Chapter 4
Studies of *Biomphalaria* Snails Infected with Larval Trematodes Using Chromatography and Atomic Spectrometry

Joseph Sherma and Bernard Fried

Abstract This chapter describes chromatographic and atomic spectrometric methods used for the analysis of *Biomphalaria* snails infected with larval trematodes. Some of the analyses are concerned directly with larval schistosomes and echinostomes, whereas other analyses consider the snail tissues and organs infected with the larval schistosomes and echinostomes. Coverage includes the chromatographic methods used for the determination of different classes of organic compounds and atomic spectrometric methods for elemental analyses. The major chromatographic methods used have been thin-layer chromatography and high performance thin-layer chromatography. Atomic absorption spectrometry and inductivity coupled plasma-optical emission spectrometry have been used for determining certain elements, including metals. These studies have contributed to a better understanding of the chemical composition of larval schistosomes and echinostomes and of *Biomphalaria* infected by these digeneans.

4.1 Introduction

This chapter describes certain methods that have been used in the analysis of larval trematodes from *Biomphalaria* snails. Considered are also studies on the analyses of *Biomphalaria* tissues infected with larval trematodes, mainly schistosomes and echinostomes. Included are the chromatographic methods for the determination of different classes of organic compounds and atomic spectrometric methods for elemental analysis. Explanations of the abbreviations used in this chapter are given in Table 4.1. The major chromatographic methods applied

B. Fried (✉)
Department of Biology, Lafayette College, 111 Kunkel Hall, Easton, PA 18042, USA
e-mail: friedb@lafayette.edu

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Table 4.1 The abbreviations used in this review

AAS	Atomic absorption spectrometry
ASW	Artificial spring water
C-18	Silica gel layer chemically bonded with octadecylsilyl groups
CE	Cholesteryl esters
DGG	Digestive gland–gonad complex
DI	Deionized water
U.S.A. EPA	United States of America Environmental Protection Agency
FAAS	Flame atomic absorption spectrometry
FFA	Free fatty acids
FS	Free sterols
GFAAS	Graphite furnace atomic absorption spectrometry
GLC	Gas–liquid chromatography
HPLC	Column high-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
ICP-AES	Inductively coupled plasma-atomic emission spectrometry
L-T	Lettuce-tetramin
MALDI-MS	Matrix assisted laser desorption ionization mass spectrometry
nano-HPLC/ESI-IT-MS	HPLC/electrospray ionization-ion trap mass spectrometry
NMID	Nonmethylene-interrupted diene
PC	Phosphatidycholine
PE	Phosphatidylethanolamine
PI	Postinfection
PIn	Phosphatidylinositol
PS	Phosphatidylserine
RP	Reversed phase
SCW	Snail-conditioned water
SE	Steryl esters
SPE	Solid phase extraction
TG	Triacylglycerols
TLC	Thin-layer chromatography
UV	Ultraviolet
Y-L	Yolk-lipid

have been TLC and HPTLC, with a few papers reporting analyses by GLC and column HPLC; AAS and ICP-AES have been reported for the determination of elements, including metals. Earlier studies of the TLC analysis of larval trematodes in *Biomphalaria* from the 1970s through the mid-1990s were reviewed by Fried and Haseeb (1996). Some TLC information on *Biomphalaria* infected with larval echinostomes has been given in Sherma and Fried (2009). This chapter includes mainly TLC-related publications from 1995 through 2009. Publications earlier than 1995 are included if they are related to our 2005–2009 coverage. No previous review of analyses of column chromatographic and spectrometric methods is available. Therefore, all references on these topics are covered here.

4.2 Thin Layer Chromatography Principles and Methods

4.2.1 Principles

TLC is a type of liquid chromatography in which the stationary phase is a layer of sorbent on a glass plate, aluminum foil, or plastic sheet. In the basic TLC procedure, standard compound and sample solutions are applied to the origin line at the bottom of the plate, and the plate is developed by placing it in a closed chamber containing a pool of mobile phase, which is a mixture of two or more solvents. As the mobile phase rises through the layer, the compounds move at different rates and are separated, forming the chromatogram. The separated zones are then visualized by their natural color in daylight or under UV light, or by an application of a detection reagent. Quantification is performed by instrumental densitometry.

Only those materials, instruments, and techniques of TLC that have been applied to studies of larval trematode infected *Biomphalaria* are described in Sects. 2.2–2.10, and the references given in these sections are examples rather than comprehensive. Additional details on TLC are available in Sherma and Fried (2003).

4.2.2 Snail Maintenance and Sample Preparation

Biomphalaria glabrata snails were maintained in aerated glass containers, 20 per container, in 800 mL of ASW. The formulation of ASW was given in Ulmer (1970). Snails were fed Romaine lettuce ad libitum, and cultures were changed twice a week. The details of snail maintenance are given in Schneck and Fried (2005). For estivation studies, snails were maintained on moist paper towels in a closed container at a relative humidity of 98% and a temperature of 24–25°C. The details of the estivation studies are given in White et al. (2007). *B. glabrata* snails maintained under conditions of estivation survived for up to 7 days. In starvation experiments, snails were maintained without exogenous food for up to 1 week. Most snails survived these conditions for the week (White et al. 2007).

Snails were infected with *Schistosoma mansoni* miracidia as described in Fried et al. (2001). Usually snails 5–8 mm in shell diameter were infected in masse with about five miracidia per snail. Infected snails were maintained as described in Fried et al. (2001). *B. glabrata* snails were infected with *Echinostoma caproni* miracidia or eggs as described in Idris and Fried (1996), and infected snails were maintained as described in that paper. Additional information on the maintenance of *B. glabrata* snails infected with echinostome larvae was given in Fried and Peoples (2009).

In our laboratory, when *B. glabrata* samples were prepared for analyses by the techniques described in this review, most of the snail tissue that was used consisted of the DGG. However, in some studies whole bodies were used, and we also occasionally used shells for analyses. The details of the procedures used for obtaining *B. glabrata* samples are described in Fried and Sherma (1990). In brief, shells were

lightly cracked with a hammer and removed to obtain either the entire snail or the DGG. For the DGG, the posterior region of the snail was severed from the visceral mass, and the DGG was placed in a Petri dish half filled with Locke's solution. The formulation of Locke's solution was given in Fried (1994). When whole bodies were used, the shell was removed, and the entire body was placed in Locke's solution. Whole snail bodies or DGGs were rinsed with several changes of Locke's solution to remove debris. When hemolymph was needed, it was obtained by gently cracking the snail's shell and allowing hemolymph to drain into the bottom half of a Petri dish. The hemolymph was removed with a Pasteur pipet, placed in an Eppendorf tube, and briefly centrifuged to separate the plasma (snail hemolymph minus the hemocytes is referred to as plasma) from the hemocytes and debris. The details of this procedure are given in Fried and Sherma (1990). In most cases, plasma was used for analysis. All tissues and fluids were used immediately after collection or within a few hours following storage at 4°C. However, some tissues were stored at −20°C and used from 2 to 14 days later.

4.2.3 Sample Extraction and Purification

The classic Folch procedure was used to extract lipids and phospholipids (polar lipids) from the DGG of *B. glabrata* infected with *S. mansoni* (White et al. 2007; Fried et al. 2001); from whole bodies, DGG, and hemolymph of snails patently infected with *Echinostoma caproni* (Bandstra et al. 2006); from rediae, cercariae, and encysted metacercariae of *E. caproni* removed from experimentally infected *B. glabrata* (Marsit et al. 2000); and from the feces of *B. glabrata* infected with *E. caproni* (Schneck et al. 2005). The Folch procedure involves extraction of the sample with chloroform-methanol (2:1) in a glass homogenizer, usually in the ratio of 20 parts solvent to 1 part sample, and then filtration through glass wool if necessary. The extraction is performed up to three times. With a ratio of four parts sample volume to one part salt solution, Folch wash (0.88% KCl in DI water) is added, and the upper, aqueous layer is discarded. The combined extracts are evaporated to dryness in a warm water bath under nitrogen gas and then reconstituted in chloroform-methanol (2:1). The reconstitution volume is chosen so that the scan areas of zones in sample chromatograms are bracketed within the scan areas of the standard zones of the calibration graph for densitometric quantification.

Neutral lipids were extracted from SCW collected from snails infected with *E. caproni* using chloroform-methanol (2:1) in a centrifuge tube, and the lower layer was filtered through glass wool, evaporated, and reconstituted for TLC analysis (Schneck et al. 2005). Neutral lipids and phospholipids were isolated from cercariae of *S. mansoni* from *B. glabrata* snails by vortexing a sample of cercariae and ASW with chloroform-methanol (2:1), refrigerating overnight to obtain two layers, and evaporating and reconstituting the lower layer for TLC analysis (Schariter et al. 2002).

Glycolipids were extracted from *B. glabrata* infected with *S. mansoni* with 20 volumes of chloroform-methanol (1:1), the extract was centrifuged (1,000 g for 10 min), and the residue was re-extracted in 10 volumes of chloroform-methanol (1:2). The extract was again centrifuged and the supernatants combined and taken to dryness on a rotary evaporator. Glycolipids were separated from other lipid classes of the total extract by Florisil column chromatography of their acetylated derivatives (Maloney et al. 1990).

Prior to TLC determination, extraction was performed using acetone in a glass homogenizer for the pigments lutein and beta-carotene from whole bodies or DGGs of *B. glabrata* infected with *E. caproni* and *Helisoma trivolvis* infected with *E. trivolvis* (Evans et al. 2004); by vortexing with 70% aqueous ethanol for free pool amino acids in cercariae, rediae, encysted and excysted metacercariae from *B. glabrata* snails experimentally infected with *E. caproni* (Ponder et al. 2003); and by homogenization in a glass tissue grinder for sugars in the DGG of estivated *B. glabrata* and those infected with *S. mansoni* (Jarusiewicz et al. 2006). *S. mansoni* cercariae from *B. glabrata* were vortexed for 10 min with absolute ethanol-DI water (7:3) to extract sugars and amino acids, followed by centrifugation, evaporation of the supernatant, and reconstitution for TLC analysis (Wagner et al. 2002).

4.2.4 Layers and Mobile Phases

Neutral and polar lipids have been analyzed on 10×20 cm HPTLC silica gel plates with diatomaceous earth preadsorbent (or concentrating zone) and 19 scored lanes (HPTLC-HLF, Analtech, Newark, DE; White et al. 2007 and LHP-KDF, Whatman Inc., Florham Park, NJ; Schariter et al. 2002 and Bandstra et al. 2006). The preadsorbent zone adjacent to the main analytical layer facilitates manual sample application of relatively high volumes of biological sample extracts, and the lanes serve as a guide for positioning of the initial zones and the densitometer source slit for scanning chromatograms. Glycolipids were analyzed on silica gel 60 HPTLC plates without preadsorbent from EMD Chemicals, Inc. (an affiliate of Merck KGaA, Darmstadt, Germany) (Maloney et al. 1990).

The usual mobile phase for separating neutral lipid classes on these plates was petroleum ether-diethyl ether-glacial acetic acid (80:20:1); steryl esters and methyl esters were better separated from each other and from unidentified nonpolar hydrocarbons using the less polar mobile phase hexane-petroleum ether-diethyl ether-glacial ether (50:25:5:1). The mobile phase chloroform-methanol-water (65:25:4) was used to separate polar lipids and glycolipids.

Lutein and beta-carotene were determined on unlaned EMD Chemicals, Inc. 10×20 cm C-18 chemically bonded silica gel RP-HPTLC plates with a preadsorbent zone. The mobile phase was petroleum ether-acetonitrile-methanol (1:1:2) (Evans et al. 2004).

Sugars were analyzed on Whatman Inc. LK5DF silica gel 20×20 cm TLC plates containing a preadsorbent zone and 19 lanes. The mobile phase was ethyl

acetate-glacial acetic acid-methanol-water (60:15:15:10) (Jarusiewicz et al. 2006; Wagner et al. 2002).

The analysis of amino acids required four layers having different separation mechanisms (Ponder et al. 2003, 2004; Wagner et al. 2002): EMD Chemicals, Inc. silica gel HPTLC with *n*-butanol-acetic acid-water (3:1:1) mobile phase (normal phase adsorption), EMD Chemicals, Inc. cellulose HPTLC with the same mobile phase (normal phase partition), Whatman Inc. 20×20 cm preadsorbent C-18 bonded silica gel with *n*-propanol-0.5 M NaCl (4:6) mobile phase (RP), and Polygram Ionex-25 SA-Na sheets (Macherey-Nagel, Bethlehem, PA) with pH 3.3 citrate buffer mobile phase (strong acid cation exchange).

4.2.5 Standard and Sample Solution Application

The neutral lipid, phospholipid, carbohydrate, amino acid, and pigment standards were purchased from commercial sources, such as Matreya, Inc. (Pleasant Gap, PA), Nu-Check Prep (Elysian, MN), or Sigma (St. Louis, MO), and dissolved in an appropriate solvent to prepare the standard solutions.

Standard solutions and reconstituted sample extracts were generally applied to the layer in 1.00–16.0 μL aliquots. It is important that the sample zone weight applied is within the weight range of the standard calibration graph for quantitative analysis. Drummond (Broomall, PA) Digital Microdispensers (10 and 25 μL) with disposable glass pipets were used to apply the initial zones of samples and standards. Solutions are applied as diffuse vertical streaks to plates containing a preadsorbent zone, and tight band-shaped initial zones are automatically formed at the preadsorbent-analytical sorbent interface. Solutions must be applied as compact spots to layers without a preadsorbent. In the analysis of glycolipids (Maloney et al. 1990), a Camag (Wilmington, NC) Linomat IV semiautomatic applicator with 100 μL syringe was used to apply bands by the spray-on technique.

4.2.6 Plate Development with the Mobile Phase

Isocratic, linear, ascending development was carried out in a large volume, covered glass TLC or HPTLC chamber (normal chamber or N-chamber) that was lined with a saturation pad (Analtech, Newark, DE) or piece of chromatography paper and pre-equilibrated with the vapors of the mobile phase for 10–15 min before insertion of the plate with the initial zones. Most used was the Camag twin trough chamber, a special N-chamber with an inverted V-shaped ridge on the bottom dividing it into two sections that allow development with a very low volume of mobile phase. The ambient temperature in the laboratory was typically 22°C and the humidity was 50%.

4.2.7 Methods for Detection of Separated Zones

After removing the mobile phase from the plate by drying inside a fumehood with cool air from a hair dryer, neutral lipids were detected as blue zones on a yellow background by spraying with PMA detection reagent (5%, w/v, in ethanol) and heating on a plate heater (Camag) for 10 min at 115°C. Polar lipids were detected as brown–black zones on a white background by spraying with a 10% solution of cupric sulfate in 8% phosphoric acid and heating at 140°C for 15 min (White et al. 2007). Glycolipids were visualized using an orcinol reagent specific for carbohydrate residues (Maloney et al. 1990).

Lutein and beta-carotene appeared as natural yellow zones in daylight (Evans et al. 2004). Sugars were detected as dark purple zones against a light yellow background by spraying with alpha-naphthol-sulfuric acid reagent and heating at 110°C for 5 min (Jarusiewicz et al. 2006), and amino acids as purple to blue zones on a pale background by spraying with ninhydrin reagent (0.3 g ninhydrin and 3 mL of glacial acetic acid in 100 mL of *n*-butanol) and heating for 10 min at 110°C (Ponder et al. 2003).

4.2.8 Documentation of Chromatograms

Chromatograms on plates were documented by scanning with a videodensitometer (VideoScan, Camag). Pigment zones fade rapidly and must be photographed (and quantified, if required) immediately after plate development.

4.2.9 Identification of Zones

Compounds in sample chromatogram zones have been identified by comparison of R_F values and detection colors with standard zones developed on adjacent lanes of the same plate. The R_F value is defined as the distance from the origin to the center of the zone divided by the distance from the origin to the mobile phase front.

4.2.10 Quantification of Analytes

Accurate and precise quantification was performed using a Camag TLC Scanner II slit scanning densitometer with which standard calibration graphs were calculated by linear regression and sample weights interpolated automatically under computer control. Typical settings of the TLC Scanner II used for measuring the bands formed on the preadsorbent plates by the aliquot scanning method were slit width 4,

slit length 4, and scanning speed 4 mm/s. Scanning wavelengths found to provide optimum quantitative results for the separated and detected standard and sample zones were 610 nm for neutral lipids, 370 nm for polar lipids, 610 nm for amino acids (except 495 nm for histidine), 515 nm for sugars, 448 nm for lutein, and 455 nm for beta-carotene. All wavelengths were provided by the tungsten densitometer source except for 390 nm (deuterium source).

The interpolated weight of analyte in a zone, extract reconstitution volume, volume applied, and sample weight (solid samples) or volume (liquid samples) were used in appropriate equations to calculate the concentration of the analyte in various samples. Up to 15 different samples could be analyzed with four standards on a single plate.

4.2.11 Statistical Analysis of Data

The accuracy and precision of densitometric quantitative data are high because calibration graphs relating scan areas vs. standard weights are established under the same TLC conditions as for sample zones separated on the same plate. In most studies, Student's *t*-test (*P* equal or less than 0.05) was used to determine the significance of data based on the mean ± standard error values of analytes from different sample populations.

White et al. (2007) did statistical analysis using a single factor analysis of variance (ANOVA) to determine whether there was a significant difference in the lipid percentage of the DGG among infected-estivated, infected-starved, and control snails. If a significant difference ($P<0.05$) was found, the data were subjected to the Bonferroni method to determine among which populations the difference occurred. The SPSS version 13.0 software was used for all data analyses.

4.3 Thin Layer Chromatography Applications

4.3.1 Introduction to Thin Layer Chromatography Applications

This section examines TLC studies on lipids, amino acids, carbohydrates, and lipophilic pigments isolated in larval stages of schistosomes and echinostomes. It also examines these analytes in *Biomphalaria* infected with the intramolluscan stages of these larval trematodes.

4.3.2 Lipids in Biomphalaria *Infected with Larval Trematodes*

This section examines the TLC of lipids in *Biomphalaria* snails infected with larval trematodes, mainly schistosomes and echinostomes. The TLC analyses are of analytes from larval stages and also include studies on effects of larval trematodes on the lipid

content of the snail hosts. Most studies are concerned with neutral lipids and phospholipids (polar lipids). Unless otherwise noted, the TLC and HPTLC studies have used silica gel layers, the Mangold solvent system, detection by spraying with PMA or cupric sulfate, and quantification by densitometry. Unless otherwise noted, the studies are arranged in chronological order.

Fried et al. (1989) determined the effects of larval *E. caproni* infection on the neutral lipid content of the DGG of *B. glabrata* using semiquantitative HPTLC. The TG fraction was greater in control than in infected DGG, as also were the SE and FFA fractions. The FS fraction did not differ between control and infected DGG samples. This study showed that larval echinostomes can reduce depot fats in the DGG of the *B. glabrata* snail.

Pérez et al. (1995) analyzed the phospholipids in the DGG of *B. glabrata* infected with *E. caproni*. The mean weight percentages of PE and PC in the DGG of the two populations of snails were not significantly different, but the mean weight percentage of PS was 1.5 times greater in the DGG of the infected snails than that of the uninfected snails. This study showed that larval echinostomes can alter the concentration of some phospholipids in the host snail.

Chaffee et al. (1996) used TLC to study neutral lipids in SCW from *B. glabrata* snails. A major lipid fraction in SCW at 2 and 4 h after snail incubation contained FFA, and the amounts of these acids were quantified by densitometry. The amount of FFA released at 4 h was significantly greater than at 2 h. Snails also released other lipids into the water. The potential of these neutral lipids to serve as chemoattractants for larval echinostomes and schistosomes remains to be determined.

Haas et al. (1997) used TLC to obtain surface lipids from the skin of humans and pigs. Such lipids provide chemical signals that stimulate the secretion of acetabular gland contents of the cercariae of *S. mansoni*. They showed that skin lipids such as FFA, phospholipids, and glucosylceramides play a role in stimulating acetabular secretions of *S. mansoni* cercariae.

Marsit et al. (2000) used HPTLC to analyze the neutral lipids in the rediae, cercariae, and encysted metacercariae of *E. caproni* removed from experimentally infected *B. glabrata* snails. Visual observations showed that the most abundant lipid fraction in all stages was FS. The concentrations of FS were determined in all stages by densitometry.

Thompson et al. (1991) reported that *B. glabrata* infected with *S. mansoni* and maintained on a diet of hen's egg yolk produced fully developed cercariae in about one half of the time taken by snails fed romaine lettuce. Fried et al. (2001) reexamined the Thompson et al. (1991) claim of rapid cercarial development as a factor of snail diet, and also examined the nutritional effects of the high fat diet on larval schistosome development. Infected snails maintained at 26°C and fed either diet produced fully developed cercariae by 4 week PI. The earlier contention that the yolk diet enhanced the time to cercarial patency was not confirmed. HPTLC analysis of neutral lipids show that the DGG of infected snails fed the yolk diet contained significantly higher concentrations of FS and CE, but not TG, than those of the infected snails fed the lettuce diet.

Schariter et al. (2002) used HPTLC to analyze neutral lipids and phospholipids in the cercariae of a Puerto Rican strain of *S. mansoni*. Visual observations of the

chromatograms showed that the most abundant neutral lipids in the cercariae were FFA and FS, and the most abundant phospholipids were PC and PE. Densitometry was used to quantify the above-mentioned lipid fractions. This was the first study to provide quantitative data on neutral lipids and phospholipids on a per cercaria basis for *S. mansoni*. The possible functions of lipids in schistosome cercariae were discussed.

Schneck et al. (2004) used HPTLC to analyze neutral lipids in chemically excysted metacercariae of *E. caproni*. The encysted metacercariae (cysts) were obtained from the kidney/pericardium of experimentally infected *B. glabrata* snails. The cysts were chemically excysted, and the excysted metacercariae were maintained in Locke's solution. The excysted metacercariae released neutral lipids into the medium, and densitometry was used to quantify these neutral lipids. Neutral lipid release in echinostomes is related to worm mediated chemoattraction (Haseeb and Fried 1988).

Schneck et al. (2005) used HPTLC to determine neutral lipids in SCW and feces from *B. glabrata* snails infected with *E. caproni*. Analysis of SCW showed the presence of FS, FFA, and TG, but significant differences in the concentrations of these lipids in SCW from infected vs. uninfected snails were not detected. The FFA fraction was the major neutral lipid detected in the feces, and the amount of this lipid was significantly lower in the uninfected samples vs. the infected samples as determined by quantitative densitometry.

Bandstra et al. (2006) examined the effects of larval echinostome infection on the neutral lipid and phospholipid content *B. glabrata* patently infected with rediae of *E. caproni*. Uninfected snails were used as controls. The major neutral lipids in whole bodies and DGGs of both groups were FS, FFA, and TG. The major phospholipids were PC and PE. Densitometric analysis showed that the concentration of TG in infected DGGs was significantly less than that of the uninfected snails.

4.3.3 Effects of Diet and Larval Trematodes on Lipids in Biomphalaria *Snails*

Beers et al. (1995) determined the effects of diet and *E. caproni* parasitism on the neutral lipids in the DGG of *B. glabrata* snails. An analysis of the snails fed on the Y-L diet showed that TG were significantly reduced in the DGG of infected snails whereas levels of FS were significantly elevated in the infected snails. In the snails fed the L-T diet, the TG level was reduced in the DGG of infected snails, but the difference was not significant. The FS level was also elevated as in snails on the Y-L diet, but again this finding was not significant. There were significant differences between TG and FS levels in both infected and uninfected snails maintained on the Y-L diet, but there were no significant differences in any lipid class in the infected vs. uninfected snails. The results of this study showed that both diet and larval trematode parasitism can influence the lipid constituents of *Biomphalaria* snails.

Thompson et al. (1991) used two-dimensional TLC to analyze neutral lipids and phospholipids in the DGG of uninfected *B. glabrata* snails and those infected with *S. mansoni*. Both snail populations were maintained on different diets, as follows. Some snails were fed either egg yolk or lettuce whereas others were not fed (starved). The neutral lipids were separated by two-dimensional TLC on 20 × 20 cm activated 250-μm layers of silica gel G. The mobile phase for the first direction was hexane-diethyl ether, 80:20, and that for the second direction was hexane-diethyl ether-methanol, 70:20:10. Each mobile phase contained 1% acetic acid. The zones were visualized by spraying the plate with 50% sulfuric acid and heating to char the lipids. Identities were established by use of co-chromatography and comparison of R_F values. The phospholipids were identified by the use of two-dimensional TLC on activated layers of silica gel G. Plates were developed with chloroform-methanol-water-40% aqueous ammonia, 86:28:1:3, in the first direction and chloroform-methanol-water-acetic acid, 76:9.5:2.5:12, in the second direction. Spots were visualized by spraying with molybdenum blue reagent, and the choline-containing phospholipids were identified with Dragendorff's reagent (Thompson and Lee 1987). The major neutral lipid fraction was TG, and this fraction was greatest in the DGG of infected or uninfected snails maintained on the egg yolk diet. FFA were present in trace amounts in all populations; likewise, the major phospholipid fraction was PC, but other polar lipids were also present. Reduced levels of PC and some other phospholipids were found in the DGG of snails infected with *S. mansoni*. These reduced lipid levels coincided with the idea that membrane phospholipids were used by the developing sporocysts and cercariae. In one trial, increased levels of lipids and a shortened period of patency (4 or 5 weeks for snails maintained on egg yolk compared with 8 or 9 weeks for snails maintained on lettuce) were observed for snails maintained on the egg yolk diet. Thompson et al. (1991) were not able to confirm this finding in a subsequent trial. Fried et al. (2001), using HPTLC-densitometry, repeated the experiment of Thompson et al. (1991) and found that maintaining *B. glabrata* infected with *S. mansoni* on a Y-L diet did not increase the time of patency compared with those maintained solely on lettuce. An analysis of snails at 6 weeks PI revealed a significant increase in the FS and SE fractions in the DGG of infected snails maintained on a Y-L diet compared with those of infected snails maintained on a lettuce diet.

White et al. (2007) examined the effects of estivation or starvation on the neutral lipid and phospholipid content of *B. glabrata* patently infected with *S. mansoni* by HPTLC densitometry. Infected-estivated snails were maintained in a moist chamber at 24 ± 1°C and a relative humidity of 98 ± 1%. Infected-starved snails were maintained in ASW at 23 ± 1°C without exogenous food. Infected snails (the controls) were maintained in ASW at 23 ± 1°C and fed lettuce ad libitum. The three groups were maintained in the laboratory for 7 days, and then the lipids from the DGG were extracted and analyzed by class. Infected-estivated snails exhibited a greater mortality rate and weight loss after 7 days than did the infected-starved snails. The SE concentration in the infected-starved snails was significantly increased ($P = 0.010$) compared with the controls but not compared with infected-estivated snails; the concentration of PC in infected-estivated snails

was significantly decreased ($P = 0.007$) compared with the controls but not when compared with the infected-starved snails. Estivation or starvation had a significant effect on the concentration of certain lipid classes in the DGG of *B. glabrata* infected with *S. mansoni*.

4.3.4 *Amino Acids in* Biomphalaria *Snails Infected with Larval Trematodes*

Wagner et al. (2002) used TLC and HPTLC to analyze the amino acids present in the cercariae of *S. mansoni*. Visual observations of the amino acid chromatograms confirmed the presence of histidine, tryptophan, isoleucene, alanine, and proline. Several other unknown ninhydrin-positive zones were also detected. Quantification of the histidine and tryptophan zones in cercarial samples by densitometry gave mean values of 3.7 and 0.59 ng/cercariae, respectively. This is the first study to report concentrations of amino acids in *S. mansoni* cercariae.

Pachuski et al. (2002) used TLC and HPTLC to determine the amino acid content in the DGG of *B. glabrata* snails infected with larval *S. mansoni* for 8 weeks. DGG of infected and control snails were pooled, extracted in ethanol, and chromatographed using various sorbent-mobile phase combinations applicable to amino acid analysis. Zones were detected with ninhydrin and quantified by densitometry. Qualitative analysis showed the presence of histidine, lysine, alanine, methionine, threonine, asparagine, proline, and leucine/isoleucine in both the infected and uninfected DGG. Quantitative analysis was done on histidine, lysine, alanine, and methionine, but only lysine showed a significant reduction in concentration (Student's *t*-test, $P < 0.05$) in the DGG of infected snails compared to the controls. Lysine, an essential amino acid for the developing schistosome larvae, is obtained in significant amounts from the snail DGG.

Ponder et al. (2003) used TLC and HPTLC to determine the free pool amino acids in the rediae, cercariae, encysted metacercariae, and excysted metacercariae of *E. caproni*. Larvae of each type were pooled and extracted in ethanol, and their free pool amino acids separated using four types of layers with different separation mechanisms. The zones were detected with ninhydrin spray reagent and quantified by densitometry. Qualitative analysis revealed the presence of valine, leucine, lysine, histidine, and alanine in rediae; histidine in cercariae; histidine and alanine in encysted metacercariae; and leucine in excysted metacercariae. Quantitative analysis showed that rediae contained 0.76 ng of lysine per organism, and excysted metacercariae contained 0.16 ng of leucine per organism.

Ponder et al. (2004) used TLC and HPTLC to analyze the free pool amino acids in the DGG of *B. glabrata* infected with *E. caproni* vs. uninfected control snails. Qualitative analysis showed the presence of histidine, lysine, serine, alanine, valine, and isoleucine in all samples. Quantitative analysis of lysine and valine gave mean weight percentages of 0.007 and 0.002, respectively, in the DGG of uninfected snails and 0.005 and 0.003, respectively in the DGG of infected snails.

However, the differences in these values between infected and uninfected snails were not statistically significant.

4.3.5 *Carbohydrates in* Biomphalaria *Snails Infected with Larval Trematodes*

Wagner et al. (2001) used TLC to analyze the effects of larval trematode parasitism by *E. caproni* on carbohydrates in the whole body of *B. glabrata* snails infected for 10 weeks. The major sugars found in snail whole bodies were glucose, maltose, and raffinose. At 10 weeks PI, infection by larval *E. caproni* caused a significant reduction in the concentrations of both maltose and raffinose in the whole bodies of *B. glabrata*. No significant reduction was observed in the concentration of glucose in the infected vs. control snails at 10 weeks PI. This paper reviewed earlier studies on the effects of larval trematode parasitism on the carbohydrate content of host snails.

Wagner et al. (2002) used TLC to analyze the carbohydrates present in the cercariae of *S. mansoni* released from *B. glabrata*. Visual observation of the chromatograms showed the presence of glucose and raffinose and three unidentified alpha-naphthol-positive zones. Quantification of the glucose zones by densitometry in three cercarial samples gave a mean value of 0.49 ng per cercaria. Raffinose was present at lower levels in different cercarial batches. This was the first quantitative study to report sugar values in the *S. mansoni* cercaria.

Jarusiewicz et al. (2006) used TLC to analyze the glucose and maltose concentrations of the DGG of uninfected-estivated *B. glabrata* snails and estivated *B. glabrata* patently infected with *S. mansoni*. All snails were estivated in a moist chamber at a relative humidity of 98 ± 1% and a temperature of 23 ± 1°C for 14 days. Carbohydrates were extracted from the DGG with 70% aqueous ethanol, and extracts were analyzed on silica gel preadsorbent plates using ethyl acetate-glacial acetic acid-methanol-water (60:15:15:10) mobile phase, alpha-naphthol-sulfuric acid detection reagent, and quantification by densitometry. The concentrations of glucose and maltose were significantly reduced in both uninfected-estivated snails and infected-estivated snails.

4.3.6 *Lipophilic Pigments in* Biomphalaria *Snails Infected with Larval Trematodes*

Evans et al. (2004) used HPTLC to quantify the concentration of beta-carotene and lutein in several planorbid snails infected with larval trematodes. An important combination was laboratory raised *B. glabrata* snails experimentally infected with *E. caproni*. Uninfected snails served as controls. The pigments were extracted from the snail whole bodies and DGG, separated by reversed phase HPTLC, and

quantified by densitometry. *B. glabrata* infected with *E. caproni* showed no significant differences in the concentrations of lutein and beta-carotene compared to the uninfected cohorts. Differences in this study compared with other previous studies on this topic reflected intrinsic differences in the larval trematode snail systems used.

4.4 Gas Chromatography

4.4.1 Principles

GLC is a method in which a liquid sample is injected through a rubber septum into a port and vaporized onto the front end of a column. Isothermal or programmed temperature GLC on a packed column with an inert solid support onto which a nonvolatile liquid stationary phase is adsorbed or on a wall coated open tubular (WCOT) capillary column has been used for studies of infected *B. glabrata*. As the inert carrier gas (mobile phase) flows through the column, the sample components move at different rates and reach the detector separated in time. In general, the compound with the lowest boiling point elutes first. The injection port, column, and detector are heated to keep the sample from condensing during the analysis. The flame ionization detector has been used in all analyses reported below.

The chromatogram is a display of the detector signal as a series of peaks at different retention times that are the basis of qualitative analysis for the separated compounds. Quantitative analysis is performed by generating a calibration graph of peak area vs. weight of a series of standards for each analyte, by dividing the peak area of a compound by the total area of all peaks if the response factors for all analytes are equal, or correcting the detector response using predetermined response factors for each analyte.

See the book by Grob and Barry (2004) for information on the theory, instrumentation, and techniques of GLC.

4.4.2 Methods and Applications

Furlong and Caulfield (1988) analyzed the sterol composition of cercariae, schistosomules, and adult *S. mansoni* from *B. glabrata* by GLC. It was found that cercariae and schistosomules contained cholesterol, desmosterol, campesterol, stigmasterol, and beta-sitosterol while adults contained only cholesterol. In all stages, cholesterol comprised greater than 50% of the total sterols, and in cercariae and schistosomules desmosterol comprised 38 and 21% of the total sterols, respectively. The other three sterols made up approximately 10% of the total. The same five sterols found in cercariae and schistosomules were present in the hepatopancreas of uninfected snails but with a much higher desmosterol concentration in the parasite (38%) than

in the snail (2%). As in cercariae and schistosomules, the three minor sterols comprised approximately 10%. Thus, the sterol composition of cercariae and schistosomules was similar but not identical to the snail host. Lipids were extracted from samples by the Folch et al. method, and neutral lipids and phospholipids were separated by silicic acid liquid chromatography. Neutral lipids were saponified and acetylated derivatives were prepared and analyzed using a PerkinElmer (Waltham, MA) Model 3920 gas chromatograph with an FID. Both free sterols and acetylated derivatives were injected onto either 3% SE-30 or 3% OV-17 packed columns operated isothermally at 244 or 262°C, respectively. Retention times and peak areas of sterol peaks were calculated with a PerkinElmer Model LCI-100 integrator operating in the default gas chromatography mode.

Shetty et al. (1992) studied the sterols in the DGG complex of *B. glabrata* infected with the patent larval stages of *E. caproni* vs. uninfected controls. The major sterol present was cholesterol at levels of 59% in the infected snails and 51% in controls. Both populations contained the phytosterols desmosterol, campesterol, stigmasterol, and beta-sitosterol, but the percentage composition was reduced in the infected snails. A Hewlett-Packard (Avondale, PA) Model 5890 gas chromatograph with a 30 m × 0.32 mm id SPB-1 methylsilicone WCOT column programmed from 200 to 280°C and an FID was used. Sterols were identified based on retention times compared to standards, and percentage composition was calculated by dividing the peak area of a particular sterol by the total area of all peaks in the chromatogram.

Fried et al. (1993) did GLC studies to determine the fatty acid composition of *B. glabrata* snails experimentally infected with the intramolluscan stages of *E. caproni*. The infection was found to reduce the amounts of saturated fatty acids in whole snail bodies but increased the amounts of these acids in the DGG complex of the snails. In both whole bodies and DGGs, infection increased markedly the amounts of palmitoleic acid (16:1n-9) and 20:2 non-methylene-interrupted diene (NMID), but reduced the amounts of 16:1n-7, 20:1n-11+9, and 22:1n-11+13; docosahexaenoic acid (DHA) concentration was markedly decreased in infected whole snail bodies. Lipids were extracted by the Folch method and then treated with 7% BF_3-methanol to convert acyl lipids to methyl esters. GLC of the fatty acid methyl esters was carried out on a PerkinElmer model 8240 gas chromatograph equipped with a 30 m × 0.25 mm id Supelcowax-10 polyethylene glycol fused silica capillary column programmed from 185 to 230°C and an FID. Peak areas were converted to % fatty acids by correcting the FID response using a previously developed computer program.

4.5 Column High-Performance Liquid Chromatography

4.5.1 Principles

HPLC involves high pressure flow of a liquid mobile phase through a metal tube (column) containing the stationary phase. The sample is applied to the column through a loop injector, and separated mixture components are eluted into a detector.

HPLC columns have a short length and small internal diameter (id) compared to classical LC. The differences of HPLC compared to GLC are that the former is usually performed at ambient temperature, and the HPLC mobile phase affects the selectivity but the GLC carrier gas does not. Detectors for HPLC include fluorescence, electrochemical, or UV. Mechanisms of separation, depending upon the type of column used, include adsorption, normal and RP phase partition, bonded phase, ion exchange, and size exclusion.

See the book by Dong (2006) for information on the theory, instrumentation, and techniques of HPLC.

4.5.2 Methods and Applications

Furlong and Caulfield (1988) used two HPLC procedures to determine the phospholipid composition of *S. mansoni* cercariae, schistosomules, and adults from *B. glabrata*. It was found that PC was the major phospholipid of all three stages (50%). The remaining phospholipids consisted of PE, PS, and PIn. In addition, in adults there were small quantities of sphingomyelin and lysophosphatidylcholine. The percentage of each phospholipid was similar among stages except for a slight increase in PS in adults compared to the other two stages. In the first protocol, HPLC was carried out on a Waters (Milford, MA) automated gradient control system. Lipid from approximately 25,000 cercariae or schistosomules or 50 adult pairs was eluted from a Waters Z-module cartridge with acetonitrile-methanol-phosphoric acid (130:1.8:1.25) at a flow rate of 5 mL/min. Phospholipids were detected by UV absorbance at 214 nm, and the major phospholipid classes were identified by comparison of retention times with standards. Quantification was performed by cutting out and weighing peaks recorded on a strip chart recorder; the total weight of all peaks exclusive of the solvent front was taken as 100%, and the percentage of a given peak was calculated accordingly. The second HPLC protocol separated and determined neutral lipids and phospholipids in a single run using a Spherisorb 10 μm silica column; a ternary gradient system with hexane-THF (99:1), chloroform-isopropanol (1:4), and isopropanol-water (1:1); and a mass selective detector using individual calibration graphs for quantification of each compound.

Manger et al. (1996) measured the biogenic monoamines serotonin, dopamine, and L-dopa using HPLC in the extracts of the central nervous system and plasma of infected *B. glabrata* in snails at 7, 14, 21, and 28 days post exposure to the miracidia of *S. mansoni*. The findings suggested that serotonin acts as a stimulant for egg production in *B. glabrata*, and that parasitic castration may be due, at least in part, to larval induced suppression of serotonin in the snails' central nervous system and plasma during the course of infection with *S. mansoni*. The HPLC system included a pump, injector with 20 μL sample loop, electrochemical (amperometric) detector with a glassy carbon working electrode and Ag/AgCl reference electrode, and BAS 3 μm ODS column (10 cm × 3.2 mm id). The mobile phase consisted of

0.1 M citrate buffer (pH 3) containing 0.7 mM Na_2EDTA, 0.25 mN octyl sulfate, and 10% acetonitrile with a flow rate of 0.6 mL/min.

During the intramolluscan life cycle stages of *S. mansoni* within the *B. glabrata* intermediate host, the larval parasites synthesize a wide array of glycoconjugates exhibiting, in part, unique carbohydrate structures. In addition, the larval parasites express definitive host-like sugar epitopes, such as Lewis X determinants, supporting the concept of carbohydrate-mediated molecular mimicry as an invasion and survival strategy. Lehr et al. 2006 investigated whether common carbohydrate determinants occur also at the level of the intermediate host by performing structural characterization of hemolymph glycoprotein-*N*-glycans of *B. glabrata*. *N*-glycans were released from tryptic peptides and labeled with 2-aminopyridine. Sugar chains serologically cross reacting with *S. mansoni* glycoconjugates were isolated by immunoaffinity chromatography using a polyclonal antiserum directed against schistosomal egg antigens, and fractionated by *Aleuria aurantia* lectin affinity chromatography and HPLC. Obtained glycans were analyzed by MALDI-MS and nano-HPLC/ESI-IT-MS as well as by monosaccharide constituent and linkage analysis. The results revealed a highly heterogeneous oligosaccharide pattern. Cross-reacting species represented about 5% of the total glycans and exhibited a terminal Fuc(alpha1-3) GalNAc unit, a (1–2)-linked xylosyl residue, or both types of structural motifs. The study demonstrated the presence of common carbohydrate epitopes in larval *S. mansoni* and in the intermediate host *B. glabrata.* HPLC was carried out at 40°C using a TSK-Amide-80 column (4×250 mm) and fluorescence detection; the mobile phase was a gradient of 0–100% of 3% aqueous triethylamine/acetic acid, pH 7.3-acetonitrile (1:1) in 50 min.

Bezerra et al. (1997) examined the profile of carboxylic acids in *S. mansoni* resistant and susceptible strains of *B. glabrata.* The acids were extracted from the hemolymph of two susceptible strains (PR, Puerto Rico and Ba, Jacobina-Bahia from Brazil) and from two resistant strains (13-16-R_1 and $10R_2$) using SPE followed by HPLC. The carboxylic acids identified in all hemolymph samples by comparison with known standards were pyruvic, lactic, succinic, malic, fumaric, acetic, propionic, beta-hydroxybutyric, and acetoacetic. Under standard conditions, the concentration of each acid varied among the strains tested and appeared to be specific for each strain. Only the concentration of fumarate was consistently different ($P<0.05$) between resistant and susceptible strains. Organic acids were recovered from centrifuged hemolymph by direct application onto SAX anion exchange quaternary amine Bond-Elut SPE column (Varian Inc., Walnut Creek, CA) and elution with 0.5 M sulfuric acid, and the eluate was subjected to HPLC on a Biorad (Hercules, CA) Aminex ion exclusion HPX-87H column (300×7.8 mm) with 0.5 mM sulfuric acid mobile phase and UV detection at 210 nm.

Massa et al. (2007) used HPLC to determine the effects of a patent *S. mansoni* infection on certain carboxylic acids in the DGG and hemolymph of *B. glabrata*. Hemolymph was analyzed for carboxylic acids using the same anion exchange SPE and ion exclusion HPLC-UV techniques as described above (Bezerra et al. 1997) with an Agilent 1100 series system (Wilmington, DE) except the mobile phase was 5.0 mM instead of 0.50 mM sulfuric acid to achieve better and more consistent

analyte separations. An analysis of DGG samples was done using extraction with 50% Locke's solution, cleanup of the extract by anion exchange SPE, and ion exclusion HPLC-UV. Acetic, fumaric, malic, and pyruvic acids were detected, confirmed, and quantified at concentrations ranging from 12 to 280 ppm in the DGG and 124–8,000 µg/dL in the hemolymph. Infection with *S. mansoni* caused a significant reduction in concentrations of acetic, fumaric, malic, and pyruvic acids in the DGG but not in the hemolymph of *B. glabrata* compared to uninfected cohort snails. Reductions in the infected DGG suggest these acids are utilized by the sporocysts and cercariae in the snail tissue, or that infection stimulates reduced production or increased utilization by the snail tissue.

4.6 Inductively Coupled Plasma-Atomic Emission Spectrometry and Atomic Absorption Spectrometry

4.6.1 Principles

ICP-AES and AAS are atomic spectrometric methods used to determine elemental analytes in samples. ICP-AES is an emission method in which the source is very hot plasma of ionized argon, into which samples and standards are introduced. Atoms are ionized and emissions are measured using a sequential, monochromator design or a simultaneous multichannel, polychromator or array-based design to isolate different wavelengths, and one or more PMT detectors. The wavelength is characteristic of a particular element, and the intensity of the emission indicates the concentration of the element in the sample.

In AAS a liquid sample is aspirated into a flame (FAAS) or pipetted into a graphite furnace (GFAAS) at high temperature, and the resulting unexcited gaseous analyte atoms absorb light emitted from a hollow cathode lamp and passed through the flame or furnace. The lamp emits exactly the best wavelength required for the analysis. A photomultiplier tube detector converts the amount of light reaching it into an electrical signal that is read out as absorption. The analyte concentration in the sample is determined from its amount of absorption using a calibration graph prepared using standards of known concentration.

See the paper by Sherma (2008) for further information on the procedures and instrumentation of atomic spectrometry.

4.6.2 Methods and Applications

Mohammad and Mostafa (2007) used FAAS to determine the alteration of Ca concentration in the soft parts and shells of *B. alexandrina*, a planorbid species related to *B. glabrata*, and in *Bulinus truncatus* due to infection with *S. mansoni*

and *S. haematobium*, respectively. The results showed significant lowering in the Ca content in the shells of infected *B. alexandrina* and *B. truncatus* relative to the shells of uninfected snails. In contrast, the Ca content in the soft parts of snails that were releasing cercariae was significantly higher than in the soft parts of uninfected snails. Generally, Ca content was significantly higher in the shells than in the soft parts of the snails, whether infected or uninfected. Samples were digested with concentrated nitric acid to extract Ca, and extracts were analyzed using the source wavelength and sample aspiration rate recommended by the manufacturer of the instrument, which was not identified. Four aqueous standards having analyte concentrations within the linear range of the instrument and containing the same amount of nitric acid as samples were used for calibration. Each standard, sample, and reagent blank was analyzed using three 10s integrations, and the blank value was subtracted to give the final concentration.

Ong et al. (2004) employed ICP-AES to study element ions in whole bodies of uninfected *B. glabrata* snails and those experimentally infected with larval stages of *S. mansoni.* Infected and cohort uninfected snails were analyzed 8 weeks PI. Of the 28 elements measured, 16 (Al, B, Ba, Ca, Cd, Cu, Fe, K, Mg, Mn, Na, Ni, Pb, Se, Sn, and Zn) were identified in infected and uninfected whole bodies at concentrations above the detection limit of ICP-AES analysis. Of these, Ca, Cd, Mn, and Na were present in significantly higher amounts in whole infected vs. whole uninfected snails. Ca was extracted from snail bodies using 2% nitric acid, and analysis was done with a Thermo Jarrell Ash (Franklin, MA) simultaneous reading spectrometer with an autosampler. The instrument was calibrated using U.S.A. EPA Method 6010B, which employs a three point calibration graph generated from the measurement of a calibration blank and two multielement standards. Interelement correction factors were applied to minimize interference between elements in samples. The samples, standards, and a reagent blank were analyzed using three 30s integrations.

4.7 Concluding Remarks

Most of the studies reported here have used TLC and HPTLC for the analyses of larval schistosomes and echinostomes and *Biomphalaria* snails infected with these digeneans. These studies have provided considerable information on the qualitative and quantitative analyses of various organic compounds, including neutral lipids, phospholipids, glycolipids, amino acids, sugars, and lipophilic pigments such as lutein and beta-carotene. Changes in *Biomphalaria* tissues and hemolymph infected with schistosomes and echinostomes have documented changes in the pathobiochemical effects of the infection on this host. The atomic spectrometry studies have provided new information on elemental analyses of schistosomes and echinostomes and on the pathochemical effects of schistosome and echinostome infection on *Biomphalaria* snails. Less information is available on GLC and HPTLC analysis of schistosomes and echinostomes associated with *Biomphalaria* snails. Further work

using these analytical tools may provide much needed information on the molecular species of compound classes in *Biomphalaria* snails, information which is not easily obtained by TLC and HPTLC alone.

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