Validated Method to Determine (±)-Gossypol in *Candida tropicalis* Culture by High-Performance Liquid Chromatography

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A validated high-performance liquid chromatography (HPLC) method has been developed to analyze the (±)-gossypol in the selection of strains of *Candida tropicalis* culture. Since gossypol was easily degraded and oxidized, the addition of antioxidant NADPH- Na_4 and acetone extraction was chosen to prevent gossypol degradation and gradient elution assay was applied to obtain gossypol resolution. Concentrations of gossypol in *C. tropicalis* ZD-3 culture 20 $\mu\text{g/mL}$ were determined, and concentration–time profiles were observed. Linearity of the gossypol standard curve by HPLC area method was ranged from 0.1 to 20 $\mu\text{g/mL}$ with $Y = 26.954 \times X - 29.547$, $R^2 = 0.9991$, and $n = 3$, with limit of detection (LOD) of 50 ng/mL and lower limit of quantification (LLOQ) of 500 ng/mL. The recovery rate is dose-dependent and ranged from 85.3% to 103.5%. It is a rapid and reliable HPLC method for gossypol quantization in microorganism culture which could be applied in solid fermentation in the feed industry.

Keywords: HPLC, gossypol, *Candida tropicalis* culture, acetone, gradient elution

Introduction

Gossypol (Figure 1) is a yellow polyphenol pigment present in the gland of cotton tissue such as leaf, seed, and stem. Gossypol exists in cottonseed products in two forms, which were defined as free gossypol (FG) and bind gossypol (BG) by the American Oil Chemists' Society (AOCS) official method. The FG with biological activity includes gossypol and gossypol derivatives. BG was bound to amine products such as amino acids and peptides during cottonseed meals' processing, which was insoluble in ether, chloroform, and aqueous acetone [1]. FG is toxic to animal [2, 3], principally because of the existence of (±)-gossypol in cotton byproducts such as cottonseed meal, cottonseed cake, and cotton hulls. The adverse effects limit the amount of the cottonseed meal as protein feed in the animal diet. Therefore, the gossypol is an enormous obstacle to increase cottonseed meal in feed supplement for livestock. Except for extruding, extraction, heating, and chemical detoxification of gossypol, the microbial fermentation is a commercially available method in China. It is an optimal measure that separates and screens strain of *Candida tropicalis* or *Aspergillus niger* and then ferments in solid-state cottonseed meal for gossypol detoxification [4–6]. The strong binding of gossypol with protein to form the Schiff base and the exoenzyme inducing by microbial was proposed to be the reason that the free gossypol was eliminated significantly in solid-state fermentation of cottonseed meal by fungi [4–6]. However, a method to determine the remaining “free gossypol” is absent to evaluate the screened bacterial strains.

The earlier spectrophotometry method which determined gossypol level according to the color reaction of the aldehyde group of aniline was time-consuming as well as gossypol content exaggerating and has less sensitivity [7, 8]. Meanwhile, the new developed report of the determination of gossypol carried out on animal plasma [9–11], tissue [11, 12], and the cottonseed [8, 13] and oil [14] was mostly based on high-performance liquid chromatography (HPLC) [11, 13], high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS)

Chemical Formula: $\text{C}_{30}\text{H}_{30}\text{O}_8$
Molecular Weight: 518.56
Gossypol

Figure 1. The chemical structure of gossypol

[9], and capillary electrophoresis [15] techniques. Both AOCS official method and the previous HPLC method were not fully validated for the quantitative analysis of gossypol in fungi culture. The instability of the gossypol and the strong binding of gossypol with protein cause the lack of a validated method for the quantitative analysis of free gossypol in fungi culture or enzyme reaction system. To rapidly screen the strains of *C. tropicalis* with biodegradability on gossypol, it is essential to build an analytical method to detect the remaining amount gossypol in the biological samples. In the present study, an accurate, suitable, and sensitive with a minimum loss of gossypol analysis method was obtained for quantification of gossypol in fungi's strain screen.

Experimental

Chemicals and Reagents. The (±)-gossypol was purchased from Solarbio Co. (Beijing, China), and its purity was analyzed as 99.5% by HPLC. Methanol and acetonitrile solvents (chromatograph grade) were purchased from Sigma-Aldrich Co. (Shanghai, China) and Thermo Fisher Scientific Co. (Waltham, USA), respectively. Methanoic acid (chromatograph grade) was purchased from Chemical Industry Research Institute (Tianjin, China). Strains of *C. tropicalis* ZD-0 and ZD-3 were screened in the Feed Science Institute, Zhejiang University in 2006 and stored in our laboratory.

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Instrument and Chromatographic Conditions. The Agilent 1200 liquid chromatography (LC) consisted of G1311 Quaternary Pump with G1322 Vacuum Degasser for the mobile phase. Samples were injected by G1329 autosampler (ALS) fitted with a 20 µL sample loops. The same solution was used to flush the needle. The complexing reactants were separated by Agilent 1200 HPLC with a SUPELCOSIL C₁₈ column (4.6*250 mm, 5 µm), while the G1316 Thermostatted Column Compartment was maintained at 30 °C and an ultraviolet–visible (UV–vis) diode array detector setting, at 380 nm. The sample was gradient eluted with acetonitrile–H₂O containing 0.3% methanoic acid at the flow rate of 1.0 mL/min. Data acquisition and quantization of gossypol were performed using Chemstation Software.

Method Development. The extract solution and gradient elution were optimized to obtain better HPLC gossypol peak resolution. Preparation of gossypol solution was done by dissolving 20 mg (±)-gossypol in 100 mL acetone solvent in a brown vial. The storage solution containing 200 µg/mL gossypol was subsequently diluted to a standard serial solution containing gossypol from 0.01 to 20 µg/mL. All the gossypol solutions were freshly prepared before analysis. The calibration curves were plotted by the area of gossypol peak with corresponding concentration.

Gradient Elution. In order to achieve a good resolution of gossypol in acetone solution, the gradient elution was attempted in which the moving phase consisted of (A) acetonitrile with (B) 0.3% solution of methanoic acid. The moving phase was delivered at a flow rate of 1 mL/min, and the injection of 20 µL samples was assayed at 380 nm. The different kinds of gradient elution, including linear, two-step, and three-step ways were attempted to obtain the optimal way. The specific gradient elution procedure was shown in Table 1.

Method Validation. The strains of two *C. tropicalis* ZD-0 and ZD-3 stored at –80 °C were allowed to grow in 5 mL yeast extract peptone dextrose medium (YPD medium 1% yeast extracts, 2% peptone, and 2% glucose) and shaken at 30 °C until the optical density (OD) reached 2.0. The *C. tropicalis* ZD-0 strain was the initial strain, which has been used as the control group. The screened ZD-3 was capable of degrading gossypol in solid cottonseed meal fermentation. Afterwards, a loop of the culture was plated in YPD agar mediums. The single clone was picked up and then further grown in 50 mL YPD medium. Treatments of 5 mL ZD-3 culture were initiated by adding 100 µg gossypol during the start of the logarithmic growth stage when OD value was 2.0. The prepared culture gossypol concentration was 20 µg/mL. Identical volumes of prepared ZD-0 without gossypol cultures were performed at the blank sample.

Determination of the selective method was tested by three individual initial strain ZD-0 cultures spiked with gossypol and diluted in a series of acetone solution. The distinctive calibration standard curves were prepared and analyzed on three separated days. The limit of detection (LOD) was defined as the signal to noise ratio of 3.0 when the lowest concentration of the sample could be measured reliably. The lower limit of quantification (LLOQ) was defined as the signal to noise ratio of 10.0 and the precision relative standard deviation (RSD) of the lowest concentration within 20%.

Stability of gossypol in *C. tropicalis* culture was assessed by spiking the gossypol into the prepared blank ZD-0 culture at four different temperatures. The freshly prepared *C. tropicalis* blank culture was spiked with gossypol and antioxidant and extracted subsequently as described above. The pooled supernatants contained three levels of gossypol concentration (namely, 0.2, 2.0, and 10.0 µg/mL) which were stored at 4 °C, room temperature (24 °C) for 24 h, respectively. Recovery of gossypol in culture was measured by comparing the response of gossypol at three concentrations (50, 200, and 1000 µg/mL) after extraction. Accuracy and precision of the method were determined from triplicate samples of standard curve concentrations. The assay was conducted on the same day and the next day for the intra- and inter-day accuracy.

Preparation of the Culture Sample and the Calibration Standards. To evaluate the ability of *C. tropicalis* ZD-3 biodegrading to gossypol, each 300 µL sample volume was collected at 0, 0.5, 1, 1.5, 2, 3, and 6 h intervals. The samples were gained with the antioxidant 25 µL 25 mM NADPH-Na₄ and 700 µL acetone extractions following high speed refrigerated centrifuge (4 °C) at 12,000 rpm for 2 min. The supernatant was filtered through 0.45 µm Whatman filter before injecting into the HPLC system. The injection volume was set at 20 µL. The standard solutions were prepared by the initial strain ZD-0 liquid. The culture was spiked with the gossypol acetone solution subsequently diluted by acetone to a series of gossypol stock solution at the concentration ranging from 0.1 to 20 µg/mL.

Results and Discussion

Method Development. The previous study indicated that the order of the gossypol stability in solvents followed acetone > acetonitrile > methanol [8]. Methanol and acetonitrile solution can only stabilize gossypol at very low temperature and complicated compounds would be formed between methanol solvent and gossypol at the analytical level [8] that had been confirmed in this study as showed in Figure 2. The small amount of gossypol remaining in *C. tropicalis* culture is unstable and

Table 1. Effects of different mobile phase components and their proportion on the separation of gossypol in culture

Elution assay	Mobile phase and its proportion (v/v)	Retention time/min	Resolution	
Gradient elution	Acetonitrile (A) and 0.3% methanoic acid solution (B)	0–0.01 min, 40 % A; 0–5 min, 40% A; 5–10 min, 50% A; 10–15 min, 60% A; 15–20 min, 70% A; 20–25 min, 80% A; 25–30 min, 90% A; 30–35 min, 40% A	28.24	None
		0–0.01 min, 40 % A; 0.01–5 min, 65 % A; 5–10 min, 65% A; 10–15 min, 85% A; 15–25 min, 85% A; 25–30 min, 40% A	17.96	None
		0–0.01 min, 40 % A; 0.01–6 min, 65 % A; 6–12 min, 75% A; 12–17 min, 75% A; 17–23 min, 40% A	8.21	0.93
		0–0.01 min, 40 % A; 0.01–6 min, 75 % A; 6–12 min, 85% A; 12–17 min, 85% A; 17–23 min, 40% A	13.12	1.89
		0–0.01 min, 40 % A; 0.01–6 min, 75 % A; 6–12 min, 90% A; 12–17 min, 90% A; 17–23 min, 40% A	12.73	1.05

Figure 2. The sample was analyzed by HPLC 3 days later and various compounds were formed between methanol and the (±)-gossypol

rarely extractable in the solvent which is the big obstacle to examine the effect of the microorganism on gossypol degradation. Thus, gossypol was extracted from fungi culture with 70% acetone aqueous in which the free gossypol would be effectively extracted. However, even under nitrogen, drying the gossypol ended up with poor recovery. Thus, the *C. tropicalis* culture added with gossypol and antioxidant NADPH- Na_4 was depoliticized and extracted with acetone and after that directly filtered in preparation for analysis.

Gradient Elution. The moving phase consisting of acetonitrile (A) and 0.3% methanoic acid solution (B) was at a flow rate of 1.0 mL/min. Acetonitrile was used as the mobile phase because the gossypol was relatively stable and had very low water solubility. The common solvent such as methanol is reported to have a poor ability to stabilize gossypol, and instead, it will react to form some complicated products during the storage at room temperature [16]. The addition of methanoic acid is useful to suppress the byproduct peaks comparing with the phosphoric acid and to reduce the gossypol oxidation of the phenolic hydroxyl. Considering column life and the resist of tailing peak [16], the concentration of methanoic acid solution was 0.3%. The conventional strategy is to evaporate the acetone and dissolve the residue in methanol solution prior to HPLC analysis. At first, gossypol acetone solution was eluted with acetonitrile– H_2O containing 0.3% methanoic acid at 254 nm, but the gossypol peak was undetectable since it was over covered by acetone signal peak. The method had been abandoned because of the anxiety about the proceeding loss of few remaining amount of gossypol during the process. The antioxidant NADPH- Na_4 [17, 18] or glutathione-reduced [11] was added to stabilize the gossypol. To separate acetone and gossypol chromatographic profile, the UV detectable wavelength was monitored at 380 nm, which were not interfered with the reagent and the relative maximum gossypol absorption [7]. The gradient elution including linear (the portion of acetonitrile from 40% to 90%), two-step (the portion of acetonitrile stayed at 65% and 85% for 10 minutes, respectively), and three kinds of three-step (the portion of acetonitrile stayed from 65% to 75%, 75% to 85%, and 75% to 90% for 6 min, respectively) were attempted to acquire the excellent gossypol peak symmetry (Table 1). The elution was attempted to find out that simultaneous peak profile of gossypol was clearer and the loss of the gossypol metabolites with the increasing of the organic phase portion. The linear and the two-step gradient elution methods were dismissed because of the absence of small peaks before the gossypol peak and too long retention time (28.24 min and 17.96 min) (Table 1). Based on the principle, that the material was eluted more quickly through increasing the portion of the organic solvent in the mobile phase, the elution program was adjusted gradually to separate the gossypol and its decomposition products in acetone

Figure 3. Comparison of three kinds of three-step gradient elution to separate gossypol profile peaks. (a) The portion of acetonitrile (A) was gradually increasing from 65% to 75% with resolution of 0.93 and retention time at 8.21 min; (b) the portion of acetonitrile (A) from 75% to 85% with resolution of 1.89 and retention time at 13.12 min; (c) the portion of acetonitrile (A) from 75% to 90% with resolution of 0.93 and retention time at 12.73 min

solution. When the portion of acetonitrile was from 65% to 75%, the poor separation with resolution (RS) ratio of 0.93 was observed between the gossypol decomposition and the gossypol profile (Figure 3a). The chromatographic peaks RS improved to 1.89 and retention time (RT) of 13.12 min when the moving phase consisting of 85% acetonitrile (Figure 3b). As the portion of acetonitrile increased from 75% to 95%, the loss of the small peaks before the gossypol peak appeared and the RS was decreased to 1.05 (Figure 3c). To achieve better resolution of gossypol peaks, the following procedure was adopted. The mobile phase composition was acetonitrile (A) with 0.3% methanoic acid solution (B) and was increased linearly to 75% A/25% B over a 6 min period, and then held at 85% A/15% B for 6 min, before returning to 40% A/60% B (Table 1). In this way, the resolution and separation of gossypol were excellent and the retention time is 13.12 min (Figure 3b).

The separation of gossypol chromatogram peak was broadened in the mobile phase consisting of 85% methanol and 15% phosphoric acid (0.3%) solution (Figure 4) at 254 nm with RS of 1.34 and RT of 8.58 min. In comparison with the optimized gradient elution method, in the constitution of the

Figure 4. The mobile phase consisted of 85% methanol and 15% phosphoric acid (0.3%) solution with resolution of 1.34 and retention time at 8.58 min

mobile phase containing 75% acetonitrile (A) gradually increased to 85% and monitored at 380 nm (Figure 3b) had a better RS of 1.89 and the RT was 13.12 minutes. Linearity of the gossypol standard curve by the optimized gradient elution method was ranging from 0.1 to 20 µg/mL with $Y = 26.954 \times X - 29.547$, $R^2 = 0.9991$, and $n = 3$, with LOD of 50 ng/mL and LLOQ of 500 ng/mL.

Method Validation. Data presented in Table 2 showed that the gossypol that was stored at -4°C has a preferable stability compared with the gossypol stored at room temperature (25°C) in which the gossypol appeared to decompose to 99.6% and 82.3% within 24 h, respectively. The gossypol was then performed at 30°C condition for 6 h as this temperature was appropriate for the growth of the *C. tropicalis*. The gossypol had a good stability when it was stored at a lower temperature (-80°C) within 1 month. The previous study demonstrated that the gossypol was only stable under very low temperature for long periods of time [8]. In general, the acetone solution is the optimal option for stock and degradation research of gossypol. The measurement of the intra- and inter-day accuracy and precision was assayed on two separate days in triplicate samples. The results of Table 3 demonstrate that the intra-day and inter-day precisions ranged from 2% to 10%. The recovery of gossypol was determined by spiking the *C. tropicalis* culture to three different concentrations of gossypol solution and extracted using acetone, which had been described above. The percent recovery was expressed by comparing the culture spiking

Table 2. Stability of gossypol in *C. tropicalis* ZD-0 culture in four treatments by HPLC assay ($n = 3$, mean $\pm$ SD)

Added concentration (µg/mL)	4°C , 24 h	Room temperature, 24 h	30°C , 6 h	-80°C , 1 month
0.2	95.6 ± 0.6	82.3 ± 0.4	82.3 ± 0.6	99.4 ± 0.5
2	98.2 ± 0.3	81.2 ± 0.3	85.3 ± 0.2	97.6 ± 0.3
10	99.2 ± 0.3	87.2 ± 0.5	87.7 ± 0.3	96.2 ± 0.4

Table 3. Intra-day and inter-day accuracy and precision of calibration standards spiked with gossypol and extracted according to the method ($n = 3$, unless otherwise mentioned)

Nominal concentration (µg/mL)	Intra-day ($n = 3$)			Inter-day ($n = 3$)		
	Measured (µg/mL) Mean $\pm$ SD	Accuracy (%)	Precision (%)	Measured (µg/mL) Mean $\pm$ SD	Accuracy (%)	Precision (%)
0.1	0.10 ± 0.01	100.3%	10%	0.10 ± 0.01	99.3%	10%
1	1.01 ± 0.10	100.7%	10%	1.04 ± 0.1	104.0%	10%
5	4.88 ± 0.49	97.7%	10%	5.22 ± 0.39	104.3%	7%
10	9.83 ± 0.21	98.3%	2%	9.73 ± 0.45	97.3%	5%
20	21.28 ± 1.01	106.4%	5%	20.85 ± 1.18	104.2%	6%
50	51.33 ± 1.01	102.7%	2%	50.27 ± 1.95	100.5%	4%

Table 4. Extraction recovery of gossypol in *C. tropicalis* ZD-0 culture

Nominal concentration (µg/mL)	Recovery (%)
50	85.3
200	88.6
1000	103.5

Figure 5. The concentration of (±)-gossypol in *C. tropicalis* ZD-3 culture after 6 h from the initial adding gossypol of 20 µg/mL

gossypol solution and the water spiking of the sample. The recovery rate is dose-dependent and ranged from 85.3% to 103.5% (Table 4).

The Application of the Method in *Candida tropicalis* ZD-3 Culture. The *C. tropicalis* ZD-3 was the screened strain with capability of detoxification on gossypol in our laboratory [5]. The proposed metabolism pathway of gossypol in *C. tropicalis* culture was as follows: the binding gossypol was produced by amine products combined with free gossypol; the detoxification of free gossypol by exoenzyme from *C. tropicalis*; and the free gossypol spontaneously oxidized [2–4]. This HPLC method was applied to determine the remaining gossypol and its metabolites in *C. tropicalis* ZD-3 culture after the addition of gossypol to 20 µg/mL concentration. Concentrations of gossypol in *C. tropicalis* ZD-3 culture (20 µg/mL) were determined as has been mentioned above and the concentration–time profiles were shown in Figure 5. The gossypol concentration decreased rapidly from 19.74 µg/mL to 0.62 µg/mL in the first 2 h after the gossypol addition. Then, the slower rate was measured from 2 to 4 h and the remaining amount of gossypol reduced from 0.62 µg/mL to 0.02 µg/mL.

Conclusion

A new HPLC method was quantitatively developed to determine the gossypol in *C. tropicalis* culture. This method could provide a rapid and reliable assay to screen the strains of the bacterium for the researchers and the application in the solid fermentation in feed industry. The acetone extraction and gradient elution were implemented in gossypol analysis via HPLC.

Linearity of the gossypol standard curve by HPLC area method was ranged 0.1 to 20 µg/mL with $Y = 26.954 \times X - 29.547$, $R^2 = 0.9991$, and $n = 3$, with LOD of 50 ng/mL, and LLOQ of 500 ng/mL. The recovery rate is dose-dependent and ranged from 85.3% to 103.5%. In general, the reliable HPLC assay is accurate and effective to quantify the amount of ($\pm$)-gossypol in *C. tropicalis* culture for the study on ($\pm$)-gossypol degrade-strains selection and the applied in fermented feed in the future.

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