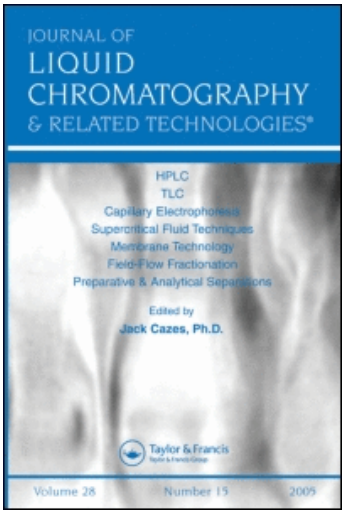

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P. M. Eidam[a]; J. J. Schariter[a]; B. Fried[b]; J. Sherma[a]
[a] Department of Chemistry, Lafayette College, Easton, PA, U.S.A. [b] Department of Biology, Lafayette College, Easton, PA, U.S.A.

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HPTLC ANALYSIS OF TISSUE AND BLOOD OF *BIOMPHALARIA GLABRATA* SNAILS TO ASSESS THE EFFECTS OF A DIET OF ROMAINE LETTUCE LEAF VERSUS MIDRIB ON THE CONCENTRATIONS OF LIPIDS, PIGMENTS, AND CARBOHYDRATES

P. M. Eidam,[1] J. J. Schariter,[1] B. Fried,[2,*] and J. Sherma[1]

[1] Department of Chemistry and
[2] Department of Biology, Lafayette College,
Easton, PA 18042, USA

ABSTRACT

HPTLC analysis was used to determine the concentrations of lipids, lipophilic pigments, and carbohydrates in the blood and tissues of *Biomphalaria glabrata* snails fed the leafy versus midrib portion of Romaine lettuce. HPTLC analysis was also used to analyze the concentrations of these analytes in the two diets. The concentrations of certain neutral lipids, phospholipids, lipophilic pigments, and carbohydrates were significantly higher in snails fed the leafy rather than the midrib diet. Correspondingly, the concentrations of these analytes were significantly greater in the leafy versus midrib portion of the lettuce. Snail growth was significantly greater when fed the leafy versus midrib portion. In a

*Corresponding author.

chemoattraction bioassay, in which snails were allowed to choose between the two diets, snails were significantly attracted to the leaf rather than the midrib portion. For optimal growth and chemical composition of *B. glabrata* maintained in the laboratory, we suggest that the midrib portion of Romaine lettuce be removed and discarded prior to feeding the snails.

INTRODUCTION

The leaf of Romaine lettuce (*Lactuca sativa longefolia*) contains two major areas, one being the mesophyll-parenchyma layer with numerous chloroplasts and the other a large basal midrib area (also known as the main vein). The midrib branches into a smaller network with a net-like venation pattern. This zone contains vascular bundles, is white, and has relatively few chloroplasts.

We have made incidental unpublished observations on feeding and behavioral preferences of planorbid snails to these two areas of Romaine lettuce. We noted that the Colorado strain of *Helisoma trivolvis* was more attracted to the midrib, and preferred feeding on this zone rather than the leafy portion. An opposite effect was noted with *Biomphalaria glabrata* snails in laboratory aquaria; these snails preferred feeding on the leafy rather than the midrib portion of the lettuce.

Romaine lettuce, by itself or with supplementation, is a major dietary constituent of *B. glabrata* in the laboratory.[1] However, no study has distinguished the feeding behavior of *B. glabrata* on the leafy versus midrib portion of the Romaine lettuce. The purpose of the study was to use high performance thin layer chromatography (HPTLC) to determine the effects of diets composed of the leafy versus the midrib portion of Romaine lettuce on the lipid composition and lipophilic pigments in the whole bodies of *B. glabrata,* as well as the carbohydrate composition in the hemolymph and digestive gland-gonad complex (DGG) of this snail. We also examined the effects of each diet on snail growth (based on wet body weights and shell diameters of the snails). To determine chemoattraction of *B. glabrata* towards either the leafy or midrib portion, chemoattraction studies were done with *B. glabrata* and Romaine lettuce as described in Fedok et al.[2] Lastly, HPTLC was used to determine lipids, lipophilic pigments, and carbohydrates in the leafy versus midrib portions of the lettuce.

EXPERIMENTAL

Snail Maintenance

B. glabrata, 7.0 – 8.0 mm in shell diameter, were obtained from Dr. Fred Lewis of the Schistosomiasis Research Laboratory, Rockville, Maryland. The

snails were maintained in glass vessels; 25 snails in 1000 mL of artificial spring water (ASW),[3] and fed ad libitum with about 500 mg of either the leafy or midrib portion of Romaine lettuce. Food and water were changed every third day.

Growth was determined by weighing 5 snails on each diet (blotted wet weight in mg) and also by measuring the maximum snail shell diameter (in mm) at 7 and 14 days after the cultures were started.

Sample Preparation for Neutral Lipids, Phospholipids, and Lipophilic Pigments

At 7 and 14 days after the cultures were initiated, 4 snails on each diet were extracted to obtain neutral lipid and phospholipid samples. Snail shells were removed, whole snail bodies were blotted dry and weighed, and the lipids were extracted by homogenizing the snail tissue with a glass homogenizer in 2 mL of chloroform-methanol (2:1). Extracts were evaporated under a stream of nitrogen gas and reconstituted in 2.00 mL of chloroform-methanol (2:1). The average neutral lipid and phospholipid sample weighed 27.1 mg.

Sample preparation was the same for the lipophilic pigments, except that the solvent for extraction was HPLC grade acetone at 4°C. All samples were stored at –20°C until analyzed. The average lipophilic pigment sample weighed 29.2 mg.

Dietary samples were prepared in the same manner as the snail tissue samples. The average diet sample weighed 503 mg.

Sample Preparation for Carbohydrates

For carbohydrate HPTLC analysis, DGG and hemolymph samples were prepared instead of the whole body used for lipid and pigment analysis. Hemolymph and DGG samples were prepared as described in Anderton et al.[4] Two DGG samples were prepared for both leaf and midrib diets. Each of these samples consisted of 3 pooled DGGs with an average weight of 100 mg. Two hemolymph samples were also prepared for each sample type. Hemolymph was extracted from 3 pooled snails for each sample, to give an aliquot of 100 μL. Both hemolymph and DGG samples were extracted with 70% ethanol and reconstituted with 200 μL of this solvent.

Chemoattraction

The procedure for the chemoattraction study followed that of Fedok et al.[2] Snails were allowed to choose a 1 cm^2 piece of either the leafy or midrib portion

of the lettuce or a 1 cm^2 piece of a filter paper blank. For details on the bioassay chamber and the design of the experiment see Fedok et al.[2]

TLC of Neutral Lipids

Neutral lipid analysis was performed on Whatman (Clifton, New Jersey, USA) LHP-KDF high performance 10 x 20 cm channeled, preadsorbent HPTLC silica gel plates, as described by Masterson et al.[5]

The standard solution was prepared from the Nu-Check Prep. (Elysian, Minnesota, USA) neutral lipid standard 18-4A containing marker compounds for free fatty acids, free sterols, triacylglycerols, methyl esters, and cholesteryl esters at a concentration of 0.200 mg/mL each in chloroform-methanol (2:1). For analysis, reconstituted samples and standards were spotted in the preadsorbent area with a 10 μL Drummond (Broomall, Pennsylvania, USA) digital microdispenser in aliquots of 2.00, 4.00, 8.00, and 16.0 μL. The mobile phase used to develop the plates was petroleum ether-diethyl ether-acetic acid (80:20:2), and development was done to a distance of 7.5 cm past the preadsorbent/silica gel interface in a glass, paper-lined Camag (Wilmington, North Carolina, USA) twin-trough HPTLC chamber that had preequilibrated for 15 min. The plates were air dried, and lipids were detected by spraying the plate with 5% ethanolic phosphomolybdic acid (PMA), and heating on a Camag Plate Heater III for 15 min at 115°C.

Lipids were quantified by linear scanning at 700 nm using a Camag TLC Scanner II with a tungsten light source, slit dimension settings of length = 4 and width = 4, and a scanning speed of 4 mm/s. The CATS-3 software linear regression program provided a calibration curve that related the standard zone weights to their optimized scan areas. The computer automatically determined the analyte weight for each sample zone from the calibration curve.

TLC of Phospholipids

Phospholipid analysis was performed on Whatman (Clifton, New Jersey, USA) KHP-KDF high performance 10 x 20 cm channeled, preadsorbent HPTLC silica gel plates as described by Albrecht *et al.*[6] The standard solution was prepared from the Matreya Inc. (Pleasant Gap, Pennsylvania, USA) polar lipid standard mix #1127. This standard contained cholesterol, phosphatidylethanolamine (PE), phosphatidylcholine (PC), and lysophosphatidylcholine, each present in the concentration of 0.250 mg/mL in chloroform-methanol (2:1). For analysis, reconstituted samples, as well as standards were spotted in the preadsorbent area with a 10 μL Drummond digital microdispenser in aliquots of 2.00, 4.00, 8.00, and 16.0 μL. The mobile phase used to develop the plates was chloroform-

methanol-water (65:25:4). The phospholipids were detected on dried plates as brown/black spots on a white background by spraying with a 10% solution of cupric sulfate in 8% phosphoric acid and heating at 160°C for 10 min. Quantification was performed by linear scanning of standard and sample zones at 370 nm as described above for lipids.

TLC of Lipophilic Pigments

Lipophilic pigment analysis was performed on Merck (EM Separations Technology, Gibbstown, New Jersey, USA) 10 x 20 cm RP-18F254s C-18 chemically bonded silica gel plates. The β-carotene and lutein standards were obtained from Sigma (St. Louis, Missouri, USA). The standard solutions were prepared by diluting the standards to concentrations of 0.0100 mg/mL for lutein and 0.100 mg/mL for β-carotene in chloroform-methanol (2:1). For analysis, reconstituted samples and standards were spotted in the preadsorbent area with a 10 μL Drummond digital microdispenser in aliquots of 2.00, 4.00, 8.00, and 16.0 μL. The mobile phase used to develop the plates for a distance of 6.5 cm was petroleum ether (35-60°C) – acetonitrile - methanol (1:2:2). The pigments were detected in visible light as colored bands on a white background. Once the plates were developed and air dried, the zones were immediately scanned due to the rapid deterioration of lipophilic pigments at room temperature when exposed to light. Scanning was performed at 448 nm for lutein and 455 nm for β-carotene.

TLC of Carbohydrates

Quantitative analysis was carried out on Merck 10 x 20 cm channeled, high performance silica gel CF_{254} plates that were impregnated with sodium sulfite and citrate buffer as described previously.[7] The mobile phase consisted of ethyl acetate - glacial acetic acid - methanol - deionized water (48:12:12:8), with double development to 7 cm past the preadsorbant-silica gel interface. Following development, a hair dryer was used to evaporate the remaining solvent until the odor of acetic acid disappeared.

For sugar identification and quantification, standard solutions of glucose and maltose (Sigma) were prepared at 1.00 mg mL^{-1} and 0.100 mg mL^{-1}. Standards were applied with a 10 mL Drummond digital microdispenser at 6.00 μL and 8.00 ML for the 0.100 mg mL^{-1} solution, and 1.20 μLcalibration curve by spotting volumes of reconstituted solutions between 3.00 μL and 9.00 μL.

Detection reagent preparation involved diluting 5.0 g of 1-naphthol in 33 ml of ethanol in a 250 mL beaker. A magnetic stir bar and 127 mL ethanol were added to the beaker. Concentrated sulfuric acid (20 mL) followed by 13 mL of

deionized water was added to the solution, which was stirred magnetically for 10 min. The detection reagent storage bottle was wrapped in aluminum foil to exclude light; the reagent was not used after its color darkened. The plates were sprayed moderately with the reagent and dried at 110°C for 10 min. Zones appeared purple on a white background. Quantitative analysis of maltose and glucose was performed by scanning the standard and sample zones at 515 nm.

Unless otherwise noted, the significance of differences between means was evaluated using the Student's t-test with significance based on a P value of < 0.05.

RESULTS

Growth

The results of the growth studies (based on snail shell diameter and body weight) are summarized in Table 1. The shell diameter and body weight of the snails maintained on the leafy portion of the lettuce was significantly greater than that of snails on the midrib diet at 7 and 14 days after the cultures were initiated.

Chemoattraction

The results of the chemoattraction studies are summarized in Table 2. In a single choice bioassay, the snails were significantly attracted to both the leaf and midrib potions of the lettuce. When given a choice between the two diets in a dual choice bioassay, snails were significantly attracted to the leafy portion.

Table 1. Growth of *Biomphalaria glabrata* (Bg) Snails on Leaf (L) vs. Midrib (M) of Romaine Lettuce

Days	Diet	Mean +/− SE of Length (mm)	Mean +/− SE of Body Weight (mg)
0	L	7.25 +/− 0.52	55.5 +/− 12.4
0	M	7.25 +/− 0.66	52.2 +/− 12.3
7	L	8.25 +/− 0.68*	89.4 +/− 26.0*
7	M	7.42 +/− 0.59	70.3 +/− 17.8
14	L	9.00 +/− 0.87*	96.5 +/− 19.6*
14	M	8.10 +/− 0.75	85.4 +/− 16.5

n = 3 or 4 samples.
*Significantly different from cohort at $P < 0.05$.
SE = Standard error.

Table 2. Chemoattraction of *Biomphalaria glabrata* (Bg) to Midrib Romaine Lettuce and Leafy Romaine Lettuce

% of Snails in Zones Diet Experiment	A	B	C	Chi-Square	P
B. vs B.	38	20	42		
B. vs L.	10	9	81	113.29	<0.0001
B. vs M.	15	11	74	105.68	<0.0001
L. vs M.	60	9	31	67.42	<0.0001

B = Blank; L = Leafy Romaine Lettuce; M = Midrib Romaine Lettuce.

Neutral Lipids

TLC analysis showed that the major neutral lipids in *B. glabrata* after 7 days on the leaf diet were free sterols (R_f = 0.22), and triacylglycerols (R_f = 0.57). Trace amounts of free fatty acids (R_f = 0.32) and cholesteryl esters (R_f = 0.87) were found, but these fractions could not be quantified because their concentrations were below the detection limit of the analysis (2.41 x 10^{-4}%). After 14 days on the leaf diet, the major neutral lipids in the snails were free fatty acids, free sterols, and triacylglycerols. Cholesteryl esters were present in trace amounts and could not be quantified. Snails maintained on the midrib diet for 7 days had quantifiable amounts of free sterols. Free fatty acids, triacylglycerols, and cholesteryl esters were also present, but quantification was not possible.

Snails maintained on the midrib diet for 14 days had free fatty acids, free sterols, and triacylglycerols, but the cholesteryl esters were not present in quantifiable amounts. The concentrations of triacylglycerols and free fatty acids in snails on the leaf diet were significantly greater than the same analytes in snails on the midrib diet for 14 days. The quantitative results for neutral lipids are summarized in Table 3.

TLC analysis also showed differences in the neutral lipid profiles in the diets (Table 3). Free sterols were detected in the midrib but not the leaf diet. Triacylglycerols were detected in the leaf but not the midrib diet. The concentration of free fatty acids in the leaf was significantly greater than that in the midrib.

Phospholipids

TLC analysis showed that the major phospholipids in snails on both diets at 7 and 14 days were PE (R_f = 0.31) and PC (R_f = 0.47); no other phospholipids were found that matched the migration of the standards. The results of the quan-

Table 3. Neutral Lipid Content (Weight %) of Romaine Lettuce and Whole Bodies of *Biomphalaria glabrata* (Bg) Snails on Leaf (L) vs. Midrib (M) of Romaine Lettuce Diets

	Days	Diet	Free Fatty Acids (Mean +/− SE)	Free Sterols (Mean +/− SE)	Triacylglycerols (Mean +/− SE)
Bg Snails	7	L	ND	0.343 +/− 0.068	0.231 +/− 0.031
	7	M	ND	0.536 +/− 0.23	ND
	14	L	8.31×10^{-3} +/− 0.81×10^{-3}	0.139 +/− 0.021*	0.116 +/− 0.023*
	14	M	8.94×10^{-3} +/− 2.4×10^{-3}	0.0471 +/− 0.018	0.0137 +/− 0.0029
Romaine Lettuce					
Leaf			0.430 +/− 0.17*	ND	0.0536 +/− 0.033
Midrib			0.0840 +/− 0.024	0.0384 +/− 0.0055	ND

n = 3 or 4 samples.
*Significantly different from cohort at $P < 0.05$.
ND = Not detectable below detection limit of 2.41×10^{-4} %.

titative studies on phospholipids are summarized in Table 4. There were no significant differences in the concentrations of either PE or PC in snails at 7 or 14 days after the cultures were started, regardless of diet. None of the phospholipids detected in the snails was found in either diet at levels above the detection limit, 9.85×10^{-5} %.

Lipophilic Pigments

TLC analysis showed that the major lipophilic pigments in *B. glabrata* snails after 7 days on either the leaf or midrib diet were β-Carotene ($R_f = 0.07$) and lutein ($R_f = 0.41$). Differences in the concentrations of lipophilic pigments in the snails at 7 and 14 days after the cultures were initiated are summarized in Table 5. β-Carotene and lutein concentrations in snails on the leaf diet were significantly greater than those in snails on the midrib diet after 14 days. The concentrations of β-carotene and lutein in the leaf were significantly greater than those in the midrib (Table 5).

Carbohydrates

Zones in both the DGG and hemolymph samples corresponded to the maltose and glucose standards at R_f values of 0.61 and 0.70, respectively. Quantitative analysis of maltose and glucose for both hemolymph and DGG samples yielded greater sugar concentrations in the leaf-fed snails compared to the midrib-fed snails (Table 6). The results also showed that the glucose concentration varied more than the maltose concentration in leaf- versus midrib-fed snails. Quantification of the diets revealed a slightly higher concentration of glucose in

Table 4. Phospholipid Content (Weight %) of Romaine Lettuce and Whole Bodies of *Biomphalaria glabrata* (Bg) Snails on Leaf (L) vs. Midrib (M) of Romaine Lettuce Diets

Days	Diet	Phosphatidylethanolamine (Mean +/− SE)	Phosphatidychoine (Mean +/− SE) Bg Snails
7	L	0.675 +/− 0.18	0.391 +/− 0.059
7	M	1.01 +/− 0.17	0.393 +/− 0.11
14	L	0.327 +/− 0.074	0.402 +/− 0.077
14	M	0.277 +/− 0.056	0.357 +/− 0.12

n = 3 or 4 samples.

Table 5. Lipophilic Pigment Content (Weight %) of Romaine Lettuce and Whole Bodies of *Biomphalaria glabrata* (Bg) Snails on Leaf (L) vs. Midrib (M) of Romaine Lettuce Diets

	Days	Diet	β-Carotene (Mean +/- SE)	Lutein (Mean +/- SE)
Bg Snails	7	L	$5.13 \times 10^{-3} +/- 3.2 \times 10^{-3}$	$3.72 \times 10^{-3} +/- 2.3 \times 10^{-3}$
	7	M	$3.69 \times 10^{-3} +/- 1.4 \times 10^{-3}$	$1.28 \times 10^{-3} +/- 0.61 \times 10^{-3}$
	14	L	$4.24 \times 10^{-3} +/- 0.12 \times 10^{-3}$*	$4.31 \times 10^{-3} +/- 0.64 \times 10^{-3}$*
	14	M	$3.13 \times 10^{-3} +/- 0.33 \times 10^{-3}$	$1.09 \times 10^{-3} +/- 0.51 \times 10^{-3}$
Romaine Lettuce				
Leaf			$18.7 \times 10^{-3} +/- 0.65 \times 10^{-3}$*	$30.4 \times 10^{-3} +/- 9.0 \times 10^{-3}$*
Midrib			$0.121 \times 10^{-3} +/- 1.4 \times 10^{-6}$	$0.351 \times 10^{-3} +/- 0.25 \times 10^{-4}$

n = 3 or 4 samples.

*Significantly different from cohort at $P < 0.05$.

Table 6. Carbohydrate Content of Romaine Lettuce and DGG (Weight %) and Hemolymph (mg/dL) of *Biomphalaria glabrata* (Bg) Snails Fed Leaf (L) vs. Midrib (M) Diets of Romaine Lettuce

Days	Diet	Maltose (Mean +/− SE)	Glucose (Mean +/− SE)
			DGG of Bg Snails
10	L	0.0414	0.0593
10	M	0.0201	0.0156
			Hemolymph of Bg Snails
10	L	88.7	77.9
10	M	66.0	30.5
			Romaine Lettuce
10	L	ND	0.135
10	M	ND	0.107

n = 3 or 4 samples.

ND = Not detectable below detection limit of 0.00750 %.

the leaf versus the midrib (Table 6). Maltose was below the detection limit of 0.00750% in both diets.

DISCUSSION

This is the first study that describes the use of the leaf versus midrib portion of Romaine lettuce as a diet for *Biomphalaria glabrata* snails. It is clear from our study, that the leafy portion is more nutritious that the midrib in terms of providing both enhanced snail growth and the accumulation of lipids, lipophilic pigments, and carbohydrates in snail blood and tissues.

Hyman[8] noted that many factors influence the growth of pulmonate snails in the laboratory, including snail density, availability of food, and accumulation of waste products. It is clear, from our study and others, that food content is very important in determining maximum snails growth. Thus, Florin et al.[9] noted that *Lymnaea elodes* showed maximal growth based on both shell length and body weight when fed leaf lettuce; diets of Tetramin fish food or freshly killed *Helisoma trivolvis* snails resulted in submaximal snail growth. Keas and Esch[10] reported that *Helisoma anceps* snails raised on a high quality diet were larger than those on a low quality diet. In accord with the findings of Keas and Esch,[10] we designate the leafy portion of Romaine lettuce as a high quality diet and the midrib portion as a low quality diet.

The concentrations of analytes in the diets reflect closely their concentrations in the blood and tissues of *B. glabrata*. Thus, a diet with relatively high concentrations of neutral lipids, phopholipids, and lipophilic pigments, e.g., the leafy portion of Romaine lettuce, will induce relatively high concentrations of these analytes in the snail compared to the midrib portion of the lettuce with its lower concentrations of these analytes. It would appear that "you are what you eat" is an important adage to the chemical makeup of *B. glabrata,* at least in terms of lipids, lipophilic pigments, and carbohydrates.

The chemoattraction studies were interesting in that snails were attracted to either the leafy or midrib portion of Romaine lettuce in a single choice bioassay, but they preferred the leaf over the midrib when allowed to choose between the two in a dual choice bioassay. Based on this study, *B. glabrata* is capable of discriminating between food substances in bioassays. In a previous study, however, Masterson and Fried[11] observed that *B. glabrata* showed no significant choice difference between lettuce and Tetramin in the same bioassay. It appears that snail chemoattraction is not necessarily an indicator of snail food preference.

As a result of this study, we recommend that the midrib be removed and discarded before Romaine lettuce is fed to the snails, in order to achieve maximal growth and optimal chemical concentrations in *B. glabrata* tissues and blood when these snails are maintained in laboratory cultures.

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