CHAPTER II

EXPERIMENTAL

2.1 Chemicals and Materials

Gibberellic acid (GA_3) and sodium dodecyl sulfate (SDS) were purchased from Fluka. Disodium tetraborate ($Na_2B_4O_7.10H_2O$) was supplied by Riedel-de Haën. 3-Amino-4-methylbenzoic acid (AMBA) used as an internal standard in CE for analysis of GA_3 was obtained from Fluka. 3-Acetamidophenol (AP) used as a surrogate for extraction before HPLC analysis was supplied by Sigma. Ethylacetate (analytical grade) and methanol (HPLC grade) were supplied from Merck. Double deionized water was used for preparation of all solutions.

CU-Gibb is a commercial GA_3 product of the Institute of Biotechnology and Genetic Engineering (IBGE), Chulalongkorn University. The fermentation broth samples containing GA_3 were obtained from IBGE. *Gibberella fujikuroi* N9-34 for production of gibberellic acid was obtained from stock culture of the Institute of Biotechnology and Genetic Engineering (IBGE), Chulalongkorn University. The strain was maintained in potato dextrose agar slant at 25 °C. 1 Littre of a production medium containing the following substances was prepared: 120 g sucrose, 2.39 g $(NH_4)_2SO_4$, acid hydrolysed cotton seed hull with nitrogen content of 1.14 g, 5.0 g KH_2PO_4, 1.0 g $MgSO_4.7H_2O$, 0.1 g Al_2O_3, 2.0 ml soy been oil and water. An inoculum of *Gibberella fujikuroi* was added to a 250 ml Erlenmeyer flask containing 50 ml of the production medium. The fermentation was carried out at 25 °C with 250 rpm rotating agitation for a desired day. The culture was filtered, and the fermentation broth was kept in a fridge at 0 °C prior to analysis of gibberellic acid.

2.2 CE Conditions and Optimisation

2.2.1 CE conditions

All CE separations were performed on a P/ACE system 5010 Beckman CE instrument. An uncoated fused silica capillary used was 57 cm in length (50 cm to detector) × 50 μm I.D., thermostatted at 25 °C. UV detection was set at 214 nm. Sample or standard solution in water was injected by 0.5 psi pressure for 4 s. The BGE was prepared by dissolving the desired amounts of $Na_2B_4O_7.10H_2O$ with water. For MEKC, SDS at desired concentration was added into the BGE solution. Finally, The BGE solution was filtered through a 0.45 μm membrane filter and degassed before use. A new capillary was conditioned with 1 M HCl for 15 min, 1 M NaOH for 30 min, 0.1 M NaOH for 30 min, water for 30 min and finally with the BGE for 30 min. Prior to analysis each day, the capillary was rinsed with 0.1 M NaOH for 15 min and then BGE for 15 min. Between consecutive analyses, the capillary was flushed with 0.1 M NaOH for 3 min, and then BGE for 3 min. After analysis each day, the capillary was rinsed with 0.1 M NaOH for 10 min, the BGE for 10 min and then water 5 min. The capillary filled with water was kept. All solutions were prepared using water and filtered through 0.45 μm membrane filters prior to analysis. Each experiment was run in triplicate.

2.2.2 CZE optimisation

To optimise CZE conditions, the BGE as a borate buffer at pH 9.2 was used for determination of GA_3 in fermentation broth. The 10-day fermentation broth was ten times diluted with water, and used for investigation on suitable CZE conditions for separation of GA_3 from other compounds in the broth. Effects of BGE concentration and applied voltage on separation of GA_3 were studied. The buffer concentration was varied in the range of 12.5 to 50 mM using the applied voltage at 25 and 30 kV. Results are discussed in Section 3.1.2.

2.2.3 MEKC optimisation

For MEKC, The separation of GA_3 and other compounds was carried out using SDS as micellar phase. The studies of effects of BGE concentration and applied voltage on separation of GA_3 were similar to CZE. Effect of SDS concentration on the separation was also investigated by varying SDS concentration in the range of 20 to 150 mM. The 10-day fermentation broth was ten times diluted with water, and used for investigation on suitable MEKC conditions for separation of GA_3 from other compounds in the broth. Results are discussed in Section 3.1.3.

2.2.4 Stability of GA_3 in water and diluted BGE

To study stability of GA_3, 100 ppm GA_3 standard solutions in water and 2.5 mM $Na_2B_4O_7$ were separately prepared, and 10 ppm AMBA was used as an internal standard. The stability of the GA_3 standard solutions in water and the 2.5 mM $Na_2B_4O_7$ solution was investigated at period of time 0 to 48 hours. The amounts of GA_3 in the solutions were determined by using suitable MEKC conditions obtained from Section 2.2.3. Results are discussed in Section 3.1.4.

2.3 Validation of MEKC Method

2.3.1 Calibration plot

A stock standard solution of 2000 ppm GA_3 in water was prepared. The desired diluted standard solutions of GA_3 were obtained by pipeting the appropriate amount of 2000 ppm GA_3. Each diluted GA_3 solution contained 10 ppm AMBA used as an internal standard. The A_{corr} ratio of GA_3 and AMBA as a function of the GA_3 concentration was plotted as shown in Figure 3.13. Each point in Figure 3.13 is the average of triplicate runs.

2.3.2 Accuracy and precision

In order to determine accuracy and precision of the method and investigate the effect of the sample matrix on accuracy and precision, the known amounts of GA_3 spiked in water and ten times diluted fermentation broth were separately prepared and determined by MEKC for ten runs. For GA_3 spiked in water, the known amounts of GA_3 at levels of 50 and 140 ppm were prepared. For GA_3 spiked in the diluted broth, the amounts of GA_3 at levels of 50 ppm in 5, 7, and 10-day fermentation broths were used. Each solution contained 10 ppm AMBA. The diluted broth solutions with and without spiking 50 ppm GA_3 were analysed by MEKC in order to determine the amount of GA_3 by measuring the A_{corr} ratio of GA_3 and AMBA, $A_{corr,ratio}$. Each experiment was carried out for 10 runs. Results are discussed in Section 3.2.2.

2.3.3 Limit of detection (LOD) and limit of quantitation (LOQ)

The limit of detection (LOD) and the limit of quantitation (LOQ) were determined by injection of diluted GA_3 standard solution until signal-to-noise ratios of 3 for LOD and 10 for LOQ were obtained. Results are shown in Section 3.2.3.

2.4 HPLC Analysis

2.4.1 HPLC conditions

All HPLC experiments were performed on a Beckman HPLC instrument (System Gold *Nouveau*) equiped with a photo array UV detector. HPLC conditions used in this work are for routine analysis of GA_3 in fermentation broth at IBGE. An analytical column was a 5 μm Betasil C_8 250 × 4.6 mm I.D. Mobile phase contained 35% methanol and 65% water adjusted to pH 3.0 with phosphoric acid. Flow rate was controlled at 1 ml min^{-1}. UV detection was set at 208 nm. A sample solution was injected using a 10 μl loop. Each experiment was run in triplicate.

2.4.2 Sample preparation

Before introduction of samples into the HPLC column, sample preparation of GA_3 in the broth was carried out using the procedure reported in Samappito's thesis [1994]. The fermentation broth was filtered through a No. 42 membrane filter, and adjusted to pH 3.0 with 0.1 M hydrochloric acid. Then 3 ml of the pH 3.0 broth was transferred to a 10 ml glass tube. 60 µl of 5000 ppm AP and 4 ml ethylacetate were added to the tube. The mixture was shaken using a vortex for 4 min to extract GA_3 into ethylacetate. The ethylacetate solution in the upper layer was transferred in another tube containing sodium sulfate anhydrous in order to remove water. 3 ml of the solution was transferred to another tube, and then evaporated using a vacuum rotary evaporator at 35 °C to remove all ethylacetate. 3 ml of mobile phase was added into the tube to dissolve GA_3 and other residuals. The sample solution was filtered with a 0.45 µm PTFE membrane filter before analysis. Each sample needs 30 min for sample preparation.

2.4.3 Calibration plot

A stock standard solution of 2000 ppm GA_3 in mobile phase was prepared. The desired diluted standard solutions of GA_3 were obtained by pipeting the appropiate amount of 2000 ppm GA_3. Each diluted GA_3 solution contained 100 ppm AP used as an internal standard. The peak area ratio of GA_3 and AP as a function of the GA_3 concentration was plotted as shown in Figure 3.16.

2.4.4 Recovery of GA_3 extraction

To determine the recovery of GA_3 extraction, the amount of GA_3 in the broth with and without spiking GA_3 were compared. For the broth with spiked GA_3, 1 ml of a 750 ppm GA_3 standard solution at pH 3.0 was added into 2 ml of the fermentation broth adjusted to pH 3.0, giving the final concentration of spiked GA_3 250 ppm in the final solution. For the broth without GA_3 spiked, 1 ml of water adjusted to pH 3.0 was added, instead of the 750 ppm GA_3 standard solution. Aliquot of 60 µl of 5000 ppm AP, a surrogate, was added in the solution. Then the solutions were extracted with

ethylacetate, and the amount of GA_3 was determined as the procedure in Section 2.4.2. Each experiment was carried out in triplicate.

2.4.5 Limit of detection (LOD) and limit of quantitation (LOQ)

In HPLC, LOD and LOQ were determined as the same procedure as in Section 2.3.3. Results are shown in Section 3.3.2.

2.5 Applications to Real Samples

2.5.1 Determination of GA_3 in fermentation broth by MEKC and HPLC

The amounts of GA_3 in 3, 5, 7, 10 and 11-day fermentation broths were determined by MEKC and HPLC. For MEKC, The fermentation broths were filtered through 0.45 μm nylon membrane filters, and then were 10 times diluted with water. Each diluted sample contained 10 ppm AMBA as an internal standard for quantitative analysis. For HPLC, The fermentation broths were extracted using a method previously mentioned in Section 2.4.2. Results obtained from MEKC and HPLC are compared in Section 3.4.1.

2.5.2 Determination of GA_3 in commercial products by CZE and MEKC

For GA_3 products, there are two types of CU Gibb. packages, a tablet of GA_3 and a tube of GA_3. The tablet GA_3 was grinded before preparation. The desired amounts of tablet GA_3 and tube GA_3 were dissolved in water. Each diluted sample contained 10 ppm AMBA as an internal standard for quantitative analysis. The sample solutions were filtered through 0.45 μm nylon membrane filters before analysis by CZE and MEKC using suitable CE conditions obtained from Sections 2.2.2 and 2.2.3, respectively. Results are shown in Section 3.4.2.