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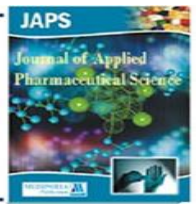

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Quantitative Estimation of Piperine in Piper nigrum and Piper longum Using High Performance Thin Layer Chromatography

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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, 1999), 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

Soxhlet 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-Methnol, 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 vaccum. 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.

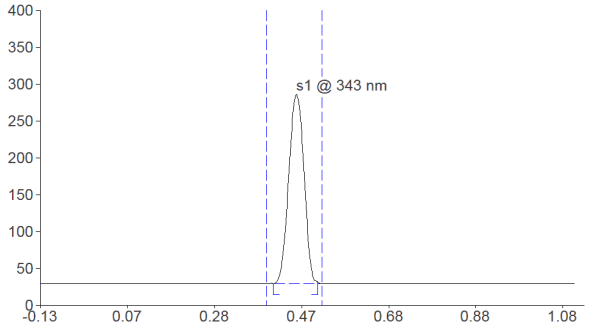
HPTLC

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 µl 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.



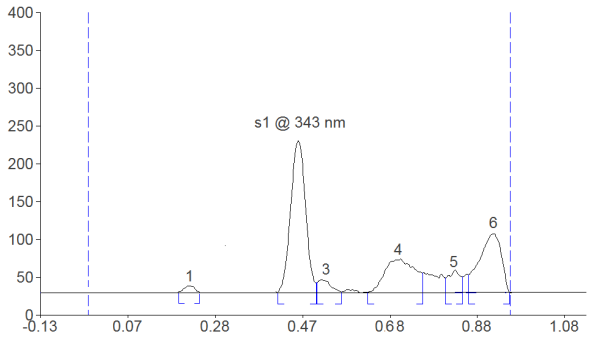
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.41	0.6	0.46	256.6	100.00	0.51	2.5	8397.0	100.00	s1

Fig1 Representative chromatogram of characterizing compound piperine.

Method Validation

The method was validated for specificity, accuracy, precision by use of calibration standards of piperine and it is applied to extracts of *P. nigrum* and *P. longum.* Limit of detection and limit of quantitation were determined by the visual method, by spotting different concentration of piperine. The lowest concentration that could be detected for three replicates spots was regarded as the limit of detection. The lowest concentration for

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 Vmeasured and precision was calculated as RSD (%).



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.18	1.5	0.22	15.8	2.58	0.24	1.6	417.0	1.80	unknown *
2	0.42	0.3	0.47	201.3	53.89	0.51	13.6	6404.1	44.90	s1
3	0.51	14.1	0.52	17.7	4.73	0.56	1.6	499.3	3.50	unknown *
4	0.63	0.9	0.70	45.2	12.09	0.75	26.9	2914.1	20.43	unknown *
5	0.80	20.0	0.82	30.9	8.26	0.84	21.6	782.4	5.49	unknown *
6	0.86	24.5	0.92	78.6	21.03	0.95	0.8	3662.6	25.68	unknown *

Fig 2 Representative HPTLC chromatogram of extract at 343 nm.

Application of the Validated Method

Extracts of *P. nigrum* and *P. longum* and formulation obtained as described in section 2.2 were diluted with methanol and later with mobile phase. All samples were analyzed in duplicate and the amount of piperine present in the sample were determined by use of calibration plots obtained as described in section above.

RESULT AND DISCUSSION

Results from general qualitative chemical testing of *P.nigrum* and *P.longum* fruits for the presence of phytoconstituents reveals the presence of alkaloids. Quantitative estimation of the physicochemical properties expressed as (% w/w) of the dry *P.nigrum* and *P.longum* fruit powder were : moisture content 4.68, water soluble extractive value 17.10, alcohol soluble extractive values 14.20, total ash 5.01, acid insoluble ash 0.79, water soluble ash 1.81, foreign organic matter 1.60 for *P.nigrum* and for *P.longum* Moisture content 2.35, water soluble extractive value 13.50, alcohol soluble extractive values 10.10, total ash 2.05, acid insoluble ash 0.20, water soluble ash 0.82, foreign organic matter 1.31. From all this data it was observed that water soluble extractive values was higher indicating the amount of polar constituent is more in water extract than alcohol extract in both the species.

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 that 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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