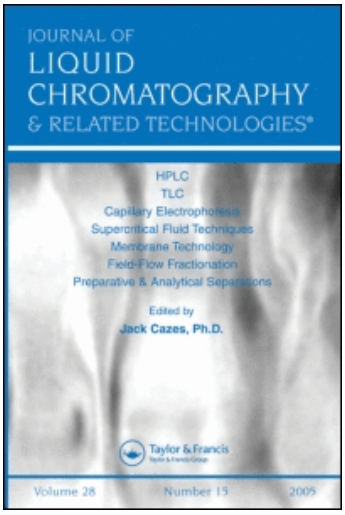

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Thin Layer Chromatographic Analysis of Neutral Lipids in Snail Conditioned Water and Feces of *Biomphalaria glabrata* Infected with *Echinostoma caproni*

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ABSTRACT

Thin layer chromatography (TLC) was used to determine neutral lipids in snail conditioned water (SCW) and feces from *Biomphalaria glabrata* snails infected with *Echinostoma caproni.* Samples were extracted in chloroform–methanol (2 : 1), and the neutral lipids were separated on silica-gel plates with a mobile phase consisting of petroleum ether–diethyl ether–glacial acetic acid (80 : 20 : 1). Zones were detected with phosphomolybdic acid reagent and quantified by densitometry. Qualitative analysis of SCW samples showed the presence of free sterols, free

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fatty acids, and triacylglycerols; qualitative analysis of fecal samples showed free sterols, free fatty acids, and unidentified pigments. Quantitative analysis of SCW with feces (SCW-WF) from infected snails showed $7.35 \pm 2.3\,\mu g\ 2^{-1}\,mL^{-1}$ free fatty acids compared with uninfected controls, which had $8.05 \pm 0.54\,\mu g\ 2^{-1}\,mL^{-1}$. Quantitative analysis of SCW without feces (SCW-WOF) showed that infected snails contained $4.76 \pm 0.64\,\mu g\,2^{-1}\,mL^{-1}$ of free fatty acids compared with uninfected controls, which had $5.88 \pm 0.34\,\mu g\ 2^{-1}\,mL^{-1}$. Free fatty acids were the major fraction in snail feces. Samples from infected snails had $0.0224\% \pm 0.0022\%$ compared with the uninfected controls, which had $0.0353\% \pm 0.0036\%$. The free fatty acids composition of the feces for infected snails was significantly lower than that of the uninfected samples (Student's *t*-test, $P < 0.05$).

Key Words: Thin layer chromatography; Neutral lipids; Snail conditioned water (SCW); *Biomphalaria glabrata*; *Echinostoma caproni*; Snail feces.

INTRODUCTION

Biomphalaria glabrata snails release substances such as lipids, sugars, and amino acids into water, which act as chemoattractants (pheromones) for larval trematodes and conspecific snails.[1–5] Water containing such chemoattractants is often referred to as snail conditioned water (SCW). Likewise, feces released from pulmonate snails may contain pheromones. To date, all such studies on SCW and snail feces have been done on uninfected *B. glabrata* and *Helisoma trivolvis* snails.[6] In a recent study on *B. glabrata* infected with *Schistosoma mansoni*, Boissier et al.[7] revealed that the behavior of infected snails was altered by larval trematode parasitism. Because behavior may be mediated, in part, by pheromones released into the water, our study examined neutral lipid fractions in SCW, and feces from *B. glabrata* snails infected with the economically important trematode *Echinostoma caproni*.[8] The purpose of this study was to determine if the neutral lipids in the SCW and the fecal contents of infected snails differed qualitatively and quantitatively from the uninfected controls. Analyses were done on SCW with feces (SCW-WF), SCW without feces (SCW-WOF), and feces alone, by use of high performance thin layer chromatography (HPTLC).

EXPERIMENTAL

Sample Preparation

Cultures of uninfected *B. glabrata* snails (8–12 mm in shell diameter) were maintained at $23^{\circ}C \pm 1^{\circ}C$ in aerated glass containers, each containing

10–20 snails per 800 mL of artificial spring water (ASW), under diffuse overhead fluorescent light for 12 hr day^{-1}. The ASW was prepared as described by Ulmer.[9] Matched cohorts of *B. glabrata* snails were infected with miracidia of *E. caproni* as described by Idris and Fried,[10] and used 7–10 weeks post infection. These snails contained patent infections of *E. caproni* and were the source of the infected snails.

To collect SCW, infected snails, 8–12 mm in shell diameter, were placed 10 snails per well in six-chamber-multiwell plates, with each well containing 5 mL of ASW, for 2 hr under overhead light at 28°C. For controls, 10 uninfected snails, 8–12 mm in shell diameter, were placed in each well as described for the infected snails. Eight experimental and eight control samples were used and divided into two groups. In the SCW-WF group, sample aliquots of 2 mL were removed from the well and placed in a 15-mL centrifuge tube. In the SCW-WOF group, sample aliquots of 2 mL were filtered through glass wool to remove the feces and placed in a 15-mL centrifuge tube. Each sample was extracted with 4 mL of chloroform–methanol (2 : 1) and filtered through glass wool, and the upper layer was removed and discarded. The lower, lipophilic layer was evaporated in a water bath (45–55°C) under nitrogen gas. All samples were reconstituted in 25.0 μL of chloroform–methanol (2 : 1) prior to HPTLC analysis.

Fecal samples were obtained from aerated glass containers, each containing 8–10 infected or uninfected snails in 800 mL of ASW for 3 days. The samples were collected by pipetting feces and ASW into a weighing dish lined with aluminum foil and then blotted dry. These samples (approximately 100–200 mg) were extracted with 2 mL of chloroform–methanol (2 : 1) and filtered through glass wool, and 1-mL Folch wash (0.88% KCl) was added to separate the filtrate into two layers. The upper hydrophilic layer was discarded, and the lower lipophilic layer was evaporated to dryness in a water bath (45–55°C) under nitrogen gas. Fecal samples from uninfected and infected snails were reconstituted in 75.0 or 60.0 μL of chloroform–methanol (2 : 1), respectively, prior to HPTLC analysis.

Thin Layer Chromatography

The standard for neutral lipid analysis was the neutral lipid standard 18-4A (Matreya, Inc., Pleasant Gap, PA), which contained 20.0% each of cholesteryl oleate, methyl oleate, triolein, oleic acid, and cholesterol, and a total lipid concentration of 29.4 mg mL^{-1}. The standard zones were used to represent cholesteryl esters, methyl esters, triacylglycerols, free fatty acids, and free sterols, respectively, in the samples. The standard was placed in a 25-mL volumetric flask and diluted with chloroform–methanol (2 : 1) to prepare a standard solution containing 0.294 μg μL^{-1} for each of the

components. HPTLC analysis was performed on Whatmann (Clifton, NJ) LHPKDF silica gel plates, 10 × 20 cm, containing 19 scored lanes and a pre-adsorbent spotting area. Plates were precleaned by development to the top with dichloromethane–methanol (1 : 1). The standards (2.00, 4.00, 8.00, and 16.00 μL) and reconstituted samples (1.00, 4.00, 8.00, and 16 μL) were applied to the pre-adsorbent zone in individual lanes with a 10-μL Drummond (Broomall, PA) digital microdispenser. Plates were developed with the Mangold mobile phase,[11] petroleum ether–diethyl ether–glacial acetic acid (80 : 20 : 1), for a distance of 8.0 cm past the pre-adsorbent-silica gel interface, in a rectangular Camag (Wilmington, NC) HPTLC twin-trough chamber containing about 25 mL of the mobile phase in each trough, and a saturation pad (Analtech, Newark, DE) in the trough not used for development. Development times were 8–9 min. Developed plates were dried in a fume hood with cool air from a hairdryer for 5 min, sprayed with 5% ethanolic phosphomolybdic acid (PMA) solution, and heated for 10 min at 115°C on a Camag plate heater until blue neutral lipid bands appeared on a yellow background.

Quantitative densitometric analysis was done with a Camag TLC Scanner II, using the tungsten light source set at 610 nm and other settings of slit width 4, slit length 4, and scanning speed 4 mm sec^{-1}. The CATS-3 software was used to create a linear regression calibration curve relating the weights of the standard zones (0.598–4.77 μg) to their peak areas. Weights of lipids in sample aliquots were automatically interpolated from the curve based on their peak areas on each peak. If the areas of more than one aliquot of a particular sample were bracketed within the calibration curve, the weight corresponding to the sample area closest to the areas of the middle two standards was used to calculate the weight percent of the lipid.

The weight of lipid in SCW samples (μg 2^{-1} mL^{-1} SCW) was calculated by multiplying the interpolated weight from the calibration curve (μg) by the reconstitution volume (μL) and dividing by the amount of sample spotted (μL). The percentage of lipid in the feces sample was calculated by multiplying the interpolated compound weight by the ratio of the reconstitution volume to the volume of sample spotted, dividing by the wet weight of the sample (μg), and multiplying by 100.

RESULTS AND DISCUSSION

Neutral lipids were identified by comparison of sample zones with the migration of standards. Standard zones yielded R_F values for cholesterol, oleic acid, trolein, methyl oleate, and cholesteryl oleate of 0.21, 0.28, 0.53, 0.67, and 0.87, respectively, in the Mangold mobile phase. Free sterols,

free fatty acids, and triacylglycerols were identified in the SCW-WF and SCW-WOF based on comigration of their zones with the corresponding standard zones.

The fecal samples from both infected and uninfected snails contained free sterols and free fatty acids. A zone that stained blue upon PMA treatment was detected in the fecal samples ($R_F = 0.79$) but could not be positively identified. Three pigments were present on the plates containing the fecal samples; an orange-yellow pigment, possibly a carotene, migrated identically compared to the cholesteryl ester standard zone ($R_F = 0.87$), and two green pigments, possibly chlorophylls ($R_F = 0.030$ and 0.13), were present that did not have R_F values equal to any standard zone.

Table 1 presents data for the concentrations of free fatty acids in SCW-WF and SCW-WOF. The SCW-WF ($n = 4$) and SCW-WOF ($n = 4$) samples contained quantifiable levels of only free fatty acids. In all SCW samples, the levels of free sterols and triacylglycerols released into the water were below the quantification limit.

Table 2 presents the percentages of free sterols and free fatty acids in fecal samples from uninfected snails ($n = 20$) and those infected with *E. caproni* ($n = 14$). Mean values of free sterols and free fatty acids in snails infected with *E. caproni* were 2.5 and 1.6 times lower, respectively, than those in the feces of uninfected snails. The concentrations of free fatty acids in the feces of snails infected with *E. caproni* were significantly lower than those in the feces of uninfected snails (Student's *t*-test, $P < 0.05$).

Previous studies found that the accumulation of chemicals in *B. glabrata* SCW are dependent on snail age, diet, media used, and time at which the SCW was collected.[2,3,5] Chaffee et al.[2] reported that the major neutral lipid fractions released by *B. glabrata* into water were free sterols and free fatty acids, as identified in the present study. Chaffee et al.[2] used deionized water to collect SCW from *B. glabrata* and their snails were fed on a lettuce-tetramin diet. We used ASW to collect substances released by the snails, and our snails were fed on the leafy portion of Romaine lettuce. These differences may account, in part, for the variations in concentrations of free fatty acids reported in the two studies.

Table 1. Concentrations ($\mu g\ 2^{-1}\ mL^{-1}$) of free fatty acids released from *B. glabrata* snails into SCW.

	SCW-WF ($n = 4$)	SCW-WOF ($n = 4$)
Uninfected snails	8.05 ± 0.54	5.88 ± 0.34
Infected snails	7.35 ± 2.3	4.76 ± 0.64

Table 2. Percent by weight (mean ± SE) of neutral lipids in feces of snails infected with *E. caproni*.

	Free sterols	Free fatty acids
Infected samples	0.00784 ± 0.00099	0.0224 ± 0.0022[a]
Uninfected control samples	0.00941 ± 0.0024	0.0353 ± 0.0036

[a]Value significantly reduced compared to control samples (Student's *t*-test, $P < 0.05$).

Conaway et al.[6] reported that free fatty acids and free sterols are the major neutral lipids in feces of uninfected *B. glabrata* snails. They found that after 7 days of snail starvation, the levels of free fatty acids in the feces decreased significantly. In our study using well-fed *B. glabrata* snails, *E. caproni*-infected snails showed a significant decrease in the amount of free fatty acids in snail feces compared to the uninfected controls. Thompson[12] has discussed the starvation hypothesis in relation to snail–larval trematode interactions. This hypothesis suggests parallels between snails infected with larval trematodes and snail starvation. According to this hypothesis, snails infected with larval trematodes, and adequately-fed, will show a depletion in nutrients such as carbohydrates and lipid nutrients, similar to what would happen if uninfected snails were starved. Decline in the free fatty acid fraction in the feces of infected snails in our study tends to support the starvation hypothesis.

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