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High-performance liquid chromatographic determination of gossypol and gossypolone enantiomers in fish tissues using simultaneous electrochemical and ultraviolet detectors

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Abstract

There is a need to measure dietary transfer of gossypol and its metabolite, gossypolone in aquatic animals because of common use of cottonseed meal as a feed ingredient and fertilizer. The analytical method for gossypol and gossypolone enantiomers, therefore, becomes important. HPLC techniques have been developed by using mainly UV detection. We simultaneously used both UV and electrochemical (EC) detectors, and found that each individual detector has its own advantage which can increase accuracy and ease of identification. EC detection (2.5 and 50 ng/ml) exhibited a significantly lower detection level for both gossypol and gossypolone enantiomers than the UV detection (40 and 300 ng/ml) in the rainbow trout tissues, while UV detectors showed more stable detection than EC. We were able to detect a very low concentration of each gossypol enantiomer by EC but not UV detection especially in seminal plasma. For the first time gossypolone enantiomers were quantified in fish tissues by HPLC and its method was described. The technique, simultaneous adoption of both UV and EC detectors, could be helpful for a very low concentration of gossypol and/or gossypolone enantiomers in tissues of other animals and humans.

Keywords: Enantiomer separation; Gossypol; Gossypolone

1. Introduction

Gossypol (1,1′,6,6′,7,7′-hexahydroxy-5,5′diisopropyl - 3,3′dimethyl - [2,2′]binaphthalenyl-[8,8′]dicarbaldehyde) is a naturally occurring polyphenolic compound present in cotton plants, *Gossypium* sp., especially in its pigment glands. Gossypol (Fig. 1) has been known to have toxic effects on terrestrial animals [1], humans [2], and fish [3–5] when ingested. Gossypolone (Fig. 1), an oxidized metabolite of gossypol [6], is also known to have similar potency as gossypol [7]. Recently, it has been reported that gossypol and gossypolone have anticancer effects in animal models [8,9]. The anticarcinogenic effects of gossypol are the result of action of the (−)-enantiomer rather than that of the (+)-enantiomer, because of higher biological activities of the (−)-enantiomer [10]. Therefore, the analytical method separating gossypol and/or gossypolone enantiomers becomes important and needs to be

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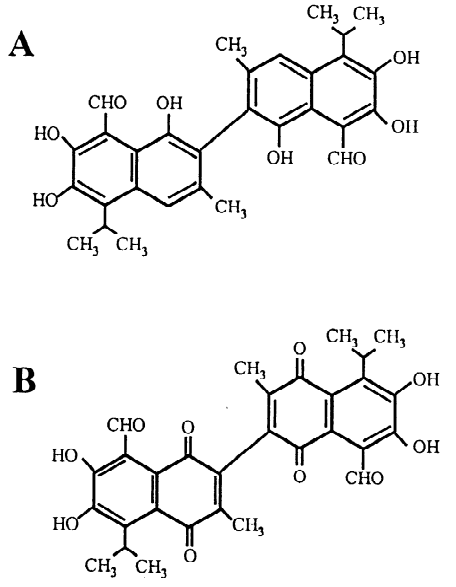

Fig. 1. The chemical structures of gossypol (A) and gossypolone (B).

accurate, particularly in the case of low concentrations of the enantiomers in specific tissues. Total gossypol was defined as the sum of free and bound gossypol, and free gossypol as ''acetone soluble gossypol'' [11]. The total gossypol in this study refers to the gossypol which was not pre-extracted by acetone.

The resolutions of racemic gossypol and/or its enantiomers have been achieved and refined for HPLC systems for more than 30 years. However, most of the methods, to our knowledge, have mainly adapted UV detection [12,13], while few used electrochemical (EC) detection [14–16]. Furthermore, no analytical description was reported for its quantitatively most important metabolite, gossypolone. The most recent methods for gossypol enantiomer analysis in cottonseed [12] and animal tissues [13] were described for HPLC systems with UV detection. Therefore, the purpose of this study is to compare and describe the modification of the precolumn-derivatization methods with a chiral reagent [12,14] by simultaneously adapting UV and EC detectors to determine gossypol and gossypolone in fish tissues (kidney, blood and seminal plasma), especially at very low concentrations (below 0.1 μg/ml) of gossypol in seminal plasma of rainbow trout, *Oncorhynchus mykiss*.

2. Experimental

2.1. Equipment

The HPLC system consisted of two delivery system pumps (Model 506A, Beckman Instruments Inc., San Ramon, CA, USA) equipped with a 20-μl injection loop connected to a 4.6 mm×150 mm Shodex C_{18} column (Showa-Denko, Shoko Co. Ltd., Tokyo, Japan) packed with octadecyl-bonded porous silica gel (5 μm). The UV detector (Programmable detector module 166) was purchased from Beckman Instruments Inc., San Ramon, CA, USA and the electrochemical detector (Model LC-4C) was purchased from BAS, West Lafayette, IN.

2.2. Materials and reagents

Acetonitrile and water (HPLC grade) were obtained from Fisher Scientific, Pittsburgh, PA. Racemic gossypolone, (*R*)-(−)-2-amino-1-propanol ($[\alpha]^{19} = -18°$), glacial acetic acid, and *N*,*N*-dimethylformamide were obtained from Sigma Chemical, St. Louis, MO. Standards of (+)- and (−)-enantiomers of gossypol were provided by Dr. Quezia B. Cass, Departamento de Química, Universidade Federal de São Carlos, São Carlos, Brazil [17].

2.3. Chromatographic conditions

Gossypol and gossypolone enantiomer derivatives were extracted by the methods described by Wu et al. [14] and Hron et al. [12] with some modifications. The mobile phase was made of 80 ml acetonitrile and 2 m*M* KH_2PO_4 (final concentration) dissolved in 100 ml water (HPLC grade) adjusted to pH 3.0 with H_3PO_4. UV detection at 254 nm was applied for the optimum wavelength, and EC detection was examined with applied potentials ranging from 0.7 to

1.0 V for the best resolution. The mobile phase was delivered at a flow-rate of 1.0 ml/min. The HPLC injection volume was 20 μl. The retention time for the (+)- and (−)-gossypol were 4.0 and 6.7 min, respectively. The retention time for the (+)- and (−)-gossypolone were 3.3 and 4.6 min, respectively.

2.4. Sample preparations

The analytical conditions for both gossypol and gossypolone extraction and analysis were the same. Blood and seminal plasma and kidney of rainbow trout broodstock (2.5 years old), fed cottonseed meal over 10 months, were used. Blood and seminal plasma samples were mixed with extraction reagent composed of 2% 2-amino-1-propanol and 10% glacial acetic acid in *N*,*N*-dimethylformamide, and vigorously vortexed. For kidney samples, homogenization (Model: Omni 5000, Omni International, Marietta, GA, USA) with the extraction reagent for 30 s was used instead of vortexing. The vortexed or homogenized samples were heated at 90–95 °C for 30 min, cooled on ice, and then centrifuged (4 °C) at 1500×*g* for 5 min. After centrifugation, an aliquot of the supernatant was diluted with the mobile phase to obtain a desirable concentration, centrifuged again at 1500×*g* for 5 min, and filtered through a syringe filter (0.45 μm, Whatman Inc., Clifton, NJ, USA) before injection into the HPLC system.

2.5. Extraction recovery rates and external standards

For the recovery, known amounts of gossypol and gossypolone standards in extract reagent solution were added into running samples at the beginning of the extraction and followed by the same procedure as described before. Five sub-samples were used for the replications. For external standard curves, each gossypol enantiomer or racemic gossypolone were mixed with the extract reagent, heated at 90–95 °C for 30 min, cooled, and then diluted with mobile phase. Each external standard curve of gossypol enantiomers for EC and UV detection was made with the concentration range from 1.0 to 200 ng/ml and from 0.05 to 2.0 μg/ml, respectively, for linear curves. External standard curve of racemic gossypolone was prepared with the concentration from 1.0 to 5.0 μg/ml. These concentrations of standards were applied for the calculation of sample gossypol or gossypolone, even though the standard curves were significantly linear for both gossypol enantiomers (r^2=0.999) and racemic gossypolone (r^2=0.996) up to the concentration of 5 μg/ml.

Table 1
Gossypol and gossypolone enantiomers in kidney, blood plasma, and seminal plasma of rainbow trout broodstock fed a diet containing 60% cottonseed meal for 10 months[a]

	Kidney (μg/g)[b]	Blood plasma (μg/ml)[b]	Seminal plasma (ηg/ml)[c]
Gossypol			
(+)-enantiomer	66.5±26.5	12.4±1.34	91±22
(−)-enantiomer	32.2±11.2	5.63±1.15	47±13
Total	98.7±35.5	18.0±2.31	138±35
Gossypolone			
(+)-enantiomer	34.7±21.9	2.85±0.45	n.d.[d]
(−)-enantiomer	6.65±1.36	1.72±0.08	n.d.
Total	41.4±20.6	4.57±0.42	n.d.

[a] Data are expressed as mean±S.E. (n=5). Dietary free and total gossypol concentrations were 463 and 9550 μg/g, respectively. The fish were fed at a feeding rate of 3% (body weight basis) per day.
[b] Data by UV detection.
[c] Data by EC detection. UV detector did not detect low concentration of gossypol in seminal plasma.
[d] Not detected.

3. Results and discussion

Extraction recovery rates ($n=5$) were higher than 92% for both gossypol enantiomers, and 74% for both gossypolone enantiomers. Coefficients of variation (within analysis) were 1.0 and 5.3% for gossypol and gossypolone, respectively.

The analytical results of the concentrations of gossypol and gossypolone in tissues of rainbow trout broodstock are provided in Table 1. We clearly quantified those enantiomers in kidney and blood plasma with UV and/or EC detection. However, gossypol in seminal plasma was detected only by EC detection which exhibited higher sensitivity than UV detection. EC detection showed higher sensitivity than UV detection when low concentrations of racemic gossypol (Fig. 2A) and gossypolone (Fig. 2B) were encountered. Detection levels for gossypol enantiomers were 40 ng/ml and 2.5 ng/ml with a signal-to-noise ratio of 3 for UV and EC detection, respectively. Gossypolone detection levels were 300 ng/ml and 50 ng/ml for UV and EC detection, respectively. For the blood plasma sample, EC detection resulted in clearer peaks for both gossypol

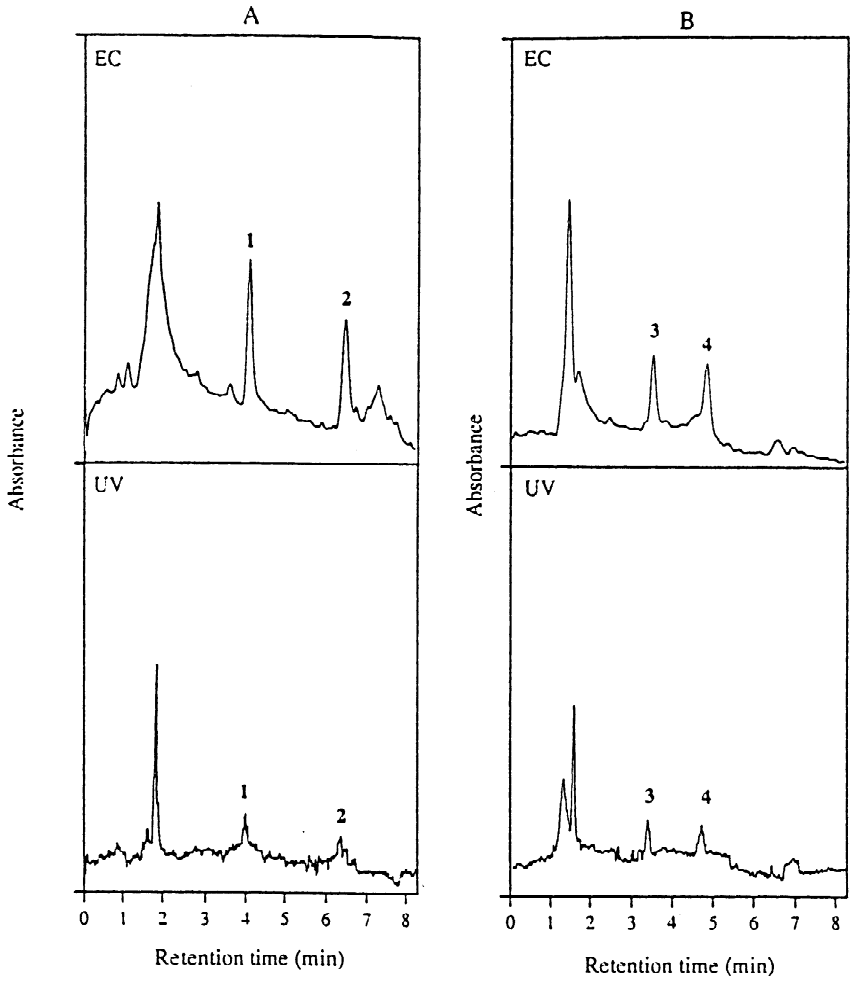

Fig. 2. Chromatogram for gossypol [A; 1 and 2 for (+)- and (−)-enantiomers, respectively] and gossypolone [B; 3 and 4 for (+)- and (−)-enantiomers, respectively] showing peaks for racemic standard gossypol (0.2 ng/20 μl injection volume) and gossypolone (4.0 ng/20 μl injection volume). EC detection (both top) shows clearly detectable peaks of (+)- and (−)-enantiomers, while UV detection (both bottom) shows undistinguishable peaks for those enantiomers.

enantiomers compared to those of UV detection (Fig. 3A). In our preliminary study (not published), the concentration of gossypol enantiomers were the lowest in seminal plasma than in other fish tissues (liver, bile, muscle, kidney, stomach, blood plasma) including spermatozoa and eggs. A very low concentration of gossypol enantiomers in seminal plasma was not detectable by UV, but clearly quantified with EC detection (Fig. 3B). The chromatogram of gossypolone and its spiking standard in kidney tissue by UV detection is shown in Fig. 4. In kidney tissue, UV detection was enough to quantify gossypolone. The proportion of gossypolone (−)-enantiomer was significantly lower than that of (+)-enantiomer, as shown in gossypol enantiomers (Fig. 3). In the dietary total gossypol, the concentrations of (+)- and (−)-enantiomer were 4955 and 4593 mg/kg, respectively, showing that the proportion of each enantiomer is almost 1:1. Kidney tissues of rainbow trout fed diets devoid of cottonseed meal did not show any corresponding peaks for gossypol or gossypolone.

We found that the resolution by UV detection is consistent and stable regardless of running times or sample numbers, whereas EC detection showed some

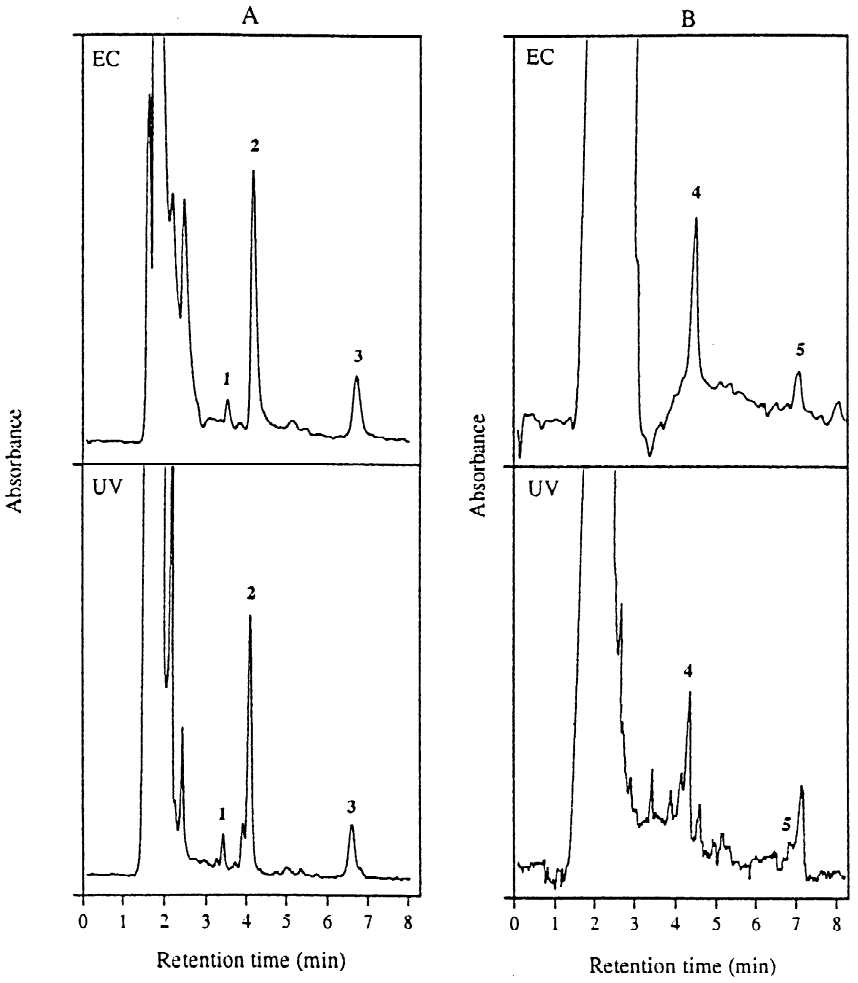

Fig. 3. Chromatogram showing peaks of total gossypol and total gossypolone enantiomers in blood plasma [A; 1, 2, and 3 for gossypolone (+)- enantiomer, gossypol (+)-, and (−)-enantiomers, respectively], and total gossypol enantiomers in seminal plasma [B; 4 and 5 for gossypol (+)- and (−)-enantiomers, respectively] of rainbow trout. EC detection (B, top) exhibits much clearer peaks than those of UV (B, bottom). Gossypolone concentration was not detectable in seminal plasma.

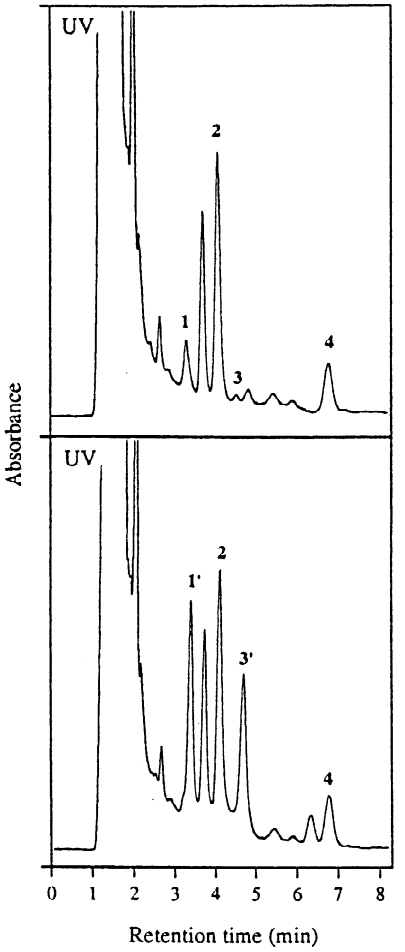

Fig. 4. Chromatogram (top) showing peaks of total gossypol [2 and 4 for (+)- and (−)-enantiomers, respectively] and total gossypolone [1 and 3 for (+)- and (−)-enantiomers] in kidney tissue of rainbow trout. Chromatogram (bottom) showing peaks of standard gossypolone spiking on the same sample extract (see 1′ and 3′ for spiked gossypolone standard).

inconsistency in resolutions by running times and sample numbers; i.e. the resolution by EC detection decreased as sample injection number increased, which means that the glassy carbon electrode becomes blunt. Wang et al. [15,16] only used the EC detector in quantifying gossypol enantiomers in rat tissues, while most analyses of gossypol were conducted by UV detection. We assumed that the individual detectors have their own problems in resolution of gossypol enantiomers. The EC detection would have the inconsistent resolutions of gossypol enantiomers in a long period of HPLC use time because of its very sensitive electrode, whereas the UV detection would have faced a high detection level in seminal plasma samples containing a low concentration of gossypol. The decreasing sensitivity of EC detection could be monitored by simultaneous running of UV detection. Therefore, we expect that simultaneous use of both UV and EC detectors could improve the separations of gossypol and gossypolone enantiomers with enhanced consistency of results in tissues having low concentrations of gossypol, such as wet muscle or seminal plasma. This technique could be specifically useful in seminal plasma, because gossypol is known as an anti-fertility agent in males [4,18–20].

The stability of extracted gossypol was also examined in this study (data not presented). The extracted gossypol enantiomers in mobile phase (used as diluent) were recovered by 95% after 96 h storage in dark at −20 °C. However, under fluorescent light and room temperature, gossypol was only recovered by 47% after 96 h storage. In the extraction method, it was observed that 15–20 min of heating samples was enough to obtain the 2-amino-1-propanol derivatives of gossypol and gossypolone enantiomers, but the heating temperature was critical and should be maintained above at least 80 °C. For the extraction of samples with the extraction reagent, more work is needed to verify the best extraction method between wet and freeze-dried samples, because the extraction results were not consistent.

We found that high voltage (over 0.95 V) resulted in unstable chromatograms although it gives higher sensitivity, and that the applied potential of 0.80–0.85 V vs. Ag–AgCl reference electrode was enough to detect gossypol enantiomers in fish seminal plasma with a relatively stable chromatogram.

In conclusion, UV detection can correct the disadvantage of using EC detection by considering its consistent resolution regardless of running time or sample numbers. EC detection can contribute in quantifying a very low concentration of gossypol enantiomer in seminal plasma and also be useful in finding a gossypol-like noise on UV chromatogram. Therefore, the findings indicate that quantification of low concentrations of gossypol and gossypolone enantiomers can be accomplished with consistent

resolution by using simultaneous UV and EC detectors.

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