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Rapid UV Spectrophotometric Method for Piperine Quantification: Evaluating Genotypic and Developmental Stage Effects in Blackpepper (*Piper nigrum* L.)

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Authors' contributions

This work was carried out in collaboration among all authors. Author MA designed the study, conducted experiments, performed the statistical analysis, wrote the protocol and wrote the draft of the manuscript. Author SR reviewed the design and result analysis of the study. Authors BNK and DP managed the analysis facilities. All authors read and approved the final manuscript.

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ABSTRACT

This study presents the development and validation of a rapid, cost-effective UV spectrophotometric method for the quantitative determination of piperine in *Piper nigrum* L. Three genotypes Panniyur 1, Panniyur 2, and Uthirankotta were studied at two critical stages of

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development (immature and mature green berries) to understand the genotypic and developmental effects on piperine accumulation. The method was highly specific and accurate and thus can be used as an effective substitute for high cost and time-consuming extraction methods for large-scale screenings. Results showed significant genotypic variation, where Panniyur 2 had the highest piperine content of 6.562 percent w/w at maturity, followed by Panniyur 1 and Uthirankotta, respectively. Piperine rises significantly from immature to mature stage in all three genotypes, which ascertains the importance of a correct harvest period to get a good yield of alkaloids. These results therefore establish that Panniyur 2 is a better genotype with regard to piperine production, and the mature green stage is the ideal period for harvest to obtain maximum bioactive principal contents. A validated UV spectrophotometric method described here will be an effective tool in quality control and breeding programs to improve the piperine yield in black pepper cultivation.

Keywords: Piper nigrum L.; piperine; UV spectrophotometer; developmental stages; alkaloid; blackpepper.

1. INTRODUCTION

Black pepper (*Piper nigrum* L.) also referred to as "King of spices" or "Black gold" belongs to the family Piperaceae and is generally believed to have originated in the sub-mountainous plains of the Western Ghats of India (Rahiman et al., 1979). It is considered a high-value crop with great economic and therapeutic importance (Bekele et al., 2025). The peculiar aroma and pungency of black pepper are attributed mainly to piperine and volatile oils. Piperine is the chief bioactive constituent of black pepper and possesses various medicinal properties such as antiplatelet, antihypertensive, anticancer, antioxidant, analgesic, antidepressant, and anti-diarrheal activity (Ashokkumar et al., 2021). Piperine content in *Piper nigrum* L. usually ranges from 2% to 9%, depending on growing conditions and environmental factors, particularly climate and geographical location (Gorgani et al., 2017a). Improving cultivation for high piperine concentration in various pepper types is important to meet the increasing demand for this spice in the international market, especially in pharmaceutical extraction, where purity and yield are crucial (Shrestha et al., 2020). A minimum concentration of 3.5 percent to 4.0 percent piperine on a dry weight basis is usually prescribed for premium black pepper grades (FSSAI, 2017). Piperine content is influenced by genotype, fruit size, developmental stage, and many other environmental factors (Sozzi et al., 2012). Black pepper berries undergo significant biochemical changes as they progress to maturity from the immature to mature green stage. During this stage, earlier studies have qualitatively confirmed an increase in piperine content (Putalun & De-Eknamkul, 1993).

Piperine, an alkaloid of black pepper is a molecule chemically characterized as the carboxamide of piperidine and piperic acid. It is highly soluble in ethanol and other organic solvents, while its water solubility is poor (Upadhyay et al., 2013). To meet the industry's demand for high-piperine pepper efficiently and to facilitate high-throughput screening in breeding programs, there is an increasing need for a rapid, reliable, and cost-effective analytical method. With high resolution and specificity, the HPLC can precisely separate piperine from interfering molecules in the extracted solvent. However, it's very low throughput, high operating costs, and dependence on specialized infrastructure severely limit its application when hundreds of samples need to be analyzed quickly and economically, such as in rapid field-based quality assessment or large-scale genotype screening. A number of studies have explored spectrophotometric methods with a view to overcoming these limitations, capitalizing on the potent UV absorbability of piperine (Parmar et al., 1997; Chauhan et al., 1998). Finally, it is known that the final yield of piperine is affected by the developmental stage of the berry or the time of harvest. Piperine has been reported to usually accumulate during the development process of the fruit (Gupta et al., 2013; Yadav et al., 2023).

Piperine has economic significance that goes well beyond its conventional use as a spice. This is why a complex industrial process is used to isolate the compound for the pharmaceutical and nutraceutical markets (Do, 2022). To produce a crude piperine from the powdered pepper fruit, large-scale extraction usually makes use of effective solid-liquid techniques like Soxhlet extraction or percolation, using solvents like ethanol or dichloromethane (Vasavirama & Upender, 2014). The main commercial use is still its potent bioenhancer properties, which were

first formally presented to the scientific community along with piperine's mode of action (Chaudhri & Jain, 2023; Patil et al., 2011). Isolated, high-purity piperine strengthens its value as a commercially significant functional ingredient by improving absorption and adding its inherent antioxidant, anti-inflammatory, and neuroprotective properties to health-focused products (Lwamba et al., 2023). Significant preclinical activity against a variety of human diseases, such as cancer and inflammatory disorders, has been proven by piperine. A few possible molecular targets were investigated in relation to various illnesses (Tripathi et al., 2022).

This research, therefore, seeks to bridge that prevailing gap in knowledge. Several chemical studies have indicated that there is variation in piperine content; however, few have employed a rapid, validated method in making direct assessment of independent and interactive effects of genotypes and developmental stage on piperine accumulation crucial for optimizing harvest timing for the piperine extraction industry and in breeding programmes developing suitable varieties.

The main objectives of this study are threefold: (i) to validate a rapid, specific, and cost-effective UV spectrophotometric method for the quantification of piperine in *P. nigrum*, (ii) to systematically apply the validated method for assessing the genotypic effect on piperine content in the prominent hybrid Panniyur1 (P1) and variety Panniyur2 (P2), in comparison with that of the cultivar Uthirankotta, also one of the parents of the hybrid P1, and (iii) to examine the developmental stage that can influence the accumulation dynamics of piperine for the selected high-performing genotypes, with the goal of pinpointing the optimal harvest time to achieve maximum alkaloid yield. This approach will help to overcome important knowledge gaps by providing a robust analytical tool and actionable insights that enable the optimization of both the choice of cultivars and agricultural practices in black pepper cultivation.

2. MATERIALS AND METHODS

The materials used for this study were three diverse genotypes of black pepper (*Piper nigrum* L.): Panniyur 1, Panniyur 2, and Uthirankotta (IISR Accession No. 1245). Panniyur 1 and Panniyur 2 were collected from the Pepper Research Station under Kerala Agricultural University located at Panniyur, Kannur, India. Uthirankotta was procured from Indian Institute of Spices Research, Appangala Research Station, Madikeri, Karnataka. These genotypes are selected based on historical chemotype data to represent the broadest range of known natural variability in piperine content. For the comparative experimental trial, samples were grouped by genotype (Panniyur 1 and Panniyur 2 as high-piperine; Uthirankotta as low-piperine) and harvested at two distinct physiological time points. The first time point, immature green stage (approximately one month after pollination), was characterized by just-formed berries that had not undergone any color break (light yellow green), representing an early developmental phase. On the other hand, the second time point, mature stage (approximately 6 months after pollination), is characterized by fully grown bold berries with dark green coloration, thus indicating peak alkaloid accumulation. All the samples were randomly collected from multiple healthy vines to ensure representation of the natural variation in the field and to minimize any possible sampling bias before the processing began.

All chemical standards and solvents used were of analytical grade. A high-purity piperine standard was obtained from Sigma-Aldrich. The primary solvent used was methanol because it is efficient for the extraction of piperine and appropriate for analysis. Major equipment included a UV spectrophotometer, 342-345 nm absorbance capability, 1 cm square silica cuvettes, and micropipettes. Other materials that were required included Whatman No. 2 filter paper, a microcentrifuge, and standard volumetric flasks.

2.1 Preparation of Piperine Standard Curve

A piperine stock solution was prepared by dissolving 0.01 g of piperine standard in methanol and making the volume up to 100 mL in a volumetric flask. A series of five working standard solutions were then prepared by dilution, resulting in a range of concentrations from 5 µg/mL to 25 µg/mL. Absorbance measurements were made using a UV spectrophotometer, which was blanked with pure methanol. The maximum absorption wavelength (λ_{max}) was recorded at 342 nm. A final calibration equation ($y = mX + c$) was derived from the linear regression of absorbance (y) versus concentration (X) data (Fig. 1).

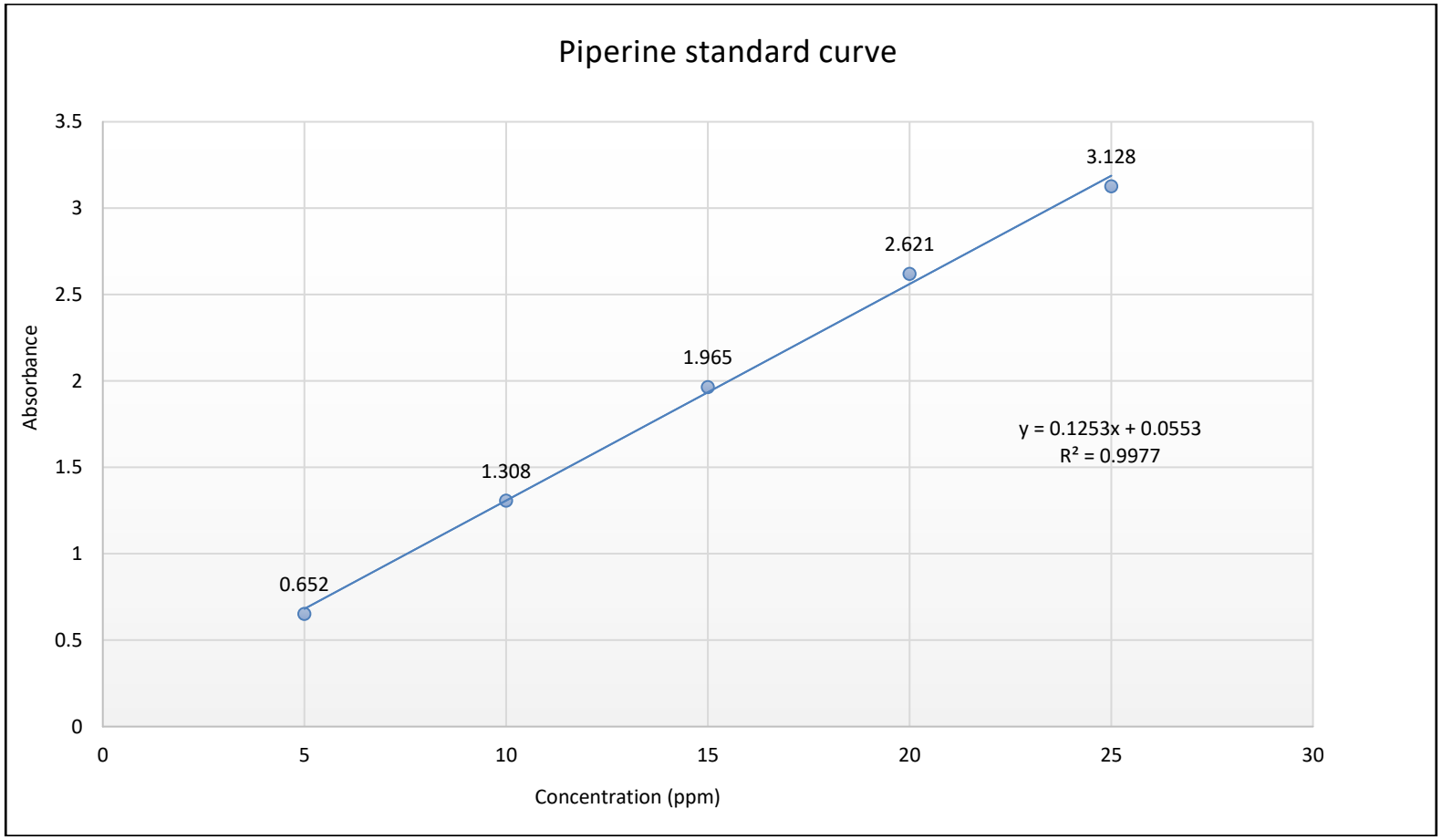

Fig. 1. Standard curve of piperine at 342 nm

2.2 Piperine Extraction and Quantification by UV Spectrophotometry

Piperine was extracted from the berries of the black pepper according to the procedure of (Dolui, 2011), with slight modifications. A sample of berries (1 g) was homogenized in a mortar and pestle with 5 mL of methanol. The crude extract was incubated in a water bath at 65˚ C for 1 hr., followed by centrifugation at 5000 rpm for 10 minutes.

The supernatant was filtered (Whatman No. 2), and a further dilution was made with the filtered extract and methanol to ensure that the concentration falls within the linear range of the standard curve. Then, the absorbance of the solution was determined spectrophotometrically at 342 nm. Piperine concentration X (in µg/mL) was calculated from the linear regression equation of the standard curve. The final concentration was adjusted for the dilution factor and reported as percent piperine content (w/w on a fresh weight basis).

2.3 Statistical Analysis

The data were gathered as per the treatment structure in a completely randomized design (CRD). Statistical analysis, including analysis of variance (ANOVA), was done using the General R-based Analysis Platform Empowered by Statistics (GRAPES) (Kerala Agricultural University, 2020) software (version 1.1.0), developed by Kerala Agricultural University. The experimental layout resulted in 3 x 2 = 6 treatment combinations replicated four times. Statistical significance was accepted at p = 0.05. The Critical Difference (CD value at P = 0.05) was calculated to compare the mean piperine content across the genotypes and stages of development.

3. RESULTS AND DISCUSSION

Piperine content (% w/w) was estimated in the three black pepper genotypes, Panniyur 1, Panniyur 2, and Uthirankotta, at two critical developmental stages of green berries, namely immature and mature stages, following the UV spectrophotometric method in 4 replicates. ANOVA performed based on CRD showed that the differences among the genotypes in both developmental stages were statistically significant ($p < 0.05$). The piperine content of genotypes, as averaged across the two developmental stages, is presented in Table 1 and Fig. 2.

3.1 Piperine Content in Immature Berries

The mean piperine content in immature black pepper berries ranged from 0.772 percent to 1.252 percent (Table 1). The highest accumulation (1.252 % ± 0.016) was observed in Panniyur 2, which was statistically superior (P = 0.05) to all other genotypes. This was followed by Panniyur 1 (1.048 % ± 0.016). The lowest piperine content was observed in Uthirankotta (0.772 % ± 0.016). The CD value for the immature stage calculated at P = 0.05 was 0.063 percent.

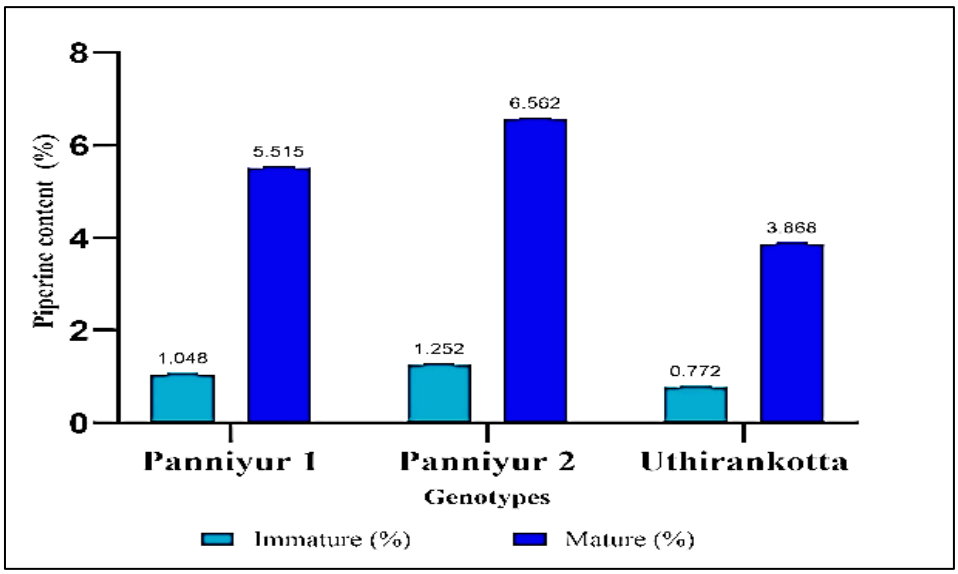

Fig. 2. Mean piperine content across two developmental stages of three genotypes

Table 1. Mean piperine content (% w/w) of black pepper genotypes across stages of development

Genotype	Piperine in immature fruit (%)	Piperine in mature fruit (%)
Panniyur 1	1.048[b]	5.515[b]
Panniyur 2	1.252[a]	6.562[a]
Uthirankotta	0.772[c]	3.868[c]
$SE_{(m)}$	0.016	0.017
CD	0.063	0.055

Note: Data are presented as means within a column followed by different letters (a, b, c) are significantly different at the P = 0.05 level according to the Critical Difference (CD).

3.2 Piperine Content in Mature Berries

At the mature stage, the mean piperine content ranged from 3.868 percent to 6.562 percent (Table 1). Panniyur 2 recorded the highest content, 6.562 % ± 0.017, a value that was statistically superior to both Panniyur 1 and Uthirankotta, with contents of 5.518 % ± 0.017 and 3.868 % ± 0.017, respectively. The CD value (at P = 0.05) was 0.055 percent for this data set.

Overall, both Panniyur 1 and Panniyur 2 showed much higher levels of piperine accumulation at both the immature and mature stages compared with the low-piperine genotype, Uthirankotta. Comparison between the two developmental stages showed that in all genotypes, there was a significant and consistent rise in accumulation as the berries went from the immature stage to the mature green stage.

The study examined the comparative mean piperine content (% w/w on a fresh weight basis) in three *Piper nigrum* L. genotypes (Panniyur 1, Panniyur 2, and Uthirankotta) at two main developmental stages: immature and mature. With maturation of berries, there was a gradual and significant increase in the piperine content in all varieties. This confirms a major trend of secondary metabolite accumulation during the later stages of fruit development in black pepper, thus agreeing with earlier reports (Mathai, 1983).

The content of piperine varied between 0.772 and 1.252 percent at the immature stage. Panniyur 2 gave the highest accumulation (1.252 %), which was statistically higher (P = 0.05) than that of both Panniyur 1 (1.048 %) and Uthirankotta (0.772 %). The recorded concentration in immature Uthirankotta was notably the lowest. The values ranged from 3.868 percent to 6.562 percent, with a significant enhancement at the mature stage. Panniyur 2 retained the maximum content, 6.56 percent, which was statistically superior (P = 0.05) to that of Panniyur 1 (5.515 %) and Uthirankotta (3.868 %), in coherence with the immature stage. The genotypic variation in the biosynthetic activity was underlined by the maximum difference of 2.694 percent between the highest (Panniyur 2) and the lowest (Uthirankotta) at maturity.

In this study, the estimated concentration of piperine ranged from 6.562 percent in mature Panniyur 2 to 0.772 percent in immature Uthirankotta. (Wood et al., 1988) and (Gorgani et al., 2017b) reported that the mature fruit values for black pepper fruits are typically within a range from 2 percent to 8 percent. The highest piperine content of 6.562 percent, obtained for mature Panniyur 2, falls within the high value range for the variety, like 6.6 percent reported (Zachariah, 2008). The slightly lower figure obtained in this study compared to some reports underscores the fact that piperine concentration tends to be highly variable with varietal character, harvest stage, and prevailing agroclimatic conditions (Chauhan et al., 2017; Pannaga et al., 2021; Jäckel et al., 2022). In mature Panniyur 1, the piperine content recorded at 5.515 percent is significantly higher compared to the earlier reported values (Sruthi et al., 2013; Deka et al., 2016; Krishnamoorthy & Parthasarathy, 2009) of this genotype, which ranged from 2.13 percent to 4.49 percent. However, it was comparable with 5.3 percent piperine content reported (Milenković & Stanojević, 2021). The 3.868 percent content in mature Uthirankotta is at the lower end of the piperine spectrum for popular black pepper cultivars, often reported to be in the range of 2.8-3.8 percent (Milenković & Stanojević, 2021).

The finding of the significantly higher accumulation of piperine in Panniyur 2 and Panniyur 1, as compared with Uthirankotta at both stages, confirms their superior genetic potential for this key secondary metabolite. This would mean that genotype is a primary factor affecting the final piperine yield. The results also accord with the generally accepted relationship

that, whereas the number of secondary metabolites like piperine increases during fruit maturation, this may be followed by a decline at the final ripening stage due to metabolism of volatile oils and other components (Milenković & Stanojević, 2021). The peak accumulation observed in the mature green stage in the present study provides evidence to support the optimal timing of harvest at this stage for maximum yield of piperine.

4. CONCLUSION

The mature green stage was indicated to be the time of maximum accumulation, and this investigation establishes beyond doubt an inherent genetic superiority of Panniyur 2 for production of piperine. The significant and reliable metabolic difference between Panniyur 2 and Uthirankotta has provided a sound scientific basis for deciding on future molecular and crop research goals. Establishment of Panniyur 2 and its crosses as top priority in breeding programs that are aimed at developing new cultivars with quality, yield, and resistance characteristics. Rapid identification and characterization of genes and regulatory elements that control biosynthesis of piperine using the Uthirankotta accession as a defined genetic tool will aid eventually in developing molecular markers for marker-assisted selection in black pepper. For large-scale screening and quality control, the validated rapid UV spectrophotometric method provides a practical solution.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declares that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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COMPETING INTERESTS

Authors have declared that they have no known competing financial interests or non-financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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