Analytical Methods

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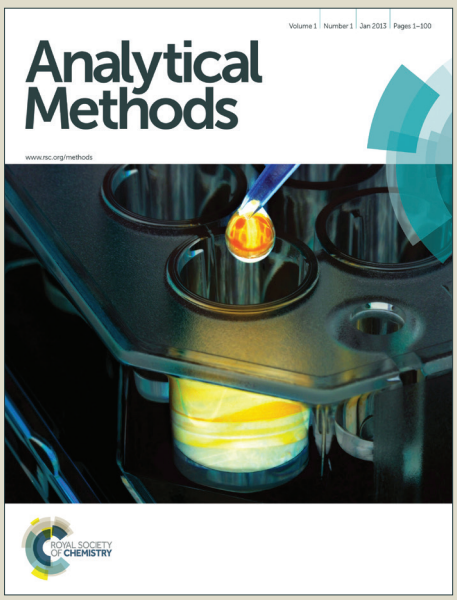

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ARTICLE

Identification and characterization of stressed degradation products of Piperine and profiling with black pepper (*Piper Nigrum L.*) Extraction by using LC/Q-TOF-dual ESI-MS experiments

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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 oxidative and hydrolysis (acid and base) stress conditions. However, it was more stable to thermal, than acidic, alkaline, neutral and photolysis stress conditions. A total of 4 degradation products were observed and the chromatographic separation of the drug and its degradation products was achieved on a C18 column (4.6 x 50 mm, 5 µm). To characterize degradation products, fragmentation pattern and accurate masses of the degradation products were established by subjecting them to LC-MS/QTOF analysis. Structure elucidation of degradation products was achieved by comparing their fragmentation pattern with that of the drug, and confirmation with profiling of black pepper extraction (*Piper nigrum L.*). The method identified Dihydropiperine, Piperylin, Piperlonguminine, trans-piperine, cis-piperine, Dihydropiperlonguminine, trans-Piperettine and cis-Piperettine. The LC-MS method was validated with respect to specificity, linearity, accuracy and precision.

Introduction:

Black pepper (*Piper nigrum L.*,)[1-2] is one of the most widely used spices in the world, 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 principle bioactive compound of Piper nigrum and Piper longum, having 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] insulin-resistance[20] activities. Zingiber officinale (ginger) also has piperine and shows some of these medicinal effets such as antioxidative[21] and ant-inflammatory[22]. It also has anti-oxidant and biotransformative effects and has been observed to enhance the absorption of drugs such as rifampicin, sulphadiazine, tetracyline, and phenytoin[23]. Piperine is also reported to inhibit enzymes (cytochrome P450, UDP-glucoronyltransferase) that catalyze the biotransformation of nutrients and drugs, thus enhancing their bioavailability and efficacies *in ViVo*[24-26].

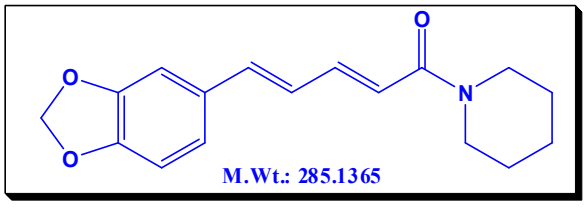


Figure 1. Chemical structures of Piperine.

Recently, they have attracted considerable attention because of the insecticidal [27], principles present in them. Thus, the genus bears great commercial, medicinal and economic potential. Of the wide array of secondary metabolites occurring in the genus Piper, principal 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 specie of this genus is *Piper nigrum.L* (black pepper) (Fig.1) which apart from culinary

applications is used in a number of ayurvedic formulations due to its various medicinal properties. Piperine is the major compound responsible for the spicy pungent flavour of pepper and has shown diverse pharmacological activities such as insecticidal, anti-inflammatory, analgesic, etc.

Piperine showed extensive degradation in acid, base hydrolysis and oxidative stress, while it was stable to neutral, thermal and photolytic stress conditions. A total of 4 degradation products were characterized with the help of the LC-MS (Q-TOF) experiment combined with accurate mass measurements of fragment ions and compare with is natural extracts profiling. We report the development of a simple, accurate and precise HPLC-DAD method for the simultaneous determination of stress degradation compounds of piperine and comparison with its plant extract of *Piper nigrum.L.* The developed method is used to compare three different species of Piper with respect to content of these compounds. Therefore, need of the hour is the development of simple and efficient analytical methods to ensure quality and consistency in the final product. High-performance liquid chromatography (HPLC) and Quadrupole Time of Flight (LCMS) methods have been used earlier to isolate, identify and quantify constituents.

Materials and methods:

Chemicals, reagents and materials:

Piperine (97.89% pure) obtained from the Sigma-Aldrich, India. Organic solvents for chromatography were purchased in LCMS grade from commercial sources, Water was obtained from ultra-purified from Elix Advantage 5 system equipped with Milli-Q Biocel (Millipore), all the chemicals used were of analytical reagent grade, and the solvents were of ACS. The purity of reference standard was determined by HPLC DAD and dual ESI (LCMS). All solvents degassed in an ultrasonic bath and then filtered through MILLEX FG (Millipore), 13 mm, 0.2 µM, fluropore, non-sterile membrane sample filter paper before injecting into system. Commercial ground black pepper was obtained from local stores, Chennai, India.

Apparatus:

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

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	200Wh/m^2	Photostability chamber	
Thermal	100 °C	Oven	4 days
Natural Sunlight	At hrs.	LUX 1,20,000-1,45,000	1 Hr

Instrumentation:

Chromatographic conditions: The LCMS system, Agilent 1200 RRLC & Q-TOF 6520 (G6520A). HPLC is equipped with Binary pump (G1312B), Auto Sampler, thermostatted column compartment (G1316B), variable wavelength detector (G1315C), Auto sampler (G1367C) coupled with thermostatted (G1330B), Computer with windows based mass hunter software version B.02.01 (B2116.20). The Effective chromatographic separation was carried out on a reverse phase Kinetex C18 core shell technology (50 x 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/%B: 0/30, 5/50, 8/50, 10/80, 10.2/30 and eluted by the following program at the flow 1 mL/min with run time of 10 min, the column temperature was maintained at room temperature(25 °C), the injection volume was 5 µl and eluents detection of the samples was carried out at 280 and 340 nm by UV detector.

High-resolution, accurate-mass spectrometry Quadrupole time-of-flight (LCMS)-analysis LC-MS equipment and conditions: LC-MS analysis was performed on quadrupole time-of-flight (Q-TOF) mass spectrometer (Q-TOF LC/MS 6520 series classic G6520A, Agilent Technologies, USA) equipped with a dual ESI (electrospray ionization) source. The data acquisition was under the control of Mass Hunter workstation software. Precisely Mass spectra were acquired by using fast polar switching mode with scan range from m/z 100 to 1000 Da with standard dynamic high resolution mode (2 GHz) and the typical operating source conditions were optimized as follows: nitrogen was used as drying (325 °C; 10 l/min); pressure of nebulizer, 50 psi gas; capillary voltage, 3500 V; Vcap-3500; Fragmentor-175; and Skimme-65 and Octopole RFPeak 750. Ultrahigh pure 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 Mass Hunter Workstation Software version B.02.01 (B2116.20) (Agilent Technologies, USA). Most of the metabolite peaks showed greater intensities in TIC as compared with UV. The protonated metabolites were also verified by extracting their corresponding masses using extracted ion chromatograms after the post run analysis.

Extraction of Piperines from Commercial Ground Black Peppers:

All operations were carried out in the dark. 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 13200 g for 10 min at 5 °C. The supernatants were then passed through a 0.45 µm Millipore 13 mm, 0.2 µM, fluropore; non-sterile membrane sample filters prior to LC-MS for piperamide analysis.

Preparation of Standard and Sample Solutions:

Stock solutions of Piperine (1 mg/mL) were prepared in the mobile phase. The serial dilutions made from stock solutions prepared with 0.001, 0.01, 0.1, 1, 10 and 100 µg/mL in the mobile phase from

stock solutions for the evaluation of the LOD, LOQ and linearity in accordance with ICH guidelines.

Stressed 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, 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. 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. Solid-state photolytic studies were carried out by exposing light to a thin layer (1 mm) of drug in a Petri dish to 1.2 106 lx h of fluorescent light and 200Wh/m2 UV-A light in a photo stability chamber (ICH, 1996). For thermal stress, the drug was kept at 100 °C in the oven for 4 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 using MILLEX FG (Millipore), 13 mm, 0.2 µM, fluropore, non-sterile membrane sample filters prior to LC-MS analysis.

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

Both the piperine and degraded samples were investigated using LC-MS/ Q-TOF mass spectrometry. The degradation products were analyzed by accurate mass measurements and compare with its profiling extract of black pepper (*piper nigrum l.*)

Results and discussion:

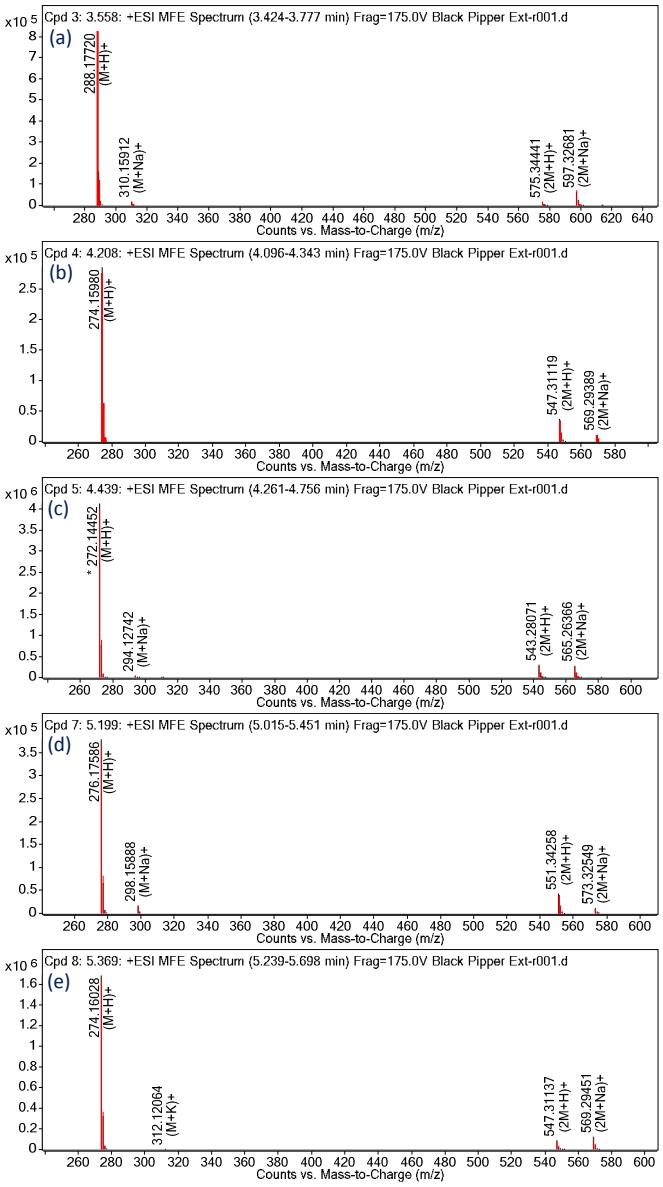
Development and optimization of LC and LC-MS method:

The main objective of the chromatographic method is to separate piperine and its degradation products. Initially, stressed sample solutions were subjected to analysis by a method involving a C18 column (250x4.6 mm i.d.; particle size 5 mm) as well as different mobile phases. The chromatographic separation was achieved on Kinetex C18 core shell technology (50 x 4.6 mm, particle size 5 mm) column. The mobile phase was prepared by mixing 0.1% Formic acid in water–acetonitrile (solvent B) (v/v), Step gradient elution was employed, T/%B: 0/30, 5/50, 8/50, 10/80, 10.2/30. The flow rate of the mobile phase was 1.0 mL/min, at ambient column temperature, the peak shape of piperine was found to be symmetrical. In optimized chromatographic conditions piperine and extract of black piperine 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; Table.3). The system suitability results are given in Table. 2 and the developed LC method was found to be specific for piperine and piperine extracts metabolites products namely Dihydropiperine, Piperylin, piperlonguminine, trans-piperine, dihydropiperlonguminine, cis-piperine, trans-Piperettine and cis-Piperettine.

Table 2. Parameters of System suitability, LOD, LOQ, Linearity, precision and Accuracy

Parameter	Value
Peak	Piperine
Capacity Factor(K')	-0.4
Plates	12638
Plates Per Meter	252760
Resolution	3.6
Symmetry	0.36
Tailing Factor	1.5
Slope	915030.8328
Intercept	1072255.356
Linearity	0.998627209
Limit of detection (ng/mL)	0.206309019
Limit of quantification(ng/mL)	0.625178846
Precision % RSD (n = 3)	1.951
Accuracy % Recovery (n = 3)	1.038829787

For LC-MS studies, same method was used as for HPLC, without replacement of buffer. The Q-TOF dual ESI source conditions were also optimized to obtain a good signal and high sensitivity. The conditions like 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.



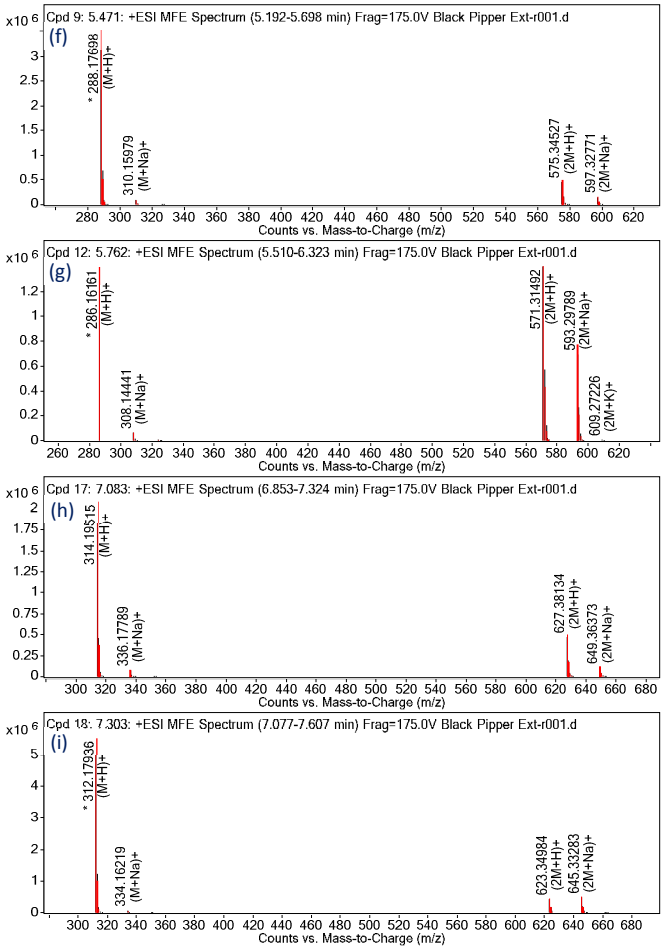


Figure 2. (a) LC-ESI-MS spectrum of $[M+H]^+$ ions(m/z 288) of Piperanine; (b) LC-ESI-MS spectrum of $[M+H]^+$ ions(m/z 274) of *trans*-Piperlonguminine; (c) LC-ESI-MS spectrum of $[M+H]^+$ ions(m/z 272) of Piperylin; (d) LC-ESI-MS spectrum of $[M+H]^+$ ions(m/z 276) of Dihydropiperlonguminine; (e) LC-ESI-MS 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 *piper nigrum l.*Extract.

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 $(2M+Na)^+$

Results of Forced Degradation Studies:

Degradation was observed in piperine samples when subjected to stress conditions like basic, neutral, 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 is homogenous and pure.

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-1M)	Piperylin	4.42	271.13974	$C_{18}H_{17}NO_3$	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 (H2O)	Piperylin	4.423	271.13954	$C_{18}H_{17}NO_3$	-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	$C_{18}H_{17}NO_3$	-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	$C_{18}H_{17}NO_3$	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-2M)	Piperylin	4.423	271.14297	$C_{18}H_{17}NO_3$	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 @ 306nm	Piperylin	4.408	271.13962	$C_{18}H_{17}NO_3$	-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 $(2M+Na)^+$
	piperetine	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	$C_{18}H_{17}NO_3$	-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 $(2M+K)^+$

Mass patterns found that the MS spectra of the piperine 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 observed the following mass spectra fragments M^+ *m/z* for piperine: 285.1 ($C_{17}H_{19}NO_3$). Although the fragment of mass 201.0 ($C_{12}H_9O_3^+$) must be formed by the indicated cleavage of the carboxyamide moiety, the mechanisms of formation of the structures with masses of 171.0 ($C_{11}H_7O_2^+$) and 143.0 ($C_{10}H_7O^+$).

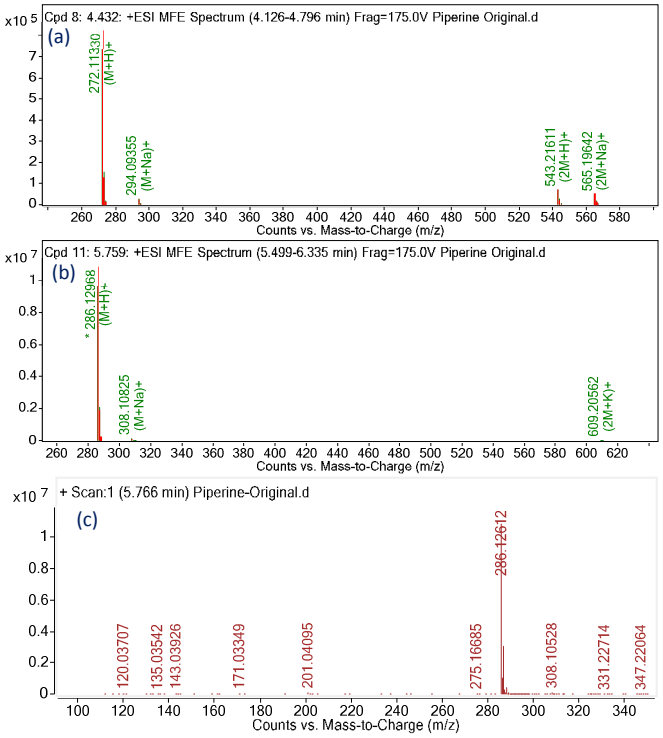


Figure 3. (a) LC-ESI-MS spectrum of $[M+H]^+$ ions(m/z 272) of Piperylin; (b) LC-ESI-MS spectrum of $[M+H]^+$ ions(m/z 286) of piperine profile of piperine sample and (c) LC-ESI-MS spectrum of Piperine possible major mass fragments ions (m/z 201.0; 171.0 and 143.0).

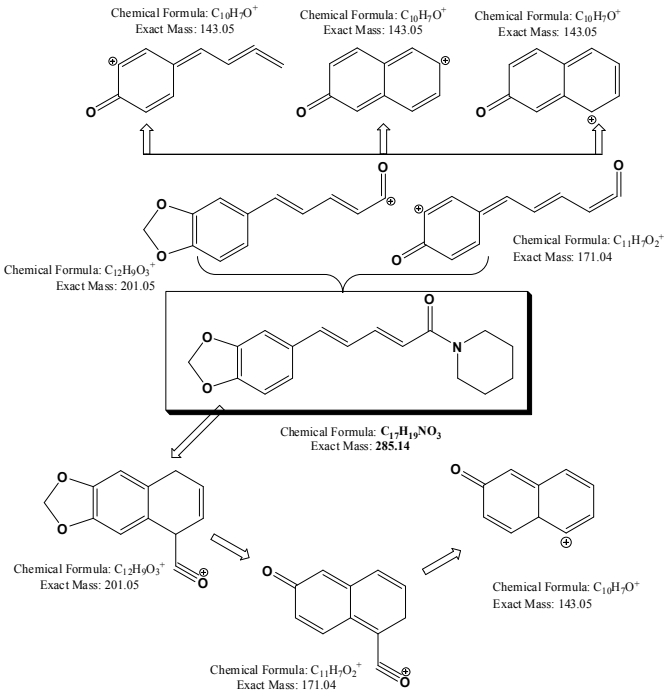


Figure 4. Piperine mass fragments: Possible structures of major mass spectral fragments of piperines.

Method Validation:

The stability-indicating method 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 piperine in mobile phase was diluted to yield solutions in the concentration range of 0.001–100 µg/mL. The solutions were prepared and analyzed in triplicate. The response for the piperine was linear in the investigated concentration (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, 10 ng/mL, 100 ng/mL, 1 ug/mL, 10 ug/mL and 100 ug/mL, 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 Diode array detector (DAD) and evaluation of the resolution factor, and was also demonstrated by subjecting all the degradation samples to LC-MS. The mass detector showed an excellent purity for piperine and every degradation product, which clearly proves the specificity of the method.

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

Conc. (µg/ml)	Intra-day precision Mean ± s.d. (n=3); %rsd; Accuracy	Inter-day precision Mean ± s.d. (n=3); %rsd; Accuracy
1 ng/mL	13827±333.799; 1.951; 1.039	15920±635.091; 3.712; 1.977
10ng/mL	47697±1414.867; 2.872; 1.044	48957±1291.214; 2.621; 0.953
100ng/mL	338506±9653.697; 2.646; 1.059	357459±11576.410; 3.173; 1.270
1ug/mL	2531862±57205.792; 2.199; 1.027	2601918±76144.317; 2.927; 1.367
10ug/mL	12323262±139279.209; 1.117; 1.018	12436613±151997.633; 1.219; 1.111
100ug/mL	85341712±2851875.313; 3.212; 1.041	88705685±2642335.284; 2.976; 0.965

Solution Stability and Mobile Phase Stability:

The % RSD (n = 3) of the assay of piperine during solution stability experiments were within 1.05%. No significant changes were observed in the content of samples during solution stability and mobile phase stability experiments when performed using the related substance method. The solution stability and mobile phase stability experiment 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 (MFE) chromatograms obtained under various stress conditions. A total of 4 degradation products were identified and characterized by mass spectrometric analysis (LC-ESI-MS). The degradation products are confirmed by comparing with profile of extract of black pepper (Table 3).

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

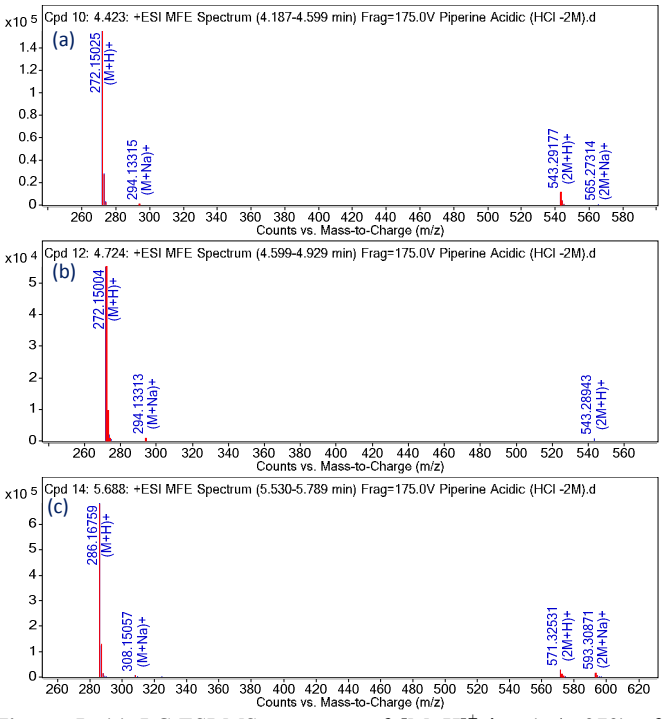
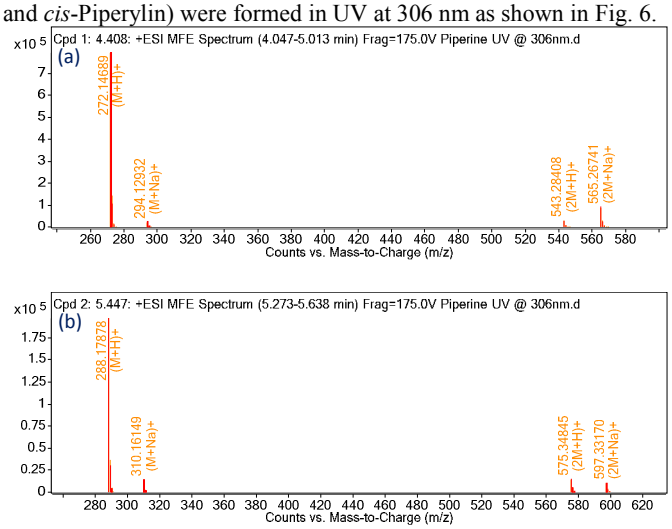


Figure 5. (a) LC-ESI-MS spectrum of $[M+H]^+$ ions(m/z 272) of Piperylin; (b) LC-ESI-MS spectrum of $[M+H]^+$ ions(m/z 272) of trichostachine; (c) LC-ESI-MS spectrum of $[M+H]^+$ ions(m/z 286) of piperine under acidic degradation condition.

Photolysis and solid-state studies: The piperine was stable to sunlight and thermal stress in the solid state, for 1hr and 4 days at 100 °C respectively, where in degradation products (trichostachine and *cis*-Piperylin) were formed in UV at 306 nm as shown in Fig. 6.



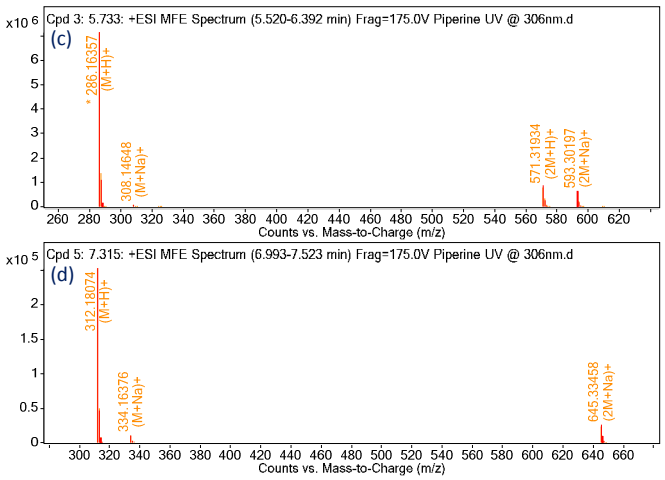


Figure 6. (a) LC-ESI-MS spectrum of $[M+H]^+$ ions(m/z 272) of Piperylin; (b) LC-ESI-MS spectrum of $[M+H]^+$ ions(m/z 288) of Piperanine; (c) LC-ESI-MS spectrum of $[M+H]^+$ ions(m/z 286) of Piperine; (d) LC-ESI-MS spectrum of $[M+H]^+$ ions(m/z 312) of Piperettine under UV degradation condition.

Conclusions:

Stress degradation studies on piperine, carried out according to ICH guidelines, provided information on the degradation behaviour of the piperine under the conditions of hydrolysis and oxidation. The liquid chromatography method described in the present study can resolve all the degradation products from the piperine as well as from each other under various stress conditions. The Piperine showed extensive degradation in acid, base hydrolysis and oxidative stress, while it was stable to neutral, thermal and photolytic stress conditions. A total of 4 degradation products were characterized with the help of the LC-MS (Q-TOF) experiment combined with accurate mass measurements of fragment ions and compare with is natural extracts profiling.

The developed method is stability indicating and can be conveniently used by quality control departments to determine the related substance and assay in regular Piperine samples and also stability samples and thereby produce formulations which are highly efficacious.

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† Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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