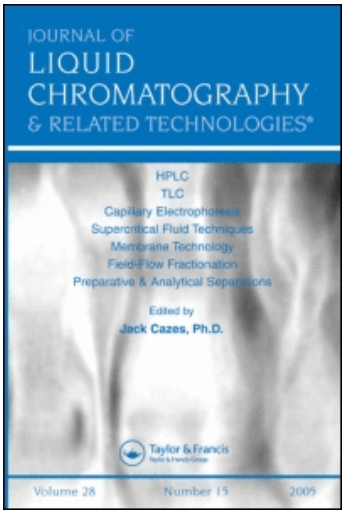

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HIGH PERFORMANCE THIN-LAYER CHROMATOGRAPHIC ANALYSIS OF NEUTRAL LIPIDS IN THE MARINE SNAILS *ILYANASSA OBSOLETUS* AND *LITTORINA LITTOREA* INFECTED WITH LARVAL TREMATODES

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ABSTRACT

High performance thin-layer chromatography analysis was used to analyze neutral lipids in the digestive gland-gonad (DGG) complex of the marine snails *Ilyanassa obsoletus* and *Littorina littorea* infected with larval trematodes. The results were compared with the DGGs of snails not infected with larval trematodes. The most abundant neutral lipid in both infected and non-infected snails was triacylglycerols, with percentages ranging from 2.8% in *I. obsoletus* snails infected with unidentified armatae cercariae to 24.7% in *I. obsoletus* snails infected with *Zoogonius rubellus*. *L. littorea* snails infected with an unidentified armatae cercaria contained 6 to 7 times the amount of cholesteryl ester than uninfected snails or those infected with *Cryptocotyle lingua* cercariae. *I. obsoletus* snails infected with *Z. rubellus* or an unidentified armatae cercaria contained 2 to 3 times more free sterol than the uninfected controls. Larval trematode parasitism was found to alter the neutral lipid profiles of the marine snails *I. obsoletus* and *L. littorea*.

INTRODUCTION

Few studies are available on the pathobiochemical effects of larval trematodes in their snail hosts.[1] The pathobiochemical effects of larval trematodes on the neutral lipid profiles of the freshwater snail *Helisoma trivolvis*, infected with various larval trematodes, have been studied.[2] The study showed that neutral lipid profiles in the snails varied both qualitatively and quantitatively as a result of larval trematode parasitism.

Few studies are available on the effects of larval trematodes on the neutral lipid profiles of marine snails. We recently obtained two species of marine snails infected with larval trematodes. The first snail species, *Ilyanassa obsoleta*, was infected with the cercariaeum of *Zoogonius rubellus* and an unidentified xiphidio cercaria in the armatae group. The second snail species, *Littorina littorea*, was infected with the heterophyid cercaria of *Cryptocotyle lingua*, and an unidentified xiphidio cercaria in the armatae group.

The purpose of this study was to use high performance thin-layer chromatography (HPTLC) to analyze the neutral lipids in marine snails infected with the above-mentioned larval trematodes and compare the lipid profiles with those of uninfected snails (i.e., snails not harboring larval trematode infections).

Results were also compared with the previous study[2] on neutral lipids in the freshwater snail *H. trivolvis* infected with various species of larval trematodes.

EXPERIMENTAL

Preparation of Samples

Snails were obtained from an intertidal zone in Saunderstown, Rhode Island and prepared for HPTLC within one day of receipt or following maintenance for up to 2 weeks in artificial sea water at 12°C. To remove the snail body, each snail was dissected individually in sea water, its shell removed, and the infection status of the snail was determined by examining the digestive gland-gonad complex (DGG). As mentioned in the Introduction, snails were identified as infected with a specific larval trematode[3] or with a cercarial type[4] when identification did not match published criteria.

Snails not infected with larval trematodes were designated as uninfected controls. Snail DGGs were handled individually and, following dissection from snail bodies, they were rinsed briefly in sea water, blotted on filter paper, and

weighed on an analytical balance. The weights of individual DGGs (both infected and uninfected) ranged from 56 to 278 mg. Weights of infected and uninfected DGGs were matched prior to their HPTLC analysis. To extract lipids, each DGG was homogenized in 2 mL of chloroform-methanol (2:1), and the extracts were filtered through a plug of glass wool contained in a Pasteur pipet. Nonlipid contaminants were removed by the addition of 0.5 mL of Folch wash (0.88% KCl). The top aqueous phase was separated from the bottom lipid phase and was discarded. The lipid phase was dried under a stream of nitrogen at 22°C, and the HPTLC sample was prepared by reconstitution with 1000 µL of chloroform-methanol (2:1).

Preparation of Standards

The standard used for HPTLC analysis was neutral lipid standard 18-4A (Matreya, Pleasant Gap, PA, USA) containing equal concentrations of cholesterol, oleic acid, triolein, methyl oleate, and cholesteryl oleate. The standard was diluted with chloroform-methanol (2:1) to contain 0.200 µg/µL of each lipid.

HPTLC Analysis

Neutral lipid analysis by HPTLC was performed on Whatman (Clifton, NJ, USA) 20 x 10 cm LK5-DF silica gel TLC plates with 19 lanes and a pre-adsorbent zone. Plates were pre-cleaned by development to the top with dichloromethane-methanol (1:1) and air-dried in a fume hood. The standard and reconstituted samples were applied in 2.0, 4.0, 8.0, and 16.0 µL aliquots to separate lanes using a 25 µL Drummond (Broomall, PA) digital microdispenser and dried with a hair dryer.

Plates were developed to a distance of 6.0 cm past the pre-adsorbent-silica gel interface with the Mangold solvent system (petroleum ether-diethyl ether-glacial acetic acid, 80:20:1) in a paper-lined Camag (Wilmington, NC, USA) twin-trough HPTLC chamber. Development took 10-12 min. The plates were dried with a hair dryer, sprayed with 5% phosphomolybdic acid in ethanol, and heated in a 110°C oven for 15 min. Neutral lipids appeared as blue zones against a yellow background.

Densitometry of sample and standard zones was performed using a Camag TLC Scanner II with the tungsten light source set at 700 nm, slit width 4, slit length 5, and scanning rate 4 mm/sec. The CATS-3 software was used to generate a calibration curve relating the weights of the standard zones (0.40-3.2 µg) to their peak areas.

Table 1

Percentage of Neutral Lipids in the Digestive Gland-Gonad Complex of *Littorina littorea* (*Ll*) and *Ilyanassa obsoletus* (*Io*)*

Snail Species	Sample Size	Infection Status	% Triacyl	% Free Sterol	% Chol. Ester	% Free Fatty Acid
LI	3	Un.	9.6 ± 4.2	0.21 ± 0.064	0.63 ± 0.064	0.049 ± 0.012
LI	3	*C.l.*	7.9 ± 3.6	0.228 ± 0.0069	0.075 ± 0.025	0.053 ± 0.020
LI	4	Un.C.	10.9 ± 3.1	0.19 ± 0.050	0.099 ± 0.050	0.076 ± 0.016
Io	1	*Z.r.*	24.7	0.21	0.26	N.D.
Io	1	Un.	2.8	0.36	0.45	N.D.
Io	5	Un.C.	5.2 ± 2.3	0.095 ± 0.021	0.28 ± 0.13	N.D.

* Abbreviations: Un. = Unidentified *armatae cercaria*; *C.l.* = *Cryptocotyle lingua*; Un.C. = Uninfected control; *Z.r.* = *Zoogonius rubellus*; N.D. = Not determined.

The analyte weight in the sample aliquot having a scan area closest to that of the middle standard was determined by automatic interpolation from the calibration curve using its peak area. The percentage of all lipids but triacylglycerols in the sample was determined by means of the following equation:

$$\text{percentage of neutral lipid} = W \times R \times 1/S \times 100,$$

where W is the interpolated weight (μg) of lipid from the calibration curve, R is the ratio of the sample reconstitution volume (1000 μL) to the volume of the sample aliquot, and S is the wet weight of the DGG sample (μg). To quantify triacylglycerols, multiplication by a dilution factor was necessary to obtain triacylglycerol zones bracketed within the calibration curve.

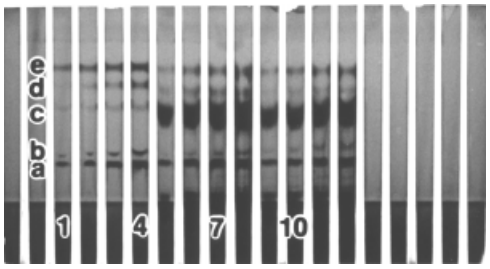

Figure 1. Photograph of chromatograms showing the neutral lipids in extracts of infected and uninfected DGG samples of *L. littorea*. Lanes 1-4 contain 2.0, 4.0, 8.0, and 16.0 μL aliquots of the 0.20 μg/μL 18-4A neutral lipid standard. Lanes 5-8 and 9-12 contain 2.0, 4.0, 8.0, and 16.0 μL aliquots of DGG samples infected with *C. lingua* and uninfected DGG samples, respectively. Abbreviations: a: cholesterol, b: oleic acid, c: triolein, d: methyl oleate, e: cholesteryl oleate.

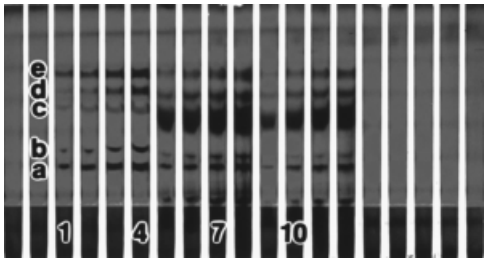

Figure 2. Photograph of chromatograms showing the neutral lipids in the extracts of infected and uninfected DGG samples of *I. obsoletus*. Lanes 1-4 contain 2.0, 4.0, 8.0, and 16.0 μL aliquots of the 0.20 μg/μL 18-4A neutral lipid standard. Lanes 5-8 and 9-12 contain 2.0, 4.0, 8.0, and 16.0 μL aliquots of DGG samples infected with *Z. rubellus* and uninfected DGG samples, respectively. Abbreviations: a: cholesterol, b: oleic acid, c: triolein, d: methyl oleate, e: cholesteryl oleate.

RESULTS AND DISCUSSION

The number of samples analyzed ranged from one to five, depending upon the availability of snails with particular infections and of uninfected controls (see Table 1). The major neutral lipid fraction detected in the DGGs of both infected and uninfected *L. littorea* and *I. obsoletus* was triacylglycerols (R_f = 0.62), as seen in Figure 1 (for *L. littorea*) and Figure 2 (for *I. obsoletus*). In order to quantify this fraction (see Table 1), samples had to be diluted with

chloroform - methanol (2:1), resulting in a dilution factor ranging from 11 to 44 in the equation given above. Lesser amounts of neutral lipids present in the DGG samples that were also quantified were free sterols (R_f = 0.26) and cholesteryl esters (R_f = 0.86) (see Table 1). Free fatty acids (R_f = 0.35) were also present but only quantifiable in samples from *L. Littorea* (see Table 1). The percentages of neutral lipids that were present in the DGGs of *L. littorea* and *I. obsoletus* are given in Table 1. Large standard errors in Table 1 indicate a high degree of variability in the lipid content of individual marine snails. Low amounts of methyl esters (R_f = 0.76) were also present but were not quantified because of the poor separation of this zone from that of the triacylglycerols.

An examination of the data in Table 1 shows some differences in the neutral lipid profiles of infected versus uninfected DGG samples in both *L. littorea* and *I. obsoletus*. There was an approximately six times increase in cholesteryl esters in the DGGs of *L. littorea* infected with the unidentified armatae cercaria compared with *L. littorea* infected with *C. lingua* or the uninfected controls. Moreover, the data on the percentage of free sterol in infected versus uninfected *I. obsoletus* snails suggested that there was more free sterol in infected snails, but since only two infected snails were available for analysis, no definitive conclusions can be made at this time.

No major patterns were seen in the triacylglycerol profiles of infected and uninfected *L. littorea* and *I. obsoletus* due to the variability of this neutral lipid in individual snails.

In the only other published study on the effects of larval trematodes on the neutral lipid content of marine snails, it was shown, using TLC, that infection of *Littorina saxatilis rudis* by sporocysts of the trematode *Microphalus similis* reduced the levels of triacylglycerols and free fatty acids in the snail digestive gland compared with uninfected controls.[5] Unpublished observations[6] based on TLC analysis of the marine snail *Cerithidia californica* infected with various larval trematodes suggested that the lipid profile of the snail varied, depending on the type of larval trematode present. Observations from the present study, along with others,[5,6] suggest that larval trematodes alter the lipid profiles of marine snails as has been reported for fresh water snails.[2]

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