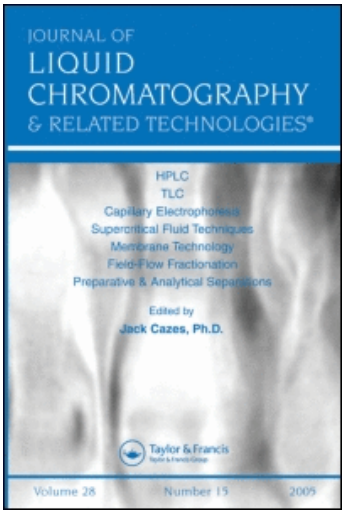


**Journal of Liquid Chromatography & Related Technologies**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# Thin Layer and High Performance Column Liquid Chromatographic Analysis of Selected Carboxylic Acids in Standards and from *Helisoma trivolvis* (Colorado Strain) Snails

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**To cite this Article** Massa, Daniel R. , Chejlava, Michael J. , Fried, Bernard and Sherma, Joseph(2007) 'Thin Layer and High Performance Column Liquid Chromatographic Analysis of Selected Carboxylic Acids in Standards and from *Helisoma trivolvis* (Colorado Strain) Snails', Journal of Liquid Chromatography & Related Technologies, 30: 15, 2221 — 2229
**To link to this Article: DOI:** 10.1080/10826070701451563
**URL:** http://dx.doi.org/10.1080/10826070701451563

*Journal of Liquid Chromatography & Related Technologies*®, 30: 2221–2229, 2007

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070701451563

# Thin Layer and High Performance Column Liquid Chromatographic Analysis of Selected Carboxylic Acids in Standards and from *Helisoma trivolvis* (Colorado Strain) Snails

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**Abstract:** Separations and detections of acetic, fumaric, lactic, malic, pyruvic, and succinic acid standards were compared using previously reported thin layer chromatography (TLC) and high performance TLC (HPTLC) systems, which consisted mainly of silica gel or cellulose layers and acid-base indicator or aniline-aldose detection reagents. The best results were obtained with an HPTLC cellulose layer, triple development with *n*-propanol-2 M ammonium hydroxide mobile phase, and aniline-xylose reagent, but resolution and sensitivity were not adequate for determination of these acids in *Helisoma trivolvis* (Colorado strain) snails.

Analysis of snail digestive gland-gonad complex (DGG) samples was carried out using extraction with 50% Locke's solution, cleanup of the extract by anion exchange solid phase extraction (SPE), and ion exclusion high performance liquid chromatography with ultraviolet detection. Hemolymph was applied directly to the SPE column. Acetic, fumaric, malic, and pyruvic acids were detected at concentrations

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ranging from 24 to 200 ppm in the DGG and less than 154 to 16,000 ppm in hemolymph.

**Keywords:** Thin layer chromatography, TLC, Ion exclusion chromatography, Carboxylic acids, Digestive gland-gonad complex, Hemolymph, *Helisoma trivolvis* snails, Solid phase extraction



## INTRODUCTION

*Helisoma trivolvis* (Colorado strain) is a useful model planorbid snail and is easy to maintain in continuous culture in the laboratory.[1] It has been used for a number of important laboratory studies on the physiology and biochemistry of pulmonate snails.[2] In both structure and function, this snail shows similarities to the medically important planorbid, *Biomphalaria glabrata*, vector of *Schistosoma mansoni*, the causative agent of schistosomiasis. This parasitic disease, also referred to as bilharziasis, is responsible for an annual morbidity and mortality of more than 200 million people globally.

Because of the ease of maintaining *H. trivolvis* (Co) in our laboratory, we have initiated studies on certain carboxylic acids of biological significance in this snail. Some of these acids, i.e., pyruvic, lactic, succinic, fumaric, malic, and acetic, and others, play important roles in both aerobic and anaerobic metabolic pathways in medically important gastropods. Further information on understanding the role of such metabolites may be important in developing rational methods to control and even eradicate medically important snails that transmit parasitic diseases to humans and animals.

Identification of key carboxylic acids in snails has only been done in two studies on *B. glabrata*. Bezerra et al.[3] found some differences in certain carboxylic acids in strains of *B. glabrata* susceptible or non-susceptible to infection with *S. mansoni*. These authors speculated that certain organic acids may be responsible in part for snail susceptibility to infections with *S. mansoni*. In a later paper,[4] Bezerra et al. reported changes in the content of certain carboxylic acids in *B. glabrata* during estivation. The method used for analysis in both of these studies was column high performance liquid chromatography (HPLC).

The purpose of this research was to study the separation and detection of the standards of selected carboxylic acids of biological significance by thin layer chromatography (TLC) and the previously reported[3,4] HPLC method and to qualitatively and quantitatively determine these acids in the hemolymph (blood) and digestive gland-gonad complex (DGG) of *H. trivolvis*. Comparisons of all previously reported TLC and high performance TLC (HPTLC) systems utilizing cellulose and silica gel layers and acid-base indicator and other post-chromatographic derivatization reagents showed that cellulose HPTLC with triple mobile phase development and

detection with aniline-xylose reagent provided the best results for the carboxylic acids, but that the sensitivity was not adequate for analysis of the snail samples. The HPLC method was modified by use of an improved extraction method and mobile phase, and quantitative data obtained for the identified carboxylic acids using this method are reported.

## EXPERIMENTAL

### Preparation of Standards

Standards of acetic, fumaric, lactic, malic, pyruvic, and succinic acids were purchased from Sigma-Aldrich (St. Louis, MO, USA) in the highest purity grade available. Individual standard solutions for TLC were prepared by weighing and dilution at concentrations of 10,000 ppm (10.0 $\mu$g $\mu$L$^{-1}$) in ultra-pure water. Two standard mixtures were each prepared at concentrations of 1,000, 500, 100, 10.0, and 1.00 ppm in 0.50 M sulfuric acid for preparation of the HPLC calibration curves: Mixture 1 contained pyruvic, succinic, and acetic acids and Mixture 2 contained malic, lactic, and fumaric acids.



### Maintenance of Snails

*H. trivolvis* snails were maintained in aerated glass jars, 10 to 20 snails in each jar, containing 800 mL of artificial spring water (ASW) at room temperature and were fed *ad libitum* on a boiled Romaine lettuce leaf diet. Water was changed in the culture jars three times each week. All snails used were sexually mature adults having mean shell diameters $\pm$ standard error (SE) of 10.0 $\pm$ 0.3 mm (n = 20 snails). About 20 snails were used to get sufficient hemolymph and tissue samples for the HPLC analyses described below. For more details on the snail maintenance procedures, including the formulation of ASW, see the paper by Schneck and Fried.[1]

### Sample Preparation

For DGG preparation, the snail shells were cracked lightly with a hammer, and the snail body was removed with forceps under a dissecting microscope. The DGG was dissected free of the snail body using fine scissors. Each DGG sample (45.0–104.0 mg) was extracted with 50% Locke's solution[5] (6.0 mL) in a 15 mL glass tissue grinder and then transferred into a 15 mL plastic centrifuge tube. The tissue grinder was washed with an additional 1.0 mL of Locke's solution, which was then added to the centrifuge tube. The sample was centrifuged for 15 min at 250 $\times$ g, and the supernatant was transferred into a clean centrifuge tube and stored frozen until solid phase

extraction (SPE). To test the effectiveness of 50% Locke's solution as an organic acid extractant, the resulting pellets from two of the centrifuged samples were re-extracted and centrifuged a second time, using the same procedure as above. In some samples suspended particulate matter remained after centrifugation, in which case the sample was centrifuged for another 15 min at 250 × g.

For hemolymph preparation, each snail shell was cracked with a hammer in the bottom of a 6 cm Petri dish, and the hemolymph was allowed to drain into the dish. The raw hemolymph plus some cellular debris (ca., 150 to 200 μL snail$^{-1}$) was collected with a Pasteur pipet and placed in a 2000 μL Eppendorf tube. Each hemolymph sample was centrifuged at 70 × g for 5 min to obtain a pellet (consisting of residual sample debris and hemocytes) that was discarded. The supernatant (plasma) was placed directly on the SPE column. In this paper the term plasma is used interchangeably with the term hemolymph.

Bond Elut-SAX (trimethylaminopropyl bonded to silica, chloride form) anion exchange SPE columns (100 mg, 3 mL; Varian Inc., Palo Alto, CA, USA) were used to cleanup and recover carboxylic acids from the DGG and hemolymph samples prepared as described above. Using a Baker-10 extraction manifold (J.T. Baker, Phillipsburg, NJ, USA) under vacuum (2 inches Hg), the columns were conditioned with 1.0 mL of 0.50 M HCl, 1.0 mL of methanol, and 2.0 mL of deionized water. A single DGG extract (ca., 7 mL) or quantity of hemolymph (3 × 200 μL or 3 × 700 μL) was applied to an SPE column with a Pasteur pipet, followed by washing with 2.0 mL of deionized water. Carboxylic acids were eluted with 500 μL of 0.50 M sulfuric acid into a tapered glass vial inside of the manifold and then transferred to an autosampler vial.



## TLC

Mobile phase development was carried out in a TLC or HPTLC twin-trough chamber (Camag, Wilmington, NC, USA) with a saturation pad (Analtech, Newark, DE, USA) in the trough opposite to the plate to provide vapor equilibration. Aliquots (0.5 to 10.0 μL) of standard solutions were applied to the origin locations using a 10 μL Drummond (Broomall, PA, USA) digital microdispenser. Detection reagents were applied by use of a Kontes (Vineland, NJ, USA) 100 mL Chromaflex sprayer or by immersing the plate in a Camag TLC or HPTLC dip tank; heating, when required, was done on a Camag plate heater.

The following previously reported systems for carboxylic acid separations and detections were tested: cellulose HPTLC plate with *n*-propanol-2 M ammonium hydroxide (7:3) mobile phase (triple development) and aniline-xylose detection reagent;[6] silica gel 60 F254 TLC plate with acetone-water-chloroform-ethanol-ammonium hydroxide (60:2:6:10:22) mobile phase and methyl red + bromophenol blue detection reagent;[7] silica gel G TLC plate

with *n*-butyl formate-90% formic acid-water (7:2:1) and ethanolic bromocresol green detection reagent;[8] cellulose F HPTLC plate with water-saturated isopropyl ether-formic acid (3:1) containing 2–3 mg 100 $mL^{-1}$ of dichlorofluorescein as the mobile phase and pyridine vapor/ultraviolet (UV) detection;[9] silica gel G TLC plate with *n*-pentyl formate-chloroform-formic acid (70:15:15 or 20:70:10) mobile phase and bromocresol green detection reagent;[10] silica gel 60F254 HPTLC plate with diisopropyl ether-formic acid-water (80:15:5) mobile phase and bromophenol blue detection reagent;[11] Sil G-25 TLC plate with diisopropyl ether-formic acid-water (90:7:3) mobile phase and aniline-glucose detection reagent;[12] silica gel 60F254 TLC plate with diisopropyl ether-formic acid-water (90:7:3) mobile phase and bromocresol green-bromophenol blue-potassium permanganate detection reagent;[13] and silica gel 60 TLC plate with diisopropyl ether-formic acid-water (90:7:3) mobile phase and bromocresol purple detection reagent.[14]



## HPLC

HPLC was performed using an Agilent 1100 series system with an autosampler, Biorad-Aminex ion exclusion HPX-87H column (300 × 7.8 mm), and ultraviolet (UV) detection at 210 nm. Sulfuric acid (5.0 mM) was used as the mobile phase with a flow rate of 0.6 mL $min^{-1}$, and the injection volume of all standards and the samples was 100 μL.

The identification of acids was made by matching peak retention times between standard and sample chromatograms. Identification was validated by comparing the standard and sample UV spectra automatically collected by the diode array detector during the separation.

For quantitative analysis of the identified acids, linear and second-order polynomial calibration curves were generated using Microsoft Excel by relating the standard concentrations of each acid to their peak areas. The concentration of each acid in snail DGGs was calculated using the equation:

$$\text{Carboxylic acid (ppm)} = \frac{(\text{I})(\text{V})}{(\text{M})}$$

where I is sample solution concentration (ppm) interpolated from calibration curve; V, original sample volume (mL); and M, mass of snail DGG (g).

For hemolymph, concentration was calculated using the equation:

$$\text{Carboxylic acid (ug/dL)} = \frac{(\text{I})(\text{V})(100)}{(\text{HV})}$$

where HV is the volume of hemolymph (mL).

## RESULTS AND DISCUSSION

### TLC

The analysis of the six carboxylic acids of interest was studied using essentially all previously-published TLC and HPTLC layers, mobile phases, and detection reagents under conditions as close as possible to the earlier work. The previous layers were all either silica gel or cellulose, and the detection reagents contained acid-base indicators or aniline-aldose mixtures designed to produce colored zones, or dichlorofluorescein to produce fluorescent zones.

Acid-base indicator detection reagents involve dipping in or spraying with an indicator solution adjusted just barely to its basic color; the carboxylic acid zones should change the indicator to its acid color at their locations, while the layer background is the basic color. In our hands, none of the indicator detection reagents was able to consistently detect the acids at levels of 100 μg or below, except for fumaric. The best system in terms of resolution, limit of detection, and reproducibility was a cellulose HPTLC plate (Art. no. 15036; EMD Chemicals, Inc., Gibbstown, NJ, USA, an affiliate of Merck KGaA, Darmstadt, Germany) with triple development using the mobile phase *n*-propanol-2 M ammonia (7:3); the initial development distance was 6.0 cm and the two subsequent developments were each 1 cm further, with air drying between developments. The detection reagent was prepared by dissolving 1 g of xylose in 3 mL of water + 1 mL of aniline in 96 mL of methanol; it was diluted 2:1 with methanol before application to the layer by submerging the plate for three seconds. All six of the acids of interest in the present study were detected as dark brown zones on a light brown background at a level of 5 μg or less. $R_F$ values are shown in Table 1.

In our hands, no other detection reagent worked as well as aniline-xylose on a cellulose layer, and no silica gel layer with any detection reagent was as good. The resolution and sensitivity of the optimum cellulose layer system was not adequate for the detection of the carboxylic acids in snail samples at their ppm concentrations, so HPLC was used for the remainder of the research.

***Table 1.*** $R_F$ values for the carboxylic acids

| Acid | $R_F$ |
|---|---|
| Acetic | 0.20 |
| Fumaric | 0.21 |
| Lactic | 0.56 |
| Malic | 0.13 |
| Pyruvic | 0.25 |
| Succinic | 0.15 |

## HPLC

Initial attempts were made to analyze DGGs for the six carboxylic acids using 12% perchloric acid extraction, SPE of neutralized (with KOH) extracts on a Bond Elut-SAX anion exchange column, and ion exclusion HPLC with UV detection exactly as described by Bezerra et al.[3,4] However, we obtained essentially zero recovery using this method.

To prove the acids were not strongly retained on the SPE column, we collected a second 500 μL portion of sulfuric acid eluent, and no acids were found to be present in this eluate.

Our conclusion was that the carboxylic acids were not detected by HPLC in the sulfuric acid eluate because they were not being retained on the SPE column. This was presumably due to the high concentration of ions present in the solution after the KOH neutralization of the 12% perchloric acid extract, which competed with the carboxylic acids for the SPE column ion exchange sites. This was confirmed by evaporating down and analyzing the sample solution that passed through the SPE column into the manifold, which was normally discarded. This solution was found to the contain carboxylic acids by HPLC analysis. Extraction of the snail DGGs with 50% Locke's solution (pH 7) allowed the neutralization step to be eliminated, and the extracted carboxylic acids were recovered quantitatively by a single 500 μL 0.50 M sulfuric acid elution of the SPE column. Hemolymph samples, with a pH of ca. 7, could be applied directly onto the SPE column without pH adjustment.

Another change made to the method of Bezerra et al.[3,4] was the use of 5.0 mM sulfuric acid, instead of 0.50 mM, as the HPLC mobile phase. This new mobile phase gave carboxylic acid peaks that were better separated and had more consistent retention time ($R_t$) values (Table 2). Carboxylic acid calibration curves consistently had $R^2$ correlation coefficients of 0.99 with linear regression for acetic, malic, and pyruvic acids, as well as an $R^2$ correlation coefficient of 0.93 with second-order polynomial regression for fumaric acid.

Acetic, fumaric, malic, and pyruvic acids were identified in the DGG and hemolymph sample chromatograms because their $R_t$ values agreed with the

***Table 2.*** Carboxylic acid standard HPLC retention times

| Acid | Retention time (min ± standard deviation)[a] |
|---|---|
| Acetic | 14.7 ± 0.2 |
| Fumaric | 15.75 ± 0.04 |
| Malic | 9.355 ± 0.004 |
| Pyruvic | 9.1 ± 0.1 |

[a]Mean of retention times for 1, 10, 100, 500, and 1000 ppm standard solutions.

***Table 3.*** HPLC analysis of the organic acid content of *Helisoma trivolvis*[a]

| Acid | DGG (ppm ± standard error) | Hemolymph ($\mu g\ dL^{-1}$ ± standard error) |
|---|---|---|
| Acetate | 180 ± 20 | 4,100 ± 700[b] |
| Fumarate | 24 ± 1 | <154[c] |
| Malate | 240 ± 60 | 16,000 ± 3,000 |
| Pyruvate | 24 ± 3 | 1,300 ± 200 |

[a]Six trials were used for each analysis unless otherwise indicated.
[b]Mean of five trials.
[c]Fumaric acid was detected with a peak area below the area of the lowest standard. The limit of quantification calculated using this lowest standard area was 154 $\mu g\ dL^{-1}$.



corresponding standard acid peaks within ±0.2 min, and the spectra of the sample and standard peaks matched. We could not confirm the presence of succinic and lactic acids, as reported by Bezerra et al.,[3,4] based on these dual requirements.

As shown in Table 3, the most abundant carboxylic acids in both the DGG and hemolymph were malic > acetic > pyruvic > fumaric. Information on the carboxylic acid content of *Helisoma* snails is not available. However, it is of interest that data from Bezerra et al.[4] on a Puerto Rican strain of *B. glabrata* show the same relative order in the concentration of malic, acetic, pyruvic, and fumaric acids in the digestive gland and hemolymph of that planorbid as we report for *H. trivolvis*. Because carboxylic acids play an important role in carbohydrate metabolism of planorbid snails, further HPLC studies on these acids are warranted.

## ACKNOWLEDGMENT

D. Massa was supported by a Camille and Henry Dreyfus Foundation Senior Scientist Mentor Initiative Program award to J. Sherma.

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Received December 15, 2006
Accepted February 1, 2007
Manuscript 6104L