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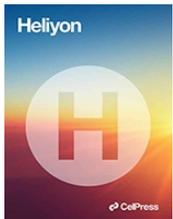
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Oleic acid as a restorative agent in alleviating adrenaline induced altered morphofunctional milieu of astric tissue and mitochondria



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Effect of oil on the performance of biopolymers as drag reducers in fresh water flow

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Particle size, penetration rate and effects of smoke and smokeless tobacco products – an invitro analysis

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Optical study to identify and quantify capsaicin in optical window

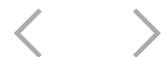
Manuel Abraham López Pacheco, José Javier Báez Rojas, Jorge Castro-Ramos, José Fabian Villa Manríquez, Karen Esmonde-White
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Evaluating the quality of care for patients with type 2 diabetes mellitus based on the HbA1c: A national survey in Iran

Ghobad Moradi, Azad Shokri, Amjad Mohamadi-Bolbanabad, Bushra Zareie, Bakhtiar Piroozi
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Discovery of Clioquinol and analogues as novel inhibitors of Severe Acute Respiratory Syndrome Coronavirus 2 infection, ACE2 and ACE2 - Spike protein interaction *in vitro*

Omonike A. Olaleye, Manvir Kaur, Collins Onyenaka, Tolulope Adebusuyi

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Evaluation of flavonoids as 2019-nCoV cell entry inhibitor through molecular docking and pharmacological analysis

Deep Bhowmik, Rajat Nandi, Amresh Prakash, Diwakar Kumar

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Prevalence of tick-borne haemoparasites and their perceived co-occurrences with viral outbreaks of FMD and LSD and their associated factors

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S.K. Yadav, U. Mehta, D. Adhikari

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Kwesi Hughes-Lartey, Meng Li, Francis E. Botchey, Zhen Qin
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Conditioned medium from mesenchymal stem cells improves condylar resorption induced by mandibular distraction osteogenesis in a rat model

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Influence of poly(*N*-isopropylacrylamide) (PIPAAm) graft density on properties of PIPAAm grafted poly(dimethylsiloxane) surfaces and their stability

Yoshikatsu Akiyama
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Biogenic synthesis and cytotoxic effects of silver nanoparticles mediated by white rot fungi

Gudikandula Krishna, V. Srileka, M.A. Singara Charya, Esraa Samy Abu Serea, Ahmed Esmail Shalan
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Properties of zinc titanates synthesized by microwave assisted hydrothermal method

Leandro Lemos Gonzales, Marlon da Silva Hartwig, Rafael Uarth Fassbender, Eduardo Ceretta Moreira, Marcelo Barbalho Pereira, Pedro Lovato Gomes Jardim, Cristiane Wienke Raubach, Mário Lucio Moreira, Sérgio da Silva Cava
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The role of house dust mite immunotherapy in Indonesian children with chronic rhinosinusitis allergy: A randomized control trial

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Non-toxic doses of modified titanium dioxide nanoparticles (m-TiO₂NPs) in albino CFW mice

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Combination of coumarin and doxorubicin induces drug-resistant acute myeloid leukemia cell death

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Coronavirus pandemic and spirituality in southwest Nigeria: A sociological analysis

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 species diversity, composition, structure and management in agroforestry systems: the case of achabira district, Southern Ethiopia



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Improving dairy performance through molecular characterization of *SREBP-1* gene in Sarda sheep breed

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The sine-Gordon expansion method for higher-dimensional NLEEs and parametric analysis

Purobi Rani Kundu, Md. Rezwan Ahamed Fahim, Md. Ekramul Islam, M. Ali Akbar

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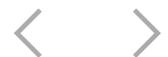
P.W. Anggoro, B. Bawono, J. Jamari, M. Tauviqirrahman, A.P. Bayuseno

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Formulation and evaluation of mucoadhesive buccal tablets of aceclofenac

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aral



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- Does restoration of sagittal cervical alignment improve cervicogenic headache pain and disability: A 2-year pilot randomized controlled trial

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- Adoption of soil and water conservation technology and its effect on the productivity of smallholder rice farmers in Southwest Nigeria

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Quynh Hoa Tran, Van Gio Nguyen, Cong Manh Tran, Minh Nam Nguyen

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- Consumer preference, growth and profitability of African catfish (
- Clarias gariepinus*
-) grown in treated and aerated wastewater fed ponds in Kumasi, Ghana

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model for the study of surface mediated drug delivery systems



Luciana Fernández, Ana Lucía Reviglio, Daniel A. Heredia, Gustavo M. Morales, Marisa Santo, Luis Otero, Fabrisio Alustiza, Ana Cecilia Liaudat, Pablo Bosch, Enrique L. Larghi, Andrea B.J. Bracca, Teodoro S. Kaufman
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Dietary factors associated with being overweight and obese among school-going adolescents in Region One, The Gambia

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Mapping chromosomal regions associated with anther indehiscence with exerted stigmas in CRI-48 and Jasmine 85 cross of rice (*Oryza sativa* L)

Samuel Oppong Abebrese, Nana Kofi Abaka Amoah, Paul Kofi Ayirebi Dartey, Isaac Kofi Bimpong, Richard Akromah, Vernon Edward Gracen, Samuel Kwame Offei, Eric Yirenyki Danquah
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Bambang Tjahjadi, Noorlailie Soewarno, Febriani Mustikaningtiyas

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 Significant lifespan difference between primary open-angle glaucoma and pseudoexfoliation glaucoma

Jon Klokk Slettedal, Leiv Sandvik, Amund Ringvold

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 Prevalence and severity of non-carious cervical lesions and dentin hypersensitivity: association with oral-health related quality of life among Brazilian adults

Anna Rachel dos Santos Soares, Loliza Luiz Figueiredo Houry Chalub, Rayssa Soares Barbosa, Deborah Egg de Paiva Campos, Allyson Nogueira Moreira, Raquel Conceição Ferreira

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 Machine learning approaches for the prediction of soil aggregate stability

Yassine Bouslih, Aicha Rochdi, Namira El Amrani Paaza

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[DNA repair pathway activation features in follicular and papillary thyroid tumors, interrogated using 95 experimental RNA sequencing profiles](#)

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[Variations in isochore thickness and depositional surface of the Dwyka, Ecca and Beaufort Groups in the Western Cape Province of South Africa as deduced from 2.5D gravity profile models](#)

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[Whole body periodic acceleration \(pGz\) improves endotoxin induced cardiomyocyte contractile dysfunction and attenuates the inflammatory response in mice](#)

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Valentina Galvani, Matthew Ackman
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Dmitry Kamashev, Maksim Sorokin, Irina Kochergina, Aleksey Drobyshev, Uliana Vladimirova, Marianna Zolotovskaia, Igor Vorotnikov, Nina Shaban, Mikhail Raevskiy, Denis Kuzmin, Anton Buzdin
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Karina Hernández Santiago, Ana Laura López –López, Fausto Sánchez-Muñoz, José Luis Cortés Altamirano, Alfonso Alfaro-odríguez, Herlinda Bonilla-Jaime

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- Association between carriers of the G allele of the + 45T> G variant of the ADIPOQ gene (rs 2241766) and the cardiometabolic profile in sickle cell trait**

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 Structural analogues of existing anti-viral drugs inhibit SARS-CoV-2 RNA dependent RNA polymerase: A computational hierarchical investigation

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 Sustainable production of *Origanum syriacum* L. using fish effluents improved plant growth, yield, and essential oil composition

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Wildlife roadkill in the Tsavo Ecosystem, Kenya: identifying hotspots, potential drivers, and affected species

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 Integration of transcriptomes analysis with spectral signature of total RNA for generation of affordable remote sensing of Hepatocellular carcinoma in serum clinical specimens

Ibrahim H. Aboughaleb, Marwa Matboli, Sherif M. Shawky, Yasser H. El-Sharkawy
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 Green tea extract increases the quality and reduced DNA mutation of post-thawed Kacang buck sperm

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 Long-term projections of economic growth in the 47 prefectures of Japan: An application of Japan shared socioeconomic pathways

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 Implementation of *in silico* methods to predict common epitopes for vaccine development against Chikungunya and Mayaro viruses

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Physical fitness and cognitive function among school-aged children in selected basic schools in the Ho Municipality of Ghana

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 Quality of life in the COVID-19 outbreak: influence of psychological distress, government strategies, social distancing, and emotional recovery

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 Volunteer public leaders' values-driven leadership: the case of village elders in Kenya

Jacqueline Nthoki Mutua, Timothy Mwangi Kiruhi
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 Deuteration enhances the anti-tumor effects and relative anti-inflammatory effects via affecting proliferation and apoptosis

Ao Li, Xiaojiao Wang, Danni Li, Xiaohong Li, Rou Li, Xuejuan Yang, Xiao Li
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 Valorization of fecal sludge stabilization via vermicomposting in microcosm enriched substrates using organic soils for vermicompost production

Rapheal Nsiah-Gyambibi, Helen Michelle Korkor Essandoh, Nana Yaw Asiedu, Bernard Fei-Baffoe
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[Influence of the characteristics of the house and place of residence in the daily educational activities of children during the period of COVID-19' confinement](#)

María Luisa Zagalaz-Sánchez, Javier Cachón-Zagalaz, Víctor Arufe-Giráldez, Alberto Sanmiguel-Rodríguez, Gabriel González-Valero

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 [Geographical variations and correlation among some chemical and thermal properties of Almaciga \(*Agathis philippinensis* Warb.\) resins from selected commercial sites in the Philippines](#)

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 [Uptake and accumulation of Cr in edible parts of *Eruca sativa* from irrigation water. Effects on polyphenol profile and antioxidant capacity](#)

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 [Detection and genetic characterization of African swine fever virus \(ASFV\) in clinically infected pigs in two districts in South Kivu province, Democratic Republic Congo](#)

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Factors associated with home births in Peru 2015–2017: A cross-sectional population-based study

Akram Hernández-Vásquez, Horacio Chacón-Torrico, Rodrigo Vargas-Fernández, Guido Bendezu-Quispe

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Quality of life of working and non-working Jordanian mothers caring for chronically ill child and its associated factors

Huda F. Gharaibeh, Muntaha K. Gharaibeh

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Depressive symptoms associated with loneliness and physical activities among graduate university students in Bangladesh: findings from a cross-sectional pilot study

Satyajit Kundu, Jhantu Bakchi, Md. Hasan Al Banna, Abu Sayeed, M. Tasdik Hasan, Mohammad Tazrian Abid, Subarna Ghosh, Nobonita Sarker, Md Shafiqul Islam Khan

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Fungal and metabolome diversity of the rhizosphere and endosphere of *Phragmites australis* in an AMD-polluted environment

Chimdi Mang Kalu, Henry Joseph Oduor Ogola, Ramganesch Selvarajan, Memory Tekere, Khayaletu Ntushelo

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Perinaphthenone and derivatives as control agents of phytopathogenic fungi: fungitoxicity and metabolism



Alisa M. Castaño, Andrés F. Gómez, Jesús Gil, Diego Durango



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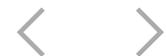
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Research article

Isocratic high-performance liquid chromatography (HPLC) for simultaneous quantification of curcumin and piperine in a microparticle formulation containing *Curcuma longa* and *Piper nigrum*Dewi Setyaningsih^{a,*}, Yosua Agung Santoso^a, Yustina Sri Hartini^a, Yosi Bayu Murti^b, Wouter L.J. Hinrichs^c, Christine Patramurti^a^a Faculty of Pharmacy, Sanata Dharma University, Indonesia^b Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia^c Department of Pharmaceutical Technology and Bio-pharmacy, University of Groningen, the Netherlands

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ABSTRACT

Poor bioavailability has been reported as a major challenge in the development of curcumin as a pharmaceutical agent. However, co-administration of curcumin with piperine has been shown to improve curcumin bioavailability. Therefore, to assure product control quality, an analytical method needs to be developed for the determination of curcumin and piperine content in a dosage form formulation. The objective of this study was to develop a simple isocratic reversed-phase HPLC (RP-HPLC) method to simultaneously quantify curcumin and piperine content in solid dispersion based microparticle formulation containing *Curcuma longa* and *Piper nigrum* extracts. The method was validated according to the International Council for Harmonization (ICH) guideline. Chromatographic separation of three curcuminoids and piperine could be achieved using acetonitrile-methanol-water of 65:5:35 %, at a flow rate of 1 mL/min and a wavelength of 353 nm for detection. Resolution (Rs) of 3.57 and 1.68 for piperine and curcumin, respectively, a theoretical plate number (N) > 8000 and a tailing factor (T) < 1.5 indicate a satisfactory separation of the compounds. The calibration curve was linear from 1.25–15 µg/mL and 2.5–30 µg/mL for piperine and curcumin, respectively, with the correlation coefficient of >0.999. The intra-day/inter-day accuracy and precision demonstrated a recovery of 99.54–101.50%/99.38–99.89% and 100.78–102.51%/101.15–102.47% with a Relative Standard Deviation (RSD) of 0.53–0.95%/0.13–1.44 % and 0.28–1.62%/0.46–1.14% for piperine/curcumin. The limit of detection (LOD) were 0.27 and 0.42 µg/mL, for piperine and curcumin, which reveals an adequate sensitivity. A solid dispersion based microparticle formulation containing *C. longa* and *P. nigrum* extracts confirmed the validity of the developed method as a recovery of 91.14% and 99.14% for piperine and curcumin, respectively. In conclusion, all the tested parameters confirm the precision, accuracy, and reliability of the method for the simultaneous analysis of curcumin and piperine within a microparticle formulation containing *C. longa* and *P. nigrum* extracts.

1. Introduction

Curcumin, a yellow polyphenolic compound isolated from turmeric (*Curcuma longa*) rhizome, has been claimed as a therapeutic agent for numerous diseases due to its anti-oxidant and anti-inflammatory properties [1, 2, 3]. However, despite extensive *in-vitro* and *in-vivo* studies, a clear therapeutic effect of curcumin in clinical studies has not been found yet [4]. These disappointing results can be ascribed to the poor bioavailability of the compound after oral intake. There are two reasons for the poor absorption of curcumin; 1) It exhibits a very low aqueous

solubility of only 11 ng/mL [4, 5], and 2) It is rapidly metabolized [1]. Therefore, a combination of two strategies addressing poor solubility and rapid metabolism is required.

An often-applied strategy to improve the dissolution behavior of a poorly water-soluble drug is formulating it as a solid dispersion [6]. Furthermore, piperine (Figure 1d), an alkaloid compound of black pepper (*P. nigrum*) extract has been identified as a bio-enhancer of various drugs, by stimulating gut amino acid transporters and inhibiting drug-metabolizing enzymes to improve drug absorption, bioavailability, and bio-efficacy [7]. Both strategies are thought to be useful to be applied

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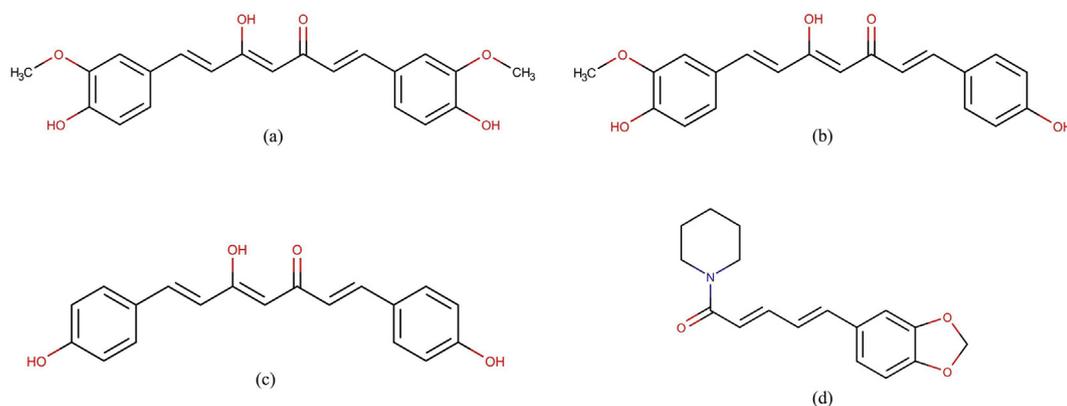


Figure 1. Molecular structure of (a) curcumin; (b) demethoxycurcumin; (c) bis-demethoxycurcumin; (d) piperine.

to curcumin. Hence, we propose a formulation based on solid dispersion technology containing *C. longa* and *P. nigrum* extracts to improve the bioavailability of curcumin. In this study, we will focus on solid dispersion microparticles prepared by spray drying using polyvinylpyrrolidone K30 (PVP K30) as a carrier.

During pre-formulation, the quality control of the product needs to be addressed. Determining compounds in the final product is a part of quality assurance in production processes. Therefore, the amount of curcumin and piperine containing in the microparticle needs to be determined accurately.

Several analytical methods for the quantification of curcumin and piperine have been developed, however, the majority of these methods concern the quantification of curcumin and piperine individually [8, 9, 10, 11]. A few methods have been developed to simultaneously determine curcumin and piperine. A spectroscopic method combining with the application of Vierordt's equation was successfully applied to allow the determination of curcumin and piperine as a mixture of *C. longa* and *P. nigrum* extracts in dissolution medium [12], however, the method is not selective.

HPLC with UV-Vis detection is one of the most common techniques used in the analysis and quality control of several formulations containing curcumin or piperine and in biological samples. Moorthi *et al.* validated an isocratic HPLC method to allow curcumin and piperine quantification in a Eudragit based nanosuspension containing pure curcumin and piperine as a binary mixture [13]. Furthermore, Sethi *et al.* reported a selective and specific HPLC method for simultaneous estimation of curcumin and piperine in human plasma [14]. Also, a sensitive LC/MS method was developed to enable simultaneous quantification of curcumin and piperine for pharmacokinetic evaluation [15]. Although the HPLC methods have been reported to be useful to simultaneously analyze curcumin and piperine in various samples, the developed methods were applied to determine curcumin and piperine as pure compounds in the samples.

To our best knowledge, no HPLC analytical method has been reported for the simultaneous analysis of curcumin and piperine in extracts. It should be emphasized that commercially available curcumin originating from turmeric rhizome is usually composed of a mixture of naturally occurring curcuminoids, namely curcumin, demethoxycurcumin, and bis-demethoxycurcumin (Figure 1a, b, c). This mixture of compounds could easily interfere with chromatographic separation if not properly resolved. Therefore, the study aimed to develop a validated HPLC method to simultaneously quantify curcumin and piperine in solid dispersion-based microparticle formulation containing *C. longa* and *P. nigrum* extracts.

2. Materials and methods

2.1. Materials

Bis-demethoxycurcumin, demethoxycurcumin, curcumin, and piperine reference standards with a purity of >98% were purchased from

Sigma-Aldrich (St. Louis, USA). *C. longa* extract of high curcuminoids content (97.56%) was a kind gift from PT Phytochemindo Reksa, Bogor, Indonesia. *P. nigrum* was extracted and purified as described by Saha *et al.* [16]. Polyvinylpyrrolidone K30 (PVP K30) was kindly given by PT Konimex, Solo, Central Java, Indonesia. Ethanol, HPLC grade acetonitrile, HPLC grade methanol, and analytical grade phosphoric acid were purchased from Merck (Darmstadt, Germany). A PTFE filter with a pore size of 0.22 μm was obtained from Whatman. Milli-Q water was prepared in the laboratory.

2.2. Preparation of solid dispersion based microparticle formulation and characterization

A PVP-30 based solid dispersion containing 30 w/w % *C. longa* extract and 10 w/w % *P. nigrum* extract was prepared by spray drying. In brief, 1800 mg *C. longa* extract and 600 mg *P. nigrum* extract were dissolved in 400 mL ethanol and 3600 mg of PVP K30 was dissolved in 50 mL ethanol. The two solutions were mixed for approximately 15 min using a magnetic stirrer.

The resulting solution was fed into a BUCHI B-290 mini spray dryer equipped with a B-295 dehumidifier via a two-way channel with a nozzle diameter of 0.7 mm. Drying was conducted at an inlet temperature of 105 $^{\circ}\text{C}$, aspiration of 100%, feeding rate of 20%, and an atomization pressure of 500 L/h. The outlet temperature was observed at 60–65 $^{\circ}\text{C}$. Micrographs of the microparticles were taken using a scanning electron microscope (Hitachi TM 3000, Japan).

2.3. Method development

2.3.1. Finding the detection wavelength

Individual methanolic solutions of curcumin (15 $\mu\text{g}/\text{mL}$) and piperine (5 $\mu\text{g}/\text{mL}$) were scanned at the range of 315–450 nm using a UV-Vis spectrophotometer (Shimadzu, Japan). The detection wavelength for the simultaneous analysis of curcumin and piperine was selected based on the crossing point of the curcumin and piperine spectra of the spectral overlay.

2.3.2. HPLC instrument and conditions

HPLC analysis was carried out using a Shimadzu LC 2010HT HPLC system (Shimadzu, Kyoto, Japan) equipped with a serial dual plunger pump, an autosampler, and an SPD-20A/20AV series UV-Vis detectors. LC-solution software was used for peak integration. Chromatographic separation was achieved on a C18 column (250 \times 4.6 mm, Eurospher 100 with 5 μm) equipped with a pre-column (Knauer, Berlin, Germany). The mobile phase was filtered through a 0.22 μm filter and degassed by ultrasonication before use. The injection volume was 20 μL . The column temperature was kept at 33 $^{\circ}\text{C}$ during chromatographic operation.

2.3.3. Selection of mobile phase

The aim was to find a mobile phase composition, which provides an acceptable peak separation of multiple components i.e. curcuminoids

(bis-demethoxycurcumin, demethoxycurcumin, curcumin) and piperine with a short running time of less than 10 min. A sample consisting of a mixture of 10 µg/mL *C. longa* and 5 µg/mL *P. nigrum* extracts in methanolic solution was used to evaluate mobile phases of different compositions.

Peak identification was carried out by running standard solutions at accordingly used mobile phase compositions. The mobile phase compositions were evaluated on the chromatographic separation parameters i.e. tailing factor (T), peak resolution (Rs), and theoretical plate number (N). The acceptance criteria are $T < 2$, $R_s > 1.5$ and $N > 2000$ [17].

2.3.4. Preparation of stock and calibration solutions

Stocks of standard solution of curcumin and piperine at a final concentration of 1 mg/mL were prepared by dissolving an appropriate amount of curcumin and piperine in methanol as an individual solution. All solutions were prepared in light-protected vials to ensure the stability of curcumin and piperine [18].

2.4. Method validation

The developed method was validated as per Q2R1 ICH guidelines [19] including selectivity, system suitability, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), linearity, and robustness.

2.4.1. System suitability

Six replicate injections of the sample containing a mixture of curcumin and piperine at a concentration of 10 and 5 µg/mL in the methanolic solution were simultaneously analysed. Retention time (Tr), peak area (AUC), Rs, T, and N were determined. Relative standard deviation (RSD) values of these parameters were calculated to evaluate the system suitability of the developed method. The suitability test was accepted when the RSD values of these parameters were less than 2%.

2.4.2. Specificity and selectivity

Specificity refers to the ability of a method to distinguish between the compound(s) of interest and impurity present in the sample matrix [20]. For the specificity study, the chromatogram of blank samples originated from the formulation matrix (no drugs) was compared to the chromatogram of microparticle formulation containing *C. longa* and *P. nigrum* extracts. Selectivity is the ability of the method to clearly show separation between the different peaks. A value of $R_s > 1.5$ is required to confirm the method to be sufficiently selective [20].

2.4.3. Preparation of calibration solutions of curcumin and piperine and linearity

Calibration solutions were prepared using solutions of curcumin and piperine in methanol at different concentrations. Stock solutions of curcumin and piperine were diluted with methanol and mixed to obtain samples with curcumin concentrations of 0.05–45 µg/mL and piperine concentrations of 0.025–20 µg/mL for. Linearity between concentration and peak area was tested using the regression line as provided by the least square analyses. The correlation coefficient (r) was used to judge linearity [21]. The concentration range with an $r > 0.99$ was considered linear. The limit of detection (LOD) and the limit of quantification (LOQ) were determined using the standard deviation approach [20]. To do so, serially diluted concentrations of the calibration solutions were prepared at nearly targeted LOD or LOQ and analyzed using least square analysis. The standard deviation (SD) of the y-intercept and slope were determined with which the LOD and LOQ were calculated based on Eq. (1) and Eq. (2) below [22].

$$LOD = 3.3 \times SD/slope \quad (1)$$

$$LOQ = 10 \times SD/slope \quad (2)$$

2.4.4. Accuracy and precision

Accuracy refers to the closeness of the obtained value with that true value of the corresponding sample concentrations, while precision denotes the closeness agreement between independent test results obtained under the developed method. In this study, accuracy and precision were carried out using the assay sample spiking method. The assay sample was the microparticle formulation as described in section 2.2; dissolved in methanol. The methanolic sample solutions at the concentration of 9.9 µg/mL (sample weight/volume) were spiked with curcumin and piperine stock solutions to result in a mixture of final addition concentrations of 2.50; 15.00; 30.00 and 1.25; 7.50; 15.00 µg/m for curcumin and piperine.

Accuracy was evaluated based on the recovery percentage, and precision was studied using repeatability assay based on the RSD values of three different sample concentrations. Accuracy and precision were conducted on one-day analysis (intra-day) and three consecutive days (inter-day) of freshly prepared samples. The analysis was done in triplicate for each concentration level. The degree of accuracy and precision were judged according to the Association of Official Analytical Chemists requirement [21].

2.4.5. Robustness

The robustness of a developed analytical method refers to its ability to remain unaffected by deliberate changes of the chromatographic conditions which indicates its reliability during normal usage. Peaks retention time and AUC values of curcumin and piperine resulted from the changed parameters of mobile phase composition, flow rates, and detection wavelengths were analyzed for the RSD values. The RSD value of less than 2% is an indication of a robust method.

2.4.6. Assay of curcumin and piperine in the microparticle formulation

A 200 mg of the microparticle formulation was accurately weighed and dissolved in 50 mL of methanol in a volumetric flask. The solution was subjected to ultrasonication for 5 min. The solution was diluted 100 times with methanol to achieve a final concentration of 40 µg/mL and then analyzed by HPLC. The content of curcumin and piperine was calculated based on the calibration curve constructed as described in section 2.4.3.

2.5. Data analysis

A Least Square Analysis supported by Excel Microsoft INC USA was used to analyze the linearity of the calibration curve. Mean and standard deviation was calculated using Excel Software (Microsoft Inc, USA).

3. Results and discussion

3.1. Preparation of solid dispersion based microparticle formulation and characterization

Microparticles of PVP-30 based solid dispersion containing curcuminoids of *C. longa* and piperine of *P. nigrum* extracts could be successfully prepared by spray drying as can be seen in the scanning electron microscopic images (see example in Figure 2).

3.2. Finding the detection wavelength

The spectral scanning of a single methanolic solution of curcumin and piperine is shown in Figure 3. As shown in Figure 3, the methanolic solution of curcumin (15 µg/mL) absorbed wavelength at the maximum value of 425 nm, while piperine dissolved in methanol at the concentration of 5 µg/mL showed a maximum absorption at the wavelength of 342 nm. Both compounds exhibited an overlapping absorption area at around 315–360 nm, where curcumin and piperine can be simultaneously detected.

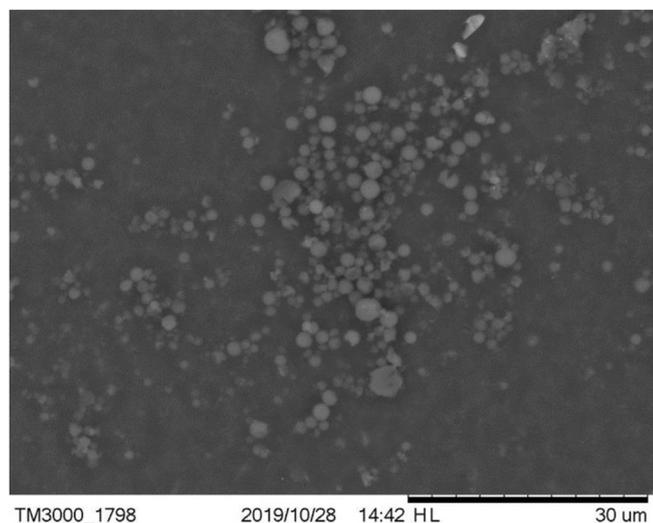


Figure 2. Scanning electron microscopic image of the spray dried PVP K30 based solid dispersion containing curcuminoids and piperine.

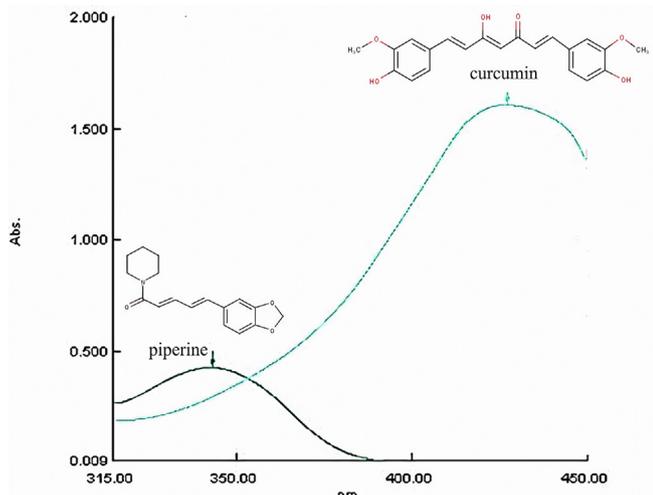


Figure 3. The overlay spectra of curcumin (15 µg/ml) and piperine (5 µg/ml) in methanolic solution.

As can be seen in [Figure 3](#), the two spectra cross each other at 353 nm. Therefore, this wavelength was used for the simultaneous detection of curcumin and piperine in this study.

3.3. Selection of mobile phase

3.3.1. The use of methanol and acetonitrile

Simultaneous analysis of a mixture contains weak acids and weak basics drugs on an RP HPLC is always challenging [23]. The target analytes in this study are curcumin (weak acid) and piperine (weak basic) which are present as co-mixture with other curcuminoids compounds (*bis*-demethoxycurcumin, demethoxycurcumin) as the active components in the microparticle formulation. The combinations methanol-water and acetonitrile-water are the most commonly used solvent mixtures in RP HPLC analysis. However, curcuminoids are poorly resolved in methanol-water mixtures at a volume ratio of 50:50 and 60:40 with calculated polarity index of 7.55 and 7.06. Methanol was therefore replaced by acetonitrile. Although with an acetonitrile-water volume ratio of 60:40 (polarity index of 7.48) a sharp peak was obtained, all three curcuminoids were co-eluted at a retention time of 12.54 min.

3.3.2. The effect of 0.1% phosphoric acid as pH modifier

A study by Espinosa et al. revealed that the retention time of acidic and basic compounds is strongly dependent on the pH of the mobile phase [24]. The addition of acid into the mobile phase can improve peak separation and reduce peak tailing by modification of interaction between the target analyte and the stationary phase of the HPLC column [25, 26].

Therefore, a mobile phase composition as reported by Sethi et al., which was used to simultaneously analyze curcumin and piperine in the plasma sample [14], with slight modifications was evaluated. The mobile phase consisted of acetonitrile-methanol-water-phosphoric acid 0.1% at a volume ratio of 20:30:47.5:2.5 (pH 4.0) and was delivered at 1 mL/min in an isocratic mode. However, under these conditions, the three curcuminoids compounds were co-eluted at a retention time of 11.80 min, whereas no piperine peak appeared within a running time of 15 min, while a maximum running time of 10 min was aimed for. To evaluate the behavior of the weakly basic compound (piperine) at a lower pH, the composition of the mobile phase was adjusted to acetonitrile-methanol-phosphoric acid 0.1 % at a volume ratio of 20:30:50 (pH 3.0) and a calculated polarity index of 7.79. However, a peak in the chromatogram that can be assigned to piperine remained absent during a run time of 15 min. Remarkably, when the mobile phase polarity was slightly modified by replacing the methanol with acetonitrile to result in a calculated polarity index of 8.00 of the mobile phase compositions of acetonitrile-water-phosphoric acid 0.1% at a volume of 50:47.5:2.5 (pH 4.0), piperine was eluted already at 0.516 min.

LoBrutto et al. reported the effect of pH on the retention time of small basic compounds, aniline, and pyridine on an isocratic HPLC mode. At the pH range of 1.3–8.6 of the mobile phase with a composition of acetonitrile-water at a volume ratio of 10:90, it was reported that as the pH of the mobile phase decreased, the retention time of the targeted compounds increased due to counterion interactions resulted from acidic mobile

Table 1. Mobile phase compositions and the observed peak characteristics.

Code	ACN	MeOH	water	Curcuminoids									Piperine		
				BDMC			DMC			C			Tr	Rs	T
				Tr	Rs	T	Tr	Rs	T	Tr	Rs	T			
1	30	25	45	-	-	-	6.30	4.66	0	6.63	0.76	0	-	-	-
2	40	30	30	8.09	1.55	0	8.25	1.08	0	8.99	1.14	0	10.72	5.34	1.17
3	30	50	20	4.26	5.61	1.11	4.86	0	0	5.35	0	0.91	8.77	3.66	1.11
4	60	10	30	5.03	6.11	1.06	5.38	1.56	1.20	5.78	1.64	1.18	7.84	3.22	1.08
5	65	5	30	4.82	4.74	0.95	5.18	1.48	1.12	5.58	1.68	1.16	7.91	3.57	1.06

Note:

1. ACN = acetonitrile; MeOH = methanol.

2. BDMC = *bis*-demethoxycurcumin; DMC = demethoxycurcumin; C = curcumin.

4. Tr = retention time; Rs = resolution; T = tailing factor.

5. - = not detected.

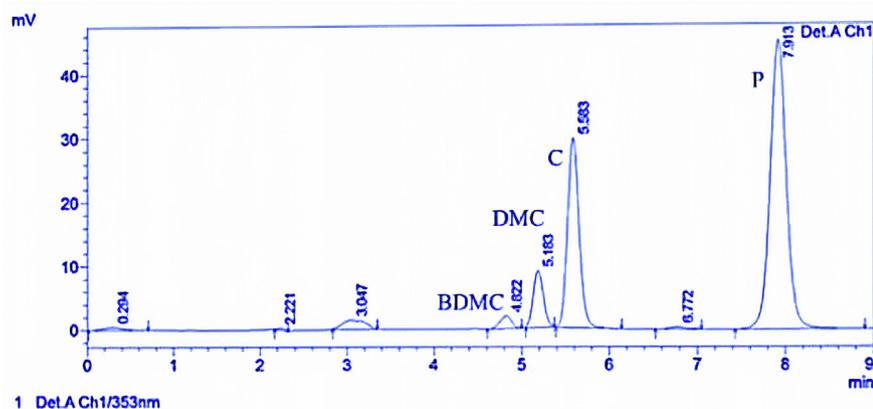


Figure 4. Chromatographic separation of bis-demethoxycurcumin (BDMC), demethoxycurcumin (DMC), curcumin (C) and piperine (P) after injection of 20 μL of the sample containing 10 $\mu\text{g}/\text{mL}$ *C. longa* and 5 $\mu\text{g}/\text{mL}$ *P. nigrum* extracts. Separation was carried out using a Knauer C18 column (250 \times 4.6 mm, Eurospher 100 with 5 μm) equipped with a pre-column and an isocratic mobile phase composed of an acetonitrile-methanol-water mixture with a volume ratio of 65:5:30 at flow rate of 1 mL/min and a detection wavelength of 353 nm.

phase modifier [27]. Guan and Palmer studied the effect of a chaotropic agent trifluoroacetic acid on the retention of four weakly basic derivatives of triazole with different pKa values [28]. Using mixtures of water and acetonitrile as the mobile phase in the gradient mode, it was found that the lower the degree of protonation, the shorter the retention time. As phosphoric acid is also categorized as a chaotropic agent [29], it is presumable that the absence of piperine during 15 min running in this study using the mobile phase composition of acetonitrile-methanol-water-phosphoric acid 0.1% at a volume ratio of 20:30:47.5:2.5 (pH 4.0) and acetonitrile-methanol-phosphoric acid 0.1% at 20:30:50 (pH 3.0) might be attributed to the effect of chaotropic effect resulting partially protonated piperine, making piperine molecules interact with the stationary phase. However, when methanol was replaced by acetonitrile, a solvent with higher elution strength than methanol at the mobile phase composition of acetonitrile-water-phosphoric acid 0.1% of 50:47.5:2.5 (pH 4.0), the previous interaction of piperine with the stationary phase might be changed, in which piperine might be more solvated in the mobile phase. As a result, piperine was eluted at an early retention time of 0.516 min.

Based on the observation that the exchange of methanol by acetonitrile in the mobile phase and the presence of phosphoric acid had a dramatic effect on the retention time of piperine, various acetonitrile-methanol-water ratios without phosphoric acid were evaluated. In these experiments, phosphoric acid was not included in the mobile phase as acids can substantially reduce the life-time of the column. The effects of the mobile phase composition on peak retention (T_r), peak resolution (R_s), and peak tailing factor (T) are shown in Table 1.

When scrutinizing this table, it can be concluded that the optimal mobile phase composition consisted of an acetonitrile-methanol-water mixture with a volume ratio of 65:5:30, which corresponds to the calculated polarity index of 7.08. Figure 4 shows the resulting baseline chromatographic separation of bis-demethoxycurcumin, demethoxycurcumin, curcumin, and piperine of the methanolic sample containing 10 $\mu\text{g}/\text{mL}$ *C. longa* and 5 $\mu\text{g}/\text{mL}$ *P. nigrum* extracts. Curcumin was eluted at 5.58 min with an R_s of 1.68 and a T of 1.16; piperine was eluted at 7.91

min with an R_s of 3.57 and T of 1.06. The obtained values of R_s , T, N of curcumin and piperine meet the requirements of qualified peak in HPLC ($T < 1.5$, $R_s > 1.5$ and $N > 2000$ [17,29]).

3.4. Specificity and selectivity

The method specificity is represented in Figure 5. There are no peaks observed at the retention time of curcumin and piperine in the blank sample (Figure 5a). The retention time for curcumin and piperine in the microparticle formulation sample (Figure 5c) is following the standard solution of curcumin and piperine (Figure 5b). The result indicates the method specificity. The method selectivity is also demonstrated by the baseline separation parameters obtained for curcumin and piperine at which the R_s values for curcumin and piperine are larger than 1.5 (Figure 5c). The curcumin peak was distinguishable from the demethoxycurcumin peak with the R_s of 1.6 and T of 1.1. These results confirm the selectivity of the developed method.

3.5. System suitability test

The optimized HPLC method was subjected to a system suitability test. A sample was injected six times in the system and the T, N, AUC, T_r , and R_s were determined. As shown in Table 2, the RSD values of all these parameters for curcumin as well as piperine were below 2% which indicates all parameters of the proposed HPLC method satisfy the USP and ICH standards. Therefore, the developed HPLC method is concluded to be suitable and effective for the analysis [17].

3.6. Linearity, range and sensitivity

Calibration curves were acquired by plotting peak area as a function of the corresponding concentration (Figure 6). Table 3 shows the regression parameters resulted from the least-square method. As shown in Figure 6 and Table 3, linearity was confirmed at concentration ranges

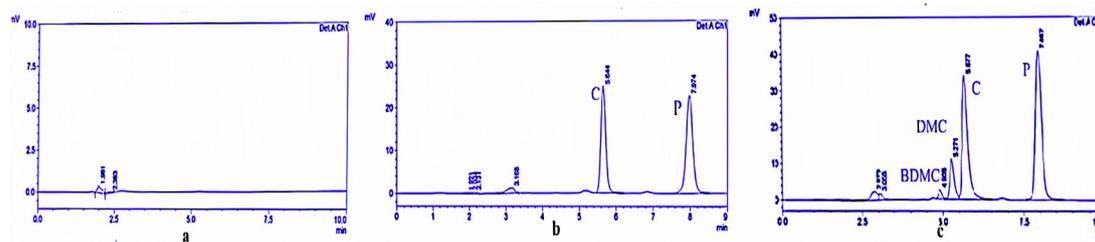


Figure 5. Chromatographic separation for specificity and selectivity assessment. (a) blank; (b) standard solution containing curcumin (10 $\mu\text{g}/\text{mL}$) and piperine (5 $\mu\text{g}/\text{mL}$); (c) solid dispersion based microparticle (9.9 $\mu\text{g}/\text{mL}$ sample) containing *C. longa* and *P. nigrum*. C = curcumin, P = piperine, BDMC = bis-demethoxycurcumin, DMC = demethoxycurcumin.

Table 2. System suitability test of the developed method.

Injection	Tr (min)		AUC		T		Rs		N	
	C	P	C	P	C	P	C	P	C	P
1	5.421	7.660	226134	283307	1.205	1.065	1.624	3.574	8167.5	9561.8
2	5.588	7.914	229048	284215	1.238	1.071	1.649	3.576	8283.6	9356.3
3	5.642	7.970	230929	284790	1.215	1.072	1.680	3.578	8144.9	9243.9
4	5.652	7.994	231654	285454	1.205	1.098	1.690	3.576	8198.7	9409.2
5	5.644	7.974	231274	284831	1.197	1.097	1.688	3.577	8300.8	9474.6
6	5.636	7.977	231993	285380	1.202	1.094	1.690	3.575	8145.6	9353.2
Mean	5.597	7.915	230172	284662	1.210	1.083	1.670	3.576	8206.8	939987
SD	0.089	0.128	2230	803	0.015	0.015	0.027	0.001	69.2	109.8
RSD (%)	1.595	1.614	0.969	0.282	1.221	1.389	1.646	0.040	0.84	1.17

C = Curcumin; P=Piperine; Tr = retention time; Rs = resolution; T = tailing factor; N = theoretical plate number; AUC = Area Under Curve.

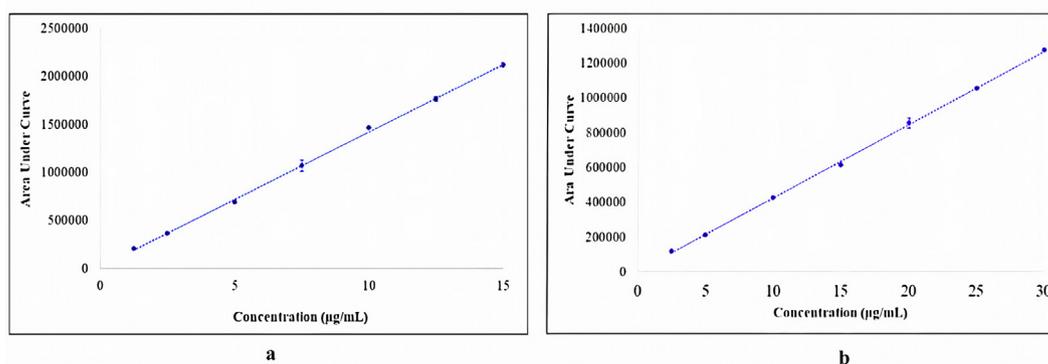


Figure 6. Calibration curves of piperine (a) and curcumin (b) standard following the method developed in this study ($n = 3$). The series concentrations of calibration samples were 1.25; 2.50; 5.00; 7.50; 10.00; 12.50; 15.00 $\mu\text{g/mL}$ for piperine and 2.50; 5.00; 10.00; 15.00; 20.00; 25.00; 30.00 $\mu\text{g/mL}$ for curcumin.

of 1.25–15.00 $\mu\text{g/mL}$ and 2.00–30.00 $\mu\text{g/mL}$ for piperine and curcumin, respectively, as the correlation coefficient, r , within these ranges were higher than 0.999. Also, linearity can be further evaluated by calculation of the RSD% of the slope values, which did not exceed 2.0 % [30].

Method sensitivity was tested by determining LOD and LOQ. The calculated LOD of piperine and curcumin was 0.27 $\mu\text{g/mL}$ and 0.42 $\mu\text{g/mL}$, respectively, and the calculated LOQ was 0.91 $\mu\text{g/mL}$ and 1.41 $\mu\text{g/mL}$ for piperine and curcumin, respectively.

3.7. Accuracy and precision

Accuracy and precision studies were conducted according to the ICH recommendations [19] on three different levels of sample concentrations which are low, medium, and high of analyte concentration. The three concentration levels used in this study were 1.25; 7.50; 15.00 and 2.50; 15.00; 30.00 for piperine and curcumin, respectively.

Table 4 summarizes the accuracy and precision data. The results show the intra-day and inter-day recovery of piperine were between 99.54–101.50% and 99.38–99.89%, respectively while the intra-day and inter-day recovery of curcumin reported were between 100.78–102.51% and 101.15–102.47%, respectively. Repeatability and intermediate precision of the developed method were evaluated by calculating RSD obtained in one day and 3 different days on freshly prepared samples. As compiled in Table 4, it is shown that intra-day and inter-day RSD of piperine were 0.53–0.95% and 0.13–1.44%, respectively; intra-day and inter-day RSD of curcumin were 0.28–1.621% and 0.46–1.14%, respectively.

According to calculated recovery and RSD values, these reported data (Table 4) show that the recovery and RSD values fully satisfy within the generally accepted range of AOAC (90–107% recovery and <5.3% of

RSD) [21], which suggests a high level of accuracy and precision of the developed method.

3.8. Robustness

The robustness parameters of the developed method were obtained by investigating the impact of slight changes in the chromatographic conditions on peak retention time and peak area. Moderate changes in volume ratios of mobile phase composition ($\pm 2\%$), flow rate (± 0.1 mL/min), and detection wavelength (± 2 nm), however, did not significantly affect the retention time and peak area as indicated by the RSD value of <2% (see Table 5).

3.9. Application of the developed method

The developed RP-HPLC method was applied to simultaneously determine the concentration of curcumin and piperine in the microparticle formulation sample containing multicomponent appears in *C. longa* and *P. nigrum* extracts. The developed method was successfully validated to separate multi-compounds in the microparticle formulation i.e. curcumin from its curcuminoids family, and piperine (Table 6). As shown in Table 6, the observed content of curcumin and piperine in the microparticles was 22.42 ± 0.67 w/w % and 9.04 ± 0.67 w/w %, respectively. Using reference standards, it was found with the developed HPLC method that *C. longa* extract contained 75.38 w/w % curcumin and *P. nigrum* extract contained 98.97 w/w % piperine. As the claimed percentages of *C. longa* and *P. nigrum* extracts in the microparticles were 30 w/w % and 10 w/w %, respectively, it can be calculated that the theoretical amounts of curcumin and piperine in the microparticles should be 22.61 w/w %

Table 3. Validation parameters for piperine and curcumin (n = 3).

Parameter	Piperine	Curcumin
Linearity range (µg/mL)	1.25–15.00	2.50–30.00
Linear equation	Y = 140779x + 11549	Y = 42144x + 1559.5
Correlation coefficient of (r)	0.9990	0.9994
RSD of slope	1.017	0.784
Limit of Detection (µg/mL)	0.27	0.42
Limit of Quantification (µg/mL)	0.91	1.41

Table 4. Results of intra-day and inter-day accuracy and precision n = 3.

Compound	Final concentration (µg/ml)	Intra-day			Inter-day		
		Recovered quantity (µg/ml)	Recovery (%)	RSD (%)	Recovered quantity (µg/ml)	Recovery (%)	RSD (%)
Curcumin	2.50	2.54 ± 0.04	101.49	1.47	2.61 ± 0.02	102.47	0.79
	15.00	15.12 ± 0.25	100.78	1.62	15.28 ± 0.17	101.88	1.14
	30.00	30.75 ± 0.09	102.51	0.28	31.39 ± 0.14	101.15	0.46
Piperine	1.25	1.27 ± 0.01	101.50	0.95	1.25 ± 0.01	99.79	1.08
	7.50	7.47 ± 0.07	99.54	0.94	7.45 ± 0.01	99.38	0.13
	15.00	15.21 ± 0.08	101.38	0.53	14.98 ± 0.22	99.89	1.44

Table 5. Robustness test of the developed method (n = 3).

Condition	Curcumin				Piperine			
	AUC		Tr (min)		AUC		Tr (min)	
	mean	RSD (%)						
Selected mobile phase composition is acetonitrile: methanol: water = 65:5:30 vol-%								
60:10:30 vol-%	21273.6	0.72	5.58	0.10	25209.7	0.308	7.84	0.09
70:5:25 vol-%	22796.7	0.54	5.66	0.10	25358.3	0.500	7.95	0.49
Flow rate of 1 mL/min								
0.9 mL/min	21320.7	0.32	5.49	0.02	25296.3	0.955	7.83	0.05
1.1 mL/min	21255.7	0.83	5.82	0.43	25164.6	0.501	7.99	0.12
Selected detection wavelength of 353 nm								
351 nm	21120.3	1.15	5.61	0.16	325481.6	0.329	7.85	0.63
355 nm	23781.4	0.45	5.63	0.07	252137.6	0.264	7.92	0.17

Tr = retention time; AUC = Area Under Curve.

and 9.90 w/w %, respectively. Using these percentages, it can be calculated that the recovery of curcumin and piperine from the microparticles was 99.14% and 91.31%, respectively, using the developed HPLC method (Table 6).

USP monograph on curcuminoids dosage form requires that the content of the active compound in the dosage form products should be

Table 6. Curcumin and piperine content in microparticles.

Sample	Amount found in microparticles	
	Curcumin (w/w %)	Piperine (w/w %)
1	21.17	8.41
2	22.90	8.39
3	22.58	8.55
4	22.90	9.98
5	22.82	9.40
6	22.17	9.50
Mean	22.42	9.04
SD	0.67	0.67
RSD (%)	3.05	7.47

Formulation claim: 30 w/w % *C. longa* and 10 w/w % *P. nigrum* extracts.

between 90.0% and 110.0% [31]. Thus, the curcumin and piperine content found in the solid dispersion-based microparticle formulation using the developed method complies with the product specification of the USP monography. The results from the assay of curcumin and piperine confirm the successful implementation of the proposed HPLC method for the simultaneous quantification of curcumin and piperine in microparticles formulation containing multicomponent i.e. curcumin and piperine of *C. longa* and *P. nigrum* extracts. Furthermore, as the running time of HPLC analysis is only 10 min, the proposed method is suitable to

be applied during routine analysis as part of quality control procedures in the formulation of the microparticles.

4. Conclusion

A suitable, rapid, accurate, and precise RP-HPLC method was developed for the simultaneous determination of curcumin and piperine in a solid dispersion-based microparticle formulation. The developed method offers a linear response across a wide range of analyte concentrations with satisfactory method sensitivity. Furthermore, the proposed RP-HPLC method guarantees the extension of the column lifetime and HPLC system due to the absence of acid in the mobile phase and the use of commonly used organic solvents, i.e. acetonitrile and methanol.

Declarations

Author contribution statement

Dewi Setyaningsih: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Yosua Agung Santoso: Performed the experiments.

Yustina Sri Hartini: Analyzed and interpreted the data.

Yosi Bayu Murti: Contributed reagents, materials, analysis tools or data.

Wouter L. J. Hinrichs: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Christine Patramurti: Conceived and designed the experiments; Performed the experiments.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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