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Full Length Research Paper

Quantitative determination of diosgenin in *Dioscorea zingiberensis* cell cultures by microplate-spectrophotometry and high-performance liquid chromatography

Peiqin Li, Yan Mou, Shiqiong Lu, Weibo Sun, Jingfeng Lou, Chunhui Yin and Ligang Zhou*

College of Agronomy and Biotechnology, China Agricultural University, Beijing 100193, China.

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Quantitative determination of diosgenin was carried out by multi-well microplate-spectrophotometry and high-performance liquid chromatography (HPLC) in this study. Diosgenin detection by microplate-spectrophotometry depended on the formed yellow substance developed by perchloric acid, and measured at 410 nm. The calibration graph was linear over the range of 2 to 10 µg diosgenin in each well with correlation coefficient (R) as 0.9988, detection limit as 0.6111 µg, and quantification limit as 1.8518 µg. For HPLC analysis, diosgenin detection was conducted at 203 nm on an Agilent TC-C_{18} column with a mobile phase of acetonitrile-water (90:10, v/v). The calibration graph was linear over the range of 0.0625 to 1.000 µg diosgenin with correlation coefficient (R) as 0.9995, detection limit as 0.0372 µg, and quantification limit as 0.1127 µg of diosgenin. Both methods were characterized by satisfactory precision and accuracy, which were then employed to detect diosgenin content in cell cultures of *Dioscorea zingiberensis*. No statistical significant differences were observed between the results obtained by microplate-spectrophotometry and HPLC (ANOVA, $p < 0.05$). Quantitative determination of diosgenin by microplate-spectrophotometry should be a rapid, accurate, simple and economic alternative method, though HPLC is more sensitive for diosgenin analysis.

Key words: Diosgenin, *Dioscorea zingiberensis*, cell cultures, microplate-spectrophotometry, high-performance liquid chromatography, quantitative determination.

INTRODUCTION

Diosgenin is an important precursor of semi-synthetic steroids such as corticosteroids, sex hormones (e.g., progesterone) and other steroidal drugs in pharmaceutical industry (Oncina et al., 2002; Qin et al., 2006), which has an estrogenic effect on the mammary gland (Aradhana et al., 1992), and plays an important role in control of cholesterol metabolism (Roman et al., 1995). Sources for diosgenin production are mainly from the plants of genera *Dioscorea*, *Costus* and *Trigonella* in the form of steroidal saponins, which attach glucose or rhamnose to aglycone by C-O glucosidic bonds (Zhang et al., 2006; Adham et al., 2009). *Dioscorea zingiberensis* C. H. Wright. is the main resource for diosgenin production in China (Wang et al., 2008). However, overexploitation of natural *D. zingiberensis* leads to rapid decrease of this plant resource and sharp shortage of diosgenin in pharmaceutical synthesis. Cell culture of *D. zingiberensis* has been regarded as an alternative means for efficient and controllable production of diosgenin (Zhou and Wu, 2006). Plant cell culture is the most useful and convenient experimental system for examining effects of various factors on the biosynthesis of desired products and for exploring effective measures to enhance their production. In order to screen appropriate elicitors or high-yield cell strains, it is very important to determine diosgenin content of the cell culture samples of *D.*

*Corresponding author. E-mail: lgzhou@cau.edu.cn.

zingiberensis quantitatively and rapidly.

Up to date, different analytical methods have been developed for diosgenin determination, mainly including gravimetric method (Morris et al., 1958), densitometric thin-layer chromatography (Brain and Hardman, 1968), gas chromatography (Savikin-Fodulovic et al., 1998), spectrometric methods (Sanchez et al., 1972; Chen et al., 2010), high-performance liquid chromatography (Huang et al., 2008; Zhang et al., 2009), and enzyme-linked immunosorbent assay (Li et al., 2010). Of them, spectrophotometry and HPLC are the two usual methods for diosgenin analysis. HPLC analysis has also been widely employed to quantitatively determine other compounds such as nimesulide (Hanif et al., 2011), levobupivacaine (Liu et al., 2011) and sumatriptan (Sheshala et al., 2012). Microplate-spectrophotometry is different from the previous original single cuvette assay, which introduced a multi-well microplate and was automatically measured with the spectrophotometer. Microplate-spectrophotometry has been widely applied in various aspects such as analysis of lipase activity (Choi et al., 2003a), detection of cytochrome P450-carbon monoxide complexes (Choi et al., 2003b), and determination of carbohydrate by phenol-sulfuric acid (Masuko et al., 2005). To the best of our knowledge, there are no reports regarding diosgenin analysis by microplate-spectrophotometry with perchloric acid as color-developing reagent. Microplate-spectrophotometry is an improvement over the classical spectrophotometric method. It can handle of a large amount of samples at the same time for high-throughout analysis of diosgenin content. Comparison studies on microplate-spectrophotometry and HPLC for diosgenin analysis have not been reported either. The purpose of this investigation was to compare the methods of microplate-spectrophotometry and HPLC in order to develop a simple, fast and economic method for diosgenin analysis of a large number of samples.

MATERIALS AND METHODS

Reagents and materials

Acetonitrile was of HPLC grade (Xilong Chemical Ltd., China). Water was purified by using a TTL-30 Mililpore instrument (Beijing Tongtailian Scientific and Technical Ltd., China). Perchloric acid (70 to 72%), sulfuric acid (98%), sodium hydroxide and petroleum ether were of analytical-reagent grade (Beijing Chemical Company, China). Diosgenin was purchased from Sigma (USA). Suspension cells of *D. zingiberensis* were cultured in MS medium supplemented with 1.5 mg/L of 6-benzyladenine (6-BA) and 1.0 mg/L of naphthalene acetic acid (NAA) at 25°C in darkness. After culturing the cells continuously at 120 rpm for 30 days, they were harvested by filtration under vacuum and lyophilized to a constant weight (Zhang et al., 2009).

Instruments and analytical conditions

Spectrophotometric analyses for diosgenin were carried out on a 96-well microplate spectrophotometer (PowerWave HT, BioTek Instruments, USA). Perchloric acid was taken as the color-developing reagent (Slack and Mader, 1961). Detection process of colorimetric assay was optimized in our previous work. The diosgenin standard or diosgenin-containing crude extract was dissolved in acetonitrile, and then an appropriate volume of solution was transferred to each well of the microplate. When acetonitrile evaporated completely at room temperarure, 200 µl of perchloric acid was added to each well. Then the microplate was shaken for 2 min at 30°C. After another 10 min, the stable absorbance of the developed yellow color solution was measured by spectrophotometer at 410 nm against blank.

The HPLC analyses were carried out on an LC-20A system (Shimadzu, Japan), which was composed of two high-pressure solvent delivery pumps, an SPD-M20A photodiode array detector (PAD), an SIL-20AC autosampler, CTO-10AS column oven, and CBM-20Alite system controller. A reversed-phase Agilent TC-C_{18} column (250 mm × 4.6 mm i.d., 5 µm particle size) was used for separation by using a mobile phase of acetonitrile-water (90:10, v/v) at a flow rate of 1 ml/min at 30°C. LC solution multi-PDA workstation was employed to acquire and process chromatographic data. Changes in absorbance at 203 nm were recorded, and spectra from 190 to 400 nm were recorded on-line for peak identification. The peak area was calibrated to diosgenin content with a standard.

Preparation of standard and sample solutions

10 mg of diosgenin was accurately weighed and transferred to a 10-ml volumetric flask. Acetonitrile (about 5 ml) was added to ensure complete solubilization with 10 to 15 min ultrasonic treatment, and the solution was diluted to the constant volume (10 ml) with acetonitrile filtered through a membrane filter (pore size, 0.22 µm). A stock diosgenin solution at 1 mg/ml was then obtained and ready for use.

Extraction of diosgenin from the cultured cells of *D. zingiberensis* was carried out as previously described with some modifications (Zhu et al., 2010). 100 mg of the ground and powdered cell sample was added to each glass tube and immersed in 20 ml of 95% ethanol, and then subjected to ultrasonic treatment for 2 h. After that, 20 ml of 1 mol/L H_2SO_4 was added to each tube to hydrolyze at 121°C for 2 h. The hydrolyte was extracted three times with petroleum ether. The combined petroleum ether solution was subsequently washed with 1 mol/L NaOH, and then washed with distilled water until it became colorless. After dehydration with anhydrous sodium sulfate, the petroleum ether fraction was concentrated to dryness under vacuum on a rotary evaporator. The extract was dissolved in acetonitrile, and then filtered through a filter (pore size, 0.22 µm) before analysis.

Validation

The spectrophotometric and HPLC methods were completely validated according to the procedures described in ICH guidelines Q2 (R1) for the validation of analytical methods (Hubert et al., 2004, 2007).

Linearity

For the method of microplate-spectrophotometry, 2 ml of standard solution containing 1 mg/ml of diosgenin was transferred to a flask, and 6 ml of acetonitrile was added to obtain a 0.25 mg/ml diosgenin solution. Then aliquots of the solution were transferred to wells of microplate, the volumes of which were 8, 16, 24, 32 and 40 µl in triplicate, corresponding to, 2, 4, 6, 8 and 10 µg diosgenin,

respectively. Calibration curve with diosgenin quantity (µg) in each well versus absorbance at 410 nm was plotted and the obtained data were subjected to regression analysis. For HPLC method, aliquots of standard solution containing 1 mg/ml of diosgenin were diluted with acetonitrile, to five different concentrations, corresponding to 6.25, 25, 50, 75 and 100 µg/ml of diosgenin gradient solutions. The injection volume was 10 µl, resulting in 0.0625, 0.250, 0.500, 0.750 and 1.000 µg diosgenin in HPLC analysis. Calibration curve was constructed by plotting the peak area versus the respective diosgenin quantity (µg) and the obtained data were also subjected to regression analysis.

Precision

The intra-day precision was examined by analysis of authentic diosgenin at levels of 3, 5 and 7 µg in each well for spectrophotometry, and 0.1, 0.4 and 0.8 µg for HPLC, which were prepared and analyzed on the same day. Each level of diosgenin was detected for three repetitions (n = 3). Similarly, the inter-day precision was assessed by analyzing the aforementioned three levels of diosgenin on three different days over a period of one week (n = 9). Diosgenin quantity (µg) and the relative standard deviations (RSD) were calculated.

Accuracy

The accuracy of the methods was determined by analyzing the mixtures which were obtained by adding known amounts of standard diosgenin to the crude extract of cells in which diosgenin content was known. The adding amounts of authentic diosgenin were at low, medium and high levels, which were 3, 5 and 7 µg in each well for spectrophotometry, and 0.1, 0.4 and 0.7 µg for HPLC. At each level, samples were prepared in triplicate, and the recovery percentages were determined for microplate-spectrophotometry and HPLC.

Detection and quantification limits

The limit of detection (LOD) and limit of quantification (LOQ) were calculated from the calibration curves as kσ/S, where k = 3.3 for LOD, and k = 10 for LOQ, σ is the standard deviation of the Y-intercept of regression line and S is the slope of the calibration curve. Both of LOD and LOQ were expressed by the detection quantity (µg) of diosgenin in the two mentioned methods.

Diosgenin analysis of the crude extracts samples

Diosgenin content of the crude extracts from cell cultures of *D. zingiberensis* was analyzed by the validated microplate-spectrophotometry and HPLC methods. The solutions of crude extracts were prepared as described earlier. For microplate-spectrophotometry, an aliquot of 200 µl sample solution was transferred to each well of the microplate, in quintuplicate. For HPLC, crude extract solution was first filtered through a 0.22 µm filter, and then an aliquot of 10 µl sample solution was analyzed, in quintuplicate. The analytical conditions for the two methods were as previously described.

Statistic analysis

All treatments were performed in triplicate, and the results were represented by their mean values and the standard deviations (SD). T-test analysis in this work was conducted out at p = 0.05 level.

RESULTS AND DISCUSSION

Determination of detection wavelength

For microplate-spectrophotometry method, diosgenin treated with perchloric acid produced a yellow color immediately. The maximum absorption wavelength of the reaction solution was determined by spectrum scanning in the range of 350 to 700 nm. The spectrum of standard diosgenin was presented in Figure 1A, which showed a single well-defined peak at 410 nm. Figure 1B presented the spectrum of cell culture extract treated with perchloric acid, from which an obvious peak at 410 nm was also observed that indicated existence of diosgenin in crude extract of the cell cultures. Thus, detection of diosgenin content in cell culture materials could be performed by this method.

In case of HPLC, a mobile phase of acetonitrile-water (90:10, v/v) at a flow rate of 1 ml/min at 30°C was applied. The detection wavelength of 203 nm was determined by spectrum scanning from 190 to 400 nm. The representative chromatogram of standard diosgenin was shown in Figure 2A, from which the typical peak of diosgenin and its retention time could be determined. The retention time for diosgenin was 18.064 ± 0.096 min (n = 5). Figure 2B showed the chromatogram of the crude extract of *D. zingiberensis* cell cultures. Compared to the chromatogram of standard diosgenin, it was concluded that there were other constituents except diosgenin in the extract, but the peak of diosgenin in crude extract could be distinguished with the retention time as 18.009 min.

Validation of microplate-spectrophotometry and HPLC

Linearity

Both methods were investigated for linearity with the regression analysis data as shown in Table 1. The linearity of the calibration graph and the adherence of the system to Beer's Law were validated by the high value of the correlation coefficient of the regression equation and by the low value of the intercept on the ordinate. The slopes of both methods were significant as the values of $t_{calculated}$ for both of the slopes were greater than those of $t_{theoretical}$ by Student's *t*-test. However, the experimental intercepts of the two methods were not significant different from theoretical zero value because the values of $t_{calculated}$ for both of the intercepts were less than those of $t_{theoretical}$. Thus, it was concluded that the two methods in this work possessed good enough linearity in the assayed range.

Precision

The intra- and inter-day precisions for both methods were

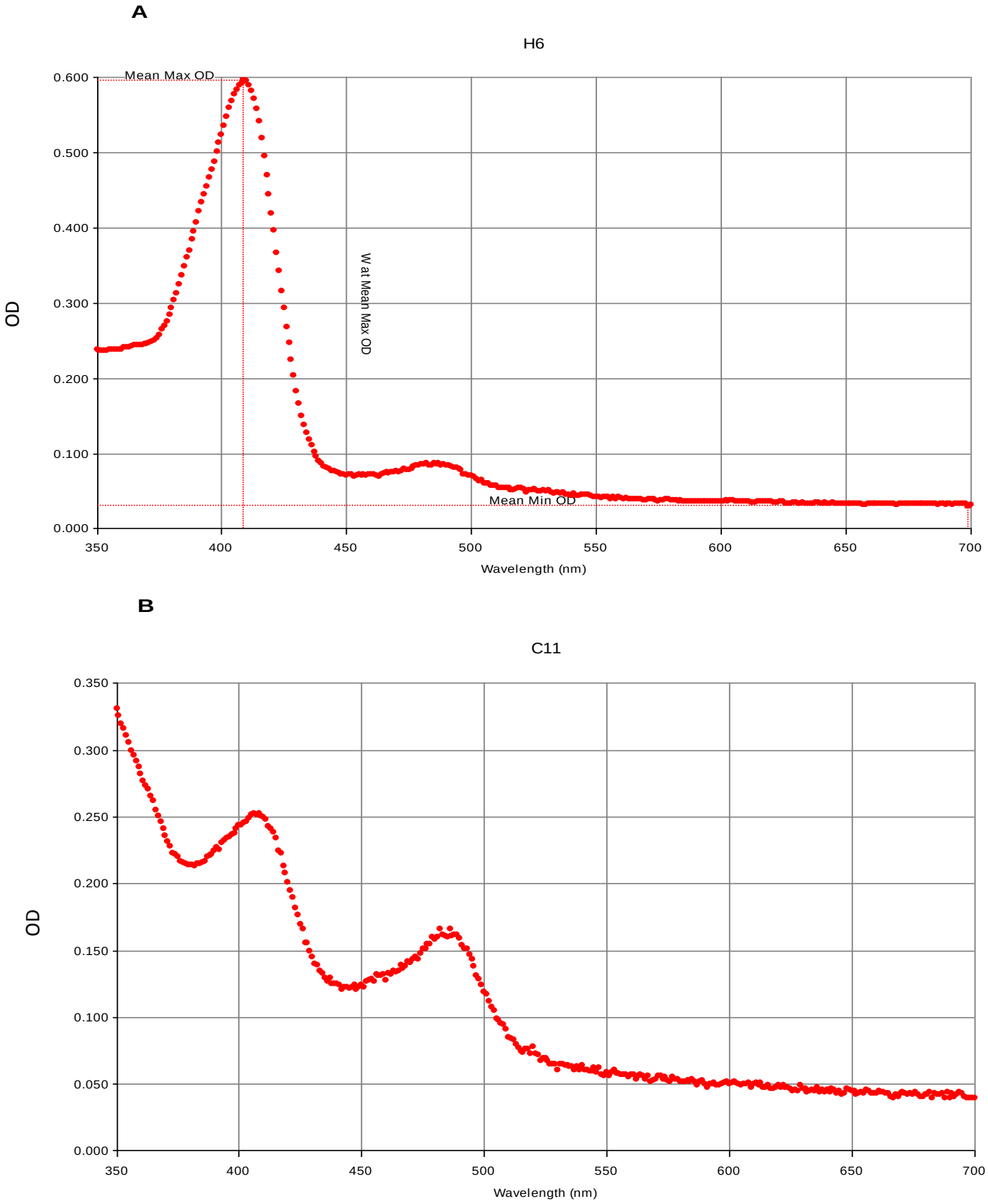

Figure 1. Absorption spectra of the mixtures of percholoric acid reacted with diosgenin (A) and cell culture extract (B), respectively.

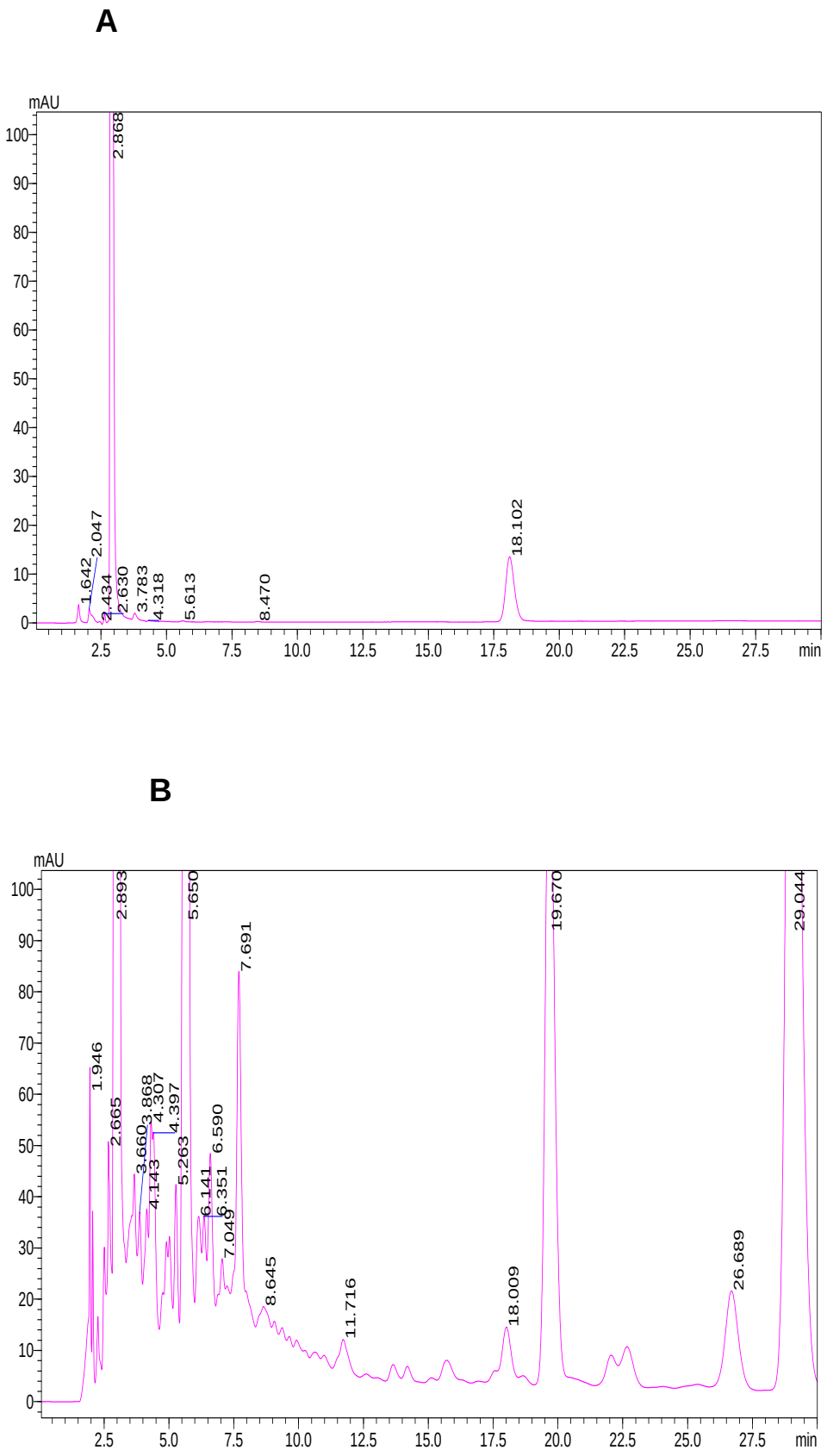

Figure 2. Chromatograms of diosgenin (A) and cell culture extract (B) obtained by HPLC. Retention time (RT) for diosgenin was 18.064 ± 0.096 min.

Table 1. Analytical parameters for the calibration curve data obtained for diosgenin by microplate-spectrophotometry and HPLC methods.

Parameter	Microplate-spectrophotometry	HPLC
Detection wavelength (nm)	410	203
Linearity range (µg)	2-10	0.0625-1.000
Regression equation	Y = 0.1218X + 0.0404	Y = 559631.1536X + 5096.0938
Correlation coefficient (R)	0.9988	0.9995
$S_{y/x}$	0.0215	7735.7323
S.D. of slope (n = 5)	0.0034	10285.7777
S.D. of intercept (n = 5)	0.0226	6305.2845
$t_{calculated}$ for slope	35.8208, significant	54.4082, significant
$t_{calculated}$ for intercept	1.7912, not significant	0.8082, not significant
$t_{theoretical}$ (n = 5, p = 0.05)	3.182	3.182
LOD (µg)	0.6111	0.0372
LOQ (µg)	1.8518	0.1127

Table 2. Evaluation of the intra- and inter-day precisions by employing microplate-spectrophotometry and HPLC for diosgenin determination.

Actual quantity (µg)	Intra-day		Inter-day	
	Quantity detected* (µg)	R.S.D. (%)	Quantity detected ** (µg)	R.S.D. (%)
Microplate-spectrophotometry				
3.000	2.900 ± 0.025	0.86	2.993 ± 0.100	3.35
5.000	5.128 ± 0.097	1.89	5.070 ± 0.135	2.71
7.000	6.978 ± 0.177	2.54	7.007 ± 0.207	1.95
HPLC				
0.100	0.112 ± 0.002	1.36	0.101 ± 0.003	2.36
0.400	0.405 ± 0.003	0.75	0.401 ± 0.011	2.74
0.800	0.795 ± 0.007	0.86	0.804 ± 0.013	1.65

*, Values represent mean ± S.D., n = 3; **, Values represent mean ± S.D.; n = 9.

summarized in Table 2. The means of R.S.D. (%) for intra-day detection at three different levels were 1.76% for spectrophotometry and 0.99% for HPLC respectively, both of which were lower than 2.0% (Cesar et al., 2008). It indicated that both methods had fairly good repeatability. The low mean values of R.S.D. (%) for inter-day detection by employing spectrophotometry and HPLC were 2.98 and 2.34%, respectively, which demonstrated both of the methods were characterized by rather good reproducibility.

Accuracy

Accuracy was calculated by means of a standard addition experiment. The results of recovery studies were shown in Table 3. The mean recovery for spectrophotometry method was 99.30% (n = 9), with low mean value of RSD as 2.03%. The mean recovery for HPLC method was 99.85% (n = 9), with low mean value of RSD as 1.31%. The satisfactory recoveries and low values of R.S.D. (%) confirmed the suitability of both proposed methods for analysis of diosgenin.

Detection and quantification limits

The limit of detection (LOD) and limit of quantification (LOQ) were presented in Table 1. For microplate-spectrophotometry, the LOD and LOQ were 0.6111 and 1.8518 µg, respectively. For HPLC, the LOD and LOQ were 0.0372 and 0.1127 µg, respectively.

Diosgenin detection in crude extracts of dry cells of *D. zingiberensis*

Diosgenin content of the cell culture samples of *D.*

Table 3. Recovery analyses obtained for diosgenin determination in the mixtures.

Amount labeled (μg)	Amount added (μg)	Amount recovered * (μg)	Recovery yield (%)	R.S.D. (%)
Microplate-spectrophotometry				
2.000	3.000	3.021 ± 0.030	98.89	2.25
2.000	5.000	5.082 ± 0.095	99.84	1.41
2.000	7.000	7.107 ± 0.148	99.19	2.42
HPLC				
0.200	0.100	0.100 ± 0.002	99.93	1.74
0.200	0.400	0.399 ± 0.004	99.83	1.01
0.200	0.700	0.698 ± 0.008	99.78	1.18

*, Values represent mean ± S.D., n = 3.

Table 4. Diosgenin analyses in cell crude extracts of *D. zingiberensis* by employing microplate-spectrophotometry and HPLC methods.

Sample no.	Diosgenin content (mg/g)	
	Microplate-spectrophotometry	**HPLC**
1	0.154	0.148
2	0.149	0.149
3	0.160	0.150
4	0.165	0.154
5	0.158	0.151
Mean ± S.D.	0.157 ± 0.006	0.151 ± 0.002
$t_{calculated}$ for means	2.115, no significant difference between two methods	
$t_{theoretical}$ (v = 8, p = 0.05)	2.306	

zingiberensis was analyzed by the validated microplate-spectrophotometry and HPLC methods. The analysis results were presented in Table 4. The diosgenin content determined by microplate-spectrophotometry was 0.157 ± 0.006 mg/g, while diosgenin content determined by HPLC was 0.151 ± 0.002 mg/g. Student's *t*-test was conducted to compare the results obtained by microplate-spectrophotometry and HPLC. It was found that $t_{caluculated}$ was 2.115 (p = 0.05), while $t_{theoretical}$ was 2.306, which demonstrated there no significant difference exist between the two methods with respect to mean values.

Spectrophotometry and HPLC for diosgenin detection were compared in this study, both of which were validated according to analytical procedures text and methodology Q2 (R1). The results showed the two methods were characterized by satisfactory linearity, precision and accuracy. When the two methods were applied to quantify diosgenin content in cultured cells of *D. zingiberensis*, the value of diosgenin content detected by spectrophotometry was slightly higher than that by HPLC. However, no significant difference was observed when we conducted *t*-test at a confidence level of 0.05 between the results obtained by the two methods, which demonstrated feasibility of the two approaches for diosgenin determination.

LOD and LOQ of the two methods were presented in Table 1, from which it was concluded that HPLC was the more sensitive method for diosgenin detection. However, we cannot discharge the analyses time and cost. When HPLC was employed to determine diosgenin content, the analysis time of each sample was at least 30 min, and the volume of acetonitrile with high price as mobile phase was 27 ml for each time. Thus, when we encounter a large number of samples detected by HPLC, too long analysis time and too high analysis cost are two problems that should not be neglected. Hence, it is necessary to develop a cheap, reliable, simple and fast assay for diosgenin determination.

The spectrophotometry for diosgenin determination referred in our study was different from the previous classical colorimetric assays performed with cuvettes, which utilized multi-well microplate for realizing high-throughout detecting. Though less sensitivity, compared to HPLC, was observed when sepctrophotometry was applied to analyze diosgenin, the results were in good agreement with those obtained by HPLC. However, the

drawback of spectrophotometry still exists, which is that the impurities in crude diosgenin extracts may interfere with the assay. The problem may be mitigated by carrying out control measurements. Thus it was suggested that multi-well microplate-spectrophotometry in our work was also suitable for diosgenin determination.

The analysis time by 96-well microplate-spectrophotometry every time was just 12 min, which is less than half of that by HPLC. That is to say, we can analyze 96 samples within 12 min. And the volume of color-developing reagent perchloric acid was just 200 µl for each sample, which reduced the analysis cost greatly. Thus, when many samples need to be analyzed, we can employ multi-well microplate-spectrophotometry to determinate diosgenin content, which can compensate the disadvantages of HPLC.

Conclusion

This is the first report to determine diosgenin content by multi-well microplate-spectrophotometry with perchloric acid as color-developing reagent. The rapidity, accuracy, simplicity and economicality of application of this proposed microplate-spectrophotometry assay make it an optimal analytical alternative for high-throughout analyzing of diosgenin content of hundreds of samples, though HPLC is more sensitive for diosgenin analysis.

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REFERENCES

Adham NZ, Zaki RA, Naim N (2009). Microbial transformation of diosgenin and its precursor furostanol glycosides. World J. Microbiol. Biotechnol., 25: 481-487.

Aradhana Rao AR, Kale RK (1992). Diosgenin-a growth stimulator of mammary gland of ovariectomized mouse. Indian J. Exp. Biol., 30: 367-370.

Brain KR, Hardman R (1968). An improved method of densitometric thin layer chromatography as applied to the determination of sapogenin in *Dioscorea* tubers. J. Chromatogr. A, 38: 355-363.

Cesar IC, Nogueira FHA, Pianetti GA (2008). Comparison of HPLC, UV spectrophotometry and potentiometric titration methods for the determination of lumefantrine in pharmaceutical products. J. Pharm. Biomed. Anal., 48: 223-226.

Chen H-G, Jin F-Y, Zhao C, Zhou X, Gong X-J (2010). Content determination of total saponins in *Triganella joenumgraecum* L. Chin. J. Spectrosc. Lab., 27: 580-582.

Choi SJ, Hwang JM, Kim SI (2003a). A colorimetric microplate assay method for high throughput analysis of lipase activity. J. Biochem. Mol. Biol., 36: 417-420.

Choi SJ, Kim M, Kim SI, Jeon JK (2003b). Microplate assay measurement of cytochrome P450-carbon monoxide complexes, J. Biochem. Mol. Biol., 36: 332-325.

Hanif M, Shoaib MH, Yousuf RI, Khan A, Anwer S, Rasul A, Sattar S, Arshad HM (2011). Reverse phase high performance liquid chromatographic (HPLC) method for nimesulide tablets dosage form prepared for *in vivo in vitro* correlation (IVIVC) studies. Afr. J. Pharm. Pharmacol., 5: 2342-2348.

Huang W, Zhao H, Ni J, Zuo H, Qiu L, Li H, Li H (2008). The best utilization of *D. zingiberensis* C.H. Wright by an eco-friendly process. Bioresource Technol., 99: 7407-7411.

Hubert P, Nguen-Huu J-J, Boulanger B, Chapuzet E, Chiap P, Cohen N, Compagnon P-A, Dewe W, Feinberg M, Lallier M, Laurentie M, Mercier N, Muzard G, Nivet C, Valat (2004). Harmonization of strategies for the validation of quantitative analytical procedures. A SFSTP proposal – part I. J. Pharm. Biomed. Anal., 36: 579-586.

Hubert P, Nguen-Huu J-J, Boulanger B, Chapuzet E, Chiap P, Cohen N, Compagnon P-A, Dewe W, Feinberg M, Lallier M, Laurentie M, Mercier N, Muzard G, Nivet C, Valat, Rozet E (2007). Harmonization of strategies for the validation of quantitative analytical procedures. A SFSTP proposal – part II. J. Pharm. Biomed. Anal., 45: 70-81.

Li J, Yang D, Yu K, He J, Zhang Y (2010). Determination of diosgenin content in medicinal plants with enzyme-linked immunosorbent assay. Planta Med., 76: 1915-1920.

Liu J, Zhou X, Jiang J (2011). A validated HPLC method for the determination of levobupivacaine in plasma and its application to pharmacokinetic studies in Chinese people. Afr. J. Pharm. Pharmacol., 5: 2232-2237.

Masuko T, Minami A, Iwasaki N, Majima T, Nishimura S-I, Lee YC (2005). Carbohydrate analysis by a phenol-sulfuric acid method in microplate forma. Anal. Biochem., 339: 69-72.

Morris MP, Roark BA, Cancel B (1958). Simple procedure for the routine assay of *Dioscorea* tubers. J. Agric. Food Chem., 6: 856-858.

Oncina R, Del Rio JA, Gomez P, Ortuno A (2002). Effect of ethylene on diosgenin accumulation in callus cultures of *Trigonella foenum-graecum,* L. Food Chem., 76: 475-479.

Qin H, Tian W, Lin C (2006). A highly efficient synthesis of 22-deoxy-OSW-1 by utilizing the intact skeleton of diosgenin, Tetrahedron Lett., 47: 3217-3219.

Roman ID, Thewles A, Coleman R (1995). Fractionation of livers following diosgenin treatment to elevate biliary cholesterol. Biochem. Biophys. Acta, 1255: 77-81.

Sanchez GL, Acevedo JCM, Soto RR (1972). Sepctrophotometric determination of diosgenin in *Dioscorea composita* following thin-layer chromatography. Analyst, 97: 973-976.

Savikin-Fodulovic K, Grubisic D, Culafic L, Menkovic N, Ristic M (1998). Diosgenin and phytosterols content in five callus lines of *Dioscorea balcanica,* Plant Sci., 135: 63-67.

Slack SC, Mader WJ (1961). Colorimetric assay for diosgenin and related compounds. Anal. Chem., 33: 625-627.

Sheshala R, Khan N, Darwis Y (2012). Validated high performance liquid chromatography (HPLC) method for the determination of sumatriptan in rabbit plasma: application to pharmacokinetic study, Afr. J. Pharm. Pharmacol., 6: 98-107.

Wang Y, Liu H, Bao J, Hong Y, Yang Z, Zhang C (2008). The saccharification- membrane retrieval-hydrolysis (SMRH) process: a novel approach for cleaner production of diosgenin derived from *Dioscorea zingiberensis*. J. Clean. Prod., 16: 1133-1137.

Zhang C, Wang Y, Yang Z, Xu M (2006). Chlorine emission and dechlorination in co-firing coal and the residue from hydrochloric acid hydrolysis of *Discorea zingiberensis*. Fuel, 85: 2034-2040.

Zhang R, Li P, Xu L, Chen Y, Sui P, Zhou L, Li J (2009). Enhancement of diosgenin production in *Dioscorea zingiberensis* cell culture by oligosaccharide elicitor from its endophytic fungus *Fusarium oxysporum* Dzf17. Nat. Prod. Commun., 4: 1459-1462.

Zhou L, Wu J (2006). Development and application of medicinal plant tissue cultures for production of drugs and herbal medicinals in China. Nat. Prod. Rep., 23: 789-810.

Zhu Y, Huang W, Ni J (2010). A promising clean process for production of diosgenin from *Dioscorea zingiberensis* C. H. Wright. J. Clean. Prod., 18: 242-247.