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Short communication

Capillary electrophoresis to quantitate gossypol enantiomers in cotton flower petals and seed

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ABSTRACT

Gossypol is a toxic compound that occurs as a mixture of enantiomers in cotton plant tissues including seed and flower petals. The (−)-enantiomer is more toxic to non-ruminant animals. Efforts to breed cottonseed with a low percentage of (−)-gossypol requires determination of the (+)- to (−)-gossypol ratio in seed and flower petals. We report a method to quantitatively determine the total gossypol and percent of its enantiomers in cotton tissues using high performance capillary electrophoresis (HPCE). The method utilizes a borate buffer at pH 9.3 using a capillary with internal diameter of 50 μm, effective length of 24.5 cm, 15 kV and cassette temperature of 15 °C. This method provides high accuracy and reproducible results with a limit of detection of the individual enantiomers of less than 36 ng/mL providing base line separation in less than 6 min.

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1. Introduction

Cottonseed is a byproduct of cotton production. After the fiber is removed the seed offers a valuable source of protein. Unfortunately, cottonseed cannot be fed to non-ruminants because of the presence of toxic gossypol, which is found naturally in the seed. However, gossypol (Fig. 1) occurs as an enantiomeric mixture due to restricted rotation around the central binaphthyl bond; of the enantiomers, (−)-gossypol is more toxic to chickens [1]. Uzbekistan scientists are developing germplasm with low levels of (−)-gossypol in the seed that can safely be fed to chickens. To follow the introduction of the low (−)-gossypol seed trait, breeders use the (+)- to (−)-gossypol ratio in flower petals as an aid in selecting plants with the low (−)-gossypol seed trait [2]. To assist Uzbek cotton breeders we have developed methodology that allows quantitative determination of total and (+)- and (−)-gossypol in cotton plant tissues, including the seed and flower petals. HPLC methods for the quantitation of (+)- and (−)-gossypol have been published [3]. However, high performance capillary electrophoresis (HPCE) provides an alternative method that has advantages compared to HPLC in that it provides good resolution of enantiomers with a shorter run time and uses less expensive solvents. Thus, the HPCE method can quantitatively and reproducibly determine the total amount of gossypol and the percentage of the (+)- and (−)-enantiomers in cotton flower petals and seed.

2. Experimental

2.1. Chemicals

Standard gossypol from cotton seed was prepared in our laboratory. Acetonitrile (R Chromasolv) for liquid chromatography, and 99.99% acetic acid were from Sigma Chemical Co. (Munich, Germany); D-alaninol was from OmegaChem Inc. (Levis, PQ, Canada), and was distilled at atmospheric pressure before use and stored at −20 °C. All reagents for capillary electrophoresis were from Agilent Technologies (Waldbronn, Germany).

2.2. Sample preparation and derivatization

Samples (seeds and flower petals) were collected from plants growing on the farm of the Uzbek Scientific Research Institute of Cotton Breeding and Seed Production, Tashkent, Uzbekistan. Seed or petals were freeze dried and then ground with a mortar and pestle. Plant tissues were ground in liquid nitrogen, and 200–300 mg of the frozen ground tissue was transferred to screw-cap test tubes and weighed. The weighed tissue was covered with 2.0 mL of derivatization reagent consisting of 88% acetonitrile (v/v), 10% glacial acetic acid (v/v) and 2% D-alaninol (v/v). The samples were gently stirred to ensure full wetting of the sample and then

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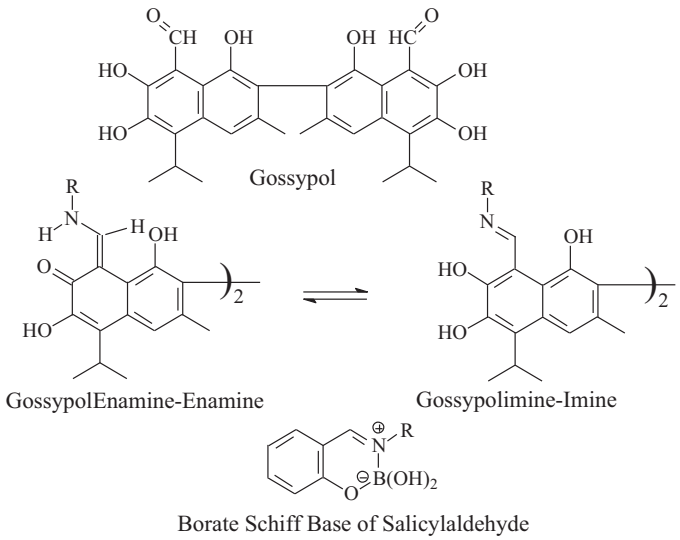

Fig. 1. Structure of gossypol, its Schiff base and the borate-Schiff base complex of salicylaldehyde.

heated in a water bath at 70 °C for 30 min. The reaction mixture was allowed to cool to room temperature and centrifuged for 3–5 min. An aliquot of the resulting clear supernatant was diluted with buffer and analyzed by HPCE. Analyses were performed within 8 h of obtaining the supernatant samples from either seed meal or flower petals.

2.3. Capillary electrophoresis

Capillary electrophoresis was performed on an Agilent Technologies HPCE (Model CE G 1600AX) (Waldbronn, Germany) equipped with an auto injector, diode array detector and computer with a HP-ChemStation-Rev.A.09.03 (1417) data analysis system. Before the analysis was initiated, capillaries were flushed with 0.1 M NaOH for 1 min, followed by 0.1 M HNO_3 for 2 min, ultrapure water for 10 min, and buffer for 4 min. The column was washed with 0.1 M NaOH for 1 min and then with buffer for 3 min between analyses. Injection was at 150 mbar for 1 s and the eluent was monitored at 238.5 nm (bandwidth of 20 nm) referenced to 550 nm (bandwidth 100 nm). UV spectra were recorded over the 220–400 nm range. A borate buffer was used as the eluent (50 mM, pH 9.3) on a capillary with effective length of 24.5 cm and an internal diameter of 50 μm. The cassette temperature was held at 15 °C, with a positive voltage of 15 kV. Under these conditions, (+)-gossypol had a retention time of 3.80 min and (−)-gossypol a retention time of 4.37 min.

3. Results and discussion

3.1. HPCE optimization

Utilizing D-alaninol as a chiral derivatizing reagent, allows one to separate the resulting diastereomeric mixture on an achiral stationary phase [4], or in this case by a difference in migration. To optimize the electrophoresis we investigated the influence of the ionic strength of the buffer, internal diameter and length of the capillary, voltage, and cassette temperature. The ionic strength of the buffer was selected to minimize the electro-ionic flow on the walls of the capillary so that the pH remains constant. A 50 μm internal diameter capillary with a length of 24.5 cm was most suitable for separation of substances with a molecular weight approximately corresponding to gossypol (MW 518). We investigated a number of different buffers including phosphate, Tris, citrate and acetate, as well as different pH values between 4 and 10. Chandler and Seshadri [5] and King [6] have shown that alkaline borate can be used to effectively extract gossypol during the refining of cottonseed oil or from leaf tissues. Since borate forms a chelate between the aldehyde group and the *ortho* phenol group, we also investigated a borate buffer that we thought might form a chelate between the nitrogen of the Schiff base and the *ortho* phenol group. Przybyski et al. have shown that the Schiff base formed between gossypol and L-phenylalanine exists in the enamine–enamine tautomer (Fig. 1) [7]. However, Nagamatsu et al. have shown that in the case of salicylaldehyde, borate accelerates the formation of the Schiff base and the preferred tautomer is the stable borate-Schiff base complex shown in Fig. 1 [8]. In our hands, we found that borate gave the best separation. Fig. 2 shows the results of the analysis of gossypol at the preferred pH of 9.3 with borate concentrations ranging from 25 mM to 100 mM. Based on these results, borate buffer pH 9.3, 50 mM was selected for all analyses. The exact tautomeric form of the borate-Schiff complex will require additional study.

We also examined the effect of capillary length (L) and internal diameter (d) on separation as follows: L = 24.5 cm, d = 50 μm;

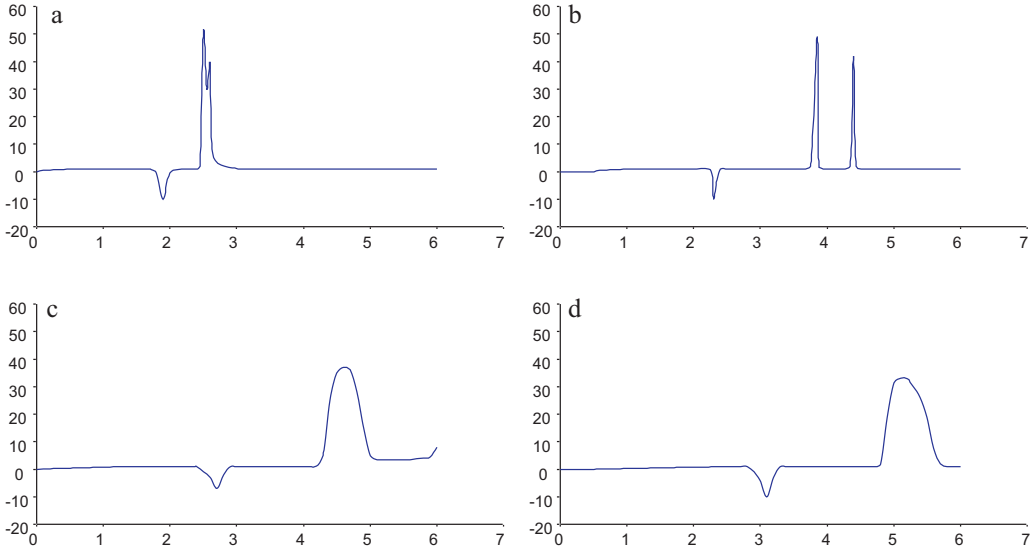

Fig. 2. Electropherograms of standard gossypol enantiomers using borate buffers (pH 9.3): (a) 25 mM; (b) 50 mM; (c) 75 mM; (d) 100 mM.

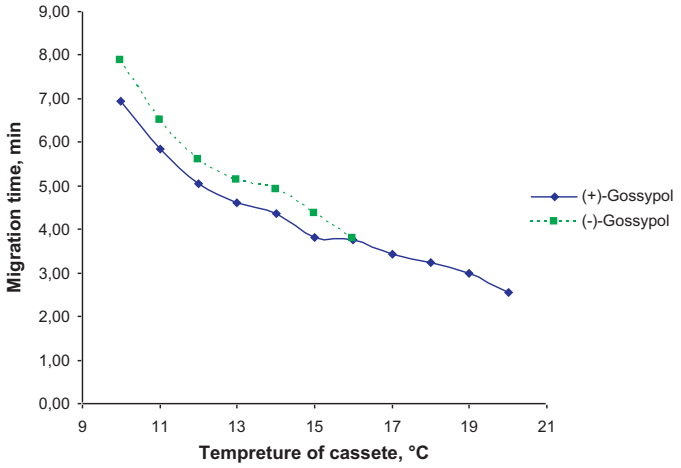

Fig. 3. Migration time of gossypol enantiomers at various cassette temperatures.

$L = 40$ cm, $d = 50$ μm; $L = 56$ cm, $d = 50$ μm; $L = 24.5$ cm, $d = 75$ μm; $L = 40$ cm, $d = 75$ μm and $L = 56$ cm, $d = 75$ μm. Using the pH 9.3, 50 mM borate buffer the enantiomers could be separated on capillaries ranging in length from 24.5 up to 56 cm with an internal diameter of 50 μm. With the pH 9.3, 50 mM borate buffer, the best results were obtained with a 24.5 cm capillary and an internal diameter of 50 μm.

With these values established, we investigated the voltage and cassette temperatures, which also influence peak separation. At a cassette temperature >17 °C, enantiomer separation did not occur, while a cassette temperature <12 °C increased the time of migration and the analysis time. The migration times of (+)- and (−)-gossypol with variation of cassette temperature are shown in Fig. 3. The optimum cassette temperature was 15 °C.

A similar study based on changes in voltage is shown in Fig. 4. A voltage above 15 kV provides acceptable enantiomer separation (Fig. 4). However, at voltages >15 kV peaks became very sharp, making integration more variable with values varying by >5%. Thus, analyses were conducted at 15 kV. Gossypol was detected and identified using a matrix diode array detector. The electropherogram of gossypol enantiomers found in an extract from cotton flower petals from a plant that exhibit the low (−)-gossypol seed trait is shown in Fig. 5. The UV absorption spectrum for the dialaninol derivative of gossypol is shown in Fig. 6 (3D UV-spectra of gossypol enantiomers from a cotton petal extract is shown in Supplementary Material).

Total gossypol in samples was determined by adding the integration values for the enantiomers and is based on a calibration curve constructed with a racemic gossypol standard. Analysis could be completed in <6 min with minimal use of buffer. Analyses were performed in parallel with HPLC for comparison purposes. Results are shown in Table 1. The comparative analysis showed a standard deviation of <5%.

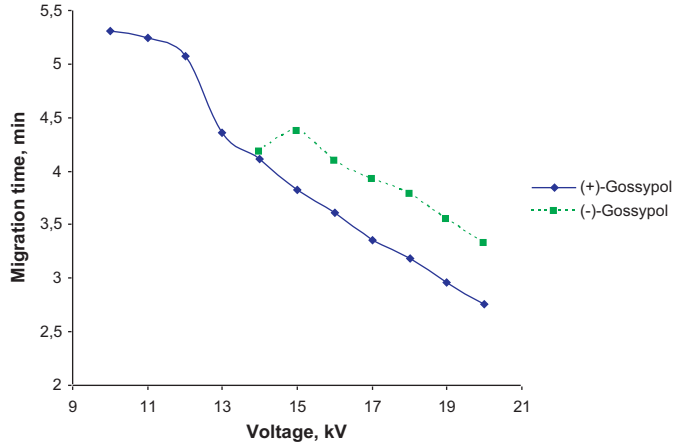

Fig. 4. Migration time of gossypol enantiomers at different voltages.

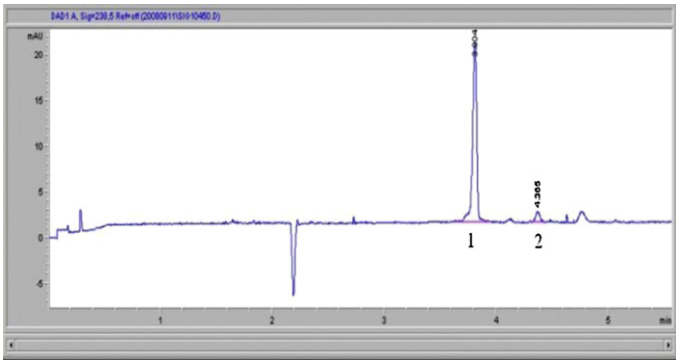

Fig. 5. Electropherogram of gossypol enantiomers [1, (+)-gossypol (3.8 min); 2, (−)-gossypol (4.4 min)] in an extract from cotton flower petals.

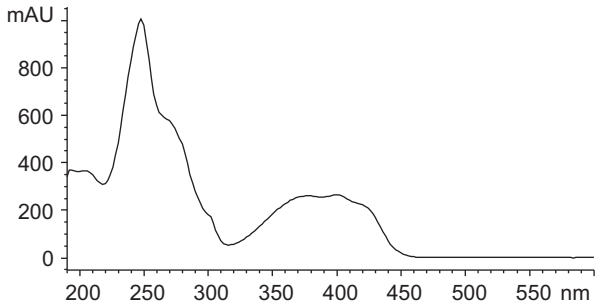

Fig. 6. UV spectrum (190–600 nm) of the D-alaninol derivative of (+)-gossypol.

Table 1
Comparative analysis between HPCE and HPLC of total gossypol and percentage (+)-gossypol in cotton flower petals.

Sample	Total gossypol (μg/mg)		% (+)-Gossypol	
	HPCE	HPLC	HPCE	HPLC
1	0.76 ± 0.04[a]	0.78 ± 0.03	75 ± 4.2	72 ± 4.6
2	1.10 ± 0.05	1.14 ± 0.05	68 ± 3.3	70 ± 3.1
3	1.85 ± 0.06	1.88 ± 0.05	93 ± 3.7	95 ± 3.4

[a] Standard deviation.

4. Conclusion

The HPCE method described herein provides a new method to quantitatively and reproducibly analyze total and (+)- and (−)-gossypol in cottonseed and flower petals that is comparable to established HPLC methods. Using a racemic mixture, the limit of detection for the individual enantiomers of (+)- or (−)-gossypol was found to be <36 ng/mL. The advantages of the HPCE technique are the low cost of buffer compared to the HPLC solvents (i.e. acetonitrile) used in published methods and the short analysis time.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.09.033.

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