Quantitative Estimation of Piperine in Piper nigrum and Piper longum Using High Performance Thin Layer Chromatography

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ABSTRACT

Piperine, a characterizing compound present in fruits of Piper nigrum and Piper longum used as bioavailability enhancer. Ingredients of antioxidant, anti-inflammatory activity has been extracted using soxhlet and supercritical fluid extraction technique. It was isolated using column chromatography. Characterization of compound was done by spectroscopic technique. A simple, rapid, accurate and specific HPTLC method developed and validated. The method proposed can be used for the routine analysis of both P.nigrum and P.longum fruit material and its formulations.

Key words: HPTLC Fingerprinting, Quantitative estimation, Piper nigrum, Piper longum, Piperine.

INTRODUCTION

Piper nigrum Linn. and Piper longum Linn. are world’s important and oldest spices, commonly known as ‘Black pepper’ and ‘Long pepper’ respectively. They belong to family piperaceae. Both plants are indigenous and cultivated in hot and moist parts of India. The fruits have variety of activity including CNS depressants, antipyretic, analgesic, hepatoprotective (Gupta et al, 1986), bioavailability enhancer (Mujumdar et al, 1999 ; Annamalai et al, 1989), Antioxidant (Khajuria et al, 1997), anti-inflammatory. The phytoconstitutes of P. nigrum and P. longum fruits include volatile oil, other minor alkaloids such as piplartin, piperlogumine, piperidine, starch, resin. and pungent alkaloid piperine (Kokate et al, 1994; Evans et al, 2004; Khare et al, 2006). Piperine is the main therapeutically active constituent of this plant. Because of its availability in the pure form it has been used as a characterizing compound in this study. Literature survey reveals that, various chromatographic methods such as HPTLC (Suthar et al, 2003; Gawas et al, 1999; Noyer et al, 1997), HPLC (Verzele et al, 1979; Rathnawathie et al, 1983) have been reported for the quantification of piperine. However these methods suffer from drawbacks such as poor resolution, lack of sensitivity and reproducibility. Literature survey further reveals that extraction of piperine has been carried out using the classical method of extraction such as soxhlet method. In this proposed work an attempt has been made to extract piperine using ‘state-of- the art’ technique i.e. supercritical fluid extraction technique. An attempt has also been made to develop and validate HPTLC method for the analysis of piperine which would be highly sensitive, having good resolution and reproducibility.

EXPERIMENTAL

Material, Chemical and Reagents

Fruits of P. nigrum and P. longum were procured from Kerala and authenticated at Agarkar
Research Institute, Pune, India. (Certificate dated 12/02/07). Tablet formulations were purchased from pharmacy in Mumbai. Analytical grade hexane, ethyl Acetate, glacial acetic acid was obtained from Merck, India whereas methanol, petroleum ether of analytical grade were obtained from Qualigens Fine Chemicals, Mumbai, India. Piperine (97%) characterizing compound was obtained from Sigma Aldrich, Switzerland and characterized by Ultra-violet (UV), Infrared, Proton nuclear magnetic resonance and by mass spectroscopy to confirm their identity and purity.

Sample Extraction

**Soxlet method for extraction**

The powdered fruit materials were extracted with methanol using Soxhlet apparatus and the extract obtained was then diluted appropriately with methanol.

**Supercritical Fluid Extraction (SFE)**

The fruit materials were powdered and passed through sieve of # 40 mesh sizes. The powdered materials were weighed and transferred to the Supercritical Fluid Extraction vessel. The materials were extracted using CO2 gas (supercritical fluid) and methanol as cosolvent. The conditions of Supercritical Fluid Extraction were optimized. The optimized conditions of Supercritical Fluid Extraction are as follows Gas: CO2, Pressure 300 bar, Temperature 45°, Modifier-Methanol, Flow rate -0.2 ml/min, CO2 flow rate- 2.5 ml/min, and extraction time-30 minutes. The extract was then concentrated on removal of solvent under vacuum. The concentrated extract was then diluted appropriately with methanol.

**Formulations**

Tablets were obtained from local market. The content of 10 tablets were weighed, triturated with glass pestle and mortar.10.0 gm of powder was weighed and extracted in methanol using soxhlet apparatus. The extract obtained was then diluted with methanol.

**Phytochemical Evaluation of P. nigrum and P. longum:**

**General Qualitative Chemical Tests for the presence of Phytoconstituents**

Sample of fruit materials were subjected to phytochemical tests for alkaloids, glycosides, phenols and tannins to confirm literature reports of phytoconstituents of the plant.

**Physicochemical properties of P.nigrum and P.longum**

Physicochemical properties were determined water soluble extractive, alcohol-soluble extractives, ash values, foreign organic matter and moisture content.

**HPTLC**

**Prewashing of plates**

HPTLC was performed on 10 cm x 20 cm aluminum backed Silica gel F254 HPTLC plates from E.Merck (Darmstadt, Germany). The adsorbent has a very large surface area, it may absorb air and other impurities from atmosphere, particularly volatile impurities, after pack has been opened. The nonvolatile impurities adsorbed by layer can lead to irregular baselines in scanning densitometry. To avoid possible interference from such impurities in quantitative analysis, plates were prewashed with methanol, dried and activated for 30 min at 110° with the plates being placed between two sheet of glass to prevent deformation of the aluminum during heating.

**Procedure**

A methanolic solution of piperine (1mg/ml) was prepared. The solution was further diluted with methanol to yield a solution containing 100 µg/ml. Different concentration of piperine were applied on plates as 6 mm bands, 6mm apart and 1 cm from edge of the plate, by means of Camag Linomat IV automatic sample applicator, fitted with 100 µ Hamilton Syringe. A methanol blank was applied to parallel track. The mobile phase, hexane: ethyl acetate: glacial acetic acid (3:1:0.1) was poured into the second trough and the plates was left to equilibrate in the chamber for 20 min at 25±2° C. The plate was then moved to the second trough containing the mobile phase and developed to a distance of 90mm. After development, the plate was removed from the chamber, dried in current of hot air, and scanned at 343 nm, using a deuterium lamp, by means of a Camag scanner III densitometer. The densitogram obtained by HPTLC of piperine characterizing compound, and extract are shown in Figure 1 and 2 respectively.

This method is followed for all quantitative analysis. CATS’ software (version 3.17) was used for data acquisition and processing of the plate. The scanning speed was 20mm s⁻¹, the offset was 10%, and the sensitivity (SPAN) was optimized to 20 min. Peak height and peak area were integrated for the entire track. The calibration plot was constructed by plotting peak area against concentration of piperine. The reproducibility of this method was ascertained by repeating the experiment three times.

![Representative chromatogram of characterizing compound piperine.](image-url)
which the RSD (%) of six replicate spots was less than 5 % was regarded as the limit of quantitation. To check the specificity of the method the in-situ reflectance spectra of peaks obtained from test sample were compared with those obtained from standards. Accuracy was determined by measurement of the recovery of piperine characterizing compound added at three different levels to plant extract, each being analyzed as described for the assay. Intraday and interday precision were determined by applying 18ng, 120ng, 240ng standard piperine. After development and densitometric scanning of the plates the peak area response was \( V_{\text{measured}} \) and precision was calculated as RSD (%).

Piperine is pungent alkaloid, insoluble in water and soluble in methanol hence methanol was used for the extraction of piperine using classical method and Supercritical fluid extraction technique. The extract obtained from different samples of \( P. nigrum \) and \( P. longum \) loaded on to HPTLC plate and developed in the optimized solvent system. Since piperine is non polar based on the various reported solvent systems comprising of petroleum ether: acetone (6:5:3.5), acetone: hexane (3:2), toluene: ethyl Acetate (7:3) were used to develop the HPTLC plate but resolution was found to be poor. Toluene was replaced with hexane due to their more nonpolar nature. Finally the solvent system hexane: ethyl Acetate (3:1) which gave resolved spots of the constituents but tailing of piperine was observed. This was reduced by adding glacial acetic acid to the mobile phase, but higher concentration of glacial acetic acid gave poor resolution hence concentration of glacial acetic acid was optimized. Thus after several trial and errors, the optimized solvent system was developed which consisted of hexane: ethyl Acetate: glacial acetic acid (3:1:0.1). The optimized system gave a good resolution of the phytoconstituents in the \( P. nigrum \) and \( P. longum \). The plate material employed was normal phase silica gel F254. Since piperine shows UV absorption 343nm so plate scanned at 343 nm. The method developed was validated for limit of detection (LOD) which was found to be 6 ng per spot, limit of quantitation (LOQ) was found to be 18 ng per spot. The method was found to linear over the range 18ng to 240 ng per spot with coefficient of regression 0.99. Intra-day and inter-day precision studies showed a % CV was less than 2.00% hence method was precise. The accuracy values obtained, in the range 98.57% to 97.25% for piperine in \( P. nigrum \) and 96.50% to 97.50% for piperine in \( P. longum \) are indicative of excellent recovery. There was no evidence of peaks coeluting at the RF of piperine (0.46). This indicates the method is specific. Stability studies were carried out for standard. It was found to be stable in sample solution, prior to development and after development.

The developed method was then validated and successfully applied for quantitation of piperine from various extracts such as soxhlet extracts and Supercritical fluid extracts of \( P. nigrum \) and \( P. longum \). The % w/w yield of piperine in Supercritical fluid extract was 8.76 for \( P. nigrum \) and for \( P. longum \) 4.96. Whereas in Soxhlet extract % w/w yield for \( P. nigrum \) 8.13 and for \( P. longum \ % w/w was 4.32. Time required for extraction of piperine by soxhlet method was 8.0 hr whereas for supercritical fluid extraction only 0.5 hr. This shows Supercritical fluid extraction has more extraction efficiency and reduced extraction time than soxhlet extraction method. Also the results showed % w/w yield of piperine was found to be higher in \( P. nigrum \) than \( P. longum \). Whereas % w/w yield of piperine in its formulation was found to be 7.41% when extracted with soxhlet method.

**CONCLUSION**

A rapid, simple, accurate, sensitive and specific HPTLC method developed and validated. This developed and validated method was used for quantitative estimation and macro and micro fingerprinting analysis of piperine from different geographical...
variety of *P. nigrum* and *P. longum* and its formulation. Also this study shows Supercritical fluid extraction has more extraction efficiency and reduced extraction time than soxhlet extraction method.

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**REFERENCES**


