DOI: 10.2478/s11686-013-0140-z

Acta Parasitologica, 2013, 58(3), 259–262; ISSN 1230-2821

VERSITA

# Effects of *Schistosoma mansoni* infection on lutein and β-carotene concentrations in *Biomphalaria glabrata* snails as determined by quantitative high performance reversed phase thin-layer chromatography

**Nicole Dieterich[1], Bernard Fried[2]*and Joseph Sherma[3]**

[1]Program in Neuroscience, [2]Department of Biology, [3]Department of Chemistry, Lafayette College, Easton PA

## Abstract

High performance thin-layer chromatography was used to determine the concentration of β-carotene and lutein in the whole body and digestive gland-gonad complex (DGG) of uninfected *Biomphalaria glabrata* snails and those infected with *Schistosoma mansoni* for 6 and 8 weeks. Pigments were extracted from the snails using acetone and separated on EMD Millipore reversed phase C-18 plates with concentration zone using petroleum ether-acetonitrile-methanol (1:1:2) mobile phase. After development, two yellow pigment zones, lutein and β-carotene, were identified with respective $R_f$ values of 0.55 and 0.13 and then quantified by densitometry. Statistical analysis of the weight percentages of each pigment showed a significant decrease ($P < 0.05$) in the concentration of β-carotene in the DGGs of infected *B. glabrata* at 6 and 8 weeks post-infection compared to the uninfected snails. No significant differences were seen in the concentrations of β-carotene in the whole body of the uninfected versus infected snail samples. Changes in the lutein concentration of the infected DGG and whole snail bodies were insignificant compared to the uninfected controls. In conclusion, larval *S. mansoni* infection caused a significant decrease in the β-carotene concentration of the DGG at 6 and 8 weeks post infection.

## Keywords

*Biomphalaria glabrata*, lutein, *Schistosoma mansoni*, high performance thin-layer chromatography, trematodes, β-carotene, densitometry

## Introduction

Carotenoids are widespread and important natural pigments in plants and animals and play vital roles in the health and well being of these organisms (Cazzonelli 2011). The most important carotenoids are α-carotene, β-carotene, β-cryptoxanthin, lutein, lycopene, zeaxanthin, violaxanthin, and neoxanthin (Zeb and Murkovic, 2010). Of these, most studies on snail-larval trematode host-parasite relationships have examined β-carotene and lutein. For instance, Fried *et al.* (1990) identified β-carotene in the digestive gland-gonad complex (DGG) of *Helisoma trivolvis* snails naturally infected with *Echinostoma trivolvis*. These authors also noted traces of lutein in the DGGs of *H. trivolvis*.

Marsit *et al.* (2000) used reversed phase (RP) high performance thin layer chromatography (HPTLC) with C-18 chemically bonded silica gel layers and densitometric scanning to show that infection with certain marine larval trematodes alters the carotenoid fractions in naturally infected *Cerithidea californica* snails. These authors reported a significant decrease in lutein of *C. californica* infected with either *Euhaplorchis californiensis* or *Mesostephanus appendiculatis* compared to the uninfected controls. The concentration of lutein in the *E. californiensis*-infected snails was more than twice that of the snails infected with *M. appendiculatis.* These authors also determined that the β-carotene content of whole *C. californica* snails was significantly reduced by infection with either trematode. The Marsit *et al.* (2000) study showed that certain larval trematode infections altered carotenoid fractions of the snail host and had different effects on lutein and β-carotene concentrations within the snails.

Evans *et al.* (2004) also used C-18 HPTLC-densitometry to study lipophilic pigments in two planorbid snails infected with echinostomatid trematodes. One study examined these

*Corresponding author: friedb@lafayette.edu

pigments in *H. trivolvis* naturally infected with *E. trivolvis* and the second study looked at *B. glabrata* experimentally infected with *E. caproni*. β-carotene and lutein concentrations were not significantly altered in the DGGs of the snails in both of these infections.

Surprisingly little information is available on the effects of infection by the medically important trematode, *S. mansoni*, on lipophilic pigments in the vector snail *B. glabrata*. Therefore, the purpose of our study was to examine the effects of experimental infection of *S. mansoni* on the β-carotene and lutein concentrations of *B. glabrata* infected with *S. mansoni*.

## Materials and Methods

*Maintenance of uninfected and S. mansoni infected B. glabrata snails*

*Biomphalaria glabrata* snails (NMRI strain) infected with *S. mansoni*, 10–14 mm in shell diameter, were obtained from Dr. Fred A. Lewis, Head, Schistosomiasis Laboratory, Biomedical Research Institute (Rockville, MD, USA) and used 6 and 8 weeks post-infection. Both stock cultures of uninfected *B. glabrata* adults and those infected with *S. mansoni* for 6 and 8 weeks were maintained at 23 ± 1°C in aerated glass jars, each containing 12–20 snails in 800 ml of artificial spring water (ASW), as described by Ulmer (1970). Cultures were fed boiled Romaine lettuce leaf ad libitum. Food and water for each culture was changed every other day. Infected snails were checked for infection by isolation in Stender dishes half filled with ASW. Only snails shedding *S. mansoni* cercariae were used. Procedures for sample preparation, HPTLC analysis, and weight percentage calculations are given below.

*Sample preparation*

For HPTLC analysis, the whole bodies of five snails (n = 5) and the DGGs of five others (n = 5) were prepared from both the uninfected and infected cultures. To do this, the shell of each snail was gently cracked with a hammer, and the snail body was removed from the shell with forceps. The DGGs were dissected free of the visceral masses with a fine scissor, and the visceral mass was discarded. Each sample (whole body or DGG) was rinsed with ASW until all extraneous matter and debris was removed. Snail samples were weighed and then frozen at –20°C until analysis could be conducted, which was usually within several weeks of sample preparation.

Individual whole body and snail DGG samples were homogenized in 2 ml of acetone in a 7 ml glass homogenizer. The supernatant was filtered through a Pasteur piper plugged with glass wool into a 6 ml glass vial covered with foil. The pellet was washed twice with 200 µl of acetone, and each washing was combined with the supernatant. The solutions were then evaporated to dryness under nitrogen in a 45°C water bath and reconstituted with 200–800 µl of heptane as necessary for the densitometric scan area of a least one sample zone to be bracketed within the scan areas of the standard zones during HPTLC analysis.

*TLC analysis*

HPTLC standards were lutein (>95%, No. 020306S, Indofine Chemical Co., Inc., Hillsborough, NJ, USA) and β-carotene (Type 1, synthetic, >93%, No. C9750, Sigma, St. Louis, MO, USA). Lutein working standard solution at 0.0100 µg/µL was prepared by dissolving 2.50 mg of the standard in 250 ml of dichloromethane. β-carotene stock standard solution was prepared at 1.00 µg/µl by dissolving 100 mg of standard in 100 ml of dichloromethane. Two consecutive 10-fold dilutions were made with the same solvent to prepare a 0.0100 µg/µl β-carotene working standard solution.

RP-HPTLC analysis was performed on EMD Millipore (Gibbstown, NJ, USA; a division of Merck KGaA, Darmstadt, Germany) 10 x 20 cm C-18 chemically bonded silica gel HPTLC plates with a concentrating zone (RP-18$F_{254}$s, Art. 15498). Plates were prewashed by development to the top with dichloromethane – methanol (1:1) and dried in a fume hood with warm air from a hair dryer. Standards of both pigments were applied with a 10.0 µl Drummond (Broomall, PA, USA) digital microdispenser onto the preadsorbent area in 4.00, 8.00, 12.0, and 16.0 µl aliquots (0.0400–0.160 µg), and reconstituted samples were applied in 4.00–16.0 µl aliquots. Initial zones were allowed to air dry for 20 sec prior to development of the plate in a CAMAG (Wilmington, NC, USA) HPTLC twin-trough chamber. A saturation pad (Analtech, Newark, DE, USA) was placed in the rear trough, and the covered chamber was equilibrated with the mobile phase for 15 min prior to development. The plates were developed with petroleum ether (37.8–53.5°C)-acetonitrile-methanol (1:1:2) mobile phase, as described by Francis and Anderson (2003), to a distance of 7 cm past the preadsorbent-C-18 layer interface. About 45 ml of the mobile phase was needed for each development, and the development time was approximately 15 min. After development, the plates were air dried for 5 min, and the pigments were visualized as yellow bands on a white background (Evans *et al*. 2004).

All experimental procedures were done rapidly in subdued light as described by Sherma *et al.* (1992) to prevent pigment degradation. Homogenization of samples, spotting and development of plates, and scanning were all done in a dark laboratory, and all samples were kept in aluminum foil-covered vials and stored at –20°C to inhibit degradation of pigments prior to analysis. Developed plates were covered in aluminum foil for transportation into lighted areas and for storage. Aluminum foil was also used to cover the developing chamber.

For quantification, densitometry of separated sample and standard zones was performed on the plates with a CAMAG TLC Scanner 3 in the absorption reflection mode using settings of slit width 4 mm, slit length 4 mm, and scanning rate 4 mm/s. The tungsten light source was set at the wavelength

of maximum absorption, which was 448 nm for lutein and 455 nm for β-carotene. The winCATS software automatically generated a linear regression calibration curve for each pigment relating the weights of the standard zones to their corresponding peak areas. From the calibration curve, the pigment weights in sample zones were automatically interpolated based on their scanned peak areas as described in Sherma *et al.* (1992). If the area of more than one aliquot of a sample was bracketed within the calibration curve, the weight corresponding to the aliquot closest to the middle of the calibration curve was used for calculation of results. The weight percentages of pigments in the snail whole body and DGG were calculated using the following equation:

$$\% \text{ pigment} = \frac{(w)(R)(\text{correction factor})(100)}{\mu\text{g snail sample}}$$

where $w$ = µg interpolated from calibration curve, and $R$ = [reconstituted volume (µl)]/ [spotted volume (µl)].

For quantification of some samples, dilution or concentration was required after reconstitution to obtain bracketed scan areas within the calibration curve. The appropriate correction factor was then included in the calculation of percent weight for each of the pigments.

## Results

Comparison of the migration of the sample and standard zones identified two major pigments in the *B. glabrata* extracts. The least polar zone had a retention factor ($R_f$) of 0.13, identical to the β-carotene standard; the most polar zone with an $R_f$ of 0.55 migrated identically to the lutein standard. A third zone was observed that was gray in color and had an $R_f$ of 0.22; this did not correspond to any of our pigment standards and, therefore, this zone could not be identified.

The mean and standard error for each analyte among the 3 sample groups (1 uninfected control, 2 infected experimental samples [6 and 8 weeks post-infection]) were calculated, and the values from the two infection types were compared to the uninfected control values using the Student's *t*-test to determine statistical differences, with $P<0.05$ being considered significant. The results are presented in Table I.

Statistical comparison of the weight percentage of each pigment showed a significant decrease, $P<0.05$, in the weight percent of β-carotene in the DGGs of the uninfected compared to the 6 and 8-week post-infection snail samples. No significant differences ($P>0.05$) were observed for the β-carotene concentrations in the whole body of the uninfected and infected snail samples at 6 and 8 weeks post-infection.

Lutein showed a decrease in the weight percentage of infected whole snails and the DGGs compared to the uninfected controls. However, the differences were not statistically significant (see Table I).

## Discussion

Fried *et al.* (1990) provided qualitative evidence to show more intense β-carotene zones on TLC plates in *H. trivolvis* snails infected with *E. trivolvis* compared to the uninfected snails. The results of our present study disagree with these previous qualitative findings since our quantitative values showed a significant decrease in β-carotene concentrations in *B. glabrata* infected with *S. mansoni* as compared to the matched uninfected controls. However, when we compared our current results with those of Marsit *et al.* (2000), our findings were in agreement with that study in that the β-carotene concentration of infected snails was significantly decreased compared to that of uninfected snails.

Our present *S. mansoni* study showed no significant differences in the lutein content of uninfected versus infected *B. glabrata* snails, a finding similar to that of Evans *et al.* (2004) for *B. glabrata* infected with *E. caproni*. However, we did note insignificant decreases in the lutein content in both the whole body and DGG of our infected versus uninfected snails. Further studies on the lutein content of infected versus uninfected snails are warranted since Marsit *et al.* (2000) found that the lutein concentrations of naturally infected *C. californica* snails infected with either *E. californiensis* or *M. appendiculates* was significantly reduced compared to the uninfected controls.

In conclusion, it is obvious from the studies reported herein that the results of larval trematode infections on lipophilic pigments in snails are quite variable and depend to a large part on the specific larval trematode snail interaction studied. Of major importance in our current study is that the

**Table I.** Weight percentage of β-carotene and lutein in the whole body and digestive gland gonad complexes (DGG) of *S. mansoni* infected (I) and uninfected (U) *B. glabrata* snails

| Pigment | Sample | U* | I*[a] | I*[b] |
|---|---|---|---|---|
| β-carotene | Whole body | 0.0549 ± 0.0062 | 0.0501 ± 0.00081 | 0.0438 ± 0.0049 |
| | DGG | 0.00731 ± 0.00070 | 0.00228 ± 0.00054** | 0.00137 ± 0.00078** |
| Lutein | Whole body | 0.00480 ± 0.0013 | 0.00199 ± 0.0074 | 0.00236 ± 0.00095 |
| | DGG | 0.00623 ± 0.00046 | 0.00494 ± 0.0033 | 0.00411 ± 0.0074 |

*Mean (wt. %) ± SE; n = 5 individual snails for each sample, **Concentration significantly reduced (Student's *t*-test, $P<0.05$), [a]Snails were 6 weeks post-infection, [b]Snails were 8 weeks post-infection.

concentration of β-carotene was significantly reduced in the DGG of *B*. *glabrata* infected with *S. mansoni* at 6 and 8 weeks post-infection.

**Acknowledgements.** Nicole Dieterich was supported by the Department of Biology and the Department of Chemistry, Lafayette College, Easton, PA, USA. We are also grateful to Dr. Fred A. Lewis, Head, Schistosomiasis Laboratory, Biomedical Research Institute, Rockville, MD, USA, for supplying the *B. glabrata* snails used in this work through NIH-NIAID contract HHSN2711010000051. The authors also thank David Lentz of EMD Millipore for providing the C-18 RP HPTLC plates.

## References

Cazzonelli C.I. 2011. Carotenoids in nature: insights from plants and beyond. *Functional Plant Biology*, 38, 833–847. DOI: 10.1071/FP11192.

Evans R.T., Fried B., Sherma J. 2004. Effects of diet and larval trematode parasitism on lutein and β-carotene concentrations in planorbid snails as determined by quantitative high performance reversed phase thin layer chromatography. *Comparative Biochemistry and Physiology*, *Part B*, 137, 179–186. DOI: 10.1016/j.cbpc.2003.11.003.

Francis G.W., Anderson O.M. 2003. Natural pigments. In: *Sherma J.*, *Fried B. (Eds.)*, *Handbook of Thin-Layer Chromatography*. *3rd ed. Marcel Dekker*, *NY*, 708–714 pp.

Fried B., Holender S., Shetty P.H., Sherma J. 1990. Effects of *Echinostoma trivolvis* (Trematoda) infection on neutral lipids, sterols, and carotenoids in *Helisoma trivolvis* (Gastropoda). *Comparative Biochemistry and Physiology*, *Part B*, 97, 601–604.

Marsit C.J., Fried B., Sherma J. 2000. High-performance thin-layer chromatographic analysis of lutein and β-carotene in *Cerithidea californica* (Gastropoda) infected with two species of larval trematodes. *Journal of Parasitology*, 86, 635–636. DOI: 10.1007/s004360050061.

Sherma J., O'Hea C.M., Fried B. 1992. Separation, identification, and quantification of chloroplast pigments by HPTLC with scanning densitometry. *Journal of Planar Chromatography-Modern TLC*, 5, 343–349.

Zeb A., Murkovic M. 2010. Thin-layer chromatographic analysis of carotenoids in plant and animal samples. *Journal of Planar Chromatography-Modern TLC*, 23, 35–39. DOI: 10.1556/JPC.23.2010.2.1.

Ulmer M.J. 1970. Notes on rearing snails in the laboratory. In: *MacInnis A.J., Voge M. (Eds.)*, *Experiments and Techniques in Parasitology*. *W.H. Freeman, San Francisco*, 143–144 pp.

(Accepted March 18, 2013)