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# Gossypol and Gossypolone Enantiomers in Tissues of Rainbow Trout Fed Low and High Levels of Dietary Cottonseed Meal

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Gossypol is an antifertilizing agent in males and females. However, gossypol and its metabolite, gossypolone, have also gained interest because of their anticarcinogenic activities. This paper examines for the first time both enantiomers of tissue gossypol and gossypolone in mature rainbow trout fed two diets containing low (15%) and high (60%) levels of cottonseed meal (CM) for 9 months. The gossypol concentration was highest in liver followed by kidney, intestine, testis, blood plasma, stomach, and muscle. Gossypol was detected in muscles of fish fed low- and high-CM diets (0.31 ± 0.03 and 1.95 ± 0.59 $\mu$g of total gossypol/g, wet basis, respectively). The (+)-gossypol enantiomer was predominantly retained in all tissues. The ratio of (−)- to total gossypol ranged from 30 to 44% in fish fed the high-CM diet and from 23 to 30% in fish fed the low-CM diet except for muscle tissue (44%). Higher gossypolone concentrations were found in intestine than in liver. Gossypolone, however, was not detected in blood plasma, muscle, and testis of fish fed the low-CM diet. The ratio of gossypolone to gossypol was highest in muscle (1.75), followed by intestine (1.59), stomach (1.50), kidney (0.43), liver (0.34), testis (0.28), and blood plasma (0.27). This study indicated that the retention of the (−)-gossypol enantiomer is dependent on dietary concentrations and that the oxidative conversion of gossypol to gossypolone occurs more actively in the digestive tract and muscle than in other tissues in rainbow trout.

**KEYWORDS: Gossypol; gossypolone; enantiomers; cottonseed meal; trout**

## 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 dialdehyde present in cotton plants, *Gossypium* sp., especially in its pigment glands. Cottonseed meal (CM) is an agricultural byproduct rich in protein. It has been extensively studied as a protein source of feed in ruminants (*1*, *2*). However, gossypol is the main antinutrient limiting the use of cottonseed in monogastric animals and humans. The U.S. Food and Drug Administration sets a limit for free gossypol at 450 mg/kg in human food products and ingredients, and the FAO and WHO set the maximum limitation at 12000 mg/kg of total gossypol (*3*). Recently, however, it has been reported that gossypol and its metabolite, gossypolone, have anticancer effects in animal models (*4*, *5*). The anticarcinogenic effects of gossypol have been reported to be 2−14 times more potent in the presence of (−)-gossypol rather than that of (+)-gossypol (*6*−*8*).

Therefore, it has been of interest that accumulations of enantiomers of gossypol and gossypolone, in vivo, be elucidated in animal models. The literature on this topic stops short of distinguishing between (−)- and (+)-gossypol enantiomers. We present for the first time data of the dietary effects of gossypolone enantiomer in animal tissues. Gossypol accumulations in tissues were determined in lambs (*9*), rats (*10*), and rainbow trout (*11*). However, those studies (*10*, *11*) were based on the spectrophotometry method for gossypol quantification in tissues except for that of Kim et al. (*9*). Therefore, the aim was to simultaneously analyze enantiomers of gossypol and gossypolone and their dynamics in tissues of mature rainbow trout fed two experimental diets containing low or high levels of cottonseed meal (CM) for several months.

## MATERIALS AND METHODS

**Animals and Diets.** The experimental fish, diets, and conditions were described in detail in our previous studies (*12*, *13*). Briefly, two groups of rainbow trout (1.5 years old) were fed one of the CM-supplemented experimental diets for 9 months. The experimental diets were formulated to contain two different levels (low and high) of cottonseed meal (solvent extracted, Southern Cotton Oil Co., Memphis, TN) by replacing fish meal protein. The levels of CM incorporation into diets were 15 and 60% for low- and high-CM diets, respectively. These diets were formulated to contain similar levels of methionine and lysine, which are limiting amino acids in CM protein (**Table 1**). The concentrations of total gossypol in the low- and high-CM diets were 2200 and 9550 mg/kg of diet, respectively, and the concentrations of free gossypol were 104 and 463 mg/kg of diet, respectively. The proportion of (+)-/(−)-gossypol in the diets did not differ significantly (50.9:49.1 and 51.9:48.1 for the low- and high-CM diets, respectively).

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**Table 1.** Compositions of the Two Experimental Diets Containing Low and High Levels of Cottonseed Meal (CM) (Percent of Dry Matter)

| | diet | |
|---|---|---|
| ingredient | low-CM | high-CM |
| menhaden fish meal | 15.00 | 0.00 |
| herring fish meal | 15.00 | 0.00 |
| cottonseed meal | 14.70 | 58.80 |
| krill hydrolysate | 5.00 | 5.00 |
| wheat middlings | 21.20 | 0.60 |
| corn gluten meal | 12.60 | 15.60 |
| yeast | 6.00 | 6.00 |
| L-methionine | 0.10 | 0.40 |
| L-lysine | 0.20 | 0.80 |
| vitamin mixture[a] | 0.50 | 0.50 |
| mineral mixture[b] | 0.50 | 0.50 |
| vitamin C[c] | 0.05 | 0.05 |
| choline chloride | 0.10 | 0.10 |
| menhaden oil | 8.90 | 11.60 |
| cellulose | 0.15 | 0.05 |
| gossypol (mg/kg) | | |
| free gossypol | 104 | 463 |
| total gossypol | 2203 | 9549 |

[a] Roche Performance Premix composition per gram of the vitamin mixture: vitamin A, 2646 IU; vitamin $D_3$, 221 IU; vitamin E, 66.1 IU; vitamin $B_{12}$, 13 $\mu$g; riboflavin, 13.2 mg; niacin, 61.7 mg; *d*-pantothenic acid, 22.1 mg; menadione, 1.32 mg; folic acid, 1.76 mg; pyridoxine, 4.42 mg; thiamin, 7.95 mg; *d*-biotin, 0.31 mg (Hoffman-La Roche, Inc., Nutley, NJ). [b] Bernhart Tomarelli salt mixture (ICN Pharmaceuticals Inc., Costa Mesa, CA) compositions (g/100 g): calcium carbonate, 2.1; calcium phosphate dibasic, 73.5; citric acid, 0.227; cupric citrate, 0.046; ferric citrate (16–17% Fe), 0.558; magnesium oxide, 2.5; manganese citrate, 0.835; potassium iodide, 0.001; potassium phosphate dibasic, 8.1; potassium sulfate, 6.8; sodium chloride, 3.06; sodium phosphate, 2.14; and zinc citrate, 0.133. Five milligrams of Se in the form of sodium selenite was added per kilogram of salt mixture. [c] L-Ascorbyl-2-phosphate magnesium (Showa Denko K.K., Tokyo, Japan).

The stomach, intestine, blood plasma, liver, muscle, kidney, and testis were sampled from four male rainbow trout per treatment for the analysis of total gossypol and its metabolite, gossypolone enantiomers, after 9 months of feeding. For the free and bound gossypol enantiomers, semen samples were collected from the fish and separated into seminal plasma and spermatozoa by centrifugation (7280*g* for 15 min at 4 °C). The results of growth rate, reproductive performance, and biochemical parameters were reported in Blom et al. (*13*) and Dabrowski et al. (*14*). All procedures and handling of fish during the feeding experiments were conducted in compliance with the guidelines of the Institutional Laboratory Animal Care and Use Committee, The Ohio State University.

**Sample Preparations and Analysis.** The free and total gossypol analyses were described in Dabrowski et al. (*14*). For the gossypolone enantiomer analysis, we adopted the same extraction methods as for gossypol enantiomers. Blood 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 the other samples (stomach, intestine, liver, muscle, kidney, and testis), homogenization (model Omni 5000, Omni International, Inc., Marietta, GA) with 5 volumes of the cold extraction reagent (0.2 g of wet tissue in 1 mL of reagent) was adopted for 30−60 s instead of vortexing. The vortexed or homogenized mixtures were warmed at 90−95 °C for 30 min in a water bath, cooled on ice, and then centrifuged at 1500*g* for 5 min. After centrifugation, an aliquot of the supernatant was diluted with mobile phase (8 volumes of acetonitrile and 2 volumes of 10 mM $KH_2PO_4$ at pH 3.0) to obtain a desirable concentration for detection. The samples were centrifuged again at 1500*g* for 5 min and filtered through a syringe filter (0.45 $\mu$m, Whatman Inc., Clifton, NJ) before injection into the HPLC system. The retention times for (+)- and (−)-gossypol were 4.1 and 6.7 min, respectively, with a flow rate at 1.0 mL/min. The retention times for (+)- and (−)-gossypolone were 3.3 and 4.6 min, respectively. Standards of (+)- and (−)-gossypol were provided by Dr. Quezia B. Cass, Departamento de Química, Universidade Federal de São Carlos, São Carlos, Brazil (*15*). Gossypolone standard was purchased from Sigma Chemicals (St. Louis, MO). External standard curves of each enantiomer were made for the calculations. The external standard curve for gossypolone enantiomers was made with racemic gossypolone.

The HPLC system consisted of two delivery system pumps (model 506A, Beckman Instruments Inc., San Ramon, CA) equipped with a 20 $\mu$L injection loop connected to a 4.6 mm × 150 mm Phenomenex C-18 column (Phenomenex USA, Torrance, CA) packed with octadecyl-bonded porous silica gel (5 $\mu$m). The UV detector (programmable detector module 166) was purchased from Beckman Instruments Inc.

Extraction recovery rates were 92.0 ± 3.5% ($n$ = 5) for both gossypol enantiomers and 74.0 ± 1.4% ($n$ = 5) for both gossypolone enantiomers. 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. The standard curve was made with the concentration range from 0.05 to 2.0 $\mu$g/mL for linearity. Coefficients of variance (within analysis) were 1.0 and 5.3% for gossypol and gossypolone, respectively. Detection levels for gossypol enantiomers were 0.8 ng/20 $\mu$L with a signal-to-noise ratio of 3 for UV detection at 254 nm. Gossypolone detection levels were 6 ng/20 $\mu$L of injection volume for UV at 254 nm.

**Statistical Analysis.** The data of gossypolone/gossypol ratio in various tissues were subjected to one-way ANOVA test, and a least significant difference (LSD) multiple-comparison test was adopted at $P$ = 0.01 by the SPSS statistical package (version 10.0, SPSS Inc., Chicago, IL).

## RESULTS

Gossypol and gossypolone enantiomers in all tissues were clearly separated in this study, and a representative HPLC chromatogram with UV detection of the small intestine is provided (**Figure 1**). The peak retention of gossypolone enantiomers was presumed by considering the retention orders of gossypol enantiomers. The results of the accumulations and (−)-enantiomer proportions of gossypol and gossypolone in tissues of rainbow trout broodstock are provided in **Figure 2**. The gossypol concentration was highest in liver among all of the tissues analyzed. Gossypol was detected even in the muscle of fish fed the low-CM diet (0.31 ± 0.03 $\mu$g of total gossypol/g). (+)-Gossypol was predominantly retained in all of the tissues. The percentage of (−)-gossypol/total gossypol ranged from 30 to 44% in fish fed the high-CM diet and from 23 to 30% in fish fed the low-CM diet except for muscle tissue (44%).

High gossypolone concentrations were found in the intestine and kidney as well as in liver tissue. In the fish fed the high-CM diet, the gossypolone concentration in the intestine was even higher than that in the liver. Gossypolone was not detected in blood plasma, muscle, and testis of fish fed the low-CM diet. The digestive tract and muscle tissues had higher concentrations of gossypolone than that of gossypol. The ratios of gossypolone to gossypol in the tissues are provided in **Figure 3**. The ratio was significantly ($P$ < 0.01) higher in muscle (1.75), intestine (1.59), and stomach (1.50) compared to that in kidney (0.43), liver (0.34), testis (0.28), and blood plasma (0.27).

Interestingly, the highest ratio of bound gossypol to total gossypol was observed in spermatozoa in comparison to blood and seminal plasma (**Table 2**). In the spermatozoa, we did not observe (−)-gossypol in free form, which means that all of the free gossypol exists as the (+)-enantiomer configuration. This is the first time that the observation of the high specificity of gossypol enantiomer binding has been made. The ratio of (−)-gossypol to total gossypol in spermatozoa (34%) was similar to that of other tissues examined above.

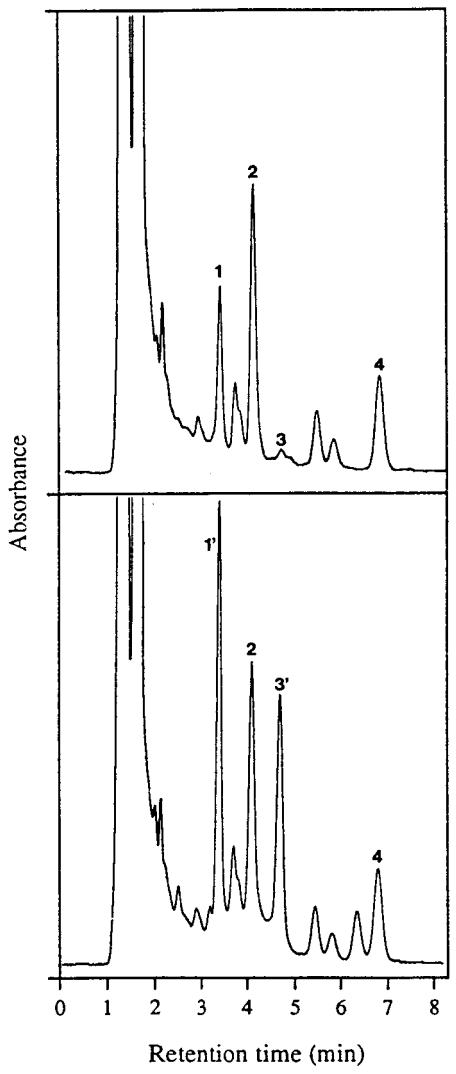


**Figure 1.** (Top) Representative HPLC-UV chromatogram showing peaks of total gossypol [2, (+)-gossypol; 4, (–)-gossypol] and gossypolone [1, (+)-gossypolone; 3, (–)-gossypolone] enantiomers in the small intestine tissue of rainbow trout fed a diet containing a high level of cottonseed meal. (Bottom) Chromatogram showing peaks of standard gossypolone enantiomers spiked on the same sample extract [see 1′ and 3′ for spiked (+)- and (–)-gossypolone standard, respectively].

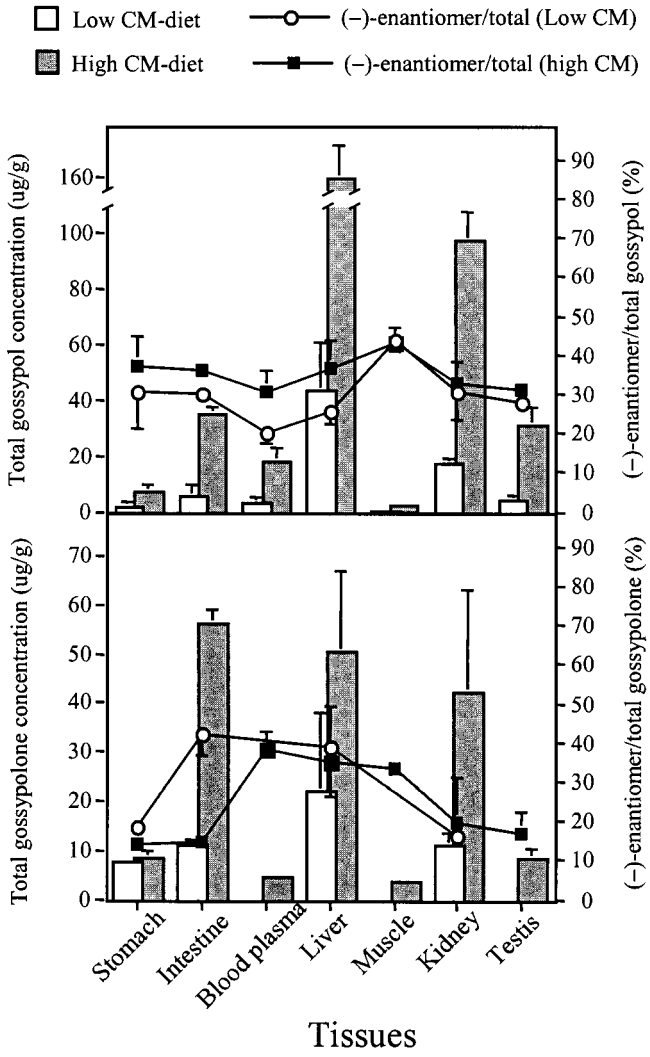


**Figure 2.** Total gossypol (top) and gossypolone (bottom) concentrations and percentage ratio of their (–)- to total gossypol in tissues of mature rainbow trout males (843 ± 86 and 792 ± 190 g for low- and high-CM treatments, respectively) fed two experimental diets formulated to contain low (15%) or high (60%) levels of cottonseed meal (CM). The values are means ± SD ($n$ = 4 fish).

## DISCUSSION

We found that all tissues in rainbow trout predominantly accumulated the (+)-gossypol enantiomer rather than the (−)-enantiomer after a prolonged feeding of CM diets that contained equal proportions of each gossypol enantiomer. This result was demonstrated in our previous studies of adult rainbow trout (*11*, *13*). In general, the proportion of (+)-/(−)-gossypol in CM is dependent on cotton cultivars (*16*). Gamboa et al. (*17*) reported that CM processed from eight oil mills (five expander solvent, two expeller, and one direct solvent) exhibited (+)-gossypol to total gossypol proportions ranging from 53.8 to 61.3%. Consistent with our result in fish, Wang et al. (*18*) reported that in rats the (−)-gossypol concentration was decreased at a higher rate than the (+)-gossypol concentration in blood plasma in vitro and in vivo. The authors suggested that the reason for the phenomenon can be attributed to more specific binding of (+)-gossypol to serum proteins, thereby resulting in a higher excretion rate of (−)-gossypol in vitro.

Bailey et al. (*16*) reported that in chickens fed a CM diet containing a gossypol (+)-/(−)-enantiomer proportion of 83:17, the ratio in liver tissues was 91:9. Other studies revealed a higher retention of (+)-gossypol in humans and dogs (*19*) and mice (*20*) and suggested that it was a result of the lower affinity of (−)-gossypol for proteins. We also previously found a higher affinity of (+)-gossypol for proteins in rainbow trout seminal plasma and spermatozoa in comparison to (−)-gossypol (*14*). The present study, therefore, supports the previous studies (*14*, *17*−*21*) in terms of a higher retention of (+)-gossypol than of (−)-gossypol in vivo. Also, we found a dependence of (−)-gossypol retention in tissues on dietary concentrations of gossypol enantiomers. The fish fed a high-CM diet showed a higher retention of (−)-gossypol than that of the fish fed a low-CM diet. This resulted in a higher ratio of (−)-gossypol to total gossypol in all tissues tested.

Current evidence suggests that (+)-gossypol has a higher affinity for serum albumin than (−)-gossypol, but there is no proof that (−)-gossypol is present predominantly in free form as hypothesized by Wang et al. (*18*) and Tanphaichitr et al.

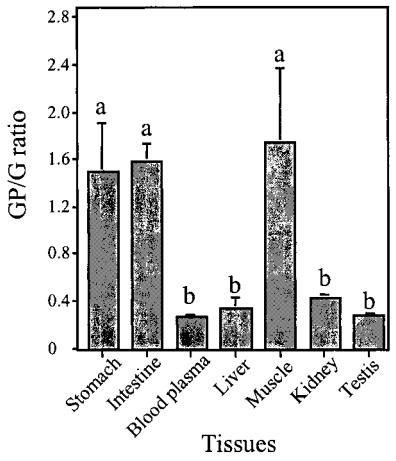


**Figure 3.** Ratio of total gossypolone (GP) to gossypol (G) in tissues of mature rainbow trout (843 ± 86 and 792 ± 190 g for low- and high-CM treatments, respectively) fed two experimental diets formulated to contain low (15%) or high (60%) levels of cottonseed meal (CM). The values are means ± SD ($n = 4$ fish).

**Table 2.** Concentrations of Free, Bound, and Total Gossypol Enantiomers in Blood, Seminal Plasma, and Spermatozoa of Mature Rainbow Trout Fed the High-CM Diet (60% Cottonseed Meal-Containing Diet)[a]

| | blood plasma (μg/mL) | seminal plasma (μg/mL) | spermatozoa (μg/g) |
|---|---|---|---|
| total gossypol | | | |
| (+)-enantiomer | 12.4 ± 2.99 | 0.091 ± 0.05 | 2.89 ± 1.39 |
| (–)-enantiomer | 5.63 ± 2.58 | 0.047 ± 0.03 | 1.51 ± 0.57 |
| free gossypol | | | |
| (+)-enantiomer | 2.18 ± 0.55 | 0.048 ± 0.032 | 0.16 ± 0.05 |
| (–)-enantiomer | 0.48 ± 0.23 | 0.021 ± 0.010 | - |
| bound gossypol | | | |
| (+)-enantiomer | 10.2 ± 2.65 | 0.043 ± 0.020 | 2.73 ± 1.38 |
| (–)-enantiomer | 5.15 ± 2.38 | 0.025 ± 0.022 | 1.51 ± 0.57 |
| ratio of bound/ total gossypol | 85.07 ± 2.82 | 48.04 ± 7.73 | 95.74 ± 2.29 |

[a] Values are mean ± SD ($n = 5$ fish).

(*20*). Therefore, we examined the proportion of each enantiomer of gossypol in blood plasma and spermatozoa (**Table 2**). However, our result in the present study did not give confirmative data that support the hypothesis by other authors (*18*, *20*). The proportions of (−)-gossypol in free and bound gossypol were almost the same in the blood plasma (**Table 2**). Therefore, it cannot be concluded that (−)-gossypol exists mainly as a free form of gossypol because of its lower concentration or accumulation in tissues compared to (+)-gossypol.

Gossypol is a well-known antifertilizing agent in males of humans (*22*−*24*), rats (*25*−*27*), bulls (*28*−*30*), monkeys (*31*), and fish (*14*, *32*, *33*). It negatively affects spermatozoa and/or steroidogenesis. The determination of free and bound gossypol enantiomers in spermatozoa of rainbow trout fed a high-CM diet exhibited a possible mechanism for a strong antifertility effect. Free gossypol is defined as "acetone soluble gossypol" (*34*), whereas bound gossypol can be estimated by subtracting the portion of free form from total gossypol. In the present study, gossypol in spermatozoa was found mostly in bound form (97%) compared to other tissue compartments, such as liver, blood, and seminal plasma (range from 48.0 to 85.1%). In previous studies in fish (*32*, *33*), gossypol impaired the motility and fertilizing ability of sperm. However, in rainbow trout fed CM-containing diets, sterility was never observed. In mammals, the cytotoxicity of gossypol on sperm has been extensively studied (*35*, *36*). It was reported that acrosin inhibition may be a mechanism in which gossypol molecules bind to the sperm membrane and prevent fertilization (*37*). Two possible reasons for the higher affinity of sperm membrane for gossypol may be considered. First, gossypol has more specific affinity for sperm proteins than other tissue proteins. It can be assumed that spermatozoa contain higher levels of α-amino groups in their chemical composition than other tissues that potentially make more Schiff bases with gossypol (*38*). It is known that spermatozoa have a higher level of histone, a basic protein molecule, in their chromosomal protein components in comparison to that of other tissues (*39*). Second, there would be a certain binding of gossypol to sperm phospholipids. The sperm plasma membrane has a relatively high level of phospholipids (*40*), specifically containing highly unsaturated fatty acids [in fish (*41*) and mammalian sperm (*37*, *42*, *43*)] compared to other tissues.

In the present study, (+)-gossypolone was more readily accumulated than its (−)-enantiomer, although differences among tissues were highly significant (**Figure 2**). No literature is available with respect to the retention of gossypolone enantiomers in vivo. However, the present study indicated that the binding properties or retention of gossypolone enantiomers are likely to be similar to those of gossypol enantiomers.

The total gossypol concentration was highest in liver and lowest in muscle tissue as demonstrated in adult rainbow trout (*11*), lambs (*9*), and chickens (*17*, *21*). Although liver was the main organ involved in the metabolic oxidation and/or elimination of gossypol (*11*, *44*), significant metabolic oxidation of gossypol to gossypolone seemed to occur in the digestive tract (**Figure 2**), where contact with ferric ions occurs (*45*) in fish as determined from the present findings. In this study, higher levels of gossypolone were found in liver, intestine, and kidney in comparison to other tissues. Interestingly, the gossypolone/gossypol ratio was higher in stomach, intestine, and muscle; we could hypothesize that significant metabolic oxidation occurs in the digestive tract in rainbow trout but deposition occurs in muscle tissue (**Figure 3**).

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