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Research Article

A Simple HPLC Method for the Analysis of [6]-Gingerol Produced by Multiple Shoot Culture of Ginger (*Zingiber officinale*)

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ABSTRACT
A simple HPLC method was developed to analyze [6]-gingerol content of *in vitro*- grown multiple shoot cultures of ginger. Separation was achieved using an Inertsil ODS-3 column. The best mobile phase was methanol:water (90:10, v/v) which was ran isocratically at a flow rate of 1 ml/min. Analysis of nine different concentrations of [6]-gingerol standards generated a calibration curve which was linear over a concentration range of 1-1000 µg/ml. Limit of detection (LOD) was found to be 0.489 µg/ml, while the limit of quantitation (LOQ) was 1.482 µg/ml. Accuracy of the proposed method was found to be 97.8%. The developed HPLC method was applied to analyze [6]-gingerol production from multiple shoots cultured under different light wavelengths. Results of the analysis revealed that different light wavelengths affected [6]-gingerol production, and that red light irradiation significantly improved [6]-gingerol accumulation in multiple shoot cultures which yielded 191.07±1.25 µg per culture bottle. The developed method is simple, reproducible and is useful for the analysis of [6]-gingerol in multiple shoots of ginger cultured *in vitro*.

Keywords: ginger, gingerol, HPLC, multiple shoot

INTRODUCTION

Zingiber officinale Roscoe, commonly known as ginger, is a tropical crop that produces a pungent aromatic rhizome valued worldwide as either spice or herbal medicine[1]. The gingerols are the major phenolic pungent principles in the fresh rhizome, with [6]-gingerol being the most abundant[2]. [6]-Gingerol possesses diverse pharmacological and physiological effects including analgesic, gastroprotective, cardiotonic, anti-inflammatory, anti-oxidant, anti-pyretic, anti-hepatotoxic, anti-angiogenic, anti-hyperglycemic, anti-cancer and anti-platelet aggregation activities[3–5]. Due to its low toxicity and attractive medicinal potential, [6]-gingerol has become increasingly in demand[6].

Several high-performance liquid chromatographic (HPLC) methods have been developed for the analysis of ginger extracts. These methods were primarily used for the analysis of gingerols from field-grown ginger[6–11], but not for the analysis of gingerols from *in vitro*- grown ginger, which is now being explored as a potential alternative source of [6]-gingerol. Recently, an HPLC method for the analysis of gingerols from both the field-grown rhizomes and *in vitro*-grown microrhizomes of ginger has been developed[12]. However, this method involves several extra steps (*e.g.* ethyl acetate extraction, drying over anhydrous sodium sulfate and filtering), which could be prone to error due to losses associated with these extra steps. In the course of our investigation for strategies that will improve [6]-gingerol production from multiple shoots of ginger, we developed an HPLC method involving a one-step extraction and directly using the extract for [6]-gingerol analysis. This paper is the first report of a simple HPLC method for the analysis of [6]-gingerol from *in vitro*-grown ginger. This is also the first report of the influence of light quality to [6]-gingerol production in ginger multiple shoots.

MATERIALS AND METHODS

Chemicals

Authentic [6]-gingerol was obtained from Sigma-Aldrich (Missouri, USA). HPLC-grade methanol was obtained from Fisher Scientific (New Jersey, USA) and HPLC-grade water was obtained from RCI Labscan Limited (Bangkok, Thailand).

Plant materials

Mature rhizome of *Zingiber officinale* cv. 'imugan' was planted in sterilized soil. After two weeks, the germinating ginger rhizome was taken and washed with sterilized distilled water. It was surface-sterilized by soaking in 50% sodium hypochlorite solution for 30 min, and then rinsed thoroughly with sterilized distilled water. Inside the laminar flow hood, the germinating shoot was aseptically excised and planted in a culture bottle containing 25 ml of solidified MS basal medium[13] supplemented with 1 mg/L naphthaleneacetic acid (NAA) and 1 mg/L 6-benzylaminopurine (BAP). After a month, the multiple shoots were formed and they were transferred individually in a similar fresh MS medium. Except for the control, all

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multiple shoot cultures were subjected to different light wavelengths by wrapping the culture bottles with cellophane of different colors, *i.e.* red, orange, yellow, green, blue, indigo and violet, which served as monochromator filters. They were incubated at 25ºC under continuous light condition for one month.

Sample preparation

One month-old multiple shoots of ginger were harvested and extracted with methanol. The methanol extract was filtered, transferred to a 100 ml round-bottom flask and concentrated *in vacuo* at 40ºC using a rotary evaporator. The residue was re-suspended in methanol, vortexed for 1 min, then filtered using 0.22 µm membrane filter, and transferred to a 1.5-ml microcentrifuge tube. All extracts were stored at 4ºC until used.

Preparation of [6]-gingerol standards

The [6]-gingerol standard was dried over silica gel for at least 3 h *in vacuo*. Sufficient HPLC-grade methanol was added to 5 mg of [6]-gingerol standard to produce a stock solution of 1000 µg/ml. Serial dilutions of this stock solution were made to produce 1, 5, 10, 25, 50, 100, 500 and 750 µg/ml working standards. All [6]-gingerol standards were capped and stored at 4ºC until used.

HPLC conditions

The ginger extracts were analyzed on a HPLC system consisting of Shimadzu LC 20-A Prominence Controller, Photodiode Array Detector (PDA), and Chromatography Manager (Tokyo 1018448, Japan). An Inertsil ODS-3 column with a dimension of 250 x 4.6 mm, 5-µm particles (GL Sciences Inc., Japan) was used. The mobile phase consisting of methanol:water was ran isocratically for 5 min at a flow rate of 1 ml/min. PDA detector was set at 282 nm. Quantitation of [6]-gingerol was achieved after comparison with a calibration curve of authentic [6]-gingerol. Except otherwise indicated, all measurements were done in five replicates.

Statistical analyses

Statistical analyses were conducted using Microsoft Excel 2010 and OpenStat Program (Version 3, 2007). Data were analyzed for significant differences using a One-way Analysis of Variance (ANOVA). Comparison among treatment means was done using Tukey's HSD Test. The significance level was set at $p < 0.05$.

RESULTS AND DISCUSSION

Optimization of the mobile phase

Previous reports on HPLC analyses of [6]-gingerol in ginger favor the use of octadecylsilane (ODS) column[6,11,12]. For the HPLC analyses of many natural products which employs ODS column, methanol is a common mobile phase being used. In this study, methanol was chosen as the mobile phase for HPLC of [6]-gingerol. Optimization was first performed to determine the right concentration of methanol. The criteria for selecting the best concentration are: 1) the ability to elute [6]-gingerol in distinct, narrow peak, and 2) the ability to elute [6]-gingerol with the shortest retention time possible. Methanol:water with volume ratios of 65:35, 70:30, 80:20 and 90:10 were used. Based on the chromatographic data as shown in Figure 1, the 90:10 methanol:water has the

Table 1: Chromatographic data for the elution of [6]-gingerol standard at different volume ratios of methanol:water

Methanol:water (v/v)	Peak area	Retention time (min)
65:35	5723747	16.2
70:30	6159755	10.6
80:20	7493793	5.6
90:10	12321526	3.8

Table 2: Repeatability of the proposed method as determined after five intra-day replicate injections of samples at three different concentrations.

Nominal concentration (µg/ml)	Measured concentration (µg/ml)	Standard deviation	CV (%)
85.0	85.90 ± 0.12	0.26	0.30
6.5	6.21 ± 0.03	0.07	1.18
5.0	5.37 ± 0.01	0.03	0.49

Table 3: Intermediate precision of the proposed method obtained after three inter-day replicate injections of samples at three different concentrations.

Nominal concentration (µg/ml)	Measured concentration (µg/ml)	Standard deviation	CV (%)
85.0	85.94 ± 0.39	0.87	1.01
6.5	6.73 ± 0.01	0.01	0.16
5.0	5.52 ± 0.06	0.14	2.44

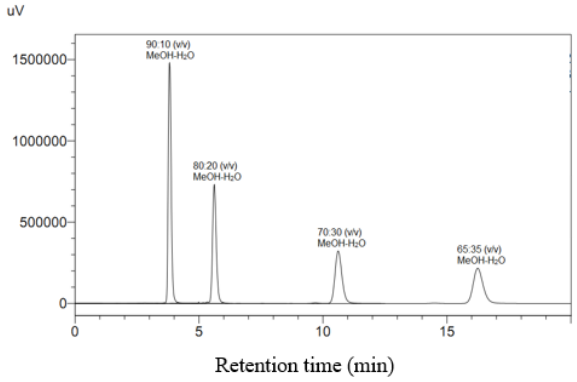

Figure 1: Overlaid chromatograms of [6]-gingerol standard eluted at different methanol:water (v/v) composition.

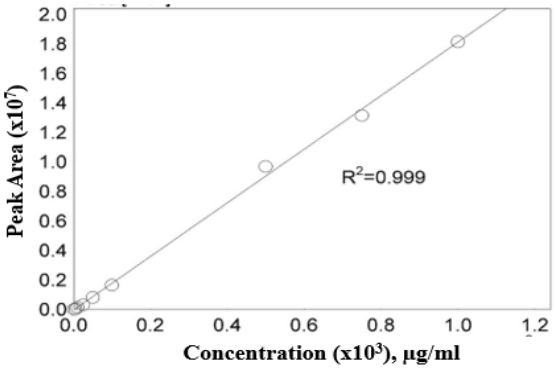

Figure 2: Standard calibration curve of [6]-gingerol

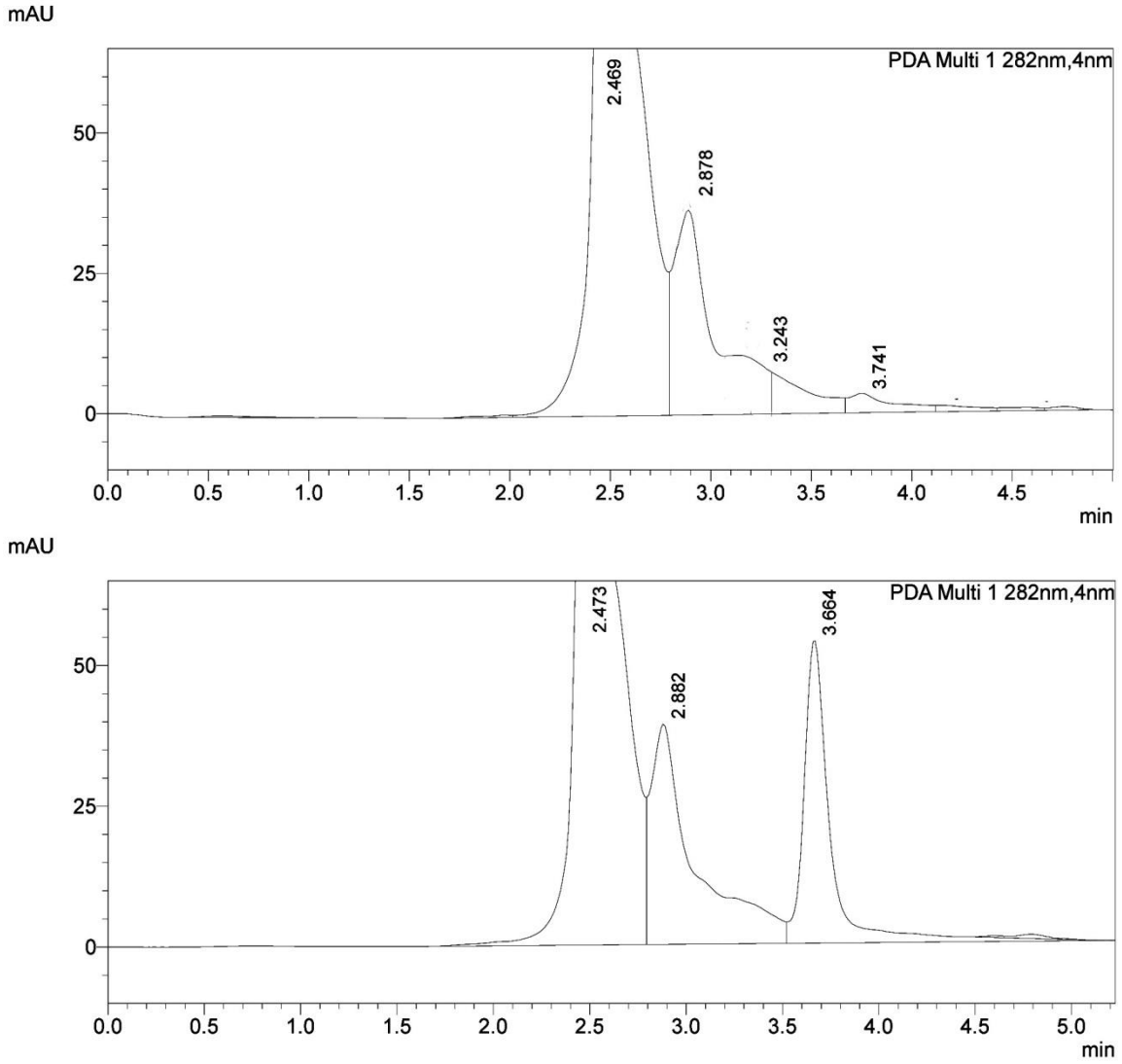

Figure 3: HPLC chromatograms of methanol extracts from multiple shoots of ginger incubated under continuous white light (control) and red light. The peak pointed by the arrow is [6]-gingerol.

shortest retention time for [6]-gingerol observed at 3.8 min. It has also the highest detection for the compound by having the largest peak area (Table 1). Hence, the optimum methanol:water ratio was chosen to be 90:10 (v/v), and was used for all the subsequent analyses in this study.

Calibration of standard curve

Nine concentrations of [6]-gingerol (1, 5, 10, 25, 50, 100, 500, 750, 1000 µg/ml) were used to establish the calibration curve. The calibration plot of peak area against amount of [6]-gingerol was linear in the range of 1–1000 µg/ml (Figure 2). The linear regression equation was y=18180x– 55635, where y is peak area and x is amount of [6]-gingerol. The retention time of the highest concentration of [6]-gingerol standard using methanol:water (90:10,v/v) was 3.8 min. All of the detected and quantitated [6]-gingerol peaks were within the 10% window (data not shown), with retention time of 3.8±0.4 min.

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated using the formula LOD=3.3σ/s and LOQ=10σ/s, in which σ is intercept standard deviation and s is the slope of calibration curve[14]. LOD and LOQ of the proposed method were 0.489 µg/ml and 1.482 µg/ml, respectively. This indicates that this method can be used in a wide range for effective detection and quantitation of [6]-gingerol in ginger multiple shoot cultures. The repeatability and intermediate precision were assessed through the coefficient of variation (CV) of five intra-day and three inter-day replicate injections of [6]-gingerol standards at three concentrations. The CV value for both tests were within the acceptance criterion of 3% (Tables 2 and 3), thereby conferring the precision of the results. The peak asymmetry factors (at 10%) of the standard peaks extended from 1.037 to 1.259, which were within the acceptable range values for test compounds[15], thereby entailing a good separation of the analyte. Accuracy of the proposed method, which was evaluated as recovery after spiking the sample with [6]-gingerol standards at two concentrations, was found to be 97.8%.

Method applicability

The developed HPLC method was applied for the quantitation of [6]-gingerol in ginger multiple shoot

Table 4: Retention time and concentration of [6]-gingerol from multiple shoots of ginger incubated under different light wavelengths.

Treatment	Retention time (min)	[6]-Gingerol concentration (µg per culture bottle)[1]
Control	3.80 ± 0.07	58.84 ± 0.06 c
Red	3.70 ± 0.01	191.07 ± 1.25 a
Orange	3.71 ± 0.03	93.75 ± 0.39 b
Yellow	3.77 ± 0.02	87.88 ± 0.71 bc
Green	3.75 ± 0.01	50.37 ± 1.07 c
Blue	3.80 ± 0.01	70.83 ± 0.35 c
Indigo	3.76 ± 0.04	80.63 ± 1.64 c
Violet	3.79 ± 0.02	54.88 ± 1.13 c

[1]Treatment means followed by a common letter are not significantly different using Tukey's HSD Test ($p< 0.05$).

cultures subjected to different light wavelengths. Results are shown in Table 4. [6]-Gingerol production in multiple shoots cultured under green, blue, indigo and violet lights were not significantly different with that of the control treatment. A slight increase in [6]-gingerol production was observed when the multiple shoots were irradiated with orange and yellow light. But the highest [6]-gingerol content was observed in multiple shoots cultured under red light, which represents a 10-fold increase in [6]-gingerol production as compared with that of the control (Figure 3). These results reveal that the synthesis of [6]-gingerol is enhanced by red light. Similar results were observed by other workers. An increased production of eleutherosides was observed when *Eleutherococcus senticosus* somatic embryos were irradiated with red light[16]. In *Aquilaria agallocha,* cucurbitacin production was observed to be significantly increased upon red light exposure than when exposed to normal conditions[17]. High accumulation of rosmarinic acid was also observed when *Ocimum basilicum* was exposed to red light[18]. Very recently, it was also demonstrated that red light stimulated curcumin accumulation in microrhizomes of *Curcuma aromatica*[19]. These results demonstrate that light quality can be controlled to stimulate biosynthetic pathways related to secondary metabolism, increasing the production not only of [6]-gingerol but other secondary metabolites as well. This has important implications for the wider use of plant tissue culture as a tool for the production of high value compounds.

CONCLUSION

A simple, high-speed and reliable HPLC method was developed for the quantitative analysis of [6]-gingerol in multiple shoot cultures of ginger. Separation was achieved using an ODS column and the best mobile phase was found to be methanol:water (90:10,v/v) with retention time observed at 3.8 min. HPLC analyses of the samples showed that light quality affect [6]-gingerol accumulation in ginger multiple shoot cultures with the highest production observed in cultures treated with red light.

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REFERENCES

1. Asghari, G., Dehghani, I., and Akbar Mostajeran, A. (2011) *In vitro* and *in vivo* production of gingerols and zingiberene in ginger plant (*Zingiber officinale* Roscoe). *Iranian J Pharm Sci 7*, 117–121.
2. Gang, D.R., Jiang, H., Xie, Z., Koo, H.J., McLaughlin, S.P., and Timmermann, B.N. (2006) Metabolic profiling and phylogenetic analysis of medicinal *Zingiber* species: tools for authentication of ginger (*Zingiber officinale* Rosc.). *Phytochem 67*, 1673–1685.
3. Kwon,Y.G., Kim, E.C., Min, J.K., Kim, T.Y., Lee, S.J., Yang, H.O., Han, S., and Kim, Y.M. (2005) [6]-Gingerol, a pungent ingredient of ginger, inhibits angiogenesis *in vitro* and *in vivo*. *Biochem Biophys Res Comm 335*, 300–308.
4. Kim, W.K., Lee, H.S., Seo, E.Y., and Kang, E.N. (2008) [6]-Gingerol inhibits metastasis of MDA-MB-231 human breast cancer cells. *J Nutrional Biochem 19*, 313–319.
5. Bukhsh, A.R., Chakraborty, D., Bishayee, K., Ghosh, S., Biswas, R., and Mandal, S.K. (2012) [6]-Gingerol induces caspase 3 dependent apoptosis and autophagy in cancer cells: drug-DNA interaction and expression of certain signal genes in HeLa cells. *Eur J Pharmacol 694*, 20–29.
6. Pawar, N.V., Pai, S.R., Nimbalkar, M., and Dixit, G. (2011) RP-HPLC analysis of phenolic antioxidant compound 6-gingerol from different ginger cultivars. *Food Chem 126*, 1330–1336.
7. Balladin, D.A., Headley, O., Yen, C., and Mcgaw, D.R. (1998) High pressure liquid chromatographic analysis of the main pungent principles of solar dried West Indian ginger (*Zingiber officinale* Roscoe). *Renewable Energy 13*, 531–536.
8. He, X., Bernart, M.W., Lian, L., and Lin, L. (1998) High-performance liquid chromatography-electrospray mass spectrometric analysis of pungent constituents of ginger. *J Chromatogr A* 796, 327–334.
9. Saha, S., Smith, R.M., Lenzb, E., and Wilson, I.D. (2003) Analysis of a ginger extract by high-performance liquid chromatography coupled to nuclear magnetic resonance spectroscopy using superheated deuterium oxide as the mobile phase. *J Chromatogr A 991*, 143–150.

10. Schwetner, H.A., and Rios, D.C. (2007) High-performance liquid chromatographic analysis of 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol in ginger-containing dietary supplements, spices, teas and beverages. *J Chromatogr B 856*, 41–47.
11. Pawar, N.V., Pai, S.R., Nimbalkar, M., Kolar, F., and Dixit, G. (2010) RP-HPLC analysis of 6-gingerol and assessment of antioxidant activities in EMS treated ginger. *J Phytology 2*, 13–23.
12. Aly, U.I., Abbas, M.S., Tahal, H.S., and Gaber, E.I. (2013) Characterization of 6-gingerol for *in vivo* and *in vitro* ginger (*Zingiber officinale*) using high performance liquid chromatography. *Global J Bot Sci 1*, 9–17.
13. Murashige, T., and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum 15,* 473– 497.
14. Shekarchi, M., Hajimehdipoor, H., Khanavi, M., Adib, N., Bozorgi, M., Akbari-adergani, B. (2010) A validated method for analysis of Swerchirin in *Swertia longifolia* by high performance liquid chromatography. *Pharmacog Mag 6*, 13–18.
15. Dolan, J.W. (2002) Peak Tailing and Resolution. LC Resources, Inc., Walnut Creek, California, USA.
16. Shohael, A.M., Ali, M.B., Yu, K.W., Hahn, E.J., Islam, R., and Paek, K.Y. (2006) Effect of light on oxidative stress, secondary metabolites and induction of antioxidant enzymes in *Eleutherococcus senticosus* somatic embryo in bioreactor. *Process Biochem 41*, 1179–1185.
17. Kuo, T.C., Chen, C., Chen, S., Lu, I., Chu, M., Hunag, L., Lin, C., Chen, C., Lo, H., Jeng, S., and Chen, L. (2015) The effect of red light and far red light conditions on secondary metabolism in agarwood. *BMC Plant Biol 15*, 139–148.
18. Shiga, T., Shoji, K., Shimada, H., Hashida, S.N., Goto, F., and Yoshihara, T. (2009) Effect of light quality on rosmarinic acid content and antioxidant activity of sweet basil *Ocimum basilicum* L. *Plant Biotechnol 26*, 255–259.
19. Wu, K., Zhang, X., Sun, S., and Wang, X. (2015) Factors affecting the accumulation of curcumin in microrhizomes of *Curcuma aromatica* Salisb. *BioMed Res Internat 2015*, 1–10.