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Enzyme-assisted supercritical carbon dioxide extraction of black pepper oleoresin for enhanced yield of piperine-rich extract

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Black pepper (*Piper nigrum* L.), the King of Spices is the most popular spice globally and its active ingredient, piperine, is reportedly known for its therapeutic potency. In this work, enzyme-assisted supercritical carbon dioxide (SC-CO_2) extraction of black pepper oleoresin was investigated using α-amylase (from *Bacillus licheniformis*) for enhanced yield of piperine-rich extract possessing good combination of phytochemical properties. Optimization of the extraction parameters (without enzyme), mainly temperature and pressure, was conducted in both batch and continuous modes and the optimized conditions that provided the maximum yield of piperine was in the batch mode, with a sample size of 20 g of black pepper powder (particle diameter 0.42 ± 0.02 mm) at 60°C and 300 bar at 2 L/min of CO_2 flow. Studies on activity of α-amylase were conducted under these optimized conditions in both batch and continuous modes, with varying amounts of lyophilized enzyme (2 mg, 5 mg and 10 mg) and time of exposure of the enzyme to SC-CO_2 (2.25 h and 4.25 h). The specific activity of the enzyme increased by 2.13 times when treated in the continuous mode than in the batch mode (1.25 times increase). The structural changes of the treated enzymes were studied by ^{1}H NMR analyses. In case of α-amylase assisted extractions of black pepper, both batch and continuous modes significantly increased the yields and phytochemical properties of piperine-rich extracts; with higher increase in batch mode than in continuous.

[**Key words:** Black pepper; Piperine; Supercritical carbon dioxide extraction; Batch and continuous mode; α-Amylase; Nuclear magnetic resonance]

Black pepper, the King of Spices is the most popular spice globally, used extensively in Ayurvedic medicines, in food products and in cosmetics. It is the dried, fully mature, unripe berry of *Piper nigrum* L., a perennial climber belonging to the family Piperaceae, native to the evergreen forests in the Western Ghats of South India (1). According to the data reported by Indian Agribusiness Systems Private Limited, 4574 tons of black pepper worth USD 9500.00 per ton has been exported from India in February 2014 (2). The characteristic aroma and flavor of black pepper is contributed mostly by its oleoresin, principally piperine, which is priced at USD 190.30 per kg (3). India alone exported piperine worth USD 24,560.00 and oleoresin worth USD 2,239,330.00 in February 2014 (3,4). Black pepper possesses several physiological effects, such as strong antioxidative effects; besides stimulating digestive capacity, reducing gastrointestinal food transit time and enhancing bioavailability of several therapeutic drugs and phytochemicals (5).

Conventional extraction of essential oil from black pepper is carried out by hydrodistillation using Clevenger apparatus. Although water is the greenest solvent, it cannot solubilize piperine, the active principle of black pepper. Extraction of piperine is therefore reportedly carried out using solvent extraction in Soxhlet assembly and in standard shake flasks. These solvent extraction techniques are relatively inexpensive; however, the drawbacks of these methods include energy and time consumption, thermal degradation, hydrolysis of desirable constituents, presence of artifacts and traces of solvents in the extracts (6). Owing to these, there are stringent global regulations on usage of these solvents. These limitations necessitate exploration of alternative green extraction techniques, such as supercritical fluid extraction (SFE), well suited for extraction of solvent-free, bioactive-rich natural extracts for food and therapeutic applications (7).

SFE uses fluids above their critical points with liquid like densities leading to high loadings of solutes. This coupled with their pressure-dependent solvating abilities, renders them excellent solvents for separations and reactions. Their low viscosities and high molecular diffusivities like gases, combined with low surface tension, makes them very amenable for mass transfer, allowing better penetration into sample matrices and faster, selective extraction of desired compounds. The most commonly used fluid for SFE is carbon dioxide ($T_c = 31.1°C$, $P_c = 73.8$ bar) which is clean, non-inflammable, non-toxic, eco-friendly and generally regarded as safe (GRAS) solvent (7).

There have been studies on fixed bed extraction of essential oil and oleoresin fractions of black pepper using supercritical carbon dioxide (SC-CO_2) extraction (8–12). Ferreira et al. (11) have reported 2.1% yield of essential oil (at 50°C and 300 bar) from the same; while Tipsrisukond et al. (12) have extracted black pepper oleoresin by SC-CO_2 at 45°C and 320 bar and reported 39.4% relative extraction rate of piperine. Sovová et al. (13) have also worked on black pepper oleoresin at 280 bar and 24–60°C and reported extraction of 30–60% of total piperine in the oleoresin fraction.

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The best Indian black pepper known worldwide for its excellent aroma, flavor and pungency is the Malabar pepper (14). In our study, we have investigated this variety for SC-CO_2 extraction of its oleoresin fraction (principally piperine). However, the yield of piperine (1.2 ± 0.1 mg/g dry black pepper which corresponds to 22.7% of total piperine in black pepper) obtained in our study was lower than that reported in literature. Proximate analysis of the raw material revealed that the main constituent of black pepper is carbohydrate (58.4 ± 0.1%). Starch (30.4 ± 0.1%) was the predominant carbohydrate in our sample, in agreement with Pruthi (15) who reported starch content of black pepper to be 34.8%. We opine that starch being one of the major constituents of black pepper coat, could possibly impede extraction of piperine by thwarting it's accessibility to solvents and would result in poor yield of the same. Therefore, for improved release of oleoresin and piperine from black pepper, hydrolysis of this starch would be necessary. Use of starch degrading enzymes, such as α-amylase (E.C. 3.2.1.1.) for pre-treatment of the pepper matrix prior to extraction would render extraction easy and improve yield of extracts. This has been affirmed by Lee et al. (16), who reported hydrolysis of corn starch by α-amylase and glucoamylase for improved recovery (40%) of reducing sugars.

There are reports on use of other enzymes under SC-CO_2 extraction conditions. Chandran et al. (17) conducted enzyme-assisted hydrodistillation of black pepper and cardamom. They have obtained improved yield of essential oil (0.9−1.8% increase) and its major components (β-caryophyllene markedly increased from 15.0% to 25.6%) by pre-treatment of the sample matrix with a mixture of cellulase, β-glucanase, pectinase and xylanase. SC-CO_2 conditions have also been employed in enzyme-assisted synthesis of dipalmitin from palmitic acid and glycerol by immobilized lipase (18); for enzymatic ring-opening polymerization of Poly (ε-caprolactone) (PCL) using lipase B (19) and in acylation of fibrous cellulose by immobilized lipase, immobilized esterase and immobilized cutinase (20), to state a few. All these authors have reported on batch mode of enzyme-assisted SC-CO_2 extractions. Senyay-Oncel and Yesil-Celiktas (21) reported an increase in activity and stability of fungal α-amylase employing dynamic (continuous) mode of SC-CO_2 conditions.

To the best of our knowledge, there is no report on use of α-amylase for SC-CO_2 extraction of black pepper oleoresin. *Aspergillus oryzae*, *Bacillus amyloliquefaciens*, *B. subtilis* and *B. licheniformis* are known to be commercial sources of α-amylase. Kılıç Apar and Özbek (22) have reported that α-amylase obtained from *B. licheniformis* showed maximum degrees of hydrolysis for corn, rice and wheat starch (40.4%, 48.1% and 58.1%, respectively) compared to that obtained by *Bacillus* species (5.5%, 19.1% and 29.1%, respectively) and *A. oryzae* (0%, 0% and 17.5%, respectively). Hence, *B. licheniformis* has been selected as the source of α-amylase in our studies.

In the present investigation, a combination of α-amylase and SC-CO_2 extraction was employed for single step hydrolysis of black pepper starch and extraction of the oleoresin fraction from the hydrolyzed matrix. The novelty of our study is that it reports for the first time on enzyme-assisted extraction of oleoresin from black pepper by SC-CO_2. Both batch and continuous modes of extraction (discussed later) were employed to enhance the yield of piperine-rich extract possessing good combination of phytochemical properties, such as total phenolic content, reducing power, antioxidant and anti-inflammatory activities. This extract would have promising usage as food and therapeutic supplements.

MATERIALS AND METHODS

Materials Malabar Garbled black pepper was procured from Spices Board, Cochin, India. Standard piperine (97% pure), α-amylase from *B. licheniformis* (lyophilized powder, 500−1500 units/mg protein, 93−100% SDS -PAGE), soluble potato starch, 1,1-diphenyl-2-picrylhydrazyl (DPPH), sodium nitroprusside ($Na_2[Fe(CN)_5NO].2H_2O$), Griess reagent and gallic acid were procured from M/s Sigma, India; Na_2SO_4, NaH_2PO_4, NaCl, NaOH, Na_2CO_3, $CuSO_4.5H_2O$, $K_3Fe(CN)_6$, $FeCl_3$, TCA, Folin-Ciocalteu's phenol reagent (FCR), potassium sodium tartrate tetrahydrate, methanol, ethanol and *n*-hexane were procured from M/s E-Merck, India. 3,5-Dinitrosalicylic acid (DNSA) was purchased from M/s Himedia, India. All chemicals were of AR grade. SPE cartridge (3 mL) and cartridge-holder were purchased from M/s Applied Separations (Allentown, USA).

Characterization of black pepper powder Black pepper berries were ground using an electric mixer grinder (HL 1618, M/s Philips, India) and particle diameters were determined using the sieve analysis method by screening the black pepper powder through a set of standard sieves in a sieve shaker in accordance to the method reported by Bhattacharjee et al. (23). Samples with mean particle diameter (d_p = 0.42 ± 0.02 mm) were subjected to proximate analyses by standard methods in which moisture (Dean and Stark method) (24); protein (Kjeldahl method) (25); fat (26); crude fiber (27); ash (28); carbohydrates (by difference) and total starch (direct acid hydrolysis) (29) were determined.

Extraction of essential oil and oleoresin from black pepper by conventional methods Ground black pepper (100 g, d_p = 0.42 ± 0.02 mm) was subjected to hydrodistillation for 8 h using Clevenger apparatus, in accordance with Politeo et al. (30), who conducted the same for 3 h. The essential oil obtained was dried over anhydrous sodium sulphate for gravimetric estimation.

Solvent extractions of black pepper oleoresin were carried out in Soxhlet apparatus, reflux heating assembly and shake flasks. For Soxhlet extraction, 5 g ground black pepper was extracted with *n*-hexane for 8 h (26). In the reflux method, 10 g ground black pepper and 50 mL ethanol were set for reflux heating for 1 h at 50 ± 2°C in accordance to the method reported by Musenga et al. (31), who conducted the same with 2.5 g pepper powder in 15 mL methanol. The solvent was filtered by Whatman no. 1 filter paper and the residue was re-extracted by the same process. We also conducted extraction using shake flask method in which 10 g ground black pepper was subjected to extraction using 50 mL ethanol at 60°C in an incubator shaker (110 rpm) (M/s Incon, India, model IS 02) for 1 h and for 3 h in separate batches. The extracts collected in all the three methods of solvent extraction were concentrated by rotary vacuum evaporator (M/s Eyela Corp., Japan) at 40−45°C and 0.05 bar Hg and stored in amber colored screw capped vials in an inert atmosphere of nitrogen at 4°C in dark, until further analyses.

SC-CO_2 extraction of black pepper oleoresin For SC-CO_2 extraction, an SPE-ED SFE 2 model of M/s Applied Separations was employed. The system comprises of a modifier pump (Speed MAX P/N 7025) fitted with refrigerated cooling bath to chill the pump head at −2°C. This maintains the required pressure inside the SFE vessel (SS 316) by pumping CO_2 into the extraction vessel placed in the oven module. An air compressor provides compressed air to build pressure inside the extraction vessel. In the static time of extraction, CO_2 was passed through the extraction vessel keeping the outlet valve closed. During the dynamic time, the outlet valve was kept open and at reduced temperature and pressure conditions, the extract precipitated in the collection vial and CO_2 in gaseous form vented out into the atmosphere. At the collection end, a micrometering valve was used to regulate the flow rate of CO_2 in the collection module and the flow rate of gaseous CO_2 was measured by a bubble flow meter under ambient conditions (1 bar and 25 ± 2°C).

Ground black pepper (20 g, d_p = 0.42 ± 0.02 mm) was charged into a 50 mL extraction vessel. Based on the preliminary trial runs, optimization of extraction temperature and pressure for maximum yield of bioactive compounds was carried out using a three-level factorial design. Extraction temperatures (40°C, 50°C and 60°C) and extraction pressures (200 bar, 300 bar and 500 bar) were investigated. The total extraction time (static time + dynamic time) was kept constant at 45 min (static time of 30 min and dynamic time of 15 min, beyond which there was no extract obtained in the collection vial). It was found that the flow rate of CO_2 above 2 L/min resulted in sputtering of the extract in the wall of the collection vial and carryover and entrainment of the same in the outlet tubing leading to loss in extract yields. Therefore, the flow rate of CO_2 was maintained constant at 2 L/min for all experiments. Extracts were collected in screw capped glass vials in an ice bath and stored in amber colored screw capped vials after dissolving in methanol. Based on the yield and phytochemical properties of the extracts, SC-CO_2 conditions were optimized at 60°C and 300 bar and these conditions were maintained in the latter experiments.

SC-CO_2 treatment of α-amylase SC-CO_2 treatment of α-amylase was conducted in our study to investigate whether the specific activity of the treated enzyme showed improved activity. The lyophilized enzyme was subjected to SC-CO_2 conditions inside an SPE cartridge (with cartridge-holder) and the specific activity of the SC-CO_2 treated enzyme was assayed.

The enzyme treatment was conducted in both batch and continuous modes. In the batch mode, both static and dynamic time of extraction were employed while continuous mode was conducted in dynamic time of extraction alone. In batch mode, CO_2 was allowed to flush through the extraction vessel for a certain period of static (equilibration) time during which the outlet valve was kept closed. After sufficient static time, the extract was recovered through the outlet and micrometering valves (discussed above). The inlet valve remained open throughout the batch mode while the outlet valve was opened only during the dynamic time of