ORIGINAL ARTICLE

Ultrasound-assisted extraction and fast chromolithic method development, validation and system suitability analysis for 6, 8, 10-gingerols and shogaol in rhizome of *Zingiber officinale* by liquid chromatography-diode array detection

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Background: Ginger, the rhizome of *Zingiber officinale* Roscoe (family *Zingeberace*) has been used as a spice throughout the world since times immemorial. Ginger has been valued as a medicinal herb in several countries and has been reported to possess carminative, anti-emetic, anti-nauseate and anti-inflammatory properties. **Aim:** Chromolith reverse phase liquid chromatographic method for simultaneous determination, quantification and validation was developed for 6, 8, 10-gingerols and shogaol in the dry rhizome of *Zingiber officinale*. **Materials and Methods:** The method was in accordance to the International Conference on Harmonisation. It is highly specific, exhibited good linearity's ($r^2 > 0.9998$) with high precision and achieved good accuracies between 97.81 and 100.84% of quantitative results. For the developed chromolith LC method, system suitability parameters like K prime, selectivity, resolution, USP resolution, asymmetry, USP tailing, symmetry factor, USP plate count and peak purity were also calculated. **Results and Conclusions:** The developed chromolith method was more sensitive, 4-times faster, generated very good number of theoretical plates (4041 to 22364) and gave better peak resolutions as compared to the earlier methods developed on normal LC columns.

Key words: Chromolith, RP-LC-PDA, rhizome, ultra-sound assisted, *Z. officinale*

INTRODUCTION

High-performance liquid chromatography (HPLC) is now among one of the most successful separation technique. It is extensively used in the phyto-chemistry and pharmaceutical industries for quality control because of its high selectivity, efficiency and speed.[1] Validation of an HPLC test method is mandatory in implementing a quality control system in any laboratory, thereby ensuring sufficient selectivity, accuracy, reproducibility and robustness over the range specified for analysis.[2] Method validation provides an assurance of reliability for providing documented evidence that the method does what it is intended to do.[3]

During the last 30 years, HPLC column-packing materials have improved with respect to particle shape and size, surface and bonding chemistry. Highly reproducible stationary phase, packed into columns, varying in particle lengths and diameters are available. The particle diameter has been reduced from 10 µm to 2.5 µm and column lengths consecutively shortened to ≤5 cm, enabling the generation of 8,000 to 10,000 theoretical plates, thereby giving high efficiency and fast separations with improved detection sensitivity. The 'fourth generation' LC material is constituted of Chromolith columns (Merck, Darmstadt, Germany), which are monolithic-type HPLC. They are operated at high flow rates and maintain high efficiency due to very flat *H*/*u* curve comparable to particulate materials <3 µm. The resulting short-retention time makes them especially interesting for high-throughput applications columns.[4-7]

Ginger, the rhizome of *Zingiber officinale* Roscoe (family *Zingeberace*), has been used as a spice throughout the world since times immemorial. It is now of considerable economical interest and is one of the important spices commercially marketed throughout the world.[8] In view of its commercial importance, it is cultivated in several countries including China, India, Australia, Sri Lanka and Nigeria. Ginger has been valued as medicinal herb

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in several countries and is reported to possess carminative, anti-emetic, anti-nauseate and anti-inflammatory properties. The main pungent principles of fresh ginger are biologically active gingerols. The compound [6]-gingerol (5-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-decan-3-one) is the most abundant constituent, which has been reported to possess various pharmacological and physiological effects. It is also used for the treatment of rheumatism, nervous disorders and asthma.[9] In the dried ginger, however, the predominant pungent principles are shogaols, which are the dehydrated products of gingerols. A number of commercial preparations of ginger containing gingerol and/or shogaols have been developed for the treatment of travel sickness and also for the treatment of chronic arthritis.[10]

A large number of HPLC and HPTLC methods for the quantification of 6-gingerol in[11,12] ginger oil,[13] crude drugs drink,[14] commercial Ayurvedic formulation[15] and rhizome[16] have been reported in the literature. The literature survey also revealed that quantification of other ginger homologues (8,10 gingerols) with 6-gingerol,[17] other diterpenes in Japanese gingers,[18] green and dried ginger,[19] freeze dried CO_2 extract,[20] three gingerols[21] with 6-shogaol in Jamaican ginger,[22] commercial ginger, fresh and dried rhizome and dietary supplements,[23-25] with have also been reported. Until date, no report on chromolithic separation of 6, 8, 10-gingerols and shogaol for the quantification, validation and system suitability in the ginger rhizome has been reported.

In this study, Chromolith column RP-18e (100 × 4.6 mm) was tested for 6, 8, 10-gingerol and shogaol [Figure 1] with respect to the validation parameters. The analysis time was shortened, as expected, by a factor of 4, and the method was found to be more eco-friendly than the conventional methods. Moreover, the repeatability of peak areas, peak symmetry and retention times were also very good.

MATERIALS AND METHODS

Plant Materials

Fresh rhizomes of ginger (*Zingiber officinale* Rosace) were collected from different places of North Western Himalayas of India. Each sample was crushed into small pieces and air-dried for 10 h. These accessions are also being maintained in the experimental fields of Indian Institute of Integrated Medicine, Jammu, India.

Extraction of 6, 8, 10-Gingerol and Shogaol from the Rhizome of *Z. officinale* Samples

Five extraction solvents were tested to extract the 6, 8, 10-gingerol and shogaol from *Z. officinale* samples. They were (i) ethanol, (ii) methanol, (iii) ethyl acetate, (iv) acetone and (v) chloroform. Mass yield data was also calculated for the above solvents. To find the optimal extraction conditions, 600 mg of dry rhizome of *Z. officinale* samples were milled using an electric mortar, then extracted after 10 min using 15 mL each of five different solvents under ultrasonication at 35°C for 45 min. The process was repeated twice. The extraction recoveries with methanol solvent were examined by spiking standards into the sample and then following the extraction method and injecting into the HPLC.

HPLC Apparatus and Chromatographic Conditions

The LC-DAD system of Waters (Milford, MA, USA) with two 515 pumps, a two-line vacuum degasser, a 717-plus auto-sampler and a PDA 2996 detector coupled with Empower workstation was used for the analysis. Analytical chromatographic separation of crude dry ginger rhizome of different solvents extracts were carried out on

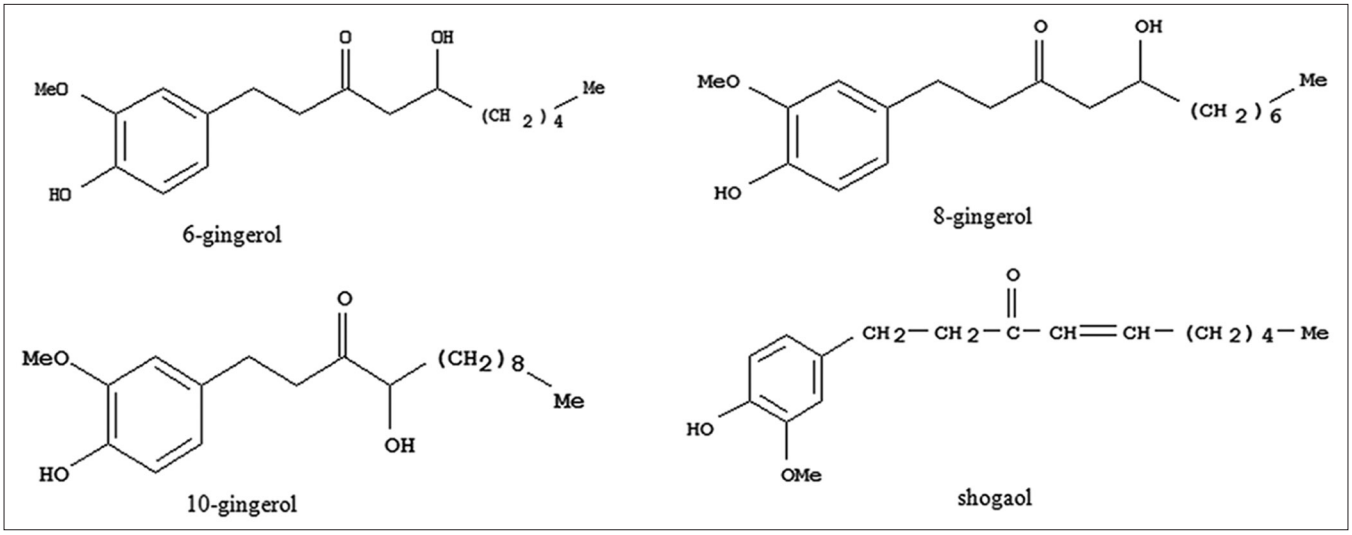

Figure 1: The chemical structures of the analyzed compounds

a chromolith performance RP-18e column (100 × 4.6 mm, Merck, Germany) protected by a chromolith guard column of the same company. The mobile phase consisted of solvent A: Solvent B (water: acetonitrile, v/v). Gradient elution was programmed as follows: 0-2 min, 60-50% A; 2-5 min, 50-30% A; 5-6 min, 30-0% A; 6-7 min, 0% A; 7-8 min, 0-60% A. UV absorption was monitored at 280 nm. The column temperature was maintained at room temperature. The flow rate was 3.0 mL/min and sample injection volume was 20 µL. The spectral data from the photodiode array detector was collected within 8 min over the 200-400 nm range of the absorption spectrum and the chromatograms were plotted at 280 nm. Peaks were assigned according to their retention times and by co-elution with authentic standards and UV spectra for all the standards and samples under the same chromatographic conditions. Before re-injection, the LC system was equilibrated for at least 2 min.

Chemicals and Reagents

ACS-certified HPLC-grade solvents supplied by JT Baker, India, were used in the chromatography studies. Water was purified using the Milli-Q-plus filter systems (Millipore, Bedford, MA, USA).

Calibration Curves

The calibration curves were prepared in the concentration range expected for each compound in *Z. officinale* samples, ranging 1080-5400 ng/µl for 6-gingerol, 1120-5600 ng/µl for 8-gingerol, 1000-5000 ng/µl for shogaol and 880-4400 ng/µl for 10-gingerol. Linearity was determined by calculating regression plots by the least square method and expressed as the correlation coefficient (R^2). The calculated concentrations of all compounds in the *Z. officinale* samples were based on peak area ratios. Limits of detection (LODs) and quantification (LOQs) were determined from the *y*-intercept standard deviation (Sb) and the slope (*a*) of the calibration curve Table 1.

Method Validation

During the optimisation steps, different compositions of mobile phase and various flow rates were tested. The primary task was to obtain values of validation parameters close to those of the particle-based column analysis using monolithic column. It was necessary to adjust the chromatographic conditions in order to get optimal results using chromolith column. Mobile phase (water: acetonitrile) with various composition and flow rates (in the range of 1-4.5 mL/min) were tested. Chromolith column with mobile phase composition of water and acetonitrile with gradient system (as discussed earlier) at a flow rate 3.0 mL/min were found to be most suitable and was used for the whole analysis [Figure 2].

Under optimal conditions, the retention time and System suitability test parameters (K prime, selectivity, resolution, USP resolution, asymmetry (@4.4), USP tailing, symmetry factor and USP plate count) and peak purity of 6, 8, 10-gingerol and shogaol were also evaluated through a software for satisfying validation requirements. Other parameters compared during the experiments included analysis time and solvent consumption. All four compounds were successfully separated using the proposed procedures, and chromatographic parameters mentioned above were calculated from the experimental data. All the results were in good agreement with values set by validation authorities.[26]

Chromatographic separation quality with chromolith column was comparable with that of a conventional particle-based column. The conventional particle-based column, under the above flow rate could not work due to excess column back pressure (more than the pump limit of 5000 psi) of the pump. In case of the monolithic column, a much lower back pressure was found (about 1200-1800 psi; depending upon the mobile phase composition at the specified flow rate of 3 ml/min). The low back pressure value for monolithic column is known to prolong the lifetime of such column, thereby lowering the analysis cost. The volumes of mobile phase spent during individual analyses were higher in case of normal length column as the running time was approximate 30-35 min and the flow rate was

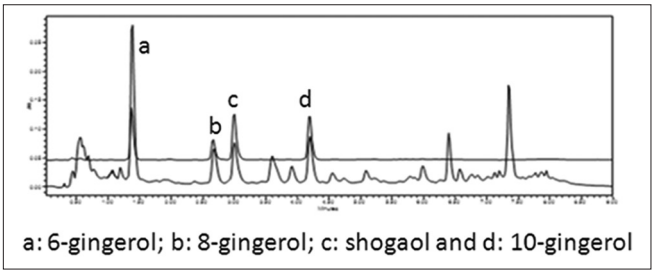

Figure 2: The overlay chromatographic profiles (recorded at 280 nm) of the methanolic extract of *Z. officinale* and the standard mixture

Table 1: Linearity of standard curves and quantification/detection limits for 6, 8, 10-gingerols and shogaol

Name of compounds	Retention time	Regression equation	r^2	Linear range (ng/µl)	LOQ (ng) at 280 nm	LOD (ng) at 280 nm
6-gingerol	1.39	Y=1.47e+002X	0.9998	1080-5400	49	16
8-gingerol	2.68	Y=9.17e+001X	0.9999	1120-5600	66	21
Shogaol	3.00	Y=1.86e+002X	0.9999	1000-5000	150	48
10-gingerol	4.18	Y=1.62e+002X	0.9999	880-4400	82	26

LOD - Limits of detection; LOQ – Limit of quantification

1 ml/min in comparison to that of the chromolith column. The significance of high flow rates that could enhance the quality of the separation process on the chromolithic columns with lower back pressure as compared to particle-based columns was accepted and appreciated.

Accuracy was evaluated by adding known quantities of pure 6, 8, 10-gingerols and shogaol to one of the pre-analysed methanolic extract of ginger rhizome in triplicate at 3 different concentration range (low, medium and high) and calculated as a recovery of the known quantity, expressed in percentage.

The specificity is judged by 'the ability to assess unequivocally the analyte in the presence of components which may be expected to be present'.[27] This parameter is important to reassure that the chromatographic signals are due to the investigated compounds and not due to impurities, degradation products and endogenous or exogenous interferences. The specificity was evaluated by verifying the purity of the chromatographic band by elaborating the UV spectral profiles with a suitable algorithm (Peak Purity) implemented in the Empower software (Water's) [Table 2]. The results of these analyses confirmed that the method was specific and no co-elution with other components occurred.

The accuracy of the method was determined by the application of the standard addition method. Accurate amounts of the pre-mixed standards (all the four) were added at three concentration levels (low, medium and high) to the pre-analysed ginger rhizome sample and then extracted and analysed in triplicate as described above. The total amount of each compound was calculated from the corresponding calibration curve and the recovery of each compound was obtained using the following equation: Recovery (%) = (amount found − amount contained)/amount spiked × 100%.

As the maximum UV absorption wavelengths of 6, 8, 10-gingerol are 280 and for shogaol is 227 nm, the detection wavelength was set at 280 nm, according to the UV spectra obtained by DAD detection. Chromatographic peaks were identified by comparing their retention times and UV absorption spectra with those acquired for standards analysed under the same chromatographic conditions through library-search facility using the Empower software. Spiking selected samples with the standard compounds was also used to assist confirmation of the peak identity.

The precision is defined by ISO as 'the closeness of agreement between independent test results obtained under stipulated conditions'.[26] It is possible to define three levels of precision (repeatability, intermediate precision and reproducibility) by keeping the factors that may affect the results unaltered (adopted method, analyzed sample, laboratory, operator and instrument). The repeatability was evaluated by running the analyses in a single day at different time, involving the same instrument, laboratory and operator, while the intermediate precision was calculated by considering a longer period of time. The precision was estimated by evaluating intra batch precision and inter-batch variation with respect to retention time and peak area of 6, 8, 10-gingerol and shogaol. The intra-batch precision was determined after 8 and 16 h from a set of six replicates in a single day by the same operator. The inter-batch precision was evaluated from a set of six replicates analyzed with the same mixture of standards on days 1, 3 and 6.

The sensitivity was evaluated by determining the LOD and LOQ. The parameters were respectively calculated by using the equations: LOD = 3.3 × Sb/a and LOQ = 10 × Sb/a, where a is the slope and Sb is the standard deviation of the y-intercept. The signal to noise ratio were calculated using the Empower software. The range of linearity was obtained from three replicates of each concentrations for 6, 8, 10-gingerols and shogaol, yielding correlation coefficients above 0.9998. The obtained values for both LOD and LOQ for 6, 8, 10-gingerols and shogaol were low, indicating that the method is capable of not only quantifying the standards but also detecting traces of these gingerol compounds.

The robustness was checked by varying the composition of the mobile phase, the temperature and the flow rate according to ICH requirements.[27] The influence of the mobile phase composition was not further investigated as it had already been checked during the development of the method. Within the tested range (25-30°C), the retention times of analytes were inversely proportional to the temperature of the column, the resolution between the four peaks remained good and the UV responses were not significantly modified.

Table 2: System suitability parameters calculated through software for chromolith LC analysis of methanolic extract of ginger rhizome

Name of compounds	Retention time	K prime	Selectivity	Resolution	USP resolution	Asymmetry @4.4	Symmetry factor	USP plate count
6-gingerol	1.39	0.3971	1.8099	1.9107	1.8091	1.8064	1.2096	4041
8-gingerol	2.68	1.6866	1.2182	2.1873	2.1364	1.3626	1.1831	10330
shogaol	3.00	2.0014	1.1877	2.1553	2.0445	1.7292	1.3561	13733
10-gingerol	4.18	3.1883	1.0953	2.0136	1.9533	0.7831	0.9011	22364

RESULTS AND DISCUSSION

To optimize the extraction of analytes, the effect of different extraction solvents were studied using ultrasound-assisted extraction as a rapid and efficient extraction tool. Five solvents with varying polarity, such as ethanol, methanol, ethyl acetate, acetone and chloroform, were used. All extractions were performed under similar conditions. The temperature for extraction was kept low (35°C) as an increase in temperature during the process may lead to conversion of gingerols to shogaols. The mass yield data suggested that methanol is the best solvent among all the solvents used for extraction. LC analysis of different solvents demonstrated that the amount of desired gingerols and shogaol were higher for the methanolic extract. For our subsequent analysis, only methanolic extracts were taken for validation and system suitability analysis. For chromolith LC-DAD method development, initially isocratic method was chosen because it is more robust and can be much more easily transferred to other laboratories for routine testing or quality control purposes than the gradient methods. However, due to large variation in the polarity of molecules, we could not succeed in our separation. A unique feature and a big advantage of silica-based monolithic reverse phase (RP_{18e}) LC columns over packed micro particulate columns is their ability to independently control the macro and mesopore diameters as well as silica skeleton diameter.

While developing LC analytical method for the gingerols analysis, different chromatographic conditions were tried to improve the separation of major compounds in suitable time with good peak separation. The best separation and resolution of peaks to allow the quantification of the major gingerol compounds present in the methanolic extract of *Z. officinale* rhizome were achieved using the parameters described in the sample preparation section of Materials and Method. The separation time was within the time frame of analysis of 8 min. The selectivity of the method was determined by comparing the chromatographic profile. The data obtained for the standards and samples included the following parameters: Retention time, maximum wavelength of absorption and UV spectrum.

High correlation coefficient values ($r^2 \geq 0.9998$) indicated good linearity between peak areas (y) and investigated compound concentration (x, ng/µl) in relatively wide concentration ranges. The LODs and LOQs for each investigated compounds are given in Table 1. The values showed high sensitivity under these chromatographic conditions. The overall intra- and inter-day precisions (RSD) for the investigated components for area and retention time were less than 1.35, 0.45 and 1.56%, 0.43%, respectively. The average recovery was in the range of 97.81-100.84% with RSD ranging from 0.40 to 2.01% for all the analysed compounds. These results indicated that the method was accurate and reproducible. When the solution was stored at 4°C, all the analytes were found to be stable within 96 h (RSD ≤ 2.0%). For checking the reproducibility of auto-sampler, pump flow and detector, repeatability of peak area and retention times were calculated by the RSD of six injections carried out on the same day. The RSD for the retention times of all peaks was <0.23% and, for the peak area, it was <1.56%.

Sufficient resolution, satisfactory peak asymmetry and higher number of USP plate counts (4041-22364) [Table 2] were obtained for all tested compounds separated on the chromolith column. The developed method differed considerably in analysis time and solvent consumption. Chromolith column permitted an analytical run to be faster (less time) than required by a conventional column. In this case, a high flow rate could be used while maintaining the validation parameters at the required levels. Results of all the samples collected from different locations and extracted using different polarity solvents are tabulated in Table 3. The results revealed that 6-gingerol is present in highest concentration in all the samples. The 6-gingerol ranges from 0.20 to 0.51%, 8-gingerol ranges from 0.07 to 0.22%, 10-gingerol ranges from 0.14 to 1.02% and Shogaol ranges from 0.07 to 0.14%. Sample collected from Sirmaur contains highest percentage of 6-gingerol, 8-gingerol and 10-gingerol. Sample collected from Pauri Garhwal contains highest percentage of shogaol.

CONCLUSIONS

The proposed method can be employed for the determination of important gingerols in baseline resolution in a shorter analysis time. All studied gingerols showed shorter retention times than those generally reported in the literature using normal LC_{18} column. The present procedure can be used to obtain the content of main gingerols in ginger, which is of great interest for the development of an analytical database of gingerol contents for quality assessment of ginger.

The method presented could achieve good results in the following parameters: Selectivity, linearity, accuracy, precision, robustness, as well as limit of detection and limit of quantitation. Therefore, this method could be considered as an analytical tool for the quality control of *Z. officinale* raw material and its products.

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Table 3: Concentration (%) of 6, 8, 10-gingerols and shogaol in the methanolic extract of dry ginger rhizomes collected from different locations of North Western Himalayas (*n*=3)

Location of sample collection	Concentration of compounds in % DWB							
	6-gingerol	% RSD	8-gingerol	% RSD	Shogaol	% RSD	10-gingerol	% RSD
Sirmaur	0.51	0.72	0.22	1.29	0.09	0.87	0.14	1.33
Mandi	0.34	0.82	0.14	1.04	0.08	1.26	0.10	0.74
Sunder Nagar	0.20	1.16	0.07	0.98	0.07	0.66	0.05	1.06
Hamirpur	0.39	1.25	0.11	0.48	0.07	1.31	0.08	0.58
Kangra	0.29	1.12	0.09	1.22	0.09	0.67	0.06	0.95
Tehri Garhwal	0.20	1.07	0.06	1.36	0.07	0.48	0.12	0.84
Bageshwar	0.48	0.76	0.20	0.58	0.12	0.55	0.13	1.27
Nainital	0.25	1.41	0.11	1.21	0.10	1.41	0.07	0.63
Udham Singh Nagar	0.39	1.25	0.15	0.59	0.10	0.83	0.11	0.73
Pauri Garhwal	0.50	0.52	0.16	0.78	0.14	0.94	0.14	1.02
Range (%)	0.20-0.51		0.07-0.22		0.07-0.14		0.06-0.14	
Average (%) (overall)	0.36		0.13		0.09		0.11	

RSD – Relative standard deviation; DWD – Dry weight basis

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