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Short communication

Identification and simultaneous quantification of five alkaloids in *Piper longum* L. by HPLC–ESI-MS^n and UFLC–ESI-MS/MS and their application to *Piper nigrum* L.

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ABSTRACT

A simple, effective and suitable UFLC–ESI-MS/MS method was firstly developed to simultaneously determine five characteristic constituents (piperine, piperlonguminine, Δα,β-dihydropiperlonguminine, pellitorine and piperanine) of *Piper longum* L. The total alkaloids of *P. longum* L. was prepared. The alkaloid contents of *Piper nigrum* L. and *P. longum* L. were compared. The analysis was carried out in multiple reaction monitoring scan mode. The method showed a good specificity, linearity ($R^2 > 0.995$), stability (RSD < 2.53%), repeatability (RSD < 2.58%), and recovery (90.0–103.5%). The limits of detection and limits of quantification of five alkaloids were in the range of 0.02–0.03 and 0.05–0.10 ng/mL, respectively. The intra- and inter-day precision was less than 9.30% and 9.55%, respectively. The validation results confirmed that the method could simultaneously determine the target alkaloids in the sample. Furthermore, the identities of the alkaloids were verified by HPLC–ESI-MS/MS. Compared with *P. nigrum*, *P. longum* had lower piperine content but was enriched in the other four alkaloids.

1. Introduction

Piper longum L. (bibo in Chinese), which belongs to the Piperaceae family, is cultivated for its fruit and commonly used as a dietary flavor in Southeast Asia (Kumar, Kamboj, Susan, & Sharma, 2011). *Piper nigrum* L., which is a close relative of *P. longum*, gives white pepper and black pepper and is a widely used spice throughout the world (Gu, Tan, Wu, Fang, & Wang, 2013). White pepper is obtained by drying the seed of *P. nigrum* and black pepper is obtained from the immature fruit of *P. nigrum*. It is important to note that *P. longum* is one of the most popular herbs in China (Bi, Liu, Luo, Wang, & Wu, 2012) and is commonly used for the clinical treatment of stomachache and vomit (Kumar et al., 2011). Recent researches also showed that it has numerous properties, such as antioxidative (Natarajan, Narasimhan, Shanmugasundaram, & Shanmugasundaram, 2006) and anti-inflammatory effects (Kumari, Ashok, Ravishankar, Pandya, & Acharya, 2012).

Pharmacological and chemical investigations have shown that alkaloids are the major active constituents of *P. longum* (Bi et al., 2012; Liu, Bi, Luo, & Wu, 2011). In our laboratory, the total alkaloids of *P. longum* (PLA) were prepared (Liu et al., 2013) and piperine (PPR), piperlonguminine (PPL), Δα,β-dihydropiperlonguminine (DPPL), piperanine (PPRA) and pellitorine (PLTR) have been isolated from PLA. White pepper and black pepper have similar alkaloid compositions with *P. longum*. Among these alkaloids, PPR can protect against glucose toxicity in mammalian neurons (Lublin et al., 2011) and partially prevent the increase of blood pressure (Hlavacková et al., 2011). PPL and DPPL can reduce intracellular and extracellular Aβ levels (Qi et al., 2009). In our preliminary study, alkaloids from *P. longum* provided neuroprotective effects in an MPTP-induced mouse model of Parkinson's disease.

In this work, five characteristic alkaloid constituents in PLA were quantified. The alkaloid contents of *P. longum* and *P. nigrum* were compared in order to investigate their neuroprotective effects related to Parkinson's disease. To our knowledge, some methods have been developed to detect PPR, such as TLC (Bhat & Chandrasekhara, 1985), HPLC (Bajad, Johri, Singh, Singh, & Bedi, 2002) and UPLC–qTOF-MS (Sachin et al., 2010). Cai, Xie, Yan, and Pan (2011) developed a RP-HPLC/UV method to analyze the contents of *P. longum* and *P. nigrum*, but this method could not identify PPR, PPL, PPRA and PLTR. Rao et al. (2011) developed an HPLC-PDA method to simultaneously determine six compounds in *P. nigrum*, including PPR and PLTR. Unfortunately, DPPL has weak UV absorption and its content in *P. longum* L. is very low. Therefore, it is necessary to develop a method to simultaneously quantify these five alkaloids, including the constituents with weak UV absorption. In the present study, a method of ultra-performance liquid chroma-

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Table 1
Contents of five alkaloids found in PLA, *P. longum* L. and *P. nigrum* L.

Batch No.	Sample type	Source	Calculated contents (mg/g)				
			PPR	PPL	DPPL	PLTR	PPRA
PLA200811	PLA	Self-prepared	347.26	14.23	7.33	20.80	47.16
PLA201009		Self-prepared	353.59	14.04	4.53	11.95	26.59
PLA201106		Self-prepared	312.44	10.25	6.11	25.97	48.16
PL201105AG	*P. longum* L.	An Guo medicinal material market (Hebei province, China)	17.17	0.65	0.35	1.20	2.58
PL201111AG		An Guo medicinal material market (Hebei province, China)	15.62	0.69	0.34	0.79	1.91
PL201204AG		An Guo medicinal material market (Hebei province, China)	14.16	0.59	0.35	0.88	1.93
PL201204TRT		Tong Ren Tang Technologies Co., Ltd. (Beijing China)	22.09	0.87	0.42	1.17	2.70
PL201204SF		Si Fang Pharmaceutical factory (Beijing, China)	13.73	0.53	0.21	0.93	1.59
PL201204GX		Pharmacy shops (Guangxi province, China)	15.17	0.59	0.25	1.05	1.86
PW201204AG	*P. nigrum* L. (white pepper)	An Guo medicinal material market (Hebei province, China)	29.20	0.19	0.05	0.83	1.09
PW201204TRT		Tong Ren Tang Technologies Co., Ltd. (Beijing China)	30.70	0.32	0.16	0.92	1.17
PW201204SF		Si Fang Pharmaceutical factory (Beijing, China)	30.71	0.16	0.05	0.66	1.22
PW201204GX		Pharmacy shops (Guangxi province, china)	33.03	0.21	0.06	0.68	1.16
PB201204AG	*P. nigrum* L. (black pepper)	An Guo medicinal material market (Hebei province, China)	31.08	0.20	0.04	0.60	1.12
PB201204SF		Si Fang Pharmaceutical factory (Beijing, China)	34.63	0.23	0.07	0.51	1.24

Table 2
Selected ions and parameters of DP, CE and CXP of five compounds and IS for MRM scan.

Compound	Q1 (*m/z*)	Q3 (*m/z*)	DP (V)	CE (eV)	CXP (V)
PPR	285.9	201.1	71	27	10
PPL	273.9	201.1	56	25	10
DPPL	276.1	134.9	66	29	10
PLTR	224.0	80.9	96	31	4
PPRA	288.0	135.1	111	39	10
IS	472.4	436.2	104	35	10

tography separation coupled with electronic spray ionization triple-quadrupole tandem mass spectrometry characterization (UFLC–ESI-MS/MS) was developed to simultaneously determine these five compounds in *P. longum* and *P. nigrum*. The method is rapid, accurate, simple, effective and suitable. The standards of the five alkaloids were isolated and purified in our laboratory. In our study, high resolution Orbitrap HPLC–MS/MS measurements were performed to verify the reliability of the standards. The HPLC–MS/MS detection provided accurate molecular weights (<5 ppm) for all standards.

2. Materials and methods

2.1. Chemicals and reagents

The alkaloids in *P. longum* (*i.e.*, PPR, PPL, DPPL, PPRA, PLTR) were isolated by the Chemistry of Chinese Material Medicine Laboratory at the Capital Medical University (Beijing, China). The purity of each alkaloid was determined to be >98% by HPLC-DAD. The alkaloid identity was confirmed by HPLC–ESI-MS^n, ^{1}H-nuclear magnetic resonance (NMR) and ^{13}C-NMR. Terfenadine was purchased from the National Institutes for Food and Drug Control (Beijing, China) and used as an internal standard (IS). Methanol and acetonitrile were purchased from Fisher (Fair Lawn, NJ, USA) and were of HPLC grade. Formic acid was purchased from MREDA (Beijing, China). Distilled water was prepared by a Milli-Q water purification system from Millipore (Molsheim, France).

2.2. Sample materials

P. longum and *P. nigrum* (white pepper and black pepper) were purchased from Anguo (Hebei, China), Tong Ren Tang Technologies Co., Ltd. (Beijing, China), Sifang Pharmaceutical Factory (Beijing, China), and pharmacy shops in Guangxi, China. All samples were authenticated by professor Gui-jun Zhang (Beijing University of Chinese Medicine, China). PLA was prepared by the Chemistry of Chinese Material Medicine Laboratory at the Capital Medical University (Beijing, China). The details were shown in Table 1. The voucher specimens were deposited at the Chemistry of Chinese Material Medicine Laboratory, Capital Medical University, Beijing, China.

2.3. Liquid chromatography

HPLC analysis was performed on a Shimadzu LC-20A liquid chromatography system (Kyoto, Japan) equipped with a binary solvent delivery system (LC-20AD), a column oven (CTO-20A), an

Table 3
MS/MS data and proposed fragmentation pathways of PPR, PPL, DPPL, PLTR, PPRA.

Sample	$[M+H]^+$	Elem. comp.	Diff. (ppm)	Fragment ions (*m/z*)	Diff. (ppm)	Fragmentation pathways
PPR	286.14325	$C_{17}H_{20}NO_3$	−1.817	201.05455	−0.351	$[M+H-C_5H_{10}N]^-$
				173.05975	0.254	$[M+H-C_5H_{10}N-CO]^-$
				135.04410	0.326	$[M+H-C_5H_{10}N-CO-C_3H_2]^-$
PPL	274.14355	$C_{16}H_{20}NO_3$	−0.802	201.05444	−0.899	$[M+H-C_4H_{10}N]^-$
				135.04399	−0.489	$[M+H-C_4H_{10}N-CO-C_3H_2]^-$
				203.07002	−0.933	$[M+H-C_4H_{10}N]^-$
DPPL	276.15903	$C_{16}H_{22}NO_3$	−1.412	161.05954	−1.031	$[M+H-C_4H_{10}N-CO-CH_2]^-$
				135.04393	−0.933	$[M+H-C_4H_{10}N-CO-C_3H_4]^-$
				151.11156	−1.202	$[M+H-C_4H_{10}N]^-$
PLTR	224.20061	$C_{14}H_{26}NO$	−1.253	133.10104	−1.029	$[M+H-C_4H_{10}N-H_2O]^-$
				81.06983	−0.578	$[M+H-C_4H_{10}N-H_2O-C_4H_4]^-$
				203.07013	−0.693	$[M+H-C_5H_{10}N]^-$
PPRA	288.15878	$C_{17}H_{22}NO_3$	−2.221	161.05926	−0.534	$[M+H-C_5H_{10}N-CO-CH_2]^-$
				135.04399	−0.489	$[M+H-C_5H_{10}N-CO-C_3H_4]^-$

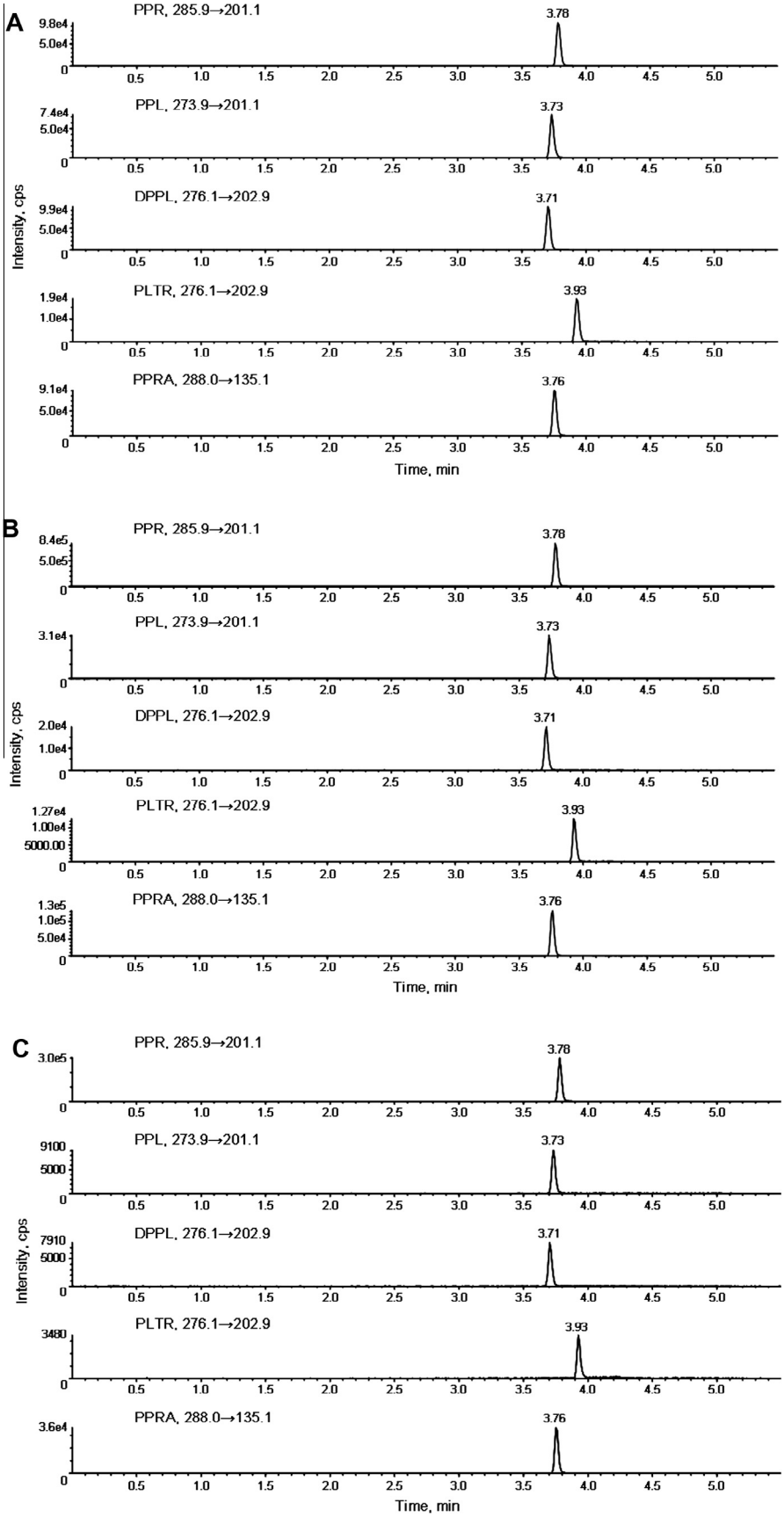

Fig. 1. Representative chromatograms of standards, PLA, *P. longum* L. and *P. nigrum* L. (A) Representative chromatograms of five standards in MRM scan mode; (B) representative chromatograms of PLA sample (PLA200811) in MRM scan mode; (C) representative chromatograms of *P. longum* L. sample (PL201105AG) in MRM scan mode; (D) representative chromatograms of *P. nigrum* L. sample (WP201204AG) in MRM scan mode.

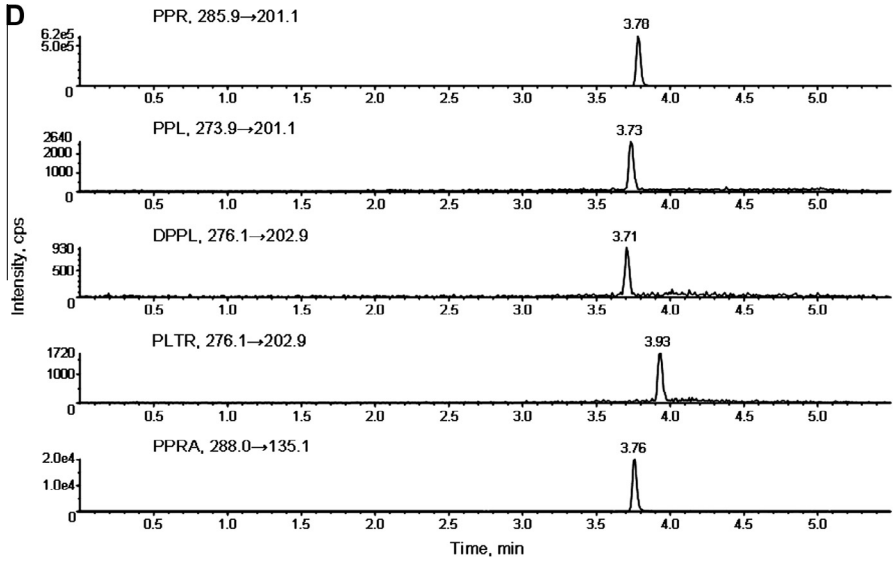

Fig. 1 (*continued*)

automatic injector (SIL-20AC) and a triple-quadrupole mass spectrometry detector (Applied Biosystems, USA).

A Phenomenex Gemini C18 reverse-phase column (50 mm × 2.00 mm, 5 μm) was used for separation. The column was maintained at room temperature. The mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The column was eluted at a flow rate of 0.55 mL/min using a linear gradient as follows: 2% (B) at 0–0.50 min, 2–98% (B) at 0.50–3.10 min, 98% (B) at 3.10–4.00 min, 98–2% (B) at 4.00–4.01 min, 2% (B) at 4.01–5.50 min. All samples were filtered through 0.45 μm membranes before a 10 μL aliquot was injected.

2.4. Mass spectrometry

The simultaneous determination of the alkaloids was performed on an API 4000 triple-quadrupole mass spectrometer (Applied Biosystems, USA). The chromatographic condition was the same as described in Section 2.3. High purity nitrogen was used as the curtain gas (CUR), nebulizer gas (GS1) and heater gas (GS2), the flow rates were set at 40, 40 and 12 units, respectively. Tandem MS analyses were performed in positive ESI mode and data were acquired by multiple reaction monitoring (MRM). Capillary temperature was 550 °C. Other parameters were as follows: ion spray voltage, 5 kV; pressure of CUR, 20 psi; pressure of GS1 and GS2, 50 psi; collision-activated dissociation (CAD), medium. To different compounds, the related selected ions of Q1 (*m/z*) and Q3 (*m/z*), and parameters of declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP) are different, they are listed in Table 2.

The identification analysis was performed on an HPLC-ESI-IT (Thermo Fisher Scientific Inc., MA, USA) equipped with an ESI source. The analysis was performed under positive ionization mode with a spray voltage of 4.5 kV. Nitrogen was used as the sheath gas, auxiliary gas and sweep gas at a flow rate of 17 arb, 3 arb and 0 arb, respectively. The capillary voltage was 40 V and the temperature was maintained at 375 °C.

2.5. Preparation of standard solutions

The standard of each alkaloid was weighed accurately and dissolved in DMSO to prepare 1 mg/mL stock solution. All stock solutions (10 μL per solution) were added into Eppendorf tubes and diluted with MeOH to give standard mixture solutions (10 μg/mL per alkaloid). Serial dilution of the standard mixture allowed to determine the calibration curves at ten different concentration levels (10.0, 5.0, 2.0, 1.0, 0.5, 0.2, 0.1, 0.05, 0.02 and 0.01 μg/mL).

The internal standard solution was prepared in MeOH (1 mg/mL) and appropriate amount of methanol/acetonitrile (1:1 v/v) was added to dilute to a final concentration of 50 ng/mL.

2.6. Preparation of sample solutions

P. longum L. and *P. nigrum* L. (white pepper and black pepper) which were pulverized (100 mesh) and PLA were sonicated in MeOH at 45 kHz for 30 min using a Shumei™ KQ-500VDE instrument (Kunshan Ultrasonic Instruments Co. Ltd., China), and passed through a 0.45 μm nylon membrane filter. The filtrate was stored as the stock solution. Sample solutions were obtained from the stock solution by serial dilution (100, 10, 1 μg/mL) with MeOH.

The sample solution (20 μL) was mixed thoroughly with IS solution (200 μL), and 10 μL of the mixed solution was diluted to 200 μL with methanol/water (1:1 v/v). A 10 μL aliquot of the diluted solution was analyzed.

2.7. Method validation

The method was validated in terms of specificity, linearity, limits of detection (LOD), limits of quantification (LOQ), precision, stability, repeatability and recovery. The sample preparation procedure was the same as described in Section 2.6.

Specificity was assessed by comparing the chromatograms of the standards with the samples. The linearity for the samples was assessed by analyzing calibration curves using least-squares linear regression. The peak area ratios (y), which was the ratio of each times, and the concentration (x) of standards were subjected with a weighted factor ($1/C^2$). The limit of detection (LOD) and the limit of quantitation (LOQ) of the analytes were determined by calculating the analyte level at signal-to-noise ratio (S/N) of 3 and 10, respectively. The intra-day precision was determined at three levels (20, 500, 8000 ng/mL) with 6 repetitions each. Six replicate samples at each concentration were determined each day over three consecutive days to determine inter-day precision. The relative standard deviation (RSD) was used as a measure of precision. The method's stability was tested the PLA solution, which was pre-

Table 4
Calibration curves, LODs, LOQs, precision, stability, repeatability and recovery of the assay.

Sample	Linearity			LOD (ng/mL)	LOQ (ng/mL)	Precision						Stability (RSD, %, $n = 6$)	Repeatability (RSD, %, $n = 6$)	Recovery (RSD, %, $n = 3$)		
	Equation	R^2	Range (ng/mL)			Intra-day (RSD, %, $n = 6$)			Inter-day (RSD, %, $n = 3$)					20 ng/mL	500 ng/mL	8000 ng/mL
						20 ng/mL	500 ng/mL	8000 ng/mL	20 ng/mL	500 ng/mL	8000 ng/mL					
PPR	$y = 0.00353x - 0.00046$	0.9955	10–10,000	0.02	0.06	6.10	5.22	6.12	6.42	5.81	5.47	0.55	1.00	3.88	1.65	0.75
PPL	$y = 0.00263x + 0.00157$	0.9960		0.03	0.10	3.77	4.82	7.53	7.57	6.29	6.22	2.53	2.58	2.13	0.74	0.58
DPPL	$y = 0.00362x + 0.00670$	0.9959		0.02	0.05	8.86	6.99	6.79	8.16	5.15	6.10	0.21	0.89	1.11	1.04	0.89
PLTR	$y = 0.00067x + 0.00326$	0.9967		0.03	0.10	9.30	3.31	1.63	7.97	2.80	2.77	1.57	1.59	7.24	0.49	1.48
PPRA	$y = 0.00327x - 0.00184$	0.9953		0.02	0.06	7.55	4.16	2.53	9.55	4.48	3.47	0.41	1.36	3.41	0.38	1.17

pared in methanol and protected from light for 24 h. Six replicate samples of PLA solution were analyzed in order to evaluate repeatability. The recovery test was used to evaluate the accuracy of the method. The sample was spiked with known amounts of mixture standard at three different levels (20, 500, 8000 ng/mL) and analyzed under optimized condition for three times, respectively.

3. Results and discussion

3.1. Identification of standards

The structures of standards were identified based on characteristic fragmentations in Thermo LTQ XL Orbitrap ion trap MS^n. The cleavage of the amide bond was detected, which resulted in the loss of the neutral amine moiety (piperidine or isobutylamine) (Sun, Pei, Pan, & Shen, 2007). The unique loss of the neutral fragments of 85 or 73 Da was characteristic of the standards. The same benzodioxole group was detected in PPR, PPL, DPPL and PPRA, and the calculated mass was 135.04410 with measurement error of 0.326 ppm, which is less than the 5 ppm threshold. All standards had a 5 ppm mass tolerance for calculating the formulae and comparing with the accurate mass detected by HPLC–ESI-MS/MS. According to the MS/MS data (Table 3), the standards were identified as PPR, PPL, DPPL, PPRA and PLTR, respectively.

3.2. Method validation

Specificity was analyzed using the API 4000 triple-quadrupole mass spectrometer as shown in Fig 1. The retention times were 3.78 min, 3.73 min, 3.71 min, 3.93 min and 3.76 min for PPR, PPL, DPPL, PLTR and PPRA, respectively (see Fig. 2). There is no endogenous interference in the samples. Table 4 shows the method's linearity, limit of detection (LOD), limit of quantitation (LOQ), precision, stability, repeatability and recovery. Linearity was determined using a series of work solutions at ten concentration levels. The calibration data showed a good linearity ($R^2 > 0.995$) in the concentration range of 10–10,000 ng/mL for the standards. The LOD and LOQ were in the range of 0.02–0.03 and 0.05–0.10 ng/mL, respectively. The precision of the method was determined through the intra-day and inter-day variations. The intra-day and inter-day variations were less than 9.30% and 9.55% at different concentrations, respectively. In the stability test, the RSD was less than 2.53%. Multiple injections showed that the repeatability variation was less than 2.58%. The recovery was in the range of 90.0–103.5%, which indicates the good accuracy of the method in the quantitative determination of the target alkaloids in the samples.

3.3. Sample analysis

The developed method was applied to quantitatively determine alkaloids in various samples. The quantitative determination was performed on LC–MS/MS using terfenadine as the internal standard (IS). PLA was analyzed using the developed method and the results are shown in Table 1. From the results, there were differences in the contents of these five constituents among PLA, *P. longum* and *P. nigrum*. The contents of the five alkaloids in PLA were determined and remarkably higher than those in the material of *P. longum* and *P. nigrum*. The reason was that PLA was isolated and purified from *P. longum*. Independent sample t-test was used to compare the mean content of each alkaloid in *P. longum* and *P. nigrum*. Compared with *P. nigrum*, the content of PPR ($p < 0.05$) in *P. longum* was less, but the contents of PPL, DPPL, PLTR and PPRA ($p < 0.05$) were relatively higher.

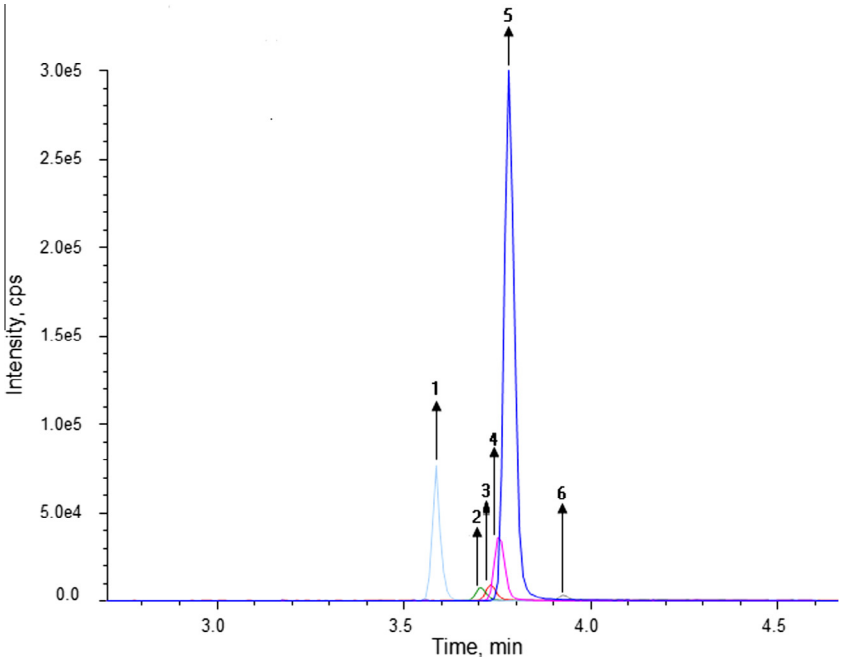

Fig. 2. The extracted ion chromatography of DPPL, PPL, PPRA, PLTR, PPR and IS in *P. longum* L. 1-IS; 2-DPPL; 3-PPL; 4-PPRA; 5-PPR; 6-PLTR.

4. Conclusions

A simple, effective and suitable method combining ultra-performance liquid chromatography separation with electron spray ionization triple-quadrupole tandem mass spectrometry (UFLC–ESI-MS/MS) was developed to simultaneously quantify five major alkaloid components (PPR, PPL, DPPL, PPRA and PLTR) in *P. longum* and *P. nigrum*. The standards of the five alkaloids were identified by high resolution Orbitrap HPLC–MS/MS measurements. The five alkaloids were successfully detected among PLA, *P. longum* and *P. nigrum*. The content of PPR in *P. longum* was significantly less than that in *P. nigrum* ($p < 0.05$) but had enriched PPL, DPPL, PLTR and PPRA ($p < 0.05$). It can be inferred that adequately seasoning with *P. longum* or *P. nigrum* in diets may assist in preventing Parkinson's disease.

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