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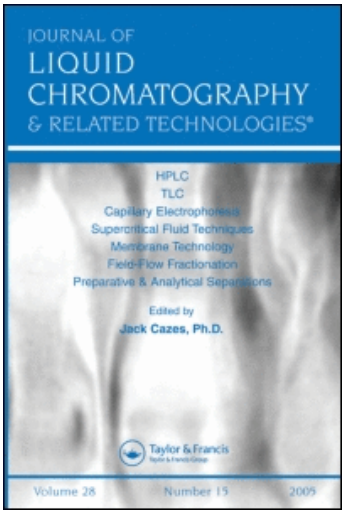

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Effects of Estivation on Lutein and β-Carotene Concentrations in *Biomphalaria glabrata* (NMRI Strain) and *Helisoma trivolvis* (Colorado Strain) Snails as Determined by Quantitative High Performance Reversed Phase Thin Layer Chromatography

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Abstract: High performance thin layer chromatrography (HPTLC) on C-18 chemically bonded silica gel layers with petroleum ether-acetonitrile-methanol (1:2:2) mobile phase and visible mode slit-scanning densitometry was used to examine the effects of estivation on β-carotene and lutein in the medically and economically important snails *Biomphalaria glabrata* (NMRI strain) and *Helisoma trivolvis* (Colorado strain). Snails were estivated for 2 weeks in moist chambers at 24°C and a relative humidity of 98%. Controls were maintained at 24°C for the same period in aerated aquaria containing artificial spring water and fed Romaine lettuce leaves *ad libitum*. As determined by HPTLC analysis, estivation had no significant effect on the weight percentage of β-carotene in the whole body or digestive gland-gonad complex (DGG) of both snail species. A significant reduction (Student's *t*-test, $P < 0.5$) was found in the percentage of lutein in the DGG of *H. trivolvis*. Whereas published and unpublished studies have demonstrated marked reductions of carbohydrates and

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lipids in estivated pulmonate snails, the effects of estivation on lipophilic pigments in these snails does not appear to be as pronounced.

Keywords: HPTLC, Estivation, β-carotene, Lutein, *Biomphalaria glabrata*, *Helisoma trivolvis* (Colorado strain), Whole body, Digestive gland-gonad complex (DGG)

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INTRODUCTION

Various studies have shown differences in lutein and β-carotene content in pulmonate snails as a function of diet and trematode parasitism.[1,2] Our laboratory has begun studies on the effects of estivation on various analytes in medically and economically important pulmonate snails. Estivation is a state of dormancy in cold-blooded animals, including gastropod mollusks, akin to hibernation in warm-blooded animals. Estivation can be induced in the laboratory by placing snails on moist filter paper in a closed chamber maintained at a relative humidity (RH) of about 98%. In the wild, snail estivation occurs naturally under conditions of drought when bodies of water dry out and snails are exposed to the air, usually on muddy or sandy substratum.

Relatively little information is available on the effects of estivation on snail analytes. Some studies have looked at depletion of carbohydrates, physiological differences[3] or changes in the organic acid[4] content of pulmonate snails; however, studies on possible changes in lipophilic pigments, i.e., lutein and β-carotene, are not available. The purpose of this study was to provide information on the effects of estivation on the whole body and digestive gland-gonad complex (DGG) of Biomphalaria *glabrata* (NMRI strain) and *Helisoma trivolvis* (Colorado strain) snails.

EXPERIMENTAL

Sample Preparation

Stock cultures of sexually mature *B. glabrata* and *H. trivolvis* were maintained in our laboratory, 20 per 800 mL of artificial spring water (ASW) for each snail species as described in Schneck and Fried.[5] During the course of this study, a total of eight *H. trivolvis* snails and eight *B. glabrata* were estivated, and the same number of snails of both species were used as the estivation controls. To induce estivation, snails of each species were maintained four per 25 cm diameter finger bowls containing a filter pad moistened with ASW and three Stender dishes each containing 5 mL of ASW. The ASW was prepared according to Ulmer.[6] Each Stender dish was covered with gauze to prevent snails from crawling into the dish.

Each finger bowl was covered loosely with another bowl to maintain a high RH and to allow for exchange of atmospheric air. The temperature in the bowl was 24°C and the RH was 98%. All eight *H. trivolvis* and seven of eight 8 *B. glabrata* snails survived estivation for 14 days under these conditions as determined by snail revival in ASW, which required an average of 20 min for *B. glabrata* and 30 min for *H. trivolvis*. Three snails of each species were used to analyze whole bodies and another three of each species were used for the analysis of DGGs. To do this, the shell was removed with forces and the whole body dissected from the shell and extracted in acetone as described below. For analysis, the DGG was dissected away from the viscera and extracted in acetone. All extracts were prepared from either a single whole snail or a single DGG. Controls were composed of the same number of snails maintained in Mason jar cultures, fed Romaine lettuce leaves *ad libitum*,[5] and extracted at the same time as the samples prepared for the estivation studies. Each sample for each species (whole body or DGG) was rinsed five times with ASW to remove shell and other debris and homogenized individually in 2 mL of acetone using a glass homogenizer. The supernatant was filtered through a Pasteur pipet plugged with glass wool into a 6 mL glass vial covered with aluminum foil. The pellet was washed twice using 200 μL of acetone, and each washing was combined with the supernatant. The solutions in the vials were evaporated to dryness using nitrogen in a 45°C water bath, and the residues were reconstituted with 100–200 μL of heptane, as necessary for the densitometric scan areas of at least one sample zone to be bracketed within the scan areas of the standard zones during HPTLC analysis.

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TLC Analysis

HPTLC analysis was performed on Merck (EMD Chemicals Inc, Gibbstown, NJ, USA, an affiliate of Merck KGaA, Darmstadt, Germany) 10 × 20 cm C-18 chemically bonded silica gel reversed plates with a preadsorbent zone (RP 184_{254}s, Art. 15498). Plates were prewashed by development to the top with dichloromethane-methanol (1:1) and dried in a fume hood with air. Standards of lutein and β-carotene were spotted with a 10.0 μL Drummond (Broomall, PA) digital microdispenser onto the preadsobent areas of separate lanes in aliquots of 4.00, 8.00, 12.0 and 16.0 μL, and reconstituted samples were applied in 2.00–16.0 μL aliquots. The initial zones were allowed to air dry for 30 s prior to development of the plate in a rectangular Camag (Wilmington, NC, USA) HPTLC twin-trough chamber. The chamber was covered in aluminum foil, lined with a saturation pad (Analtech, Newark, DE, USA), and equilibrated with the mobile phase for 15 min prior to development. The mobile phase used was petroleum ether (37.8–53.5°C)-acetonitrile-methanol (1:1:2) as described by Francis and Anderson.[7] The plates were developed in the mobile phase to a distance of

7 cm past the preadsorbent-C-18 layer interface. About 45 mL of mobile phase was needed for each development, and the development time averaged 19 min. The plates were air dried for 35 sec after development, and the pigments appeared as yellow bands on a white background.

All experimental procedures were done rapidly in subdued light as recommended by Sherma et al.[8] to prevent pigment degradation. Homogenization of samples, spotting of plates, development, and scanning were done in a dark room, 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 when transported into lighted areas and for storage. Aluminum foil was also used to cover the developing chamber.

Quantification was performed by densitometry of the separated sample and standard zones on the plates with a Camag TLC Scanner II in the reflection mode using settings of slit width 4, slit length 4, and scanning rate 4.0 mm s^{-1}. The tungsten light used was set at the maximum absorbance wavelengths of 448 nm for lutein and 455 nm for β-carotene. CATS-3 software was used to generate a linear regression calibration curve relating the weight of the standard zones (0.0400–0.160 μg) to their scanned peak areas, and the pigments in the sample zones were automatically interpolated based on their scan areas. When more than one aliquot of the same sample was bracketed within the calibration curve, the mass corresponding to the aliquot closest to the middle of the calibration curve was used to calculate the results. The percentages of pigments in the snail whole body and DGG were calculated using the following equation:

$$\%\ \text{Pigment (w/w)} = \frac{(w)(R)(\textit{Correction factor})}{\mu\text{g } \textit{snail sample}}$$

where $w = \mu$g 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 subsequently used in the calculation of the percent of each pigment.

RESULTS AND DISCUSSION

By comparing the migration of the pigment standard and sample zones, β-carotene and lutein were both identified in the chromatograms of the whole body and DGG extracts for snails of species that had estivated for two weeks and for the controls. The R_F values for the lutein and β-carotene bands were 0.45 and 0.07, respectively. Other dark green pigment bands

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Table 1. Prcentage of β-carotene and lutein in the whole body and DGG of *H. trivolvis* snails estivated for 14 days and the nonestivated controls

Pigment	Sample	Control[a]	Estivated[a]	*P*
β-Carotene	Whole body	0.00364 ± 0.00023[b]	0.00400 ± 0.00083[b]	0.89
Lutein	Whole body	0.0103 ± 0.0034[b]	0.00944 ± 0.0050[b]	0.89
β -Carotene	DGG	0.00344 ± 0.0014[b]	0.00421 ± 0.0024[b]	0.43
Lutein	DGG	0.0313 ± 0.0018[b]	0.0117 ± 0.0027[b,c]	0.0017

[a]Mean (%, w/w) ± standard error; n = 4 individual snails for each sample.
[b]n = 3 individual snails after elimination of one value with the statistical Q-test.
[c]Concentration significantly reduced (Student's *t*-test, $P < 0.05$) compared with control snails.

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with R_F values between those of lutein and β-carotene were seen but not characterized.

Table 1 lists quantitative data for lutein and β-carotene in the whole body and DGG extracts of *H. trivolvis*. The mean values of β-carotene in the whole body and DGG of *H. trivolvis* controls were 1.1 times and 0.9 times, respectively, compared with the estivated snails; values for lutein in the whole body and DGG of the *H. trivolvis* were 1.1 times and 2.6 times, respectively, for the controls compared with the estivated snails. The only value that was significantly different (Student's *t*-test, $P < 0.05$) was that of lutein in the DGG of *H. trivolvis*, for which the percent of lutein in the estivated snails was significantly reduced compared to the control.

Table 2 lists quantitative data for lutein and β-carotene in the DGG and whole body of *B. glabrata*. The mean values of β-carotene in the whole body and DGG of the control *B. glabrata* compared to the estivated samples were 0.5 times and 0.8 times, respectively. The mean values of lutein for the whole body and DGG of the control *B. glabrata* compared to the estivated samples were 0.3 times and 7.7 times for the DGG, respectively. Even though the lutein content in the DGG of the *B. glabrata* control sample

Table 2. Percentage of β-carotene and lutein in the whole body and DGG of *B. glabrata* estivated for 14 days and the nonestivated controls

Pigment	Samples	Control[a]	Estivated[b]	*P*
β−Carotene	Whole body	0.00216 ± 0.0010[b]	0.00421 ± 0.0018[b]	0.31
Lutein	Whole body	0.00182 ± 0.014[b]	0.00727 ± 0.0015[b]	0.25
β−Carotene	DGG	0.00549 ± 0.00098[b]	0.00670 ± 0.0017[b]	0.58
Lutein	DGG	0.0184 ± 0.0051[b]	0.00237 ± 0.00092[b]	0.09

[a]Mean (%, w/w) ± standard error; n = 4 individual snails for each sample.
[b]n = 3 Individual snails after elimination of one value with the statistical Q-test.

was approximately 7.7 times greater than that of the estivated samples, Student's *t*-test revealed no significant difference in their mean values.

The overall effects of two weeks of estivation on lutein and β-carotene are not very pronounced in *B. glabrata* and *H. trivolvis* as indicated by the results of this study. In fact, only lutein showed a significant reduction in concentration in the DGG of *H. trivolvis*. The effects of estivation on lipophilic pigments will depend in part on the species of snail examined. For instance, in this study depletion of lutein was seen in estivated *H. trivolvis* (Colorado strain) but not in the NMR strain of *B. glabrata*.

The significance of the reduction in lutein in the DGG of estivated *H. trivolvis* is not apparent from the results of the study. Lutein is sequestered in the snail DGG; in snails maintained on a lettuce diet, this pigment reaches a particular level of concentration. During estivation, snails do not feed and are, therefore, not capable of obtaining lutein from a dietary source. In the absence of feeding, *H. trivolvis* probably uses some of its lutein for metabolic purposes and in the absence of a dietary source can not replenish it. The function of luetin in gastropods is speculative, although Hoskin and Cheng[9] suggested that lipophilic pigments such as carotene and lutein may serve as electron acceptors in snails exposed to anoxic conditions.

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