dilution method

Fabio Mazzotti,[a] Leonardo Di Donna,[a] Hicham Benabdelkamel,[a] Bartolo Gabriele,[b] Anna Napoli[a] and Giovanni Sindona[a]*

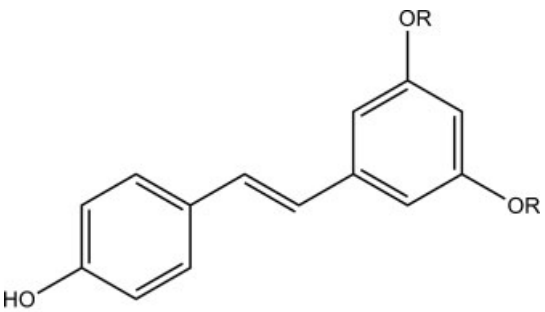
Pterostilbene (*trans*-3,5-dimethoxy-4-hydroxystilbene) is an active component found in several plant species, exhibiting important pharmacological properties. A new and reliable method of assaying this phyto compound in various matrices is presented; the assay is based on (1) the selectivity of liquid chromatography (LC) hyphenated with electrospray ionisation (ESI), (2) the specificity of a two-step mass spectrometric analysis (MS/MS) and (3) the accuracy of the isotope dilution method. The labelled analogue may be conveniently synthesised in a few steps. The sensitivity of the method is confirmed by the very low limit of detection (LOD) and limit of quantitation (LOQ) values achieved in the assay of pterostilbene in two distinct fortified matrices, and is further supported by the observed accuracy values.

Keywords: pterostilbene; ESI-MS/MS; isotope dilution method; multiple reaction monitoring; phytoalexin

Introduction

Pterostilbene (*trans*-3,5-dimethoxy-4-hydroxystilbene), (**1**, chart), a dimethylether analogue of resveratrol, is a naturally occurring phytoalexin identified in several plant species, exhibiting important pharmacological properties.[1] It belongs to a group of phenolic compounds known as stilbenes, found in deerberry and rabbiteye blueberries[2] and several varieties of grapes[3,4]; it has also been identified in the leaves of *Vitis vinifera*,[5] in *Pterocarpus marsupium*[6] and in the *Guibourtia Tessmanii* plant,[7] used in traditional medicine. In particular, the extract of *P. marsupium*, used in Ayurvedic medicine, is known for its antiglycemic effect. It is a strong antioxidant, and is reported to scavenge 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radicals and to inhibit the oxidation of citronellal and lipid peroxidation in rat liver microsomes.[8] Pterostilbene is cytotoxic against a number of cancer cell lines, including human breast cancer and murine lymphoid neoplasma cells[9,10]; moreover, it has been demonstrated that it might reduce cholesterol levels.[11,12] Animal studies have shown that this natural compound can lower blood glucose and may be a potent anti-diabetic agent.[6] The quantitation of pterostilbene in biological fluids is currently performed by high performance liquid chromatography (HPLC) coupled to fluorimetric detection (FL).[13] Furthermore, pterostilbene has been successfully assayed, together with other phytoalexins, in grapevine leaf extract and berries using the HPLC-FL methodology.[3,14]

In this work, we present a rapid method of determination of pterostilbene in blueberry juice and human serum by liquid chromatography mass spectrometry (LC-MS) under multiple reaction monitoring (MRM) condition and isotope dilution. The hexadeutero analogue of pterostilbene (**2**, chart) is used as the labelled internal standard. Isotope dilution represents an extremely accurate method of quantitation of analytes in complex natural and biological matrices by mass spectrometry and is best suited to improve precision and accuracy by reducing the problems arising from calibration procedure, sample preparation and matrix effects.[15–18]



1. R=CH_3 Pterostilbene
2. R=CD_3 Pterostilbene-d_6

Experimental

Chemicals

Pterostilbene (97% purity) was used for the assay. ACS-grade solvents and reagents (see below) were used for synthesis, while HPLC-grade MeOH and H_2O were used for analyses. All the chemicals were purchased from Sigma–Aldrich, St Louis, MO.

* *Correspondence to: Giovanni Sindona, Dipartimento di Chimica, Università della Calabria, Via P. Bucci cubo 12/C, I-87036 Arcavacata di Rende (CS), Italy. E-mail: sindona@unical.it*

a *Dipartimento di Chimica, Università della Calabria, cubo 12/C, I-87036 Arcavacata di Rende (CS), Italy*

b *Dipartimento di Scienze Farmaceutiche, Università della Calabria, cubo 12/C, I-87036 Arcavacata di Rende (CS), Italy*

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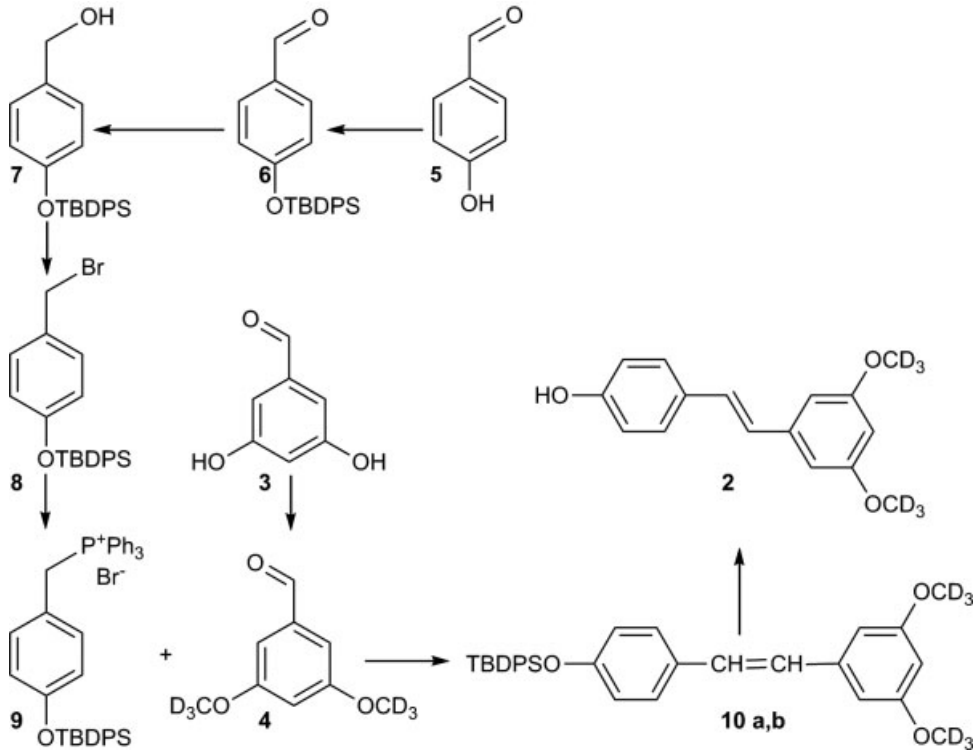

Scheme 1. Synthesis of compound **2**.

Synthesis of d_6-pterostilbene

3,5-Dimethoxybenzaldehayde (**4**, Scheme 1): 3,5-dihydroxybenzaldehyde (**3**) (1 g, 7.2 mmol) and anhydrous K_2CO_3 (20 g, 145 mmol) were stirred in dry acetone (50 ml) and 7 ml (97 mmol) of CD_3I were then added. The mixture was heated under reflux for 45 min, then cooled to room temperature, filtered and evaporated to dryness. The residue dissolved in CH_2Cl_2 was washed twice with water, dried over anhydrous Na_2SO_4, filtered and purified by flash chromatography (silica gel, hexane/ethyl acetate 4 : 1, v/v) affording **4** with 93% yield.

4-(*tert*-Butyldiphenylsilyloxy)-benzyltriphenylphosphonium bromide **9** (3 g, 11.9 mmol), synthesised as reported[19] was treated at −78 °C, in anhydrous tetrahydroufan (35 ml), with *n*-butyllithuim (2.5 M, 3 ml). The dialdehyde **4** (1.5 g, 9.3 mmol), dissolved in 5 ml of tetrahydrofuran, was then added. The crude product purified by flash chromatography (silica gel, hexane/ethylacetate 8 : 2, v/v) yielded 90% of a mixture of the *E*/*Z* isomers (**10a, 10b**).

Trans-pterostilbene-d_6 (**2**): To 1.2 g (2.45 mmol) of (**10a,b**) dissolved in 20 ml of anhydrous tetrahydrofuran, 3.4 ml of tetrabutylammonium fluoride (1 M) were added. The pale yellow solution, stirred for 45 min and poured into water, was extracted with dichloromethane. The organic phase, dried on anhydrous sodium sulfate, was evaporated to dryness to afford the *E*/*Z trans*-pterostilbene-d_6 isomeric mixture. The latter, after separation by flash chromatography (silica gel, hexane/ethyl acetate 9.5 : 0.5, v/v), yielded 70%, based on **4**, of pure *trans*-pterostilbene-d_6 (**2**,Scheme 1).[19]

The isotopic distribution, checked by high-resolution mass spectrometry, was $d_5 = 2\%$ and $d_6 = 98\%$.

The structure of deuterated pterostilbene **2** was confirmed by MS and ^{1}H nuclear magnetic resonance (NMR).

^{1}H NMR (300 MHz, $CDCl_3$): δ_H 7.39 (2H, m, $J = 8.6$ Hz, H-2′, H-6′), δ_H 7.02 (1H, distorted d, $J = 16.5$ Hz, H-β), δ_H 6.87 (1H, distorted d, $J = 16.5$, H-α), δ_H 6.82 (2H, m, $J = 8.6$ Hz, H-2, H-6), δ_H 6.63 (2H, m, $J = 8.6$ Hz, H-3′, H-5′), δ_H 6.37 (1H, m, $J = 2.1$ Hz, H-4), δ_H 5 (1H, br s, OH).

HRESI-MS: *m/z* 263.1553 $[M + H]^+$ calculated for $C_{16}H_{11}D_6O_3$ 263.1548.

Sample preparation

Blueberry juice: 12.5 µl of a solution (4 µg/ml) of the internal standard (**2**) was added to 1 ml of juice, spiked with **1** and diluted 1 : 1 with water; the resulting solution was passed through a 0.22-µm HPLC filter and used for subsequent analysis.

Plasma: 12.5 µl of a solution (4 µg/ml) of the internal standard (**2**) was added to 1 ml of human plasma, spiked with **1**; the mixture was treated with 1.5 ml of cold acetonitrile (in order to precipitate proteins) and centrifuged at 8000 rpm for 5 min. The supernatant, transferred to an Eppendorf tube, was evaporated to dryness under nitrogen gas, reconstituted with 0.5 ml of HPLC mobile phase (see below), passed through a 0.22-µm HPLC filter and used for the assay.[13]

Mass spectrometry

The LC-MS analysis was carried out on a triple-quadrupole mass spectrometer LC 320 (Varian Inc., Palo Alto, CA), equipped with an ESI source interfaced with an HPLC Prostar 210 instrument (Varian Inc.). The chromatographic analysis was performed using a Discovery C_{18} column (75 × 2.1 mm, Supelco, Bellefonte, PA), injecting a volume of 20 µl. The flow rate was 0.25 ml/min using the following eluants and linear gradients: solvent A (H_2O, 0.025% ammonium hydroxide), solvent B (MeOH); from 25% B to 98% B

in 7 min; 2 min at 98% B isocratic; from 98% B to 25% B in 2 min. A re-equilibration time of 3 min was used after each analysis. The instrument parameters of needle, shield and capillary voltages were set to −4.5 kV, 600 V and 70 V, respectively; The drying (N_2) pressure and temperature were set to 20 psi and 250 °C, respectively, while the nebulising gas (air) pressure, the housing temperature and the electron multiplier voltage were set to 45 psi, 60 °C and 1350 V, respectively. The dwell time was 0.200 s/scan, and the quadrupole resolution was set using a mass peak width of 0.8 amu. The collision gas pressure (Ar) was fixed to 2 mTorr, and the collision energies were set to 30 eV for the transitions *m/z* 255 → *m/z* 197 and *m/z* 261 → *m/z* 197 and 18 eV for the transitions *m/z* 255 → *m/z* 240 and *m/z* 261 → *m/z* 243.

High-resolution electrospray ionisation (HR-ESI) experiments were carried out on a hybrid Q-Star Pulsar-i (MSD Sciex Applied Biosystem, Toronto, Canada) mass spectrometer equipped with an ion spray (IS) ionisation source. Samples were introduced by direct infusion (3 µl/min) of the sample containing the analyte (5 ppm), dissolved in a solution of 0.1% acetic acid, acetonitrile/water 50 : 50 at the optimum IS voltage of 4800 V. The source nitrogen (GS1) and the curtain gas flows were set at pressures of 20 and 25 psi, respectively, whereas the first declustering potential (DP1), the focusing potential and the second declustering potential (DP2) were kept at 50, 220 and 10 V relative to ground, respectively.

Nuclear magnetic resonance

The NMR spectra were recorded using an AC 300 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) on samples dissolved in $CDCl_3$; chemical shifts were measured in ppm and coupling constants (*J*) in hertz.

Analytical Parameters

The limit of detection (LOD) and the limit of quantitation (LOQ) for each matrix were calculated by applying Eqns (1) and (2), according to the directives of International Union of Pure and Applied Chemistry (IUPAC) and the American Chemical Society's Committee on Environmental Analytical Chemistry.

$$S_{LOD} = S_{RB} + 3\sigma_{RB} \quad (1)$$

$$S_{LOQ} = S_{RB} + 10\sigma_{RB} \quad (2)$$

S_{LOD} is the signal at the limit of detection, S_{LOQ} is the signal at the limit of quantitation, S_{RB} is the signal of the matrices without analyte (blank) and σ_{RB} is the standard deviation for same matrices calculated on seven measurements. The values of the unknown concentrations were obtained from the appropriate calibration curves.

Results and Discussion

Tandem mass analysis provides the specificity that allows unambiguous correlation between parent and product ions; moreover, the good to excellent signal-to-noise (S/N) ratio confers the methodology the sensitivity to perform trace analysis in complex mixtures through the well-established MRM technique. Atmospheric pressure chemical ionisation tandem mass spectrometry (APCI-MS/MS) was recently applied to recognise the product of combinatorial synthesis of stilbenoids.[20]

In the case under investigation, ESI method was chosen to ionise the species eluted in the first chromatographic stage, and both

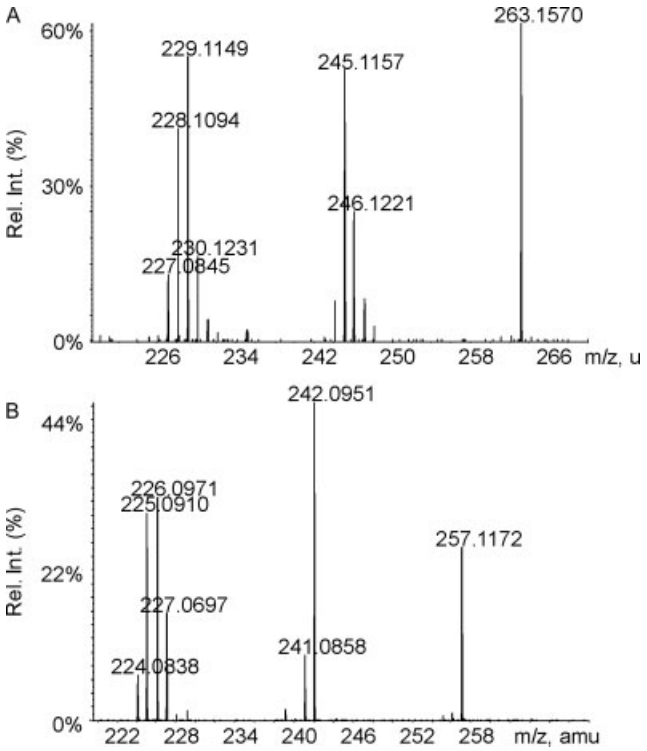

Figure 1. Partial ESI (+) MS/MS spectra of compounds **1** (A) and **2** (B).

positive (+) and negative (−) modes were checked. As expected, the ESI (+) MS/MS spectrum performed on the $[M + H]^+$ derived from unlabelled compound **1** provides a wealth of information, displaying a complex fragmentation pattern, whose first diagnostic species is represented by the unimolecular elimination of methyl radical to give the ion at *m/z* 242 (Fig. 1(A)).

The number of peaks (*m/z* 224 → *m/z* 227) arising from competitive and consecutive hydrogen and methyl radical losses from the ion at *m/z* 242 (Fig. 1(A)) represents the major drawback for a straightforward application of the MRM quantitation method.

This observation is supported by the chemistry of the $[M+H]^+$ of the d_6 reference compound **2** (Fig. 1(B)). It can be easily observed, in fact, that the elimination of d_3-methyl radical from the precursor species at *m/z* 263 competes with an extensive H/D isomerisation, giving rise to the elimination of CHD_2 and CH_2D radicals, besides the formation of $[(M - CD_3) + H]^{+\bullet}$ species at *m/z* 245. The fragmentation pattern is even more complex for the other consecutive and competitive reaction paths taken by the reactive species.

The ESI (−) MS/MS spectrum of the $[M - H]^-$ deprotonated ion of the unlabelled compound **1** shows only a few diagnostic fragment ions at *m/z* 240, 239, 225, 224 and 197 (Scheme 2 and Fig. 2(A)).

The gas-phase chemistry of the labelled isomer **2** shows the same fragmentation pattern as that of of compound **1** (Fig. 2(B)). The deprotonated species at *m/z* 261 undergoes competitive and consecutive radical and neutral losses not involving H/D isomerisation. The elimination of $CH_3\cdot$ ($CD_3\cdot$) radicals gives rise to a resonance-stabilised distonic radical anion.[21] The consecutive elimination of the second methyl group likely leads to the species suggested in Scheme 2, which further fragments through carbon monoxide elimination to give the species at *m/z* 197, which is common to both labelled and unlabelled pterostilbene. This

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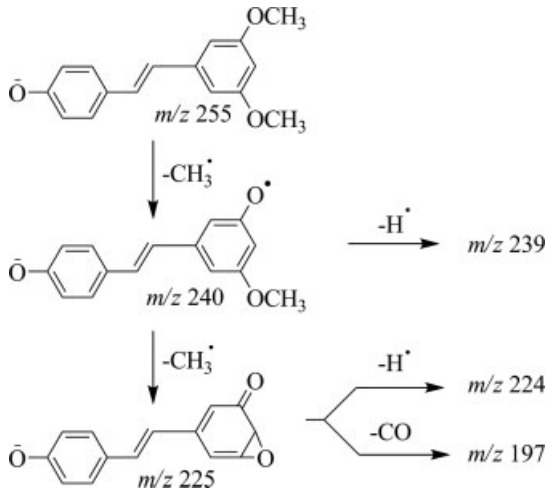

Scheme 2. Fragmentation pattern of protonated compound **1**.

fact clearly shows that no hydrogen/deuterium isomerisation competes with the observed fragmentation pathways.

The assay of analyte **1** was, therefore, performed under MRM condition in the negative ion mode selecting the transitions with the highest ion current. Accordingly, the reaction pathways *m/z* 255 → *m/z* 240 for **1** and *m/z* 261 → *m/z* 243 for the labelled internal standard were chosen. In addition, the transitions *m/z* 255 → *m/z* 197 for **1** and *m/z* 261 → *m/z* 197 for **2** were used to confirm the chromatographic elution of the analytes.

The assay of the analyte in the spiked matrices was performed by means of a calibration curve ($y = 1.3191x - 0.2974$; $R^2 = 0.9982$) built using triplicate samples of six standard solutions at different concentrations of pterostilbene ranging from 5 to 200 ng/l, and fixed concentration (50 ng/ml) of the internal standard (Table 1).

Table 1. Analytical parameters of standard solution[a]

Standard solution (ng/ml)	Area ratio	Average area ratio	Standard deviation	RSD%
	0.10000			
5	0.11630	0.111	0.009	8.40
	0.11593			
	0.23820			
10	0.23797	0.239	0.001	0.58
	0.24047			
	0.43060			
20	0.42763	0.434	0.008	1.80
	0.44237			
	1.18421			
50	1.11022	1.169	0.052	4.48
	1.21136			
	2.35510			
100	2.35294	2.375	0.036	1.50
	2.41568			
	4.55239			
200	4.52085	4.564	0.050	1.10
	4.61905			

[a] To each sample of the calibration curve ($y = 1.3191x - 0.2974$; $R^2 = 0.9982$) was added a fixed quantity of internal standard (50 ng/ml).

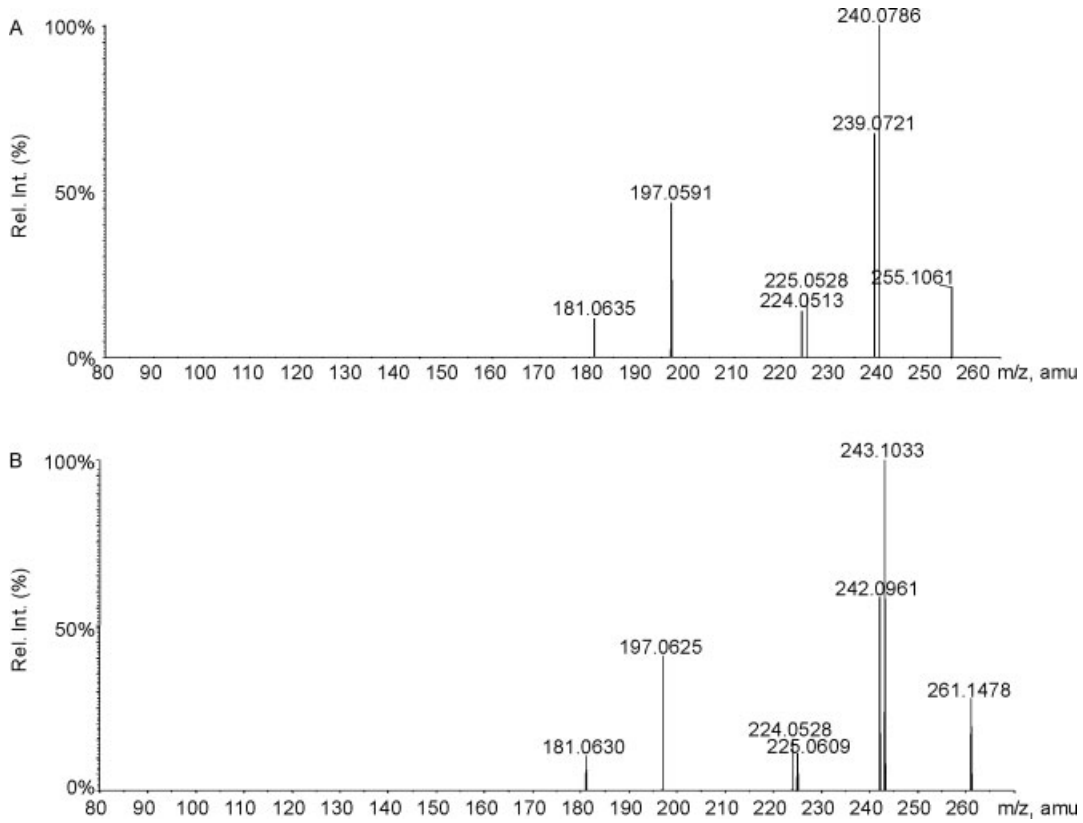

Figure 2. ESI (−) MS/MS spectra of compound **1** (A) and **2** (B).

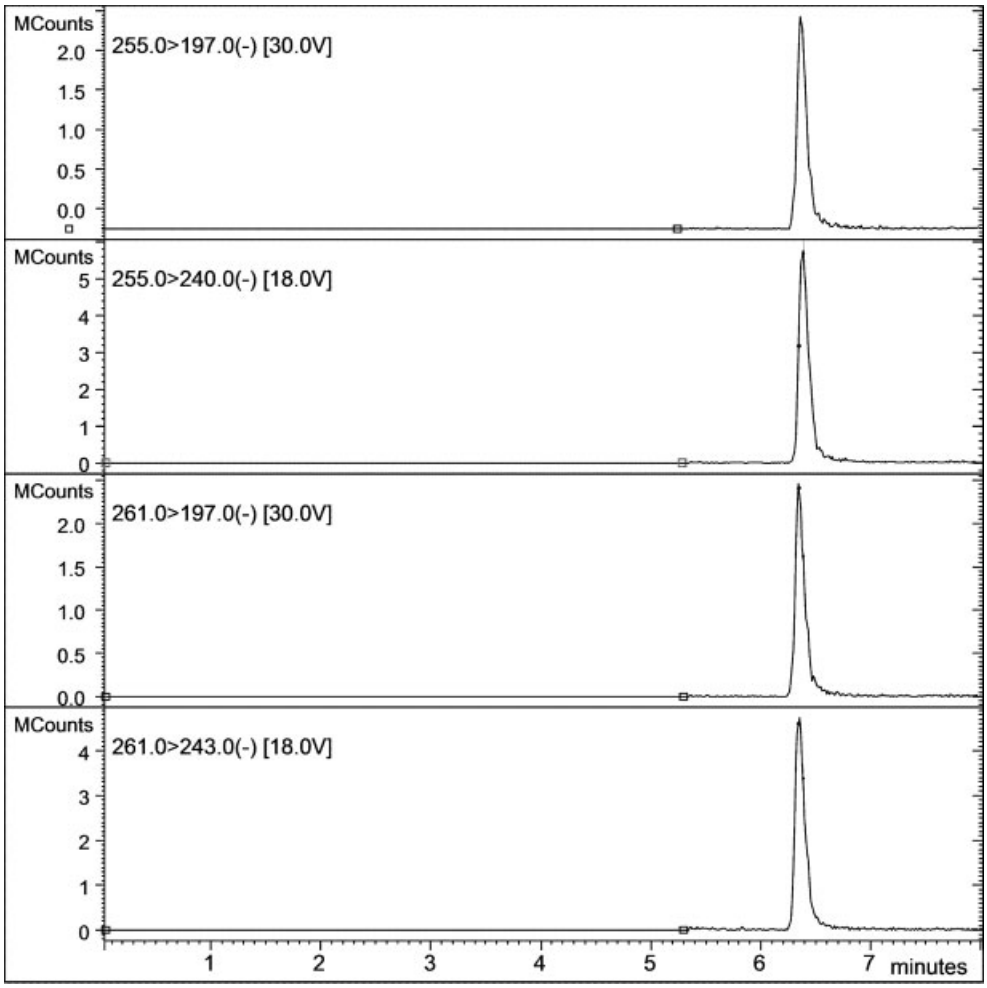

Figure 3. HPLC-MRM chromatogram of compounds **1** and **2**.

Figure 3 shows an MRM chromatogram of the standard solution at 50 ng/ml of **1** and 50 ng/ml of **2**.

The standard solutions were proven to be stable for more than 30 days at −20 °C (freezing–thaw cycle) and in sunlight for more than 48 h.

The new protocol has been applied to spiked samples of blueberry juice and human plasma. The relative standard deviation (RSD%) value was in all cases under 7%, thus showing a good repeatability of the measurements. The accuracy of the method was determined from samples prepared by adding known amounts of the analyte to blank matrices. In the three examined points (Table 2), which represent the boundaries of the calibration curve, the accuracy values ranged from 92% to 112%.

The calculated analytical parameters confirmed the appropriateness of the proposed approach. The values of LOD and LOQ were 1.5 and 3.9 ng/ml, respectively, for blueberry juice and 2.2 and 3.8 ng/ml for human plasma, suggesting that the method is suitable for evaluating very low amounts of pterostilbene in different matrices. The values of recovery are quantitative for both the juice and the plasma.[13] In particular, in the case of the juice, the samples are directly injected into the instrument, after dilution; finally, the reproducibility is, in all cases, below 10% (Table 3).

Table 2. Analytical parameters of precision and accuracy

Spiked concentration (ng/ml)	Calculated concentration (ng/ml)	RSD% (average)	Accuracy%
Juice			
4	4.5 ± 0.6	13.3	112.5
15	16.0 ± 0.5	3.0	109.6
150	146.8 ± 4.7	4.7	97.9
Plasma			
4	4.2 ± 0.5	11.9	105.0
15	13.9 ± 0.9	6.4	92.6
150	152.3 ± 6.1	4.0	101.3

The standard deviation (RSD%) was calculated over three measurements.

Conclusions

Pterostilbene (**1**) exhibits a wide spectrum of biological functions. Assessment procedures for the determination of this natural drug in natural matrices are based on the conventional use of chromatographic techniques linked to fluorescence detection. We now propose a different analytical protocol centred on the

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Table 3. Reproducibility (RSD%) and analytical parameters LOQ, LOD and recovery of the proposed method

Blueberry juice		
LOQ (ng/ml)	3.9	Reproducibility (RSD%)
LOD (ng/ml)	1.5	9.2
Recovery	98%	
Plasma		
LOQ (ng/ml)	4.1	Reproducibility (RSD%)
LOD (ng/ml)	2.2	8.5
Recovery	98%	

The reproducibility of the measurements was obtained by extracting each sample three times over a period of 1 week.

specificity of tandem mass spectrometry and on the reliability of the isotope dilution method. The procedure here presented allows the determination of the analyte with excellent specificity because of the application of MRM methods, and excellent LOQ and LOD parameters because of the use of the labelled internal standard **2**.

Acknowledgements

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