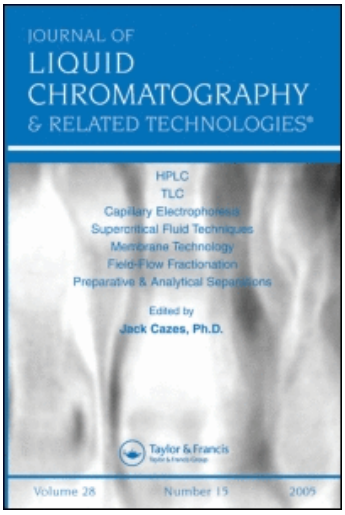

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DETERMINATION OF ESTIVATION-INDUCED CHANGES IN THE AMINO ACID CONTENT OF *BIOMPHALARIA GLABRATA* SNAILS BY HIGH PERFORMANCE THIN-LAYER CHROMATOGRAPHY-DENSITOMETRY

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□ *High performance thin-layer chromatography-densitometry (HPTLC-Dens) was used to determine changes in the amino acid content of the digestive-gland gonad complex (DGG) of* Biomphalaria glabrata *snails as a function of estivation. Amino acids were extracted in ethanol-water (70:30) from the DGG of* B. glabrata *snails estivated for 7 days, and determined on silica gel or cellulose layers developed with either 2-butanol-pyridine-glacial acetic acid-deionized water (39:34:10:26) or 2-butanol-pyridine-25% ammonia-deionized water (39:34:10:26). Separated zones were detected by postchromatographic derivitization with ninhydrin and quantified by visible mode slit-scanning densitometry at 610 nm. Alanine, arginine, glycine, leucine/isoleucine, lysine, serine, and valine were unequivocally identified in chromatograms from both estivated and unestivated snails. HPTLC-Dens showed a significant increase in alanine in estivated snails compared to unestivated controls (Student's t-test,* $P < 0.05$*). The finding of increased alanine levels in the DGG during estivation is in agreement with the hypothesis that anaerobic pathways are an important part of overall metabolism in* B. glabrata *during estivation.*

Keywords alanine, amino acids, *Biomphalaria glabrata*, estivation, high performance thin-layer chromatography, HPTLC, planorbid snails, pulmonate snails

INTRODUCTION

Estivation (also referred to as aestivation in some papers) is a biologically important state of dormancy used by a variety of organisms to minimize the adverse effects of high heat and desiccation during drought-like conditions. This phenomenon is ubiquitous among the pulmonate snails, in which estivation results in water loss and metabolic depression.[1,2] Regulation of metabolism during estivation has been well characterized in the

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model pulmonate snail *Otala lactea* and involves the reversible phosphorylation of key enzymes such as pyruvate dehydrogenase, 6-phosphofructo-1-kinase, and pyruvate kinase, among others.[1] Moreover, it has been reported that metabolic depression in *O. lactea* during long term estivation results in decreased glycoloytic activity and adjustments to amino acid metabolism.[1]

In the case of the planorbid snail *Biomphalaria glabrata*, the invertebrate intermediate host of the medically important trematode *Schistosoma mansoni*, estivation can be sustained for up to seven months.[3] Of particular importance is the finding that larval stages of *S. mansoni* can survive for long periods of time in estivating *B. glabrata* snails.[4] Thus, the parasite can use its snail intermediate host to survive long periods of drought and increase its chance of returning to an aquatic environment where it can continue its life cycle.

An increased understanding of the host-parasite interactions that occur during estivation can be obtained by determining the overall metabolic changes that occur in the snail host during this state. However, such studies of the molecular mechanisms of estivation in planorbid snails have been limited. Bezerra et al. reported a significant increase in succinate, malate, lactate, pyruvate, and acetate during estivation in *B. glabrata*.[2] Work in our laboratory using high performance thin-layer chromatography-densitometry (HPTLC-Dens) or high performance column liquid chromatography (HPLC) to study estivation in *Helisoma trivolvis* and *B. glabrata* has shown that the concentrations of triacylglycerols, glucose, maltose, lutein, succinate, fumarate, and malate in the digestive gland-gonad complex (DGG) are significantly decreased during estivation, while the levels of free sterols are increased.[5–8]

Recently, the amino acid (AA) profile of the DGG of *B. glabrata* snails has been characterized.[9] However, the effects of estivation on the amino acid content of *B. glabrata* have not been reported, and little is known about the effects of estivation on amino acid metabolism in planorbid snails. Therefore, the purpose of this study was to determine the effects of estivation on the amino acid profile of the DGG of *B. glabrata* using HPTLC-Dens.

EXPERIMENTAL

Snail Maintenance and Estivation

B. glabrata snails (NMRI strain) were maintained in the laboratory as described by Fried et al.[10] Cultures of 10 to 20 snails were maintained at $24 \pm 1°C$ in mason jars containing about 800 mL artificial spring water (ASW), prepared as described by Ulmer,[11] under diffuse fluorescent lighting for a photoperiod of 12 h per day. Snails were maintained on a

diet of boiled romaine lettuce ad libitum, and cultures were changed 3 times per week.

To induce estivation, 12 snails with a shell diameter of 7–10 mm were placed in a moistened filter paper lined finger bowl with four stender dishes, each containing 3 mL ASW. Stender dishes were covered with cheesecloth to allow vapor equilibration while limiting snail access to the water. An additional finger bowl was placed loosely on top of the above apparatus to leave a slight opening for air transfer and maintenance of aerobic conditions. This experimental design has been shown previously to produce an environment with a relative humidity of $98 \pm 1\%$ at $24 \pm 1°C$, which is sufficient to induce *B. glabrata* snails to estivate.[7] Snails were maintained under estivation conditions for 7 days without food. A 7-day estivation period has been shown previously to allow for optimal snail recovery post estivation.[5] Cultures of control snails were maintained in mason jars and fed *ad libitum*.

Following estivation, snails were placed 12 per finger bowl in 250 mL ASW to examine revival. Revival time was measured as the time required for the snail to extend its foot from the shell opening. After revival, each snail was blotted dry with a paper towel, after which the shell was cracked lightly with a hammer and teased away from the body with forceps. The DGG was dissected from the body with fine scissors, rinsed with deionized water (DI), blotted dry with a paper towel, and transferred to a microcentrifuge tube. An equivalent number of control snails were necropsied and the DGG prepared identically to those of estivated snails.

Sample Preparation

After dissection, the blotted wet weight of each DGG was recorded, and individual DGGs (8 ± 4 mg) were pooled to produce samples with a mass of 30 ± 10 mg. Samples were ground in a 7 mL glass homogenizer (Wheaton, Millville, NJ) with 3 mL ethanol-water (70:30), transferred quantitatively to 1 mL microcentrifuge tubes, and centrifuged at $8{,}000 \times g$ for 5 min. For each sample, the corresponding supernatants were quantitatively washed (2×100 µL) into a glass vial with ethanol-water (70:10) and evaporated to dryness in a water bath ($50 \pm 5°C$) using a stream of air. Evaporated extracts were reconstituted in a volume (0.5 ± 0.2 mL) of ethanol-water (70:30) chosen to produce densitometric scan areas that were bracketed within calibration plots.

Thin-Layer Chromatography

HPTLC and spiking analyses were performed as described previously for the determination of AAs in complex mixtures.[12,13] Standards of the

AAs alanine (Ala), arginine (Arg), asparagine (Asn), aspartate (Asp), citrulline (Cit), glutamic acid (Glu), glutamine (Gln), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), ornithine (Orn), phenylalanine (Phe), proline (Pro), serine (Ser), taurine (Tau), threonine (Thr), tryptophan (Trp), tyrosine (Tyr), and valine (Val) were obtained from Sigma (St. Louis, MO, USA). Individual stock standard solutions were prepared in ethanol-water (70:30) at a concentration of 1.00 $\mu g\,\mu L^{-1}$. Mixed stock standard solutions were prepared at a concentration of 50.0 $ng\,\mu L^{-1}$ for each acid. Mixed HPTLC standard solutions were prepared by diluting the mixed stock standards to either 12.5 $ng\,\mu L^{-1}$ or 25.0 $ng\,\mu L^{-1}$ with ethanol-water (70:30).

Both silica gel and cellulose layers were employed for the identification and quantification of the AAs in the DGG of *B. glabrata* snails. The silica gel layer was 20 × 10 cm HPTLC silica gel with a concentration zone (CZ), no. 13728–6 (EMD Chemicals, Inc., Gibbstown, NJ, USA, an affiliate of Merck KGaA, Darmstadt, Germany) and the cellulose layer was 20 × 10 cm HPTLC cellulose F, no. 15036–6 (EMD Chemicals, Inc.). All layers were prewashed by development to the top with dichloromethane-methanol (1:1) and air dried before use. In qualitative AA determinations and spiking analyses, the above layers were developed with either of the following two mobile phases: 2-butanol-pyridine-glacial acetic acid-deionized water (39:34:10:26; mobile phase A) or 2-butanol-pyridine-25% ammonia-deionized water (39:34:10:26; mobile phase B). Quantification of Ala, Val, Ser, and Leu/Ile was performed on the cellulose layer developed with mobile phase B and quantification of Arg and Lys was performed on the cellulose layer developed with mobile phase A.

In qualitative AA determinations, 2.00–8.00 μL aliquots of HPTLC standard or reconstituted sample were applied to the layer (cellulose) or CZ (silica gel) using a Linomat IV (Camag, Wilmington, NC, USA) fitted with a 100 uL syringe and operated with the following settings: band length 6 mm, application rate 15 s uL^{-1}, table speed 10 mm s^{-1}, distance between bands 4 mm, distance from plate side edge 7 mm, and distance from the bottom of the plate 1.0 cm. Plates were developed with the appropriate mobile phase in a solvent vapor equilibrated CAMAG 20 × 10 cm plate format twin-trough chamber with a saturation pad (Analtech, Newark, DE, USA) for a distance of 7 cm beyond the origin. Development required about 1 h for silica gel layers and about 2 h for cellulose layers. Plates were air dried in a fumehood, sprayed to saturation with ninhydrin solution (0.3 g ninhydrin in 100 mL of *n*-butanol plus 3 mL of glacial acetic acid), and heated on a Camag plate heater at 110°C for 10 min to detect the amino acids as zones of various colors on a white to pink background.

Spiking analyses were performed as described previously[13] to test the susceptibility of the HPTLC systems to matrix effects and confirm the

identities of certain ninhydrin-positive zones. Briefly, all spiking analyses used the over-spotting technique.[14] For each AA confirmed by spiking, a 2.00 or 4.00 μL aliquot of stock standard solution of the AA was spotted side-by-side with a 4.00 μL aliquot of DGG sample solution, after which a lane was spotted containing an identical aliquot of both of the previous solutions.

In quantitative AA determinations, the mixed HPTLC standards described above were spotted in 2.00, 4.00, 6.00, and 8.00 μL aliquots (25.0, 50.0, 75.0, and 100 ng for Ala, Val, Ser, and Leu and 50.0, 100, 150, and 200 ng for Arg and Lys) to create a calibration plot, followed by 2.00–8.00 μL of reconstituted sample chosen so that the scan areas of zones in sample chromatograms would be bracketed within the standard areas of the calibration plot. The areas of the bands were measured using a CAMAG TLC Scanner II with the tungsten light source set at 610 nm. The settings were slit width 4, slit length 4, and scanning speed 4 mm s^{-1}. The CATS-3 software was used to create a linear regression calibration plot relating the weights of the standard zones to their peak areas. The correlation coefficient (r-value) for each calibration plot was at least 0.99 in all analyses.

Leu standard was used to quantify mixed zones containing Leu and/or Ile that were inseparable by the four HPTLC systems used. This was a valid approach because these AAs produce zones of identical color and preliminary calibration plots created from pure zones of each AA had slopes, intercepts, and correlation coefficients (r-values) that were practically identical, indicating that the chromophore of their respective ninhydrin derivates had similar light absorption properties under the conditions used in this study.

The equation used for calculation of the concentration weight percent (Wt%) of AA in DGG tissue was

$$\mathrm{Wt\%} = (\mathrm{w} * \mathrm{R})/(10,000 * \mathrm{M})$$

where w = ng interpolated from calibration plot, R = [reconstitution volume (μL)]/[spotted volume (μL)], and M = mass of DGG sample (mg). If a dilution or concentration step was required in order to produce sample zone scan areas bracketed within the scan areas of the calibration plot, the final concentration calculated using the above equation was modified by multiplying by a dilution factor (DF), where the DF was calculated by dividing the diluted or concentrated sample volume by the original sample volume.

The statistical package R (freely available at http://www.r-project.org/) was used in all statistical analyses. Student's *t*-test was used to determine the statistical significance of quantitative data based on the mean ± standard

deviation of the Wt% of AAs in DGG samples from estivated and control snails, with $P < 0.05$ considered significant.

RESULTS AND DISCUSSION

Estivation

Following estivation of 12 *B. glabrata* snails, 11 (92%) were revived within 20 min of placement in ASW. This finding for percent survival is similar to the 87% survival reported by Muller et al.[15] for a 7-day estivation period of *B. glabrata*.

Amino Acid Determinations

Four HPTLC systems were used to determine AAs in the DGG of estivated and unestivated snails. Spiking analyses confirmed the absence of matrix effects for DGG samples and validated the use of comigration for identification. Table 1 provides retention data for all AAs unequivocally identified in the DGG of estivated and control snails based upon comigration and color similarity.

Comigration and spiking analyses identified Ala, Gly, Arg, and Lys as the principle AA components of the DGG of *B. glabrata* snails, with Ser, Val, and Leu/Ile present at slightly lower levels. Depending on the specific HPTLC system used, these AAs were visible as purple to pink zones in sample chromatograms. In addition, the AAs Gln and Met may have been present in samples at low levels, although the faintness of putative zones and overall complexity of the sample prevented the unequivocal identification of these AAs. Asn, Asp, Cit, Glu, His, Phe, Pro, Tau, Thr, Trp, and Tyr appeared to

TABLE 1 hR_F[a] Values for the Amino Acids Determined in the DGG of *B. glabrata* Snails

Amino Acid	System 1[b]	System 2[c]	System 3[d]	System 4[e]
Alanine	37	46	36	27
Arginine	22	24	21	6
Glycine	25	35	27	23
Leucine/Isoleucine	70	70	58	48
Lysine	18	25	16	4
Serine	27	40	31	28
Valine	56	60	49	44

[a] $hR_F = R_F \times 100$.
[b] System 1 = cellulose layer developed with mobile phase A.
[c] System 2 = cellulose layer developed with mobile phase B.
[d] System 3 = silica gel layer developed with mobile phase A.
[e] System 4 = silica gel layer developed with mobile phase B.

be absent in chromatograms of DGG samples in all four systems, which suggests that these AAs are not present at appreciable levels in the DGG of *B. glabrata* snails, or that they are present at levels below the limit of detection of the HPTLC methods used in this study. The limit of quantification (LOQ) for Ala, Val, Ser, and Leu/Ile was 25 ng and the LOQ for Arg and Lys was 50 ng.

Our findings of Ala, Lys, and Leu/Ile are in agreement with those of Pachuski et al.[9] and our findings of Ala, Lys, Leu/Ile, Ser, and Val are in agreement with those of Ponder et al.,[16] both studies of which determined the amino acid content of the DGG of *B. glabrata* snails using thin-layer chromatographic methods. In addition to the four AAs mentioned above, Ponder et al.[16] also reported the presence of His, and Pachuski et al.[9] reported the presence of His, Thr, Asn, Met, and Pro, although neither of the two studies reported the presence Gln, Gly, or Arg. The discrepancies between our findings and those of Pachuski et al.[9] and Ponder et al.[16] likely reflect differences in the chromatographic systems used for AA determination in all three studies. Previously, we compared modern HPTLC systems for the determination of AAs in complex mixtures, and found the four HPTLC systems used in this study to be the optimal systems for the determination of AAs based upon enhanced efficiencies and selectivities of zones.[12] Moreover, we employed automated, band-wise sample application using a Linomat IV, which has been shown to provide increased resolution of AAs compared to manual application to the layer, especially for cellulose layers that lack a CZ.[12] The above enhancements in the HPTLC methodology, in conjunction with the use of spiking analyses to confirm the validity of comigration analyses, provides further support for the identifications made in this study.

In addition to the AAs identified above, DGG sample chromatograms contained a variety of other unidentified ninhydrin-positive zones (Table 2). Because these bands were not identifiable among the AAs used in this study, they were not further characterized.

Among the AAs identified in DGG samples, only Ala, Val, Ser, Lys, Arg, and Leu/Ile were resolved enough for quantification. Table 3 provides quantitative data (Wt% $\pm$ standard deviation) for Ala, Val, Ser, Leu/Ile (quantified on the cellulose layer developed with mobile phase B), as well as Lys, and Arg (quantified on the cellulose layer developed with mobile phase A). Statistical analyses of these data show that Ala is significantly increased (Student's *t*-test, $P < 0.05$) in the DGG of estivated snails compared to that of unestivated snails. Also, it is worth noting that a *t*-test comparison of the increase in Val in estivated DGG samples gave a *P* value of 0.0547, which is just on the boarder of statistical significance. Figure 1 provides chromatograms displaying the typical AA profile of the DGG of estivated and unestivated *B. glabrata* snails. Zones corresponding to Ala

TABLE 2 hR_F Values of Additional Unidentified Ninhydrin-Positive Zones found in the DGG of Estivated and Control *B. glabrata* Snails in each HPTLC System[a]

System 1	System 2	System 3	System 4
1	7	11	1
2	10	68	19
5	12	72	51
28	16	74	52
16	18		56
49	65		
74	74		
98	82		
	93		
	98		

[a]See Table 1 for definitions of the systems.

are visually more dense in DGG samples from estivated snails compared to those of unestivated controls, which supports the quantitative data and statistical comparisons reported above.

Implications for Metabolism During Estivation

It is well known that estivation results in a significant decrease in the rate of oxygen consumption in *B. glabrata* (referred to as *Australorbis glabratus* in that study),[17] as well as significant decreases in triacylglycerols, glucose, and maltose in the DGG.[5,6] Moreover, Bezerra et al.[2] reported increases in lactate, succinate, malate, and acetate in the DGG, as well as increases in pyruvate and acetate in the hemolymph of estivating *B. glabrata*, and suggested a possible role for anaerobic metabolism during this state of aerobic stress. Given that alanine is known to accumulate in gastropods during anaerobic metabolism,[1,18] our findings, in conjunction with those above, suggest that anaerobic pathways may play a prominent role in overall metabolism during estivation in *B. glabrata* and other planorbid snails.

TABLE 3 Weight Percent (Mean ± Standard Deviation) of Amino Acids in the DGG of Estivated and Unestivated *B. glabrata* Snails

Amino Acid	Unestivated	Estivated
Alanine	0.050 ± 0.009	0.106 ± 0.003[a]
Arginine	0.05 ± 0.02	0.06 ± 0.02
Leucine/Isoleucine	0.012 ± 0.002	0.017 ± 0.002
Lysine	0.04 ± 0.01	0.045 ± 0.005
Serine	0.022 ± 0.003	0.030 ± 0.003
Valine	0.0073 ± 0.0007	0.0098 ± 0.0006

[a]Value significantly increased compared to samples from unestivated snails (Student's *t*-test, $P < 0.05$).

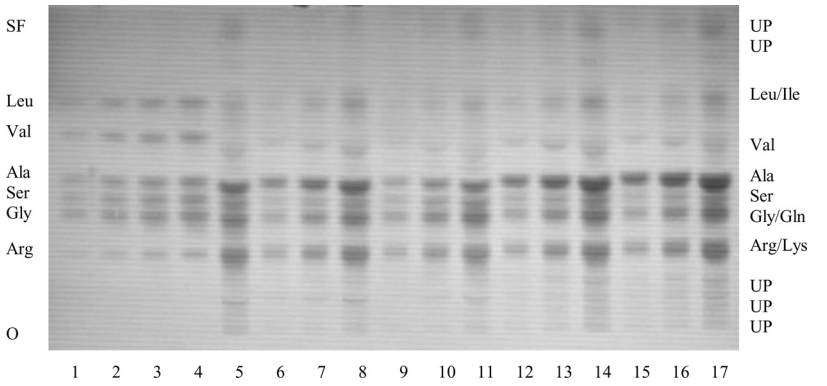

FIGURE 1 Chromatograms on a cellulose HPTLC plate developed with 2-butanol-pyridine-25% ammonia-deionized water (39:34:10:26) photographed in white light with a Camag VideoStore documentation system showing the typical amino acid profile of the DGG of estivated and unestivated *B. glabrata* snails. Lanes 1–4 contain mixed HPTLC standard solution containing Arg, Gly, Ser, Ala, Val, and Leu spotted in 2.00, 4.00, 6.00, and 8.00 μL aliquots, respectively. Lanes 5–11 contain 3 reconstituted DGG samples from unestivated snails. Lanes 12–18 contain 2 reconstituted DGG samples from snails estivated for 7 days. Ala was found to be significantly decreased ($P < 0.05$) in estivated snails compared to unestivated controls. SF = solvent front, UP = unidentified ninhydrin-positive compound, O = origin.

CONCLUSIONS

This study examined the effects of estivation on the amino acid content of the DGG of the planorbid snail *B. glabrata*. Using HPTLC-Dens, we positively identified the presence of Ala, Arg, Lys, Gly, Ser, Val, and Leu and/or Ile. These findings are generally in agreement with previous reports on the amino acid content of the DGG of *B. glabrata*. Moreover, we determined that there was a significant increase in alanine levels in the DGG after 7 days of estivation. This finding is consistent with previous reports that anaerobic metabolism may operate during estivation in *B. glabrata*.

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REFERENCES

1. Storey, K.B. Life in the slow lane: molecular mechanisms of estivation. Comp. Biochem. Physiol. A **2002**, *133*, 733–754.
2. Bezerra, J.C.B.; Kemper, A. Profile of organic acid concentrations in the digestive gland and hemolymph of *Biomphalaria glabrata* under estivation. Mem. I. Oswaldo Cruz. **1999**, *94*, 779–784.

3. Barbosa, F.S.; Barbosa, I. Dormancy during the larval stages of the trematode *Schistosoma mansoni* in snails estivating on the soil of dry natural habitats. Ecology **1958**, *39*, 763–764.
4. Richards, C.S. Estivation of *Biomphalaria glabrata* (Basommatophora, Planorbidae). Am. J. Trop. Med. Hyg. **1967**, *16*, 797–802.
5. White, M.M.; Fried, B.; Sherma, J. Determination of the effects of estivation and starvation on neutral lipids and phospholipids in *Biomphalaria glabrata* (NMRI strain) and *Helisoma trivolvis* (Colorado strain) snails by quantitative high performance thin layer chromatography-densitometry. J. Liq. Chromatogr. Rel. Technol. **2006**, *29*, 2167–2180.
6. Jarusiewicz, J.A.; Fried, B.; Sherma, J. Thin layer chromatographic analysis of glucose and maltose in estivated *Biomphalaria glabrata* snails and those infected with *Schistosoma mansoni*. Comp. Biochem. Physiol. **2006**, *145B*, 346–349.
7. Aurthur, B.; Fried, B.; Sherma, J. Effects of estivation on lutein and beta-carotene concentrations in *Biomphalaria glabrata* (NMRI strain) and *Helisoma trivolvis* (Colorado strain) snails as determined by quantitative high performance reversed phase thin layer chromatography. J. Liq. Chromatogr. Rel. Technol. **2006**, *29*, 2159–2165.
8. Brown, M.A.; Chejlava, M.J.; Fried, B.; Sherma, J. Effects of estivation on the concentration of selected carboxylic acids of two strains of *Helisoma trivolvis*.
9. Pachuski, J.; Fried, B.; Sherma, J. HPTLC analysis of amino acids in *Biomphalaria glabrata* infected with *Schistosoma mansoni*. J. Liq. Chromatogr. Rel. Technol. **2002**, *25*, 2345–2349.
10. Fried, B.; Muller, E.; Broadway, A.; Sherma, J. Effects of diet on the development of *Schistosoma mansoni* in *Biomphalaria glabrata* and on the neutral lipid content of the digestive gland-gonad complex of the snail. J. Parasitol. **2001**, *87*, 223–225.
11. Ulmer, M.J. Notes on Rearing of Snails in the Laboratory, in *Experiments and Techniques in Parasitiology*; MacInnis, A.J., Voge, M., Eds.; W. H. Freeman and Co.: San Francisco, 1970, 143–144.
12. Vasta, J.D.; Cicchi, M.; Sherma, J.; Fried, B. Evaluation of thin-layer chromatography systems for analysis of amino acids in complex mixtures. Acta Chromatogr. **2009**, *21*, 29–38.
13. Vasta, J.D.; Fried, B.; Sherma, J. Determination and quantification of amino acids in the urine of BALB/c mice infected with *Echinostoma caproni* by high-performance thin-layer chromatography-densitometry. J. Liq. Chromatogr. Rel. Technol. **2009**, *32*, 1210–1222.
14. Massa, D.R.; Vasta, J.D.; Fried, B.; Sherma, J. High-performance thin-layer chromatographic analysis of the polar lipid content of human urine and urine from BALB/c mice experimentally infected with *Echinostoma caproni*. J. Planar Chromatogr.-Mod. TLC **2008**, *21*, 337–341.
15. Muller, E.E.; Fried, B.; Sherma, J. HPTLC analysis of neutral lipids in *Biomphalaria glabrata* snails infected with *Schistosoma mansoni* (Trematoda). J. Planar Chromatogr.-Mod. TLC **2000**, *13*, 228–231.
16. Ponder, E.L.; Fried, B.; Sherma, J. Free-pool amino acids in *Biomphalaria glabrata* infected with *Echinostoma caproni* as determined by thin-layer chromatography. J. Parasitol. **2004**, *90*, 665–666.
17. von Brand, T.; McMahon, P.; Nolan. M.O. Physiological observations on starvation and desiccation of the snail *Australorbis glabratus*. Biol. Bull. **1957**, *113*, 89–102.
18. Kuytmans, J.H.; Zandee, Z.I. Comparative study of the formation and excretion of anaerobic fermentation products in bivalves and gastropods. Comp. Biochem. Physiol. B **1983**, *75*, 729–732.