

SIMPLE HPLC METHOD FOR THE QUANTIFICATION OF GINGEROLS (4-, 6-, 8-, AND 10-) AND SHOGAOLS (6-, 8-, AND 10-) IN *Zingiber officinale* var. *rubrum* SUPERCRITICAL CARBON DIOXIDE (SC-CO₂) EXTRACT

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ABSTRACT

Zingiber officinale var. *rubrum* is known as ‘Halia bara’ in the Southeast Asia region. Many herbal products containing Halia bara are in the market are being used for either cosmetic purposes or well-being. The present study aims to develop a simple, high-performance liquid chromatography (HPLC) method using a diode array detector for the quantification of predominant gingerols (4-, 6-, 8-, and 10-) and shogaols (6-, 8-, and 10-) in supercritical carbon dioxide extract of Halia bara. The optimum conditions for elution of seven compounds were: column, ODS Genesis; column temperature, 25 °C; elution, gradient elution consisting of acetonitrile and aqueous formic acid; detection wavelength, 282 nm; flow rate, 1 mL/min; injection volume, 20 µL; and run-time, 38 min. The optimized HPLC method was validated for linear range, accuracy, precision (repeatability and reproducibility), sensibility (limit of detection and quantification) and recovery. All the validation parameters were within the permissible limits stipulated by the International Council of Harmonization guidelines. In Halia bara supercritical fluid-carbon dioxide extract, the amounts of bioactive constituents are in the decreasing order of 10-gingerol, 6-gingerol, 6-shogaol, 8-gingerol, 8-shogaol, 10-shogaol and 4-gingerol.

Keywords: *Zingiber officinale* var. *rubrum*, HPLC-DAD, Halia bara, Gingerol, Shogaol, Supercritical Carbon Dioxide Extract

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INTRODUCTION

Zingiber officinale var. *rubrum*, belonging to the Zingiberaceae family, is known as ‘Halia bara’ in traditional medicine. It is widely used in the South East Asia region for immunomodulatory activity¹, bacterial infections² and the treatment of tumours.³ Halia bara contains characteristic pungent constituents, namely gingerols and shogaols.^{4,5} The bioactivity of Halia bara extract was scientifically proven to be from the presence of gingerols and shogaols.⁶ The chemical structures of the bioactive constituents, gingerols (4-, 6-, 8-, and 10-) and shogaols (6-, 8-, and 10-), were shown in Fig.-1.

There are many reports in the literature on the simultaneous quantification of bioactive molecules in *Zingiber officinale* var. *officinale* (ginger), such as gingerols and shogaols. A high-performance liquid chromatography (HPLC) method⁷ for 6-gingerol and 6-shogaol; ultra-high performance liquid chromatography (UHPLC)-tandem mass spectrometry method⁸ for 3 gingerols (6-, 8-, and 10-); UHPLC method⁹ for two gingerols (6-, and 8-) and 6-shogaol; HPLC method¹⁰ for shogaols (6-, 8-, and 10-); HPLC¹¹⁻¹³, high performance thin layer chromatography (HPTLC)¹⁴, HPLC-mass spectrometry (MS)¹⁵ and LC-MS/MS¹⁶ methods for gingerols (6-, 8-, and 10-) and 6-shogaol; Ultra-performance liquid chromatography/quadrupole-time-of-flight mass spectrometry (UPLC/QTOF-MS) method¹⁷ for

zingerone, gingerols (6-, 8-, and 10-) and 6-shogaol; HPLC electrochemical array method¹⁸ for gingerols (6-, 8-, and 10-), shogaols (6-, 8-, and 10-), 6-paradol and 1-dehydrogingerdione; and liquid chromatography-time-of-flight mass spectrometry (LC-TOF/MS) method¹⁹ for gingerols (4-, 6-, 8-, 10-, and 12), shogaols (6-, 8-, and 10-), dehydrogingerols (6-, 8-, 10-, and 12-), methylgingerols (6-, and 8-), and dehydrogingerdiones (6-, 8-, 10-, 12-, and 14-).

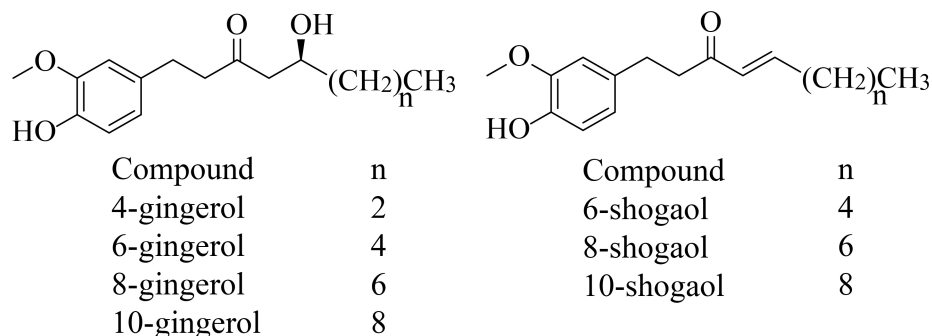


Fig.-1: Chemical Structures of Gingerols (4-, 6-, 8-, and 10-) and Shogaols (6-, 8-, 10-)

All the reported HPLC methods in the literature were carried out on organic solvent extracts of ginger. The supercritical fluid extracts were proven superior to organic solvent extracts in terms of the extracts' quality and safety.²⁰ Supercritical fluid extraction is the best method for extracting the oleoresins. In ginger, all the bioactive compounds are present in oleoresins. Although HPLC-MS has the advantages of high sensitivity, resolution, and selectivity,²¹ requires more financial resources than the HPLC-diode-array detector (DAD). The majority of the companies dealing with herbal products are either small or medium enterprises; thus, they may find difficulties in affording HPLC-MS equipment in their production facilities. There are no reports on simultaneous quantification of gingerols and shogaols in *Halia bara* supercritical fluid extract. The aim of this study is to develop an economical, reproducible, and sensitive HPLC-DAD method for the simultaneous quantification of gingerols (4-, 6-, 8-, and 10-) and shogaols (6-, 8-, and 10-) in *Halia bara* supercritical carbon dioxide (SC-CO₂) extract.

EXPERIMENTAL

Material and Methods

The reference standard gingerols (6-, 8-, and 10-) and shogaols (6-, 8-, and 10-) were purchased from Solarbio Life Science, China. Another reference standard, 4-gingerol, was isolated in our lab. All the reference standards are of > 98% purity. *Zingiber officinale* var. *rubrum* was procured from the locally grown farms from the marketplace in Kuala Lumpur, Malaysia. HPLC 1260 system (Agilent Technologies, Inc. USA) equipped with auto-injector and DAD detector was used in this study. All the HPLC solvents and reagents were purchased from ThermoFisher, USA. Ultra-pure water was attained from the UV Water Purification system, Sartorius, Germany. The carbon dioxide of purity 99.999% was purchased from Linde Malaysia.

Preparation of *Halia bara* Supercritical Carbon Dioxide (SC-CO₂) Extract

A plant taxonomist authenticated *Halia bara* rhizomes, and a voucher specimen (IMU/2019/Ginger/Bara-0819) was prepared. The rhizomes were washed with water to remove dirt, diced into small pieces, and placed in an oven at 30 ± 2 °C to dryness. Then it was grounded using a cutting mill (Rong Tsong Precision, Taiwan) equipped with a mesh of 2 mm. The plant material with a particle size of 0.2 to 0.5 mm was used for the SC-CO₂ extraction.

A commercial-scale supercritical carbon dioxide extractor (50 L x 4, Fayecon, Netherland) was used. About 25 kg of grounded *Halia bara* were loaded into the SC-CO₂ extractor (50 L). The extraction conditions were maintained at 450 bar, 50 °C and the settings of separators one and two were set at 60 bar 50 °C and 40 bar, 40 °C, respectively. The CO₂ flow was maintained at 120 L/h at 40 bar, 5 °C. The oleoresin was collected at separator 1, and oil was collected at separator 2. The total yield was 4.2 % (w/w), and the extraction time was 5 h.

Preparation of Working Standard Solutions

Accurately weighed standards were dissolved in HPLC grade acetonitrile to prepare the stock solution (100 µg/mL). Subsequently, serial dilution was performed using acetonitrile at a 1:1 ratio to prepare the working solutions of the concentration range 50 to 0.02 µg/mL.

HPLC Method Development

The HPLC-DAD was used in this study. The spectrum of the standard solutions was scanned between 190-400 nm using DAD. Three columns; ODS Genesis (250 × 4.6 mm, 5 µm), Zorbax C8 (250 × 4.6 mm, 5 µm) and ODS Hypersil (150 × 4 mm, 5 µm) were examined in this study. The chromatograms were recorded at different flow rates ranging from 0.8 to 1.2 mL/min (increments of 0.1 mL/min); temperatures: 15, 25, and 30 °C; injection volumes: 10, 15, and 20 µL; and detection wavelengths: 210, 224, 225, 254, 281, 282, and 360 nm. Two organic phases: methanol and acetonitrile, were examined in method development. The aqueous phase consisting of varying concentrations of formic acid, including 0.01, 0.1, 1.0, 10, and 50 mM in ultrapure water, was employed in method development. Different gradient programs were tried to obtain the optimum resolution of all the peaks in the shortest possible run-time.

Simultaneous Quantification of Gingerols and Shogaols

The SC-CO₂ extract was dissolved in acetonitrile to prepare a 1 mg/mL solution. The 20 µL of the filtered (0.22 µm) solution was injected. The analysis was performed using the optimum condition. The peak areas were used to determine the concentration of the gingerols and shogaols from the calibration curves. The calibration curve for each compound was constructed using the working standard solutions. The experiment was repeated six times. The compound concentration in SC-CO₂ extract was expressed as mg/g of the extract, and the values are represented as mean ± S.D of 6 readings.

HPLC Method Validation

Several series of measurements of standard solutions were carried out to validate the HPLC-DAD method. The parameters include linear range, precision (repeatability and reproducibility), accuracy, sensibility (limit of detection (LOD) and limit of quantification (LOQ)). ICH guidelines were followed to calculate the LOD and LOQ.

Triplicate analyses of standard solutions injections were performed to construct the calibration curve and find the relevant regression line equation. The areas under the peaks (AUC) were plotted versus the concentrations, and the regression line equations were obtained. Precision (reproducibility and repeatability) was reported as percent relative standard deviation (%RSD, n=3). For repeatability, three different concentrations of the standards were analyzed on the same day. For reproducibility, three varying concentrations were analyzed over three consecutive days. The sensibility was evaluated by calculating the LOD and LOQ, performed by diluting a series of concentrations until the analyte peaks were not distinguished from the noise. The calibration curve of working standard solutions was plotted. The standard deviation (SD) and Slope_{standard} were calculated from the calibration curves. The LOD and LOQ were calculated using the following formula:

$$\text{LOD} = [(SD \times 3.3) / \text{Slope}_{\text{standard}}]$$

$$\text{LOQ} = [(SD \times 10) / \text{Slope}_{\text{standard}}]$$

Three varying concentrations of standards were added to the extract. Recovery was calculated using the following formula:

$$\text{Recovery}(\%) = [(\text{Amount Found} - \text{Original Amount}) / (\text{Amount Spiked})] \times 100$$

RESULTS AND DISCUSSION

In the South-East Asia region, *Z. officinale* var. *rubrum* is widely used for various health benefits, prepared in herbal formulations.³ However, there are no reports on HPLC analysis for simultaneous quantification of gingerols and shogaols in *Halia bara*. In herbal formulations, organic solvent-free extracts are preferred over organic solvent extracts because the traces of organic solvents in herbal formulations may pose health risks.²² In this study, a simple HPLC method was developed to

simultaneously quantify gingerols (4-, 6-, 8-, and 10-) and shogaols (6-, 8-, and 10-) in *Halia bara* SC-CO₂ extract.

Optimization of Chromatographic Conditions

The HPLC method was optimized using different conditions, solvents, run times, flow rates, detection wavelengths, temperature, injection volumes, and column types to obtain a good resolution of each standard in the shortest run time. Mobile phases composed of acetonitrile, methanol, and aqueous formic acid were tested. The concentrations of aqueous formic acid tested were 0.01 mM (pH 5.02), 0.10 mM (pH 4.15), 1.0 mM (pH 3.47), 10 mM (pH 2.91), and 50 mM (pH 2.54). The mobile phase system, acetonitrile/aqueous formic acid (10 mM), gave better peak shape and resolution for all the standards. Besides, various flow rates: 0.8 to 1.2 mL/min; and column types: ODS-Genesis, Zorbax, and ODS Hypersil were employed. The 1 mL/min flow rate produced a good peak shape, and no tailing was observed. The flow rates of less than 1 mL/min showed peak tailing. The flow rates of greater than 1 mL/min caused the overlap of the peaks. ODS Hypersil and Zorbax C8 columns did not produce a good resolution. The ODS-Genesis column produced a good resolution ($R_s > 1.5$) and the asymmetry factor for all the peaks is closer to 1.0. Scanning of the standard solutions using DAD showed appreciable absorption peaks at 210, 224, 225, 254, 281, 282, and 360 nm. The wavelength selected was 282 nm because, at this wavelength, the signal/noise (S/N) ratio for all the peaks is higher compared to that at other wavelengths. The chromatograms were run at different temperatures: 15, 25, and 30 °C. There were no significant differences observed in run-time and peak shape. However, 25 °C was selected as column oven temperature mainly because the normal room temperature is 25 °C. Different injection volumes (10, 15, and 20 µL) were examined. The injection volume at 20 µL provided peaks with an asymmetry factor closer to 1.0. The optimum HPLC chromatographic conditions are shown in Table-1, and the representative chromatogram is shown in Fig.-2.

Table-1: Optimised HPLC-DAD Conditions for the Analysis of Gingerols and Shogaols

Variable	Condition
Column	ODS Genesis (250 × 4.6 mm, 5 µm)
Flow rate	1.0 mL/min
Detection wavelength	282 nm
Solvent system	Solvent A: 10 mM aqueous formic acid Solvent B: Acetonitrile
Elution method	Gradient elution
Elution Program	0 – 5 min: Linear gradient, 50 – 60%B 5 – 18 min: Linear gradient, 60 – 78% B 18 – 29.5 min: Isocratic, 78%B 29.5 – 30.5 min: Linear gradient, 78 – 100% B 30.5 – 38 min: Isocratic, 100%B (Column equilibration)

HPLC Method Validation

The validation of the optimized HPLC method was performed in these aspects: accuracy, linearity, selectivity, precision, and sensibility according to the International Council of Harmonization (ICH) guidelines (ICH, 1996/2005;2002/657/EC)²³. The parameters evaluated for validation are tabulated in Table-2.

The evaluation of method selectivity was performed by superimposing chromatograms of standard solutions and SC-CO₂ extract (Fig.-2). The purity of each peak was assessed by determining the homogeneity across the peak using the DAD. Evaluation of linearity was performed using standard working solutions of concentration range, 100 – 6.25 µg/mL. Every standard at each concentration was analyzed thrice. The reference compounds' peak areas against their concentration were used to construct calibration curves. The analyses showed that the correlation coefficients were found to be more than 0.9990. Repeatability and reproducibility were assessed to determine the method's precision and were expressed as percent relative standard deviation (%RSD), whose values are shown in Table-3 and found to be lower than 3.01%. Recovery was determined by spiking the standards into SC-CO₂ extract and is shown in Table-3. The recovery values were between 86.55 - 113.82%. Based on the guidelines in ICH, 1996/2005;2002/657/EC,²³, both recovery and precision are acceptable. LOD and LOQ were calculated

from the calibration curves of the standard solutions whose concentrations are in the range of lower limits of detection. The LOD values are in the range of 0.180 and 0.799 $\mu\text{g/mL}$, and the LOQ values range from 0.547 to 2.422 $\mu\text{g/mL}$. The LOD and LOQ values indicate that this HPLC method is very sensible for quantifying all the gingerols (4-, 6-, 8-, and 10-) and shogaols (6-, 8-, and 10-) in *Halia bara* SC-CO₂ extract.

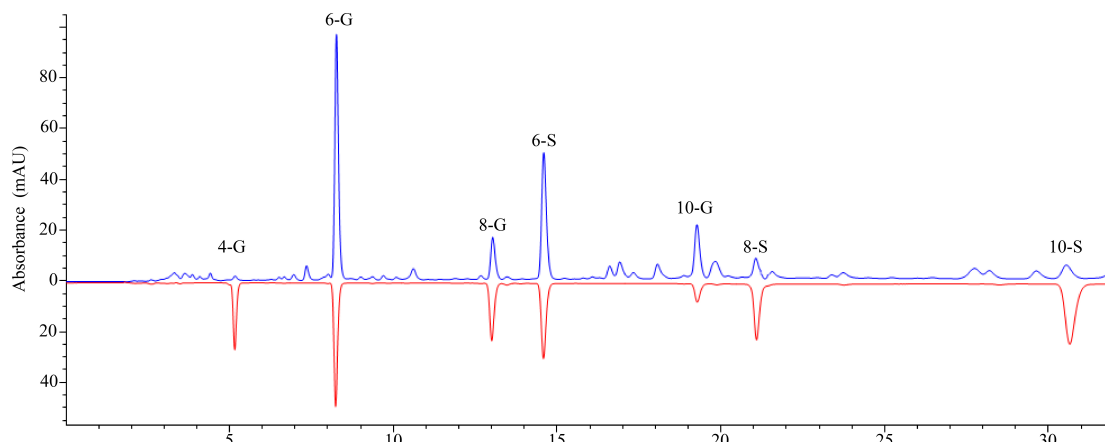


Fig.-2: HPLC Overlay Chromatogram of the Standard Mixture and SC-CO₂ Extract of *Halia bara*. The Blue Line is the Chromatogram of the Standard Mix, and the Red Line is the Chromatogram of SC-CO₂ Extract.

Table-2: Retention Time, Linearity, LOD and LOQ

Analyte	t_R (min)	Linear Range ($\mu\text{g/mL}$)	r^2	Linear Regression Equation	LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)
4-Gingerol	5.1	6.25 - 100	0.9999	$y = 7.3653x - 0.8557$	0.1806	0.5474
6-Gingerol	8.2	6.25 - 100	1.0000	$y = 15.182x - 5.161$	0.1836	0.5564
8-Gingerol	13.0	6.25 - 100	1.0000	$y = 8.6985x - 3.7239$	0.1986	0.6019
10-Gingerol	19.2	6.25 - 100	1.0000	$y = 3.2389x - 1.0055$	0.5451	1.651
6-Shogaol	14.5	6.25 - 100	1.0000	$y = 12.018x - 4.7831$	0.2887	0.875
8-Shogaol	21.0	6.25 - 100	1.0000	$y = 10.388x + 10.825$	0.7993	2.422
10-Shogaol	30.2	6.25 - 100	1.0000	$y = 20.321x - 6.9589$	0.2389	0.724

Table-3: Precision and recovery of the analytes

Analyte	Precision (%RSD)						Accuracy		
	Repeatability			Reproducibility			Recovery		
	C_H	C_M	C_L	C_H	C_M	C_L	C_H	C_M	C_L
4-Gingerol	1.51	1.66	2.61	0.32	1.23	0.18	104.5	113.3	100.6
6-Gingerol	1.71	0.39	1.41	0.29	0.96	0.34	96.6	91.1	98.5
8-Gingerol	1.81	0.11	1.21	0.27	0.88	0.07	86.9	92.9	95.0
10-Gingerol	1.76	0.04	0.83	0.29	0.96	2.72	94.0	89.6	95.1
6-Shogaol	1.45	0.61	1.60	0.25	1.01	0.34	96.8	90.0	97.5
8-Shogaol	1.50	1.42	1.96	0.33	0.90	0.84	88.7	89.3	90.4
10-Shogaol	1.08	1.20	2.56	0.41	0.06	0.51	93.7	100.9	98.4

Note: C_H , High concentration (100 $\mu\text{g/mL}$); C_M , Medium Concentration (25 $\mu\text{g/mL}$); C_L , Low concentration (6.25 $\mu\text{g/mL}$)

Quantification of Gingerols (4-, 6-, 8-, and 10-) and Shogaols (6-, 8-, 10-) in *Halia bara* SC-CO₂ Extract

In *Halia bara* SC-CO₂ extract, the concentration of gingerols and shogaols are in the order: 10-gingerol (73.17 ± 2.84 $\mu\text{g/mL}$) > 6-gingerol (63.18 ± 3.12 $\mu\text{g/mL}$) > 6-shogaol (48.39 ± 2.97 $\mu\text{g/mL}$) > 8-gingerol (15.27 ± 0.99 $\mu\text{g/mL}$) > 8-shogaol (13.27 ± 0.93 $\mu\text{g/mL}$) > 10-shogaol (11.03 ± 0.28 $\mu\text{g/mL}$) > 4-gingerol (1.53 ± 0.09 $\mu\text{g/mL}$).

Discussion

The Halia bara rhizomes were dried at room temperature under shade and extracted with supercritical carbon dioxide. Under these conditions, the total percentage of gingerols (4-, 6-, 8- and 10-) and shogaols (6-, 8-, and 10-) in Halia bara SC-CO₂ was 22.5% consisting of 15% gingerols and 7.5% shogaols. In general, the gingerols are higher in fresh ginger, whereas the shogaols are higher in dried ginger.²⁴ The shogaols are the dehydrated products of gingerols and are formed during the drying process.²⁵ However, in Halia bara SC-CO₂ extract, despite the dried rhizomes being used for extraction, the content of gingerols is higher than shogaols. In addition, it is well reported that 6-gingerol is the principal component among gingerols in *Z. officinale*.²⁶ However, in the SC-CO₂ extract of Halia bara, 10-gingerol was the main constituent of gingerols. It is also well reported that the constitution and composition of gingerols and shogaols depends on *Z. officinale* variants, drying conditions, extraction solvent and extraction method¹⁷. In addition, the medicinal value of *Z. officinale* varies among different variants, and the activity depends on the chemical composition.^{12,13} This study reveals that the chemical composition of Halia bara is different from other variants; thus, it may provide a plausible explanation on why Halia bara is preferred over other variants of ginger for general well-being.

CONCLUSION

There is a gradual escalation in using various types of ginger as health supplements, food products, and cosmetic purposes.²⁷ With the growing number of herbal products containing ginger, there is a need for efficient and cheaper methods to evaluate the quality of ginger. In general, herbal products contain a mixture of bioactive compounds and thus, developing an efficient analytical method for determining the quality of herbal products is challenging. Overall, in this study, a simple, precise, accurate, sensitive, robust, and validated RP-HPLC-DAD method at 282 nm was developed for the simultaneous quantification of gingerols (4-, 6-, 8- and 10-) and shogaols (6-, 8-, and 10-). This method can be translated to the herbal industry for assessing the quality of herbal products containing ginger.

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