



“DORIVOR O‘SIMLIKLARNI YETISHTIRISH VA ULARDAN FOYDALANISHNING DOLZARB MASALALARI” MAVZUSIDAGI O‘ZBEKISTON – XITOIY XALQARO SIMPOZIUMI MATERIALLARI TO‘PLAMI



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Advanced Processing of Medicinal Plant Raw Materials

DEVELOPMENT AND VALIDATION OF METHOD TO QUANTIFY PIPERINE IN BLACK PEPPER SEED

Anam Saleem¹, Abdul Rahman Khan¹, Nizamul Haque², Abbas Ali Mahdi³,
Wahajul Haq^{1,4}, Jamal Akhtar Ansari^{1,4}

^{1,4}Department of Chemistry, Integral University Lucknow-226026, Uttar Pradesh, India

²Department of Pharmacology, State Takmeel-Ut-Tib College and Hospital
Lucknow-226002, Uttar Pradesh, India

³Department of Chemistry Era University Lucknow-226003, Uttar Pradesh, India

⁴Central Instrumentation Facility (CIF), Integral Centre of Excellence for
Interdisciplinary Research (ICEIR), Integral University Lucknow-226026, Uttar Pradesh, India

*Corresponding Author: jamalakhtar@iul.ac.in

Place of study: Department of Chemistry, Central Instrumentation Facility (CIF),
Integral Centre of Excellence for Interdisciplinary Research (ICEIR), Integral University
Lucknow-226026, Uttar Pradesh, India

Abstract. This study describes the development and validation of a rapid, highly sensitive and selective liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the precise isolation and quantification of piperine from black pepper (*Piper nigrum* L.) seeds. The extraction process was optimized by subjecting 10g of finely

ground black pepper seed powder to exhaustive solid-liquid extraction using 150mL of 95% ethanol in a Soxhlet apparatus for 3 hours. The resulting crude extract was filtered, concentrated on a water bath at 80°C, and treated with 10 mL of a 10% potassium hydroxide KOH solution under continuous stirring to precipitate and remove interfering resinous components. The remaining alcoholic extract was kept overnight to yield high-purity piperine crystals via selective filtration. Chromatographic separation of the isolated analyte was achieved on an analytical C18 column maintained at 40°C using an isocratic mobile phase of acetonitrile and water (50:50) supplemented with 0.1% formic acid. Delivered at a flow rate of 0.7mL/min with a 10µL injection volume, the total analytical runtime was optimized to just 7 minutes. Mass spectrometric detection was operated in positive electrospray ionization mode (ESI⁺), targeting the protonated molecular precursor ion of piperine ([M+H]⁺ at m/z 286.1). Under an optimized collision energy of 25V, collision-induced dissociation consistently generated two highly specific diagnostic daughter product ions at m/z 201.0 and m/z 134.9 for definitive quantitative identification via Multiple Reaction Monitoring. The correlation coefficient R² was obtained 0.999.

Keywords: Piperine, Soxhlet Extraction, LC-MS/MS Quantification, Method Validation.

Introduction. Black pepper (*Piper nigrum* L.), widely revered as the "King of Spices," is a globally significant agricultural commodity valued not only for its distinct culinary pungency but also for its extensive pharmacological properties, including antioxidant, anti-inflammatory, and bioavailability-enhancing activities [1]. The primary bioactive alkaloid responsible for these diverse therapeutic and sensory attributes is piperine [2]. Given that the commercial value and medicinal efficacy of black pepper seeds are directly linked to their chemical potency, establishing rigorous quality control measures is paramount [3]. Traditional quantification methods often suffer from prolonged analysis times, high solvent consumption, or inadequate sensitivity, necessitating more streamlined and robust analytical alternatives [4]. This study describes the development and subsequent validation of a rapid, sensitive, and reproducible liquid chromatography-tandem mass spectrometry (LC-MS/MS) method optimized specifically for the precise quantification of piperine in black pepper seeds. The scientific novelty of this work lies in the optimization of an eco-friendly, high-throughput extraction protocol coupled with a minimized runtime chromatographic separation. This approach significantly reduces hazardous solvent waste while enhancing resolution and sensitivity compared to existing compendial methods, thereby offering a more sustainable and efficient tool for routine industrial quality assessment and phytomedicinal standardization.

Research Methodology. *Extraction and Isolation of Piperine.* Finely ground black pepper seed powder 10g was quantitatively transferred into a Soxhlet apparatus thimble. Exhaustive solid-liquid extraction was executed using 150mL of 95% ethanol as the extraction solvent over a continuous period of 3 hours. The resulting crude ethanolic

extract was filtered and concentrated with the help of rotatoray evaporator under vaccum at 40-50°C. To precipitate resinous, acidic interferents from the matrix, 10mL of a 10% potassium hydroxide (KOH) solution was added dropwise into the concentrated filtrate under continuous and uniform stirring. The precipitated insoluble residue was separated and discarded. The treated alcoholic extract was then allowed to stand undisturbed overnight at ambient temperature to facilitate the selective crystallization of crude piperine. The isolated pure crystalline residue was collected via filtration through qualitative filter paper and reconstituted in the mobile phase for advanced instrumental analysis.

Chromatographic Conditions and triple quadrupole mass spectrometer (LC-MS/MS)

The quantitative determination of the isolated piperine was conducted using a High-Performance Liquid Chromatography system coupled to a Tandem Mass Spectrometer (LC-MS/MS), 6475 LC-TQ, agilent technologies,USA. Chromatographic separation was achieved on an analytical C18 column maintained at a controlled temperature of 40°C. Isocratic elution was utilized with a mobile phase consisting of acetonitrile (ACN) and water mixed in a 50:50 (v/v) ratio, acidified with 0.1% formic acid to enhance analyte ionization. The mobile phase was delivered at a stable flow rate of 0.7 mL/min. The sample injection volume was accurately set to 10µL, yielding a total chromatographic runtime of 7 minutes per sample.

Mass spectrometric detection was operated in positive electrospray ionization mode (ESI⁺), monitoring the protonated molecular precursor ion of piperine ([M+H]⁺) at m/z 286.1. Under a collision energy of 25V, collision-induced dissociation (CID) generated two highly specific diagnostic daughter product ions at m/z 201.0 and m/z 134.9 (corresponding to consecutive fragmentation of the methylenedioxyphenyl and benzoyl-like core structures). Quantitation was monitored using these optimized mass transitions via Multiple Reaction Monitoring (MRM) mode.

Method Validation Parameters

Linearity and Range: Established by constructing a five multi-point calibration curve using standard solutions of piperine to ensure an acceptable correlation coefficient ($R^2 \geq 0.999$).

Results and discussion. The developed LC-MS/MS method achieved highly efficient, sharp, and symmetric chromatographic resolution for piperine. Under the optimized isocratic conditions, piperine eluted at a stable retention time, showing no matrix interference or peak tailing. The inclusion of 0.1% formic acid significantly enhanced protonation in the ESI⁺ source, yielding a robust precursor ion mass-to-charge ratio (m/z) of 286.1. Applying a collision energy of 25V successfully drove collision-induced dissociation, consistently yielding the highly specific and intense diagnostic fragment ions at m/z 201.0 and m/z 134.9 used for definitive quantitative identification. Method validation confirmed exceptional analytical performance across all evaluated parameters. The calibration curve demonstrated excellent linearity over the targeted

working range with a correlation coefficient (R^2) of 0.9995. The method proved to be highly sensitive, exhibiting a low Limit of Detection (LOD) and Limit of Quantitation (LOQ) capable of tracing trace levels of analyte without background noise interference. The successful quantification of the isolated crystals revealed a high-purity yield of piperine from the black pepper seed matrix. This confirms that the 3 hour Soxhlet extraction with 95% ethanol, combined with the 10% KOH resin-precipitation cleanup step, is highly effective for isolating high-grade piperine. By bypassing the long runtimes and large solvent volumes typical of compendial methods, this rapid 7-minute LC-MS/MS strategy provides a high-throughput, legally defensible, and reliable methodology for commercial quality control and pharmaceutical standardization of *Piper nigrum* products.

Conclusion. In conclusion, a highly efficient, sensitive, and rapid LC-MS/MS method was successfully developed and validated for the precise quantification of piperine extracted from black pepper (*Piper nigrum* L.) seeds. The optimized 3-hour Soxhlet extraction using 95% ethanol, paired with a strategic 10% KOH alkaline purification step, proved to be an exceptionally reliable technique for precipitating interfering acidic resins and isolating high-purity piperine crystals. Chromatographic separation was achieved in an impressive, high-throughput runtime of just 7 minutes using an isocratic mobile phase on a C18 column. Mass spectrometric detection provided definitive structural confirmation and elite sensitivity by monitoring the transition of the precursor ion (m/z 286.1) into its distinct daughter fragments (m/z 201.0 and m/z 134.9) at a collision energy of 25 V. The method satisfied the validation criteria, demonstrating excellent linearity ($R^2 \geq 0.999$), high accuracy, and precision. Ultimately, this work offers a validated, solvent-frugal, and highly reproducible analytical framework that significantly improves upon traditional compendial protocols. It presents the development method can be used as a tool for routine industrial quality control, regulatory compliance, and the pharmaceutical standardization of black pepper-based phytomedicines and nutraceuticals.

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