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Identification and characterization of stress degradation products of piperine and profiling of a black pepper (*Piper nigrum* L.) extract using LC/Q-TOF-dual ESI-MS

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A rapid, specific and reliable high-performance liquid chromatography combined with quadrupole time-of-flight dual electrospray ionization mass spectrometry (LC/Q-TOF-dual ESI-MS) method has been developed and validated for the identification and characterization of stressed degradation products of piperine. Piperine, an anti-hypertensive drug, was subjected to hydrolysis (acidic, alkaline and neutral), oxidation, photolysis and thermal stress, as per ICH-specified conditions. The drug showed extensive degradation under oxidation and hydrolysis (acid and base) stress conditions. However, it was more stable under thermal stress than under acidic, alkaline, neutral and photolysis stress conditions. A total of four degradation products were observed and the chromatographic separation of the drug and its degradation products was achieved on a C_{18} column (4.6 × 50 mm, 5 μm). To characterize the degradation products, fragmentation patterns and accurate masses of the degradation products were established by subjecting them to LC-MS/Q-TOF analysis. Structure elucidation of the degradation products was achieved by comparing their fragmentation patterns with that of the drug, and confirmation was achieved through profiling a black pepper extract (*Piper nigrum* L.). The method identified dihydropiperine, piperylin, piperlonguminine, *trans*-piperine, *cis*-piperine, dihydropiperlonguminine, *trans*-piperettine and *cis*-piperettine. The liquid chromatography-mass spectroscopy method was validated with respect to its specificity, linearity, accuracy and precision.

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Introduction

Black pepper (*Piper nigrum* L.)[1,2] is one of the most widely used spices in the world, and is well-known for its pungent constituent, piperine. White pepper is produced from the same species, but whereas black pepper is prepared by briefly cooking and drying the unripe fruits, white pepper consists of the dried, naked, ripe seeds. Interest in piperine arises from the fact that, the principal bioactive compound of *P. nigrum* and *Piper longum*, has been reported to have immunomodulatory, antibacterial/antiprotozoan,[3–6] anticarcinogenic/antigenotoxic,[7–10] antiasthmatic, antidepressant,[11,12] stimulatory, hepatoprotective, antioxidative,[13,14] anti-inflammatory,[15] antimicrobial,[16] antidiarrheal,[17] antiulcer[18,19] and insulin-resistance[20] activities. *Zingiber officinale* (ginger) also contains piperine and shows some of these medicinal effects, such as being antioxidative[21] and anti-inflammatory.[22] It also has anti-oxidant and biotransformative effects and has been observed to enhance the

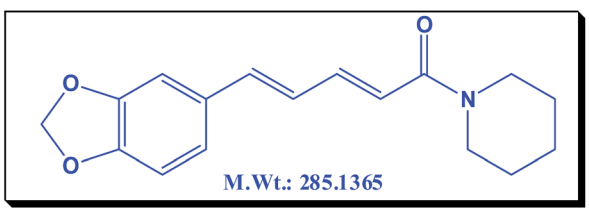

Fig. 1 Chemical structure of piperine.

Table 1 Optimized stress conditions

Stress condition		Exposure	Duration
Hydrolysis			
Acid	2 M HCl	80 °C	24 h
Base	1 M NaOH	80 °C	48 h
Neutral	H_2O	80 °C	48 h
Photolysis			
UV-light	200 W h m^{-2}	Photostability chamber	
Thermal	100 °C	Oven	4 days
Natural sunlight	At lux h	120 000–145 000	1 h

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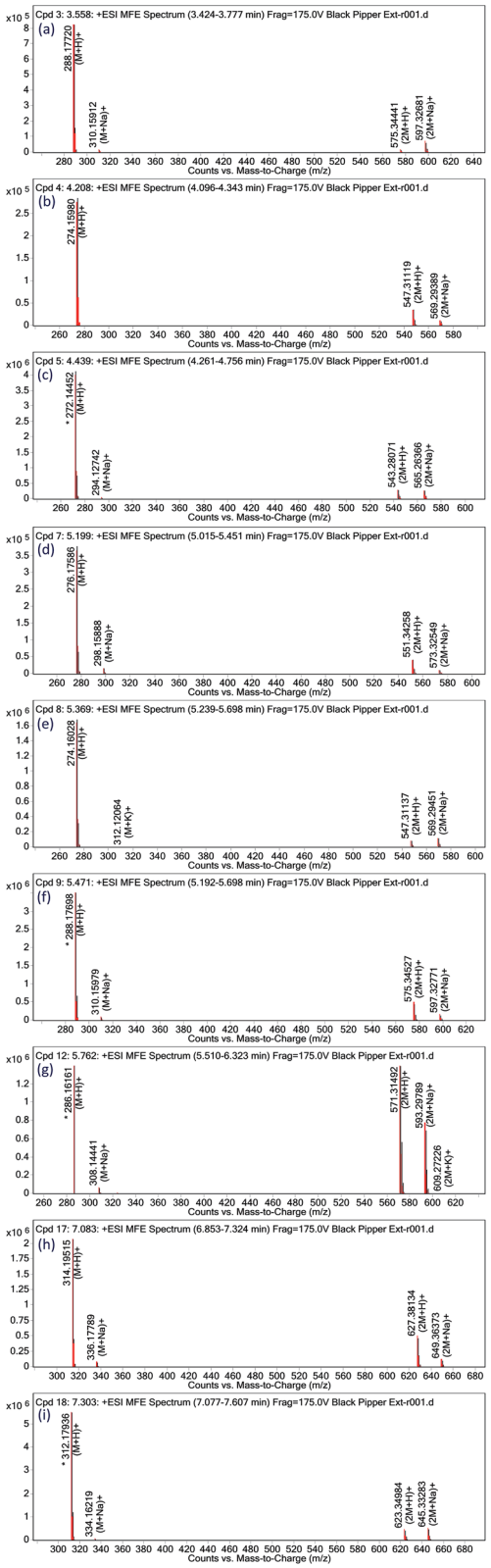

Fig. 2 (a) LC-ESI-MS spectrum of $[M + H]^+$ ions (*m/z* 288) of piperanine; (b) LC-ESI-MS spectrum of $[M + H]^+$ ions (*m/z* 274) of *trans*-piperlonguminine; (c) LC-ESI-MS spectrum of $[M + H]^+$ ions (*m/z* 272) of piperylin; (d) LC-ESI-MS spectrum of $[M + H]^+$ ions (*m/z* 276) of dihydropiperlonguminine; (e) LC-ESI-MS spectrum of $[M + H]^+$ ions (*m/z* 274) of *cis*-piperlonguminine; (f) LC-ESI-MS spectrum of $[M + H]^+$ ions (*m/z* 288) of *trans*-piperanine; (g) LC-ESI-MS spectrum of $[M + H]^+$ ions (*m/z* 286) of piperine; (h) LC-ESI-MS spectrum of $[M + H]^+$ ions (*m/z* 314) of piperdardine; (i) LC-ESI-MS spectrum of $[M + H]^+$ ions (*m/z* 312) of *cis*-piperettine profile of *P. nigrum* L. extract.

absorption of drugs such as rifampicin, sulfadiazine, tetracycline, and phenytoin.[23] Piperine is also reported to inhibit enzymes (cytochrome P450, uridine 5′-diphospho-glucoronyltransferase) that catalyze the biotransformation of nutrients and drugs, thus enhancing their bioavailability and efficacies *in vivo*.[24–26]

Recently, both species have attracted considerable attention because of the insecticidal[27] principles present in them. The genus *Piper* in particular offers great commercial, medicinal and economic potential. Of the wide array of secondary metabolites occurring in the genus *Piper*, the principal ones of interest are the alkaloids and amides. The compounds with the greatest insecticidal activity are perhaps the piperamides. Thus, *Piper* extracts can be effectively used as a unique source of biopesticide material. The most widely recognised species of this genus is *P. nigrum* L. (Fig. 1) which, apart from culinary applications, is used in a number of ayurvedic formulations because of its various medicinal properties.

Piperine showed extensive degradation in acid hydrolysis, base hydrolysis and under oxidative stress, whereas it was stable to neutral, thermal and photolytic stress conditions. A total of four degradation products were characterized using a liquid chromatography-quadrupole time-of-flight mass spectroscopy (LC-MS/Q-TOF) technique combined with accurate mass measurements of fragment ions and the results obtained were compared with results from the profiling of natural extracts. We report the development of a simple, accurate and precise high-performance liquid chromatography with diode array detection (HPLC-DAD) method for the simultaneous determination of the stress degradation compounds of piperine and compare this with a plant extract from *P. nigrum* L. The method developed was used to compare three different species of *Piper* by determining the content of these compounds. Thus, a simple and efficient analytical method to ensure quality and consistency in the final product was developed. HPLC and QTOF (LC-MS) methods have been used previously by other researchers to isolate, identify and quantify the constituents of *Piper* species.

Materials and methods

Chemicals, reagents and materials

Piperine (97.89% pure) was obtained from Sigma-Aldrich, India. Organic solvents for chromatography (LC-MS grade) were purchased from commercial sources, Water was ultra-purified using the Elix Advantage 5 equipped with a Milli-Q Biocel system (Millipore). All the chemicals used were of analytical reagent grade, and the solvents were ACS grade. The purity of the reference standard was determined using HPLC-DAD and dual electrospray ionisation (ESI; LC-MS) method. All the solvents were degassed in an ultrasonic bath and then filtered through Whatman 0.2 μm Diameter 47 mm, Nylon nonsterile membrane filter before injection into the system. Commercial

Table 2 Parameters of system suitability, LOD, LOQ, linearity, precision and accuracy

Parameter	Value
Peak	Piperine
Capacity factor (K')	−0.4
Plates	12 638
Plates per meter	252 760
Resolution	3.6
Symmetry	0.36
Tailing factor	1.5
Slope	915 030.8328
Intercept	1 072 255.356
Linearity	0.998627209
LOD (ng mL^{-1})	0.206309019
LOQ (ng mL^{-1})	0.625178846
Precision (% RSD, $n = 3$)	1.951
Accuracy (% recovery, $n = 3$)	1.038829787

ground black pepper was obtained from local stores in Chennai, India.

Apparatus

Degradation studies were carried out in an oil bath equipped with a temperature controller. A temperature controlled oven (830 V; Mack Pharmatech Private Ltd) was used for solid-state thermal stress studies. A MK-10PH, 230 V single phase photostability chamber (Mack Pharmatech Private Ltd) was used for the photodegradation study. The photostability chamber consisted of both UV and fluorescent lamps. A calibrated lux meter and UV meter were used to measure the energy. All pH measurement was done using a 780 pH meter (Metrohm Schweiz AG, Germany). Other equipment used included a sonicator and an ultra-sensitive APX-200 balance (Denver Instruments, USA).

Instrumentation

Chromatographic conditions. The LC-MS system was an Agilent 1200 RRLC and an Agilent Q-TOF G6520A. The Agilent HPLC system is equipped with a binary pump (G1312B), auto sampler, thermostatted column compartment (G1316B), variable wavelength detector (G1315C), auto sampler (G1367C) coupled with a thermostat (G1330B), and a PC with Windows-based MassHunter software version B.02.01 (B2116.20). The effective chromatographic separation was carried out on a reverse phase Kinetex C_{18} core shell column (50 × 4.6 mm, particle size 5 mm). Step gradient elution was employed using 0.1% formic acid in water (solvent A) and acetonitrile (solvent B), T (min)/B (%): 0/30, 5/50, 8/50, 10/80, 10.2/30 and eluted at a flow rate of 1 mL min^{-1} with a run time of 10 min. The column temperature was maintained at room temperature (25 °C), the injection volume was 5 μL and detection was carried out at 280 and 340 nm using UV detection.

High-resolution, accurate mass spectrometry quadrupole time-of-flight (LC-MS)-analysis – LC-MS equipment and conditions. LC-MS analysis was performed using Q-TOF MS (Q-TOF LC/MS 6520 series classic G6520A, Agilent Technologies, USA)

Table 3 *Piper nigrum* L. extract metabolites profile

Compound name	RT	Mass	Formula	Diff. (ppm)	Adduct ions (m/z)		
Piperanine	3.56	287.16993	$C_{17}H_{21}NO_3$	2.3	288.17720 $(M + H)^+$	310.15912 $(M + Na)^+$	575.34441 $(2M + H)^+$	597.32681 $(2M + Na)^+$
trans-Piperlonguminine	4.21	273.15253	$C_{16}H_{19}NO_3$	−2.85	274.15980 $(M + H)^+$	547.31119 $(2M + H)^+$	569.29389 $(2M + Na)^+$	
Piperylin	4.44	271.13724	$C_{16}H_{17}NO_3$	−4.22	272.14452 $(M + H)^+$	294.12742 $(M + Na)^+$	543.28071 $(2M + H)^+$	565.26366 $(2M + Na)^+$
Dihydropiperlonguminine	5.2	275.16858	$C_{16}H_{21}NO_3$	−4.29	276.17586 $(M + H)^+$	298.15888 $(M + Na)^+$	551.34258 $(2M + H)^+$	573.32549 $(2M + Na)^+$
cis-Piperlonguminine	5.37	273.15253	$C_{16}H_{19}NO_3$	−4.59	274.16028 $(M + H)^+$	547.31137 $(2M + H)^+$	569.29451 $(2M + Na)^+$	
trans-Piperanine	5.47	287.16971	$C_{17}H_{21}NO_3$	3.06	288.17698 $(M + H)^+$	310.15979 $(M + Na)^+$	575.34527 $(2M + H)^+$	597.32771 $(2M + Na)^+$
Piperine	5.76	285.1559	$C_{17}H_{19}NO_3$	−14.56	286.16161 $(M + H)^+$	308.14441 $(M + Na)^+$	571.31492 $(2M + H)^+$	593.29789 $(2M + Na)^+$
Piperdardine	7.08	313.18788	$C_{19}H_{23}NO_3$	−0.96	314.19515 $(M + H)^+$	336.17789 $(M + Na)^+$	627.38134 $(2M + H)^+$	649.36373 $(2M + Na)^+$
cis-Piperettine	7.3	311.1721	$C_{19}H_{21}NO_3$	−0.53	312.17936 $(M + H)^+$	334.16219 $(M + Na)^+$	623.34984 $(2M + H)^+$	645.33283 $(2M + Na)^+$

Table 4 Degradation of piperine under various stress conditions

Types of stress conditions	Compound name	RT	Mass	Formula	Diff. (ppm)	Adduct ions (*m*/*z*)			
Basic (NaOH-1 M)	Piperylin	4.42	271.13974	$C_{16}H_{17}NO_3$	3.27	272.14701 $(M + H)^+$	294.13006 $(M + Na)^+$	543.28569 $(2M + H)^+$	565.26557 $(2M + Na)^+$
	Piperine	5.742	285.15698	$C_{17}H_{19}NO_3$	2.23	286.16425 $(M + H)^+$	308.14697 $(M + Na)^+$	571.31879 $(2M + H)^+$	593.30160 $(2M + Na)^+$
Neutral (H_2O)	Piperylin	4.423	271.13954	$C_{16}H_{17}NO_3$	−0.92	272.14681 $(M + H)^+$	294.12965 $(M + Na)^+$	543.28520 $(2M + H)^+$	565.26786 $(2M + Na)^+$
	Piperine	5.742	285.15683	$C_{17}H_{19}NO_3$	2.77	286.16410 $(M + H)^+$	324.12253 $(M + K)^+$	593.30276 $(2M + Na)^+$	
Sunlight	Piperylin	4.409	271.13946	$C_{16}H_{17}NO_3$	−0.64	272.14673 $(M + H)^+$	294.12947 $(M + Na)^+$	543.28435 $(2M + H)^+$	565.26745 $(2M + Na)^+$
	trans-Piperine	5.736	285.15652	$C_{17}H_{19}NO_3$	−0.83	286.16379 $(M + H)^+$	308.14690 $(M + Na)^+$	571.32001 $(2M + H)^+$	593.30302 $(2M + Na)^+$
Thermal@100 °C	Piperylin	4.421	271.14147	$C_{16}H_{17}NO_3$	1.84	272.14874 $(M + H)^+$	294.13203 $(M + Na)^+$	543.28895 $(2M + H)^+$	565.27196 $(2M + Na)^+$
	trans-Piperine	5.711	285.15889	$C_{17}H_{19}NO_3$	0.25	286.16616 $(M + H)^+$	308.14947 $(M + Na)^+$	593.30770 $(2M + Na)^+$	
Acidic (HCl-2 M)	Piperylin	4.423	271.14297	$C_{16}H_{17}NO_3$	1.24	272.15025 $(M + H)^+$	294.13315 $(M + Na)^+$	543.29177 $(2M + H)^+$	565.27314 $(2M + Na)^+$
	Trichostachine	4.724	271.14276	$C_{16}H_{17}NO_3$	2.02	272.15004 $(M + H)^+$	294.13313 $(M + Na)^+$	543.28943 $(2M + H)^+$	
	Piperine	5.688	285.16032	$C_{17}H_{19}NO_3$	−0.04	286.16759 $(M + H)^+$	308.15057 $(M + Na)^+$	571.32531 $(2M + H)^+$	593.30871 $(2M + Na)^+$
UV@306 nm	Piperylin	4.408	271.13962	$C_{16}H_{17}NO_3$	−1.22	272.14689 $(M + H)^+$	294.12932 $(M + Na)^+$	543.28408 $(2M + H)^+$	565.26741 $(2M + Na)^+$
	Piperanine	5.447	287.1715	$C_{17}H_{21}NO_3$	1.48	288.17878 $(M + H)^+$	310.16149 $(M + Na)^+$	575.34845 $(2M + H)^+$	597.33170 $(2M + Na)^+$
	Piperine	5.733	285.1563	$C_{17}H_{19}NO_3$	−0.07	286.16357 $(M + H)^+$	308.14648 $(M + Na)^+$	571.31934 $(2M + H)^+$	593.30197 $(2M + Na)^+$
	Piperettine	7.315	311.17346	$C_{19}H_{21}NO_3$	−0.61	312.18074 $(M + H)^+$	334.16376 $(M + Na)^+$	645.33458 $(2M + Na)^+$	
Piperine metabolites	Piperylin	4.432	271.10602	$C_{16}H_{17}NO_3$	−1.62	272.11330 $(M + H)^+$	294.09355 $(M + Na)^+$	543.21611 $(2M + H)^+$	565.19642 $(2M + Na)^+$
	trans-Piperine	5.759	285.12239	$C_{17}H_{19}NO_3$	0.63	286.12968 $(M + H)^+$	308.10825 $(M + Na)^+$	609.20562 $(2M + K)^+$	

equipped with a dual ESI source. The data acquisition was under the control of MassHunter workstation software. Precise mass spectra were acquired by using the fast polar switching mode with a scan range from *m*/*z* 100 to 1000 with a standard dynamic high resolution mode (2 GHz) and the typical operating source conditions were optimized as follows: nitrogen was used for drying (325 °C; 10 L min^{-1}); pressure of nebulizer was 50 psi gas; capillary voltage was 3500 V; Vcap was 3500; fragmentor voltage was 175 V, skimmer voltage was 65 V and Octopole RF peak was 750. Ultra high purity nitrogen was used as collision gas. All the spectra were recorded under identical experimental conditions and were an average of 20–25 scans. The elemental compositions from the accurate mass measurements of *m*/*z* values and data processing of extracted ion chromatograms (EICs) were carried out by using the MassHunter Workstation Software version B.02.01 (B2116.20). Most of the metabolite peaks showed greater intensities in the total ion current chromatograms when compared to those obtained with UV detection. The protonated metabolites were also verified by extracting their corresponding masses using EICS after the post-run analysis.

Extraction of piperines from commercial ground black peppers

All operations were carried out in the dark. A sample of black pepper was ground in a coffee blender for 2 min and passed through a 100 mesh screen. The resulting powder (0.1–0.15 g) was then placed into a 5 mL vial to which was added 2 mL of 80% ethanol. The suspension was sonicated for 60 min in an ultrasonic bath and then centrifuged at 13 200 × *g* for 10 min at 5 °C. The supernatant solutions were then passed through a 0.2 μm Millex®-FG (Millipore) 4 mm; Fluoropore, nonsterile membrane filter, prior to LC-MS for piperamide analysis.

Preparation of standard and sample solutions

Stock solutions of piperine (1 mg mL^{-1}) were prepared by dissolving piperine in the mobile phase. The serial dilutions made from the stock solutions prepared with 0.001, 0.01, 0.1, 1, 10 and 100 μg mL^{-1} in the mobile phase were used for the evaluation of the limit of detection (LOD), limit of quantitation (LOQ) and linearity in accordance with the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines.

Stress degradation studies

Stress degradation studies of piperine were carried out under hydrolysis (acid, base and neutral), oxidation, dry heat and photolytic conditions, as per ICH (2003) guidelines. Acidic and basic hydrolysis was carried out in 2 M HCl, or 1 M NaOH, for 24 and 48 h, respectively, whereas neutral hydrolysis was carried out in water for 48 h. All the hydrolytic studies were conducted at 80 °C with a drug concentration of 1 mg mL^{-1}. The oxidative degradation study was carried out with 15% H_2O_2 at room temperature for 25 days at a concentration of 1 mg mL^{-1}. Solid-state photolytic studies were carried out by exposing a thin layer (1 mm) of drug in a petri dish to 1.2 × 106 lx h of fluorescent

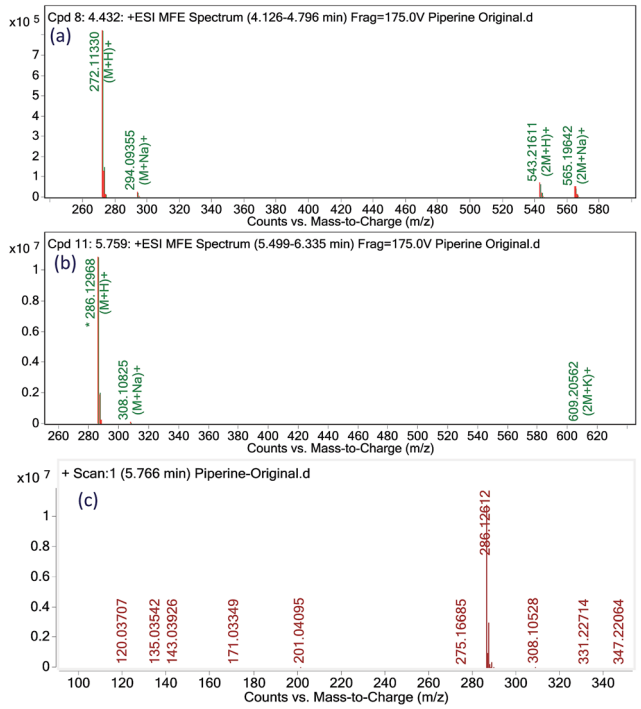

Fig. 3 (a) LC-ESI-MS spectrum of $[M + H]^+$ ions (*m/z* 272) of piperylin; (b) LC-ESI-MS spectrum of $[M + H]^+$ ions (*m/z* 286) of piperine profile of piperine sample and (c) LC-ESI-MS spectrum of piperine showing the possible major mass fragments ions (*m/z* 201.0, 171.0 and 143.0).

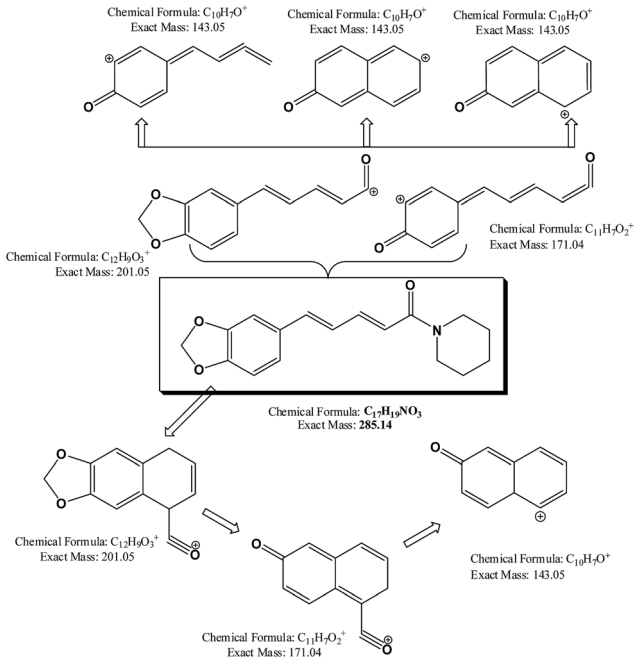

Fig. 4 Piperine mass fragments: possible structures of major mass spectral fragments of piperines.

light and 200 W h m^{-2} UV-A light in a photo stability chamber (ICH, 1996). For thermal stress, the drug was kept at 100 °C in the oven for four days. The optimized stressed conditions are outlined in Table 1. All stressed samples were withdrawn at suitable time intervals and diluted 10 times with mobile phase. All the samples were filtered by using 0.2 μm Millex®-FG (Millipore) 4 mm; Fluoropore, nonsterile membrane filter, prior to LC-MS analysis.

LC-MS/Q-TOF studies of piperine and its degradation products

Both the piperine and the degraded samples were investigated using LC-MS/Q-TOF mass spectrometry. The degradation products were analyzed using accurate mass measurements and the results were compared with those from a profiling extract of *P. nigrum* L.

Results and discussion

Development and optimization of the LC and LC-MS method

The main objective of the chromatographic method was to separate piperine and its degradation products. Initially, stressed sample solutions were subjected to analysis by a method involving a C_{18} column (250 × 4.6 mm i.d, particle size: 5 mm) and different mobile phases. The chromatographic separation was achieved on a Kinetex C_{18} core shell technology (50 × 4.6 mm, particle size 5 mm) column. The mobile phase was prepared by mixing 0.1% of formic acid in water–acetonitrile (solvent B) (*v*/*v*), step gradient elution was employed, *T* (min)/*B* (%): 0/30, 5/50, 8/50, 10/80, 10.2/30. The flow rate of the mobile phase was 1.0 mL min^{-1}; at ambient column temperature, the peak shape of piperine was found to be symmetrical. Under optimized chromatographic conditions, piperine and an extract of piperine from black pepper were separated with a resolution greater than 2. Typical retention times were about 3.558, 4.208, 4.439, 5.199, 5.369, 5.471, 5.762, 7.083 and 7.303 min, respectively, (Fig. 2 and Table 3). The system suitability results are given in Table 2 and the LC method developed was found to be specific for piperine and Piper extract metabolite products, namely: dihydropiperine, piperylin, piperlonguminine, *trans*-piperine, dihydropiperlonguminine, *cis*-piperine, *trans*-piperettine and *cis*-piperettine.

For LC-MS studies, the same method was used as for HPLC, without replacement of the buffer. The Q-TOF dual ESI source conditions were also optimized to obtain a good signal and high sensitivity. The conditions such as drying gas flow, nebulizing gas flow, drying gas temperature, capillary voltage, spray voltage and skimmer voltage were optimized to maximize the ionization in the source and sensitivity even at a very low concentration to identify and characterize the degradation products.

Results of forced degradation studies

Degradation was observed in piperine samples when subjected to stress conditions such as basic hydrolysis, neutral hydrolysis, sunlight and thermal hydrolysis. Piperine was degraded to trichostachine and *cis*-piperylin under acid hydrolysis and was degraded to piperanine and piperettine under UV conditions (Table 4). Peak purity test results obtained by using a DAD detector confirmed that the piperine peak was homogeneous and pure.

Table 5 Data of intra-day and inter-day precision studies ($n = 3$)

	Intra-day precision	Inter-day precision
Conc.	Mean ± SD ($n = 3$); % RSD; accuracy	Mean ± SD ($n = 3$); % RSD; accuracy
1 ng mL^{-1}	13 827 ± 333.799; 1.951; 1.039	15 920 ± 635.091; 3.712; 1.977
10 ng mL^{-1}	47 697 ± 1414.867; 2.872; 1.044	48 957 ± 1291.214; 2.621; 0.953
100 ng mL^{-1}	338 506 ± 9653.697; 2.646; 1.059	357 459 ± 11 576.410; 3.173; 1.270
1 μg mL^{-1}	2 531 862 ± 57 205.792; 2.199; 1.027	2 601 918 ± 76 144.317; 2.927; 1.367
10 μg mL^{-1}	12 323 262 ± 139 279.209; 1.117; 1.018	12 436 613 ± 151 997.633; 1.219; 1.111
100 μg mL^{-1}	85 341 712 ± 2 851 875.313; 3.212; 1.041	88 705 685 ± 2 642 335.284; 2.976; 0.965

Mass patterns of the MS spectra of the piperine as seen in Fig. 3 [(m/z 286 $(M + H)^+$, 308 $(M + Na)^+$, 571 $(2M + H)^+$, 593 $(2M + Na)^+$)] and Fig. 4 shows proposed structures of mass spectral fragments[28–30] of the isomers and the following mass spectra fragments M^+ m/z for piperine: 285.1 ($C_{17}H_{19}NO_3$) were observed. Although the fragment of mass 201.0 ($C_{12}H_9O_3^+$) must be formed by the indicated cleavage of the carboxyamide moiety, the mechanism of possible formation of the structures with major fragment of the MS spectra of the piperine with mass m/z 171 $[M - C_6H_{12}NO]^+$ and m/z 143 $[M - C_7H_{12}NO_2]^+$ are described.

Method validation

The method used for assessing stability was validated for linearity, precision (inter-day, intra-day and intermediate precision), accuracy and specificity. The optimized LC-MS method was validated with respect to various parameters summarized in the ICH (2005) guidelines. To establish linearity and range, a stock solution containing 1 mg mL^{-1} piperine in mobile phase was diluted to yield solutions in the concentration range of 0.001–100 μg mL^{-1}. The solutions were prepared and analyzed in triplicate. The response for piperine was linear over the investigated concentration range ($r^2 = 0.9986$) and the % RSD for each investigated concentration was <1.05%. The linearity data are given in Table 2. The intra- and inter-day precisions were determined at six different concentrations, 1 ng mL^{-1}, 10 ng mL^{-1}, 100 ng mL^{-1}, 1 μg mL^{-1}, 10 μg mL^{-1} and 100 μg mL^{-1}, on the same day ($n = 3$) and consecutive days ($n = 3$). Table 5 shows that the % RSD for intra- and inter-day precision was <3.3 and <3.8% respectively, indicating that the method was sufficiently precise. The specificity of the method was established by determining peak purity for piperine in a mixture of stressed samples using a DAD system and evaluation of the resolution factor, which was also demonstrated by subjecting all the degradation samples to LC-MS. The mass detector showed excellent purity for piperine and for every degradation product, which clearly proves the specificity of the method.

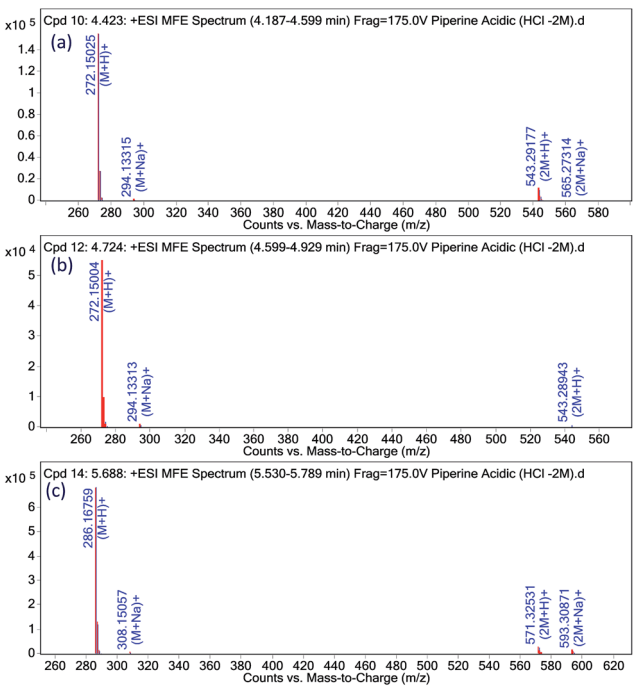

Fig. 5 (a) LC-ESI-MS spectrum of $[M + H]^+$ ions (m/z 272) of piperylin; (b) LC-ESI-MS spectrum of $[M + H]^+$ ions (m/z 272) of trichostachine; (c) LC-ESI-MS spectrum of $[M + H]^+$ ions (m/z 286) of piperine under acidic degradation conditions.

Solution stability and mobile phase stability

The % RSD ($n = 3$) of the assay of piperine during solution stability experiments was within 1.05%. No significant changes were observed in the content of the samples during solution stability and mobile phase stability experiments when performed using the related substance method. The solution stability and mobile phase stability experimental data confirms that the sample solutions and mobile phases used during assay and the related substance determination were stable for at least 48 h.

Degradation behaviour

The optimized LC-MS method is applicable for identifying the degradation products. The LC-ESI-MS molecular feature extraction chromatograms were obtained under various stress conditions. A total of four degradation products were identified and characterized by mass spectrometric analysis (LC-ESI-MS). The degradation products are confirmed by comparing them with the profile of an extract of black pepper (Table 3).

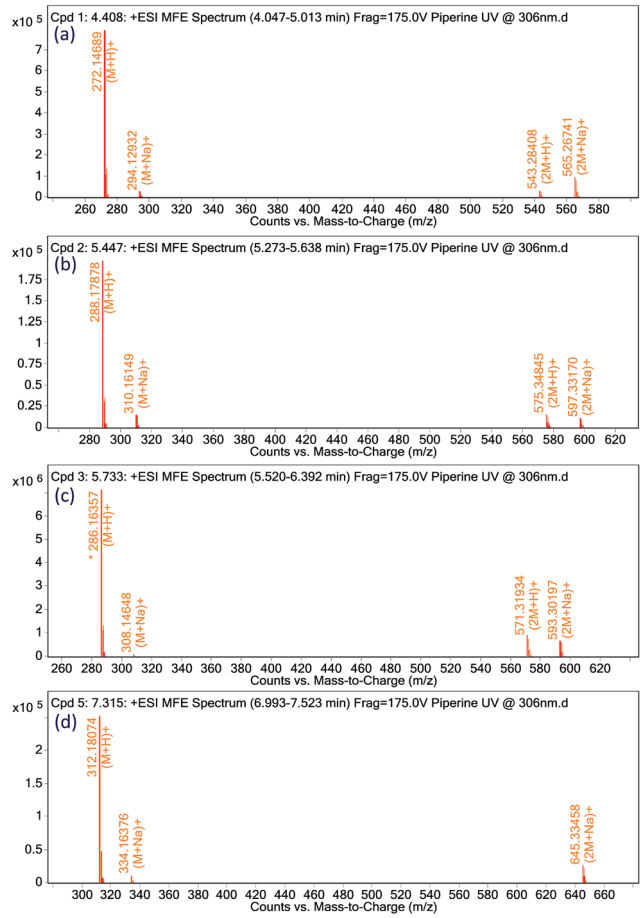

Fig. 6 (a) LC-ESI-MS spectrum of $[M + H]^+$ ions (*m/z* 272) of piperylin; (b) LC-ESI-MS spectrum of $[M + H]^+$ ions (*m/z* 288) of piperanine; (c) LC-ESI-MS spectrum of $[M + H]^+$ ions (*m/z* 286) of piperine; (d) LC-ESI-MS spectrum of $[M + H]^+$ ions (*m/z* 312) of piperettine under UV degradation conditions.

Hydrolysis. The piperine showed detectable degradation in 2 M HCl at 80 °C (Fig. 5) and after 24 h, two degradation products (trichostachine and *cis*-piperylin) were formed. In neutral conditions, on heating the drug in water for 48 h at 80 °C, no degradation products were formed. In addition to the neutral stress conditions, no degradation product were observed on treatment of the drug in 1 M NaOH for 48 h at 80 °C.

Photolysis and solid-state studies. The piperine is said to be stable at sunlight and thermal conditions in the solid state for 1 h and 4 days at 100 °C, respectively, yet degradation products (trichostachine and *cis*-piperylin) are formed in UV at 306 nm as shown in Fig. 6.

Conclusions

Stress degradation studies on piperine, carried out according to ICH guidelines, provided information on the degradation behaviour of piperine under hydrolysis and oxidation conditions. The LC method described in the present study can be used to resolve all the degradation products from piperine, as well as resolving them from each other, under various stress conditions. The piperine showed extensive degradation under acid hydrolysis, base hydrolysis and oxidative stress conditions (whereas it was stable to pH-neutral conditions), thermal and photolytic stress conditions. A total of four degradation products were characterized with the help of the LC-MS (Q-TOF) experiments combined with accurate mass measurements of the fragment ions and comparison with natural extract profiling.

The method developed is stability-indicating. It can be conveniently used by quality control departments to analyse related substances and to assay routine piperine samples. It can be used to determine the stability of samples and thereby help to produce formulations which are highly efficacious.

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