AN ABSTRACT OF THE THESIS OF

Joseph A. Kraai for the degree of Master of Science in Chemical Engineering presented on October 9, 2019.

Title: Nanostructured Diatom Biosilica Stationary Phase for Thin-layer Chromatography Separation of Polar, Ionic Analytes.

Abstract approved: _____________________________________________________

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Despite an expansive selection of available thin-layer chromatography (TLC) stationary phases, almost none are well-suited for separation of polar, ionic analytes. This work demonstrated that a highly porous stationary phase layer comprised solely of nanostructured biosilica frustules, isolated from living *Pinnularia* sp. diatom microalgae, improved TLC separation of the polar, ionic analytes Malachite Green (MG) and Fast Green FCF (FG) relative to silica gel. Intact biosilica frustules were isolated from *Pinnularia* sp. cell culture via oxidation with acidified hydrogen peroxide. Diatom biosilica TLC layers were fabricated using a facile, binder-free, drop-cast technique. FT-IR spectroscopy was employed to compare surface chemistries of biosilica vs. commercial silica gel TLC layers. Pore structure parameters of the stationary phases were characterized via SEM imaging as well as experimental measurement and analytical modeling of capillary flow through the films. TLC separations of MG and FG were performed on both stationary phases using two different mobile phase mixtures (9:1:1 v/v 1-butanol:ethanol:water and 5:1:2 v/v 1-butanol:acetic acid:water). Plate height versus solvent front migration distance relationships were measured and mathematically modeled for the reference analytes MG and FG on both stationary phases to characterize TLC separation efficiencies. Although both stationary phases were composed of amorphous silica rich in silanol groups with particle size of 10–12 µm, diatom biosilica frustules were highly porous, hollow shells with surface structure dominated by 200 nm pore arrays. Diatom
biosilica significantly improved the mobility of both MG and FG, enabling the resolution of these analytes. The diatom biosilica layer had a high void fraction of 96% but reduced the flow velocity and permeability constant by a factor of two relative to silica gel. TLC performance was enhanced, as evidenced by ten-fold reduction in theoretical plate height for both analytes using the 1-butanol:acetic acid:water mobile phase, and an increased difference in retardation factor between MG and FG ($\Delta R_f = 0.26$) using the 1-butanol:ethanol:water mobile phase. Analysis of plate height vs. solvent front position by the modified van Deemter equation suggested that dispersive mass transfer was reduced, leading to improved analyte resolution, and that the pore-perforated surface topology of the frustule decreased boundary layer resistance, leading to increased analyte flux. Overall, the basis for improved chromatographic performance is believed to be the unique micro- and nanostructure of the diatom biosilica frustule.
Nanostructured Diatom Biosilica Stationary Phase for Thin-layer Chromatography
Separation of Polar, Ionic Analytes

by
Joseph A. Kraai

A THESIS
submitted to
Oregon State University

in partial fulfillment of the requirements for the degree of
Master of Science

Presented October 9, 2019
Commencement June 2020
Master of Science thesis of Joseph A. Kraai presented on October 9, 2019.

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Joseph A. Kraai, Author
ACKNOWLEDGEMENTS

I express sincere appreciation to my graduate school advisors Dr. Gregory Rorrer and Dr. Alan Wang. Their valuable insights and continued support have made it possible for me to succeed as a student, teacher, and researcher at Oregon State University. I also acknowledge the National Science Foundation (grant numbers 1701329 and 1240488) and the United States Department of Agriculture (grant number 2017-67021-26606) for research funding. Lastly, I acknowledge and thank my family for their unconditional encouragement through my time at OSU.
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DEDICATION

I dedicate this work to my family.
Chapter 1: Introduction

For over a century of separation science, chromatography has remained the most ubiquitous and versatile technique for separation of analytes in complex matrices ranging from wastewater to biological samples.\(^1\) With the main functioning principle being affinity-based molecular separation, chromatography offers a highly tunable separation platform for analyte identification, extraction, and purification purposes. As such, a multitude of chromatography subtypes exist, each with its own set of applications and limitations. Several modern chromatographic techniques include thin-layer chromatography (TLC), high-pressure liquid chromatography (HPLC), and gas chromatography (GC). HPLC and GC are readily automated on-line methods with wide ranges of selective detector compatibility, thus are favored in trace analysis separation scenarios requiring high plate numbers. Meanwhile, significantly lower cost, complexity, and sample preparation of TLC generally make it the method of choice for rapid, qualitative determination of analytes in various sample matrices.\(^2,3\) In industry, TLC is often employed as a preliminary screening step to qualitatively identify suspect target analytes, followed by a confirmation step to quantitatively determine sample composition by means of a more precise analytical method such as HPLC.\(^2\) In this context, TLC can be complementary to HPLC or GC, with major industrial applications in clinical, pharmaceutical, and food testing.\(^4\)

Competitive advantages of TLC over other chromatographic systems principally arise from its facile experimental design/operation, inexpensive instrumentation, and high throughput capabilities. TLC operations and analyses can be readily performed in even the most basic research laboratories, with no need for specialized or costly equipment. TLC stationary phases are single-use, lessening sample preparation and eliminating the possibility of the “memory effect” in which analytes retained from past separations interfere with future chromatographic performance.\(^2\) Multiple samples can be separated in parallel on the same stationary phase plate, under virtually identical conditions. Each resulting chromatogram contains a complete set of sample analytes, and may be analyzed independent of time and solvent effects via subsequent staining, image analysis, or densitometry.\(^3\) A priori, TLC enables simple high throughput sample analysis and convenient means of
fine-tuning process parameters like stationary/mobile phase chemistries and flow velocity. This is in contrast to more expensive and complex on-line techniques like HPLC and GC which re-use stationary phases and require separate sample injections for each chromatogram. Additionally, a given HPLC or GC chromatogram only contains successfully eluted analytes and intrinsically runs the risk of mobile-phase/analyte interactions affecting detection because mobile phase is eluted with analytes. These differences demonstrate why TLC is universally used for time- and cost-efficient high throughput sample screening.

Since its inception in the early 20th century, TLC has seen several advances in instrumentation and separation efficiency. Most influential among these advances were the standardized production of pre-coated stationary phase layers (e.g. silica, alumina) in the 1960s, the advent of high-performance TLC (HPTLC) in the 1970s, the introduction of chemically-modified layers and layers comprised of spherical microparticles from the 1980s to the 1990s, and the development of ultra thin-layer chromatography (UTLC) at the turn of the century. As most of these advances resulted from stationary phase improvements (e.g. high film uniformity, minimization of mean particle diameter and size distribution), it is unsurprising that TLC offers the widest choice of stationary phases of any liquid chromatography (LC) method. Other routes to enhancing TLC separation efficiency, such as overpressured TLC, electroosmotically-driven TLC, and automated multiple development, have yet to be convincingly demonstrated and remain under-investigated. A likely explanation for this is because although forced-flow TLC typically affords superior separation efficiency, capillary-flow TLC remains popular owing to its rapid, simple, and cheap technique.

Numerous types of TLC stationary phases exist for separating different sample matrices. Conventionally, average particle size ranges from 5 – 15 µm, pore size from 40 to 120 Å, and film thickness from 100 – 250 µm. Silica gel, by far the most common TLC stationary phase, is most effective for separation of small nonpolar compounds, such as fatty acids and esters. Another common adsorbent layer is alumina, which has similar chromatographic behavior to silica gel but with higher adsorption affinity for C=C bonds. This makes alumina better-suited for
separating aromatic hydrocarbons. A multitude of surface-functionalized silica gel derivatives are also employed as TLC stationary phases. For example, cyanopropyl-silica and diol-silica layers have been used to separate the psychotropic drugs desipramine, olanzapine, and mirtazapine.

Despite the abundance of different TLC layers available, surprisingly few are reported for high resolution separation of highly polar ionic analytes. Very polar compounds do not migrate far across normal phase TLC films, particularly silica gel, due to their high adsorption affinity for the stationary phase. This mobility hindrance can be mitigated to some extent by using polar mobile phases with added electrolytes and/or modified pH to increase analytes’ relative affinities for the mobile phase. Regardless, minimal migration of polar analytes on conventional TLC films coupled with analyte band broadening over long separation times typically prevents sufficient resolution to practically separate multicomponent mixtures of polar ionic compounds. Herein, triphenylmethane (TPhM) dyes are considered as references to exemplify this fact. TPhM dyes are a class of intensely colored dyes, usually ionic, containing the TPhM structural motif. Past work using conventional silica gel TLC plates with similar solvent systems has shown that polar, ionic TPhM dyes (e.g. fast green FCF, malachite green, methyl violet) commonly exhibit small retardation factor ($R_f$) values ranging from less than 0.1 to 0.3, with $R_f$ values for less polar TPhM derivatives rarely exceeding 0.5. Recently, Sednev and Berezkin have demonstrated increased mobility and resolution of TPhM dyes on unmodified silica gel TLC plates by employing a complex combination of active gas flow over the stationary phase with multiple developments and counter-current drying. Furthermore, modern methods used for separation of polar, ionic dyes are typically complex and costly, such as capillary electrophoresis, high-speed counter-current chromatography (HSCCC), and ultra-performance liquid chromatography (UPLC). It is thereby apparent that a simple, rapid, and inexpensive route to high resolution separation of structurally similar, polar, ionic analytes would be beneficial for various industrial applications.

The overall goal of this study is to compare the TLC separation performance of porous, thin layers made of diatom biosilica frustules and silica gel particles of
similar size. Diatoms are single-celled algae that take up and biomineralize dissolved silicon in the form of Si(OH)$_4$ to biologically fabricate nano-patterned, micron-scale silica shells called frustules rich in silanol groups. The frustules are hollow with approximately 100 nm wall thickness, and consist of upper and lower halves. *Pinnularia* sp. diatoms have ellipsoidal frustules with major axis of nominally 20 µm and minor axis of 5–10 µm. Each frustule is intricately patterned with a periodic rectangular array of nominally 200 nm diameter prime pores penetrating fully through the shell, spaced approximately 300 nm apart. The base of each prime pore typically contains 4 – 6 secondary pores nominally 50 nm in diameter. In contrast, 10–12 µm silica gel particles for TLC possess a random, internal mesoporous structure with average pore size of 6 nm.

A recognized issue with porous layers for conventional TLC is that capillary forces cannot generate sufficient flow to minimize the effects of zone broadening, and silica gel particle layers in particular suffer from inadequate mass transfer. We hypothesize that the open, porous structure of the diatom biosilica frustules may promote local mobile phase interaction with the hydrated silica stationary phase, leading to improved mass transfer and chromatographic separation of these analytes. The nanostructure is provided by the diatom biosilica particle itself, whereas the microstructure is provided by the random packing of the ellipsoidal particles in the stationary phase.

The reference analytes chosen for this study are the TPhM dyes malachite green (MG) and fast green FCF (FG). MG is less polar and weakly cationic with two N-dimethyl groups, whereas highly polar FG additionally possesses anionic benzene and phenol sulfonate groups (Figure 1). MG has been extensively used to prevent and treat infections in aquaculture products, but has toxic effects on fish. There is significant interest in the development of rapid and simple methods for the detection of MG in seafood products, and TLC is one simple method to enable the separation component of a detection platform. Planar chromatography on silica gel stationary phases has been used for analysis of TPhM dyes, including malachite green and fast green, but not within the same sample matrix.
The TLC-based separation performance of malachite green and fast green on diatom biosilica and silica gel will be assessed through characterization of solvent front migration distance vs. time, analyte retardation factor, and the height equivalent to a theoretical plate. We report that nanostructured diatom biosilica offers significant enhancements in performance for TLC-based separation of malachite green and fast green relative to on silica gel, and merits continued investigation as a stationary phase for TLC.

**Figure 1.** Molecular structures of malachite green and fast green.
Chapter 2: Materials and Methods

2.1 Analytes and mobile phase

Malachite green chloride, $\text{C}_{23}\text{H}_{25}\text{ClN}_2$, molecular weight 364.917 g/mol, CAS Number 569-64-2, was obtained from Sigma-Aldrich (38800, analytical standard grade, > 96% purity). Fast Green For Coloring Food (FCF), $\text{C}_{37}\text{H}_{34}\text{N}_2\text{Na}_2\text{O}_10\text{S}_3$, molecular weight 808.843 g/mol, CAS Number 2353-45-9, was obtained from Glentham Life Sciences (42053). Two solvent systems known for separation of malachite green on silica gel,$^{13,24}$ consisting of 9:1:1 (v/v) 1-butanol:ethanol:water (solvent system 1), and 5:1:2 (v/v) 1-butanol:acetic acid:water (solvent system 2) served as the mobile phases. All solvents were analytical grade purity.

2.2 Diatom cell culture

Diatom culturing methods were adapted from those previously described.$^{25}$ Axenic cultures of the photosynthetic marine pennate diatom *Pinnularia* sp. were obtained from the UTEX Culture Collection of Algae (UTEX #B679). Diatoms were cultivated in 500 mL foam-stoppered borosilicate flasks containing 100 mL of Harrison’s Artificial Seawater Medium (ASM) with a modified Guillard’s f/2 nutrient enrichment.$^{26}$ Culture flasks were incubated at 22 °C under a light intensity of 100 $\mu$E/m² s on a 14h:10 h light:dark photoperiod, and subcultured every 21 days at 10:1 dilution in fresh medium. Typical cell number density at cell harvest was $1 \times 10^6$ cells/mL. Cell number density of *Pinnularia* sp. culture was measured using a Beckman Coulter Z2 Particle Counter at a lower threshold of 6 $\mu$m using a 100 $\mu$L aliquot of the cell suspension diluted in 10 mL of diluent (10 g/L sterile-filtered NaCl, 171 mM).

2.3 Diatom biosilica frustule isolation

Intact biosilica frustules were isolated from *Pinnularia* sp. cell culture using an acidified hydrogen peroxide treatment method adapted from previous work.$^{25}$ Approximately 1200 mL of three week-old *Pinnularia* sp. flask cultures were pooled and allowed to settle. After decanting the majority of the cell-free supernatant, the cell density was measured, and a 120 mL aliquot of the concentrated cell suspension ($\sim 3 \times 10^8$ cells) was centrifuged (20 min at 2500g). The cell pellet was washed with
40 mL Nanopure water three times to remove culture medium salts. The washed cell pellet was re-suspended in 15 mL of 8.1 M HCl in a 50 mL centrifuge tube, gently mixed by inverting five times, allowed to gravity settle, and the supernatant was pipetted out. In the first oxidation treatment, 5 mL of the concentrated slurry was added to 32 mL of 0.76 M HCl in 28% (v/v) H$_2$O$_2$, gently mixed by inverting the centrifuge tube five times, and then allowed to sit in an unsealed centrifuge tube for 72 h, with daily mixing via five inversions for the first two days. In the second oxidation treatment, this process was repeated. After removing the supernatant, the crude frustule isolate slurry was re-suspended in 40 mL H$_2$O and allowed to gravity settle, and then washed two additional times using 40 mL water. The supernatant was removed, and the washed frustule slurry was re-suspended in 40 mL anhydrous methanol (MeOH), allowed to gravity settle, and then washed two more times using 40 mL MeOH. The final frustule isolate was stored in MeOH suspension within a sealed centrifuge tube at room temperature. Frustule biosilica yield was typically ~20 mg.

2.4 Fabrication of diatom biosilica and silica gel stationary phases

Glass microscope slides (VWR, 75 × 25 mm, 1.0 mm thick) were cut into 75 × 3 mm substrates using a diamond-tipped scribe. Substrates were sonicated in a 1% (v/v) solution of Liquinox detergent for 60 min at 40 °C using a Branson model 2510 ultrasonic cleaner, rinsed with Nanopure H$_2$O and 100% ethanol (EtOH), dried under flowing nitrogen (N$_2$), cleaned in a Novascan PSD-UVT UV-Ozone cleaner at 90 °C for 1 h, then stored in a desiccator until use.

Diatom biosilica porous layers were prepared by a drop-casting technique. Cleaned 75 × 3 mm glass substrates were placed on a hot plate surface at a temperature of 45 °C. Then, 100 µL of 11.3 mg/mL Pinnularia sp. frustule suspension in methanol (MeOH) was evenly dispensed on the substrate surface. After evaporation of methanol (20–30 s), the drop-cast diatom biosilica porous layers were treated in a UV-Ozone cleaner at 90 °C for 24 h to fix the frustule porous layer to the substrate surface and oxidize residual organic contaminants. The diatom biosilica layers were stored in a desiccator.
Silica gel porous layers on an aluminum backing were obtained from Whatman (Silica gel 60, 4420-222), and cut into 75 × 3 mm lanes. The silica gel layer has an internal pore size of nominally 6 nm, pore volume of 0.8 cm$^3$/g, and film thickness of nominally 250 µm. Stationary phase porous layers of silica gel or diatom biosilica were activated by heating at 110 °C for 50 min in an oven to remove adsorbed water.

2.5 Stationary phase characterization

Diatom biosilica and silica gel particles were ground into powder and analyzed with a Nicolet 6700 FT-IR spectrophotometer equipped with a Smart iTR Attenuated Total Reflectance (ATR) sampling accessory. FT-IR transmittance (%) spectra were acquired from 500 cm$^{-1}$ to 4000 cm$^{-1}$ using 125 scans per sample analysis at 4 cm$^{-1}$ resolution.

Scanning electron microscopy (SEM) images of silica gel and frustule biosilica TLC porous layers were obtained using an FEI Quanta 600 FEG-SEM at an accelerating voltage of 5.00 kV. Porous layers were scored with a diamond-tipped glass scribe to visualize cross sectional morphologies. Prior to imaging, the porous layers were sputter-coated in gold for 35 s using a Cressington 108 auto sputter coater to deposit a 9 nm gold coating. Energy dispersive X-ray spectroscopy (EDX) was also performed during SEM imaging for determination of elemental composition.

The stationary phase mass loading was estimated by weighing the net mass of stationary phase on the plate divided by the total surface area. The stationary phase thickness was determined from analysis of light microscopy images of the porous layer cross section. The void fraction ($\phi$) of the silica gel and diatom biosilica layer was estimated from the amount of material deposited, the grain density of amorphous silica (2.2 g/cm$^3$), and the average thickness of the porous layer. The void fraction included the void space within the particles (intraparticle porosity) and between the particles (interstitial porosity). Particle dimensions of the silica gel and diatom biosilica were determined by analysis of SEM images using ImageJ software. The equivalent diameter of the particles ($d_p$) was estimated from area and perimeter measurements.
2.6 Thin layer chromatography (TLC) experiments

The TLC developing chamber consisted of a capped 250 mL borosilicate bottle containing 15 mL of the mobile phase. A clip fixed the TLC plate to a ruler that served as the reference scale. A 200 nL aliquot of analyte sample solution (0.79 g/L of malachite green, 0.79 g/L fast green in ethanol) was dispensed onto a given TLC plate at a sample origin distance of 5 mm. Analytes were added individually to determine migration distance and in a mixture to assess chromatographic separation. After ethanol completely evaporated, the TLC plate was placed within the developing chamber at an inclination angle ($\psi$) of 70°, but the bottom of the TLC plate was initially positioned above mobile phase surface. The sealed chamber was allowed to sit for 5 min to promote mobile phase vapor-liquid equilibrium. The TLC experiment was initiated by allowing the bottom side of the stationary phase to contact the mobile phase liquid surface. All experiments were performed at room temperature (22 °C). The calculated amount of liquid retained on the stationary phase was 45 µL for the silica gel TLC layer, and 11 µL for the diatom biosilica TLC layer.

The visible mobile phase and analyte migration on the developing TLC porous layer was captured with time by digital video (1080p resolution at 30 frames per second). The solvent front migration distance ($z_f$), sample migration distance ($z_s$), and sample spot width ($w_B$) were estimated from this data for a given image frame using ImageJ image analysis software (National Institutes of Health). The distance scale was calibrated using the reference ruler. After generating an intensity plot of the analyte spot, a Gaussian curve was fitted to the intensity map to determine $z_s$ (distance at maximum intensity) and $w_B$ (4$\sigma_s$). Triplicate TLC separation experiments were performed with silica gel and diatom biosilica stationary phases. On diatom biosilica, the initial spot size was 2.55 ± 0.26 mm for malachite green vs. 2.74 ± 0.21 mm for fast green (1.0 S.D., $n = 3$). On silica gel, the initial spot size was 1.72 ± 0.16 mm for malachite green vs. 2.00 ± 0.20 mm for fast green (1.0 S.D., $n = 3$). Microscopic analysis verified that the analyte spot completely penetrated the porous film.

The mobile phase viscosity ($\mu$) was measured using a Cannon-Fenske viscometer at 22 °C (triplicate measurement). The density ($\rho$) was measured with a
pycnometer, and the solvent surface tension (\(\sigma\)) was estimated by mole fraction average of known surface tension values of the pure components comprising the mobile phase at 22 °C. The molecular diffusion coefficient (\(D_M\)) of each analyte in the mobile phase solvent was estimated by the Scheibel equation at 22 °C. For solvent system 1 (9:1:1 v/v 1-butanol:ethanol:water), \(\mu\) was 2.46 ± 0.015 centipoise (cP), \(\rho\) was 0.828 g/cm\(^3\), \(\sigma\) was 40 dyne/cm, and \(D_M\) was 2.05x10\(^{-6}\) cm\(^2\)/s for malachite green and 1.45x10\(^{-6}\) cm\(^2\)/s for fast green. For solvent system 2 (5:1:2 v/v 1-butanol:acetic acid:water), \(\mu\) was 2.86 ± 0.017 cP, \(\rho\) was 0.895 ± 0.0027 g/cm\(^3\), \(\sigma\) was 54 dyne/cm, and \(D_M\) was 3.06x10\(^{-6}\) cm\(^2\)/s for malachite green and 2.21x10\(^{-6}\) cm\(^2\)/s for fast green.

2.7 Data analysis

Capillary flow of the mobile phase within the thin porous layer of silica particles (stationary phase) is described by the Lucas-Washburn wicking-flow model, where the advancing solvent front migration distance (\(z_f\)) varies with time (\(t\)) according to

\[
Z_f^2 = \frac{4\sigma}{\mu} \frac{K}{\phi R_s} t = \chi t \quad (1a)
\]

or

\[
Z_f^2 = 2k_o d_p \frac{\sigma}{\mu} t = \chi t \quad (1b)
\]

where \(K\) is the permeability of capillary solvent flow through the porous layer (cm\(^2\)), \(R_s\) is the equivalent capillary radius (cm), and \(\chi\) is the mobile phase flow velocity constant (cm\(^2\)/s). In Equation 1b, \(k_o\) is a lumped parameter for the permeability (dimensionless), which includes the \(R_s\), \(K\), and \(\phi\) terms. The key assumption of the Lucas-Washburn model is that it ignores the effect of gravity on capillary flow. The parameter \(\chi\) was estimated by linear regression from the least-squares slope of \(z_r^2\) vs. \(t\) data, and \(k_o\) was obtained from this slope. The linear velocity of the mobile phase (\(u\)) is estimated from the derivative of Equations (1a) and (1b)

\[
u = \frac{\partial Z_f}{\partial t} = \frac{\sqrt{\chi}}{2t^{1/2}} = \frac{\chi}{2z_f} \quad (2)
\]
The height equivalent to a theoretical plate (H) and the number of theoretical plates (N) required to resolve a given analyte within the porous TLC layer was estimated from the retardation factor (Rf) for a given solute. The retardation factor is defined by

$$R_f = \frac{z_s}{z_f - z_o}$$  \hspace{1cm} (3)

where $z_o$ is the initial position at which the analyte is added, and $z_s$ is analyte migration distance at solvent migration front position $z_f$. From $R_f$, N and H are respectively estimated by

$$N = \frac{1}{R_f} (\frac{z_s}{\sigma_s})^2 = 16z_s (\frac{z_f - z_o}{w_B})$$  \hspace{1cm} (4)

and

$$H = \frac{z_f - z_o}{N}$$  \hspace{1cm} (5)

where $\sigma_s$ is the Gaussian distribution associated with analyte spot position $z_s$, and $w_B$ is the measured width of the analyte spot.

The modified van Deemter Equation [20] for capillary action-driven planar adsorption chromatography describes the local variation of plate height (H) with mobile phase velocity

$$H = Ad_p \left( \frac{d_p}{D_M} u \right)^{1/3} + BD_m \frac{d_p^2}{D_m} u$$  \hspace{1cm} (6)

In terms of solvent front migration distance $z_f$, the integral form of the van Deemter equation for the average plate height is

$$H = \frac{3}{2} A \left( \frac{d_p^4 \chi}{2D_m} \right)^{1/3} \left( \frac{z_f^3 - z_0^3}{z_f - z_0} \right) + BD_m \frac{z_f + z_0}{\chi} + \frac{C \chi d_p^2}{2D_m(z_f - z_0)} \ln \left( \frac{z_f}{z_0} \right)$$  \hspace{1cm} (7)

where $d_p$ is the average particle diameter (cm), and A, B, and C are the Knox constants. The first term in Equations (6) and (7) represents eddy diffusion, and is scaled by Knox constant A; the second term represents dispersive mass transfer of the solute in the mobile phase, and is scaled by Knox constant B; the third term represents convective mass transfer resistance and is scaled by Knox constant C. Knox parameters A, B, and C were estimated by fitting plate height (H) vs. solvent
front migration distance (z) data to Equation (7) by nonlinear, least-squares regression (Marquardt method) using Statgraphics XVI (Statpoint Technologies) software. Equations (1a)–(7) above were not dependent on the thickness of the stationary phase, as long as the initial analyte spot fully penetrated the porous film, which was experimentally verified.
Chapter 3: Results and Discussion

3.1 Characterization of TLC layer morphology and surface chemistry

Scanning electron microscopy (SEM) images of the silica gel and diatom biosilica TLC porous layers are presented in Figure 2.

![Figure 2](image_url)

**Figure 2.** SEM images of TLC porous layers. Silica gel (a, b); diatom biosilica (c–e).

Both diatom biosilica and silica gel particles were randomly deposited on the glass substrate as a uniform, porous layer. However, SEM images revealed important
structural differences between the diatom biosilica and silica gel porous layers, including particle shape, size, orientation, and packing density. The diatom biosilica frustules deposited in the porous layer were largely intact. These diatom biosilica particles were ellipsoidal in shape with a highly porous internal structure, whereas silica gel particles possessed an irregular, granular morphology with a microporous internal pore structure.

Table 1
Structure and flow properties of TLC porous layers. All reported errors are ± 1.0 S.E.

<table>
<thead>
<tr>
<th>Property</th>
<th>Silica Gel</th>
<th>Diatom Biosilica</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equivalent particle diameter, ( d_p ) (( \mu )m)</td>
<td>10.1 ± 0.4</td>
<td>11.9 ± 0.2</td>
</tr>
<tr>
<td>Porous layer thickness, ( L ) (( \mu )m)</td>
<td>250 ± 5</td>
<td>53 ± 3</td>
</tr>
<tr>
<td>Stationary phase loading, ( m_s ) (( \mu )g/cm²)</td>
<td>11117</td>
<td>500</td>
</tr>
<tr>
<td>Void fraction, ( \theta ) (cm³ void/cm³ bulk)</td>
<td>0.80</td>
<td>0.96</td>
</tr>
<tr>
<td>Solvent system 1 velocity constant, ( \chi ) (cm²/s)</td>
<td>0.020 ± 0.0001</td>
<td>0.012 ± 0.00008</td>
</tr>
<tr>
<td>Solvent system 1 permeability constant, ( k_0 ) (dimensionless)</td>
<td>0.0061 ± 0.0003</td>
<td>0.0030 ± 0.00006</td>
</tr>
<tr>
<td>Solvent system 2 velocity constant, ( \chi ) (cm²/s)</td>
<td>0.019 ± 0.00005</td>
<td>0.015 ± 0.00003</td>
</tr>
<tr>
<td>Solvent system 2 permeability constant, ( k_0 ) (dimensionless)</td>
<td>0.0050 ± 0.0002</td>
<td>0.0033 ± 0.00006</td>
</tr>
</tbody>
</table>

Table 1 compares the particle properties of the TLC porous layers. For the diatom biosilica particles, the average major and minor axis lengths were 19 ± 6 (1.0 S.D., n=158) \( \mu \)m by 8 ± 3 (1.0 S.D., n = 158) \( \mu \)m. However, the mean equivalent particle diameters (\( d_p \)) for the diatom biosilica and silica gel particles were comparable (12 \( \mu \)m vs. 10 \( \mu \)m). Due to their ellipsoidal shape, the diatom biosilica particles were randomly packed into the TLC porous layer more loosely relative to silica gel particles, leading to higher porosity (void fraction) and lower mass loading. The BET surface area of *Pinnularia* sp. frustules isolated by hydrogen peroxide treatment of cultured cells is reported to be 28 m²/g,²⁷ vs. 500 m²/g for silica gel 60.²⁰ The solid biosilica portion of a *Pinnularia* sp. frustule contains mesopores ranging from 1 to 10 nm with pore volume of 0.15 – 0.20 cm³/g,²⁷ whereas the silica gel 60 contains mesopores with an average diameter of 6 nm and pore volume of nominally 0.8 cm³/g.²⁰ However, as revealed by the SEM images, the diatom biosilica frustules
were largely hollow shells containing 200 nm pores on a nominal 300 nm rectangular pitch, and it was these structural features that contributed most significantly to the high porosity of 96%. SEM/EDX analysis confirmed that the elemental (atomic) composition of diatom biosilica (65.6% O, 34.4% Si) was similar that of TLC silica gel 60 (66.6% O, 33.4% Si), and did not contain any significant levels of metal impurities.

The IR spectra of the silica gel and diatom biosilica are compared in Figure 3. Both IR spectra are characteristic of hydrated, amorphous silica. The broad peak at ~3400 cm\(^{-1}\) is attributed to adsorbed water. The prominent peaks at 1069 cm\(^{-1}\), 829 cm\(^{-1}\), and 580 cm\(^{-1}\) are attributed to asymmetric stretching, symmetric stretching, and bending vibrations of siloxane groups. Silanol vibrations are responsible for the absorbance peak at 947 cm\(^{-1}\).

Figure 3. FT-IR spectra of silica gel 60 and diatom biosilica.
The average ratio of silanol (Si—OH) peak at 946 cm\(^{-1}\) to siloxane (Si—O—Si) peak at 1069 cm\(^{-1}\) was 0.24 for silica gel and 0.27 for diatom biosilica. It is well known that silanol groups possess enhanced acidity that affect analyte-stationary phase interactions in silica-based supports for liquid chromatography.\(^{28}\) However, since both stationary phases consisted of hydrated silica rich in silanol groups of similar density, they were assumed to possess similar adsorption chemistry for a given analyte.

### 3.2 Comparison of capillary flow characteristics within TLC porous layers

Solvent front migration distance (z\(_f\)) vs. time profiles for wicking flow of the mobile phase through silica gel and diatom biosilica TLC stationary phases are presented in Figure 4 for solvent system 1 (9:1:1 v/v 1-butanol:ethanol:water) and solvent system 2 (5:1:2 v/v 1-butanol:acetic acid:water).

**Figure 4.** Solvent front migration distance (z\(_f\)) vs. time profiles for capillary flow of mobile phase through silica gel and diatom biosilica porous layers. (a) Solvent system 1 (9:1:1 v/v 1-butanol:ethanol:water); (b) Solvent system 2 (5:1:2 v/v 1-butanol:acetic acid:water). Solid lines represent fit of data to Equations (1a) and (1b), fitted parameters are presented in Table 1.

The rate of capillary rise decreased due to viscous effects and increasing gravitational hydrostatic pressure, leading to a monotone decreasing solvent migration front vs.
time profile. The solvent front migrated more rapidly through silica gel porous layer compared to the diatom biosilica porous layer.

The data presented in Figure 4 were fitted to the Lucas-Washburn wicking flow model (Equations (1a) and (1b)). Estimated flow parameters are presented in Table 1. The diatom biosilica TLC porous layer was highly porous with a void fraction of 0.96, compared to 0.80 for the silica gel porous layer. For both solvent systems, the mobile phase flow velocity constant ($\chi$) was lower for the diatom biosilica layer relative to the silica gel porous layer. The silica gel TLC porous layer flow velocity constant agreed with previously reported values. $^{21}$ Similarly, the permeability factor ($k_0$) was lower by a factor of two for the diatom biosilica vs. the silica gel porous layer.

### 3.3 TLC separation of Malachite green and Fast green FCF

Malachite green is relatively nonpolar and weakly cationic with two N-dimethyl groups. In contrast, fast green has two anionic benzyl sulfonate groups, one attached to each of the amine groups, and a third anionic phenol sulfonate group (Figure 1). Representative chromatograms in Figure 5 demonstrate TLC separation of malachite green and fast green analytes on silica gel and diatom biosilica stationary phases. Malachite green eluted faster than fast green on both solvent system 1 (9:1:1 v/v 1-butanol:ethanol:water) and solvent system 2 (5:1:2 v/v 1-butanol:acetic acid:water) mobile phases. On silica gel, fast green did not elute when solvent system 1 was used as the mobile phase. However, the more polar mobile phase (solvent system 2) promoted migration of both analytes. In contrast, diatom biosilica promoted migration of both analytes for each solvent system. Fast green had a lower mobility than malachite green, because the benzene and phenol sulfonate groups on fast green presumably interacted more strongly with the silanol groups. The relative retardation factor ($R_f$) defined by Equation (3) did not change significantly past a solvent front migration distance of 3.0 cm. Average values of $R_f$ for malachite green and fast green on diatom biosilica and silica gel are compared in Table 2 for solvent systems 1 and 2. The difference between $R_f$ values for malachite green and fast green, $\Delta R_f$, as a function of solvent front position ($z_f$) are presented in Figure 6. For solvent system 1, $\Delta R_f$ values for diatom biosilica leveled off with increasing $z_f$,
Figure 5. TLC plates showing analyte spots for malachite green and fast green samples on diatom biosilica and silica gel after development to solvent front migration distance of $z_f = 5.0$ cm. (a) Photographic image, solvent system 1 (9:1:1 v/v 1-butanol:ethanol:water) (b) optical signal intensity normalized to 1.0 for highest peak, solvent system 1; (c), optical density, solvent system 2 (5:1:2 v/v 1-butanol:acetic acid:water).

whereas $\Delta R_f$ for the silica gel layer decayed rapidly toward zero. However, for solvent system 2 $\Delta R_f$ was largely constant with $z_f$. Since fast green did not elute
significantly in solvent system 1, its $\Delta R_t$ was higher relative to solvent system 2. Overall, enhanced analyte migration and complete separation resolution was observed on the diatom biosilica layer, but not on the silica gel layer. Values of $R_f$ for both malachite green and fast green were much higher on the diatom biosilica relative to the silica gel, suggesting that both analytes were bound more strongly within the silica gel stationary phase. Silica gel has a random-mesoporous internal structure whereas diatom biosilica does not. Given the likely diffusional limitations of the analyte within the internal pores within silica gel, capillary flow velocity was not sufficient to promote separation of the two analytes on this stationary phase. For diatom biosilica stationary phase, the $R_f$ data shown in Figure 6 and Table 2 were based on average values of three trials on separate TLC plates using two separately prepared samples of diatom biosilica. Analysis of Variance (ANOVA) confirmed that at $z_f$ values greater than 3.0 cm, there was no statistically significant difference in $R_f$ values between biosilica isolation batches at 95% confidence (malachite green, $p = 1.5 \times 10^{-9} < 0.05$; fast green, $p = 2.1 \times 10^{-3} < 0.05$).

![Figure 6](https://doi.org/10.1016/j.chroma.2019.01.037)

**Figure 6.** Relative difference in retardation factor ($\Delta R_f$) between malachite green and fast green vs. solvent front migration distance ($z_f$) on silica gel and diatom biosilica porous layers. (a) Solvent system 1 (9:1:1 v/v 1-butanol:ethanol:water), average ($\pm$1.0 S.D., $n = 7$) $\Delta R_f = 0.262 \pm 0.007$ (diatom biosilica), $\Delta R_f = 0.027 \pm 0.023$ (silica gel); (b) solvent system 2 (5:1:2 v/v 1-butanol:acetic acid:water), $\Delta R_f = 0.067 \pm 0.009$ (diatom biosilica), $\Delta R_f = 0.211 \pm 0.016$ (silica gel).
Table 2

<table>
<thead>
<tr>
<th>TLC Parameter</th>
<th>Malachite Green</th>
<th>Fast Green</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Silica Gel</td>
<td>Diatom Biosilica</td>
</tr>
<tr>
<td>Solvent System 1 (9:1:1 v/v 1-butanol:ethanol:water)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Knox Constant B (± 0.5 SE)</td>
<td>36 ± 8.3</td>
<td>6.2 ± 1.2</td>
</tr>
<tr>
<td>Knox Constant C (± 0.5 SE)</td>
<td>4.4 ± 1.9</td>
<td>1.5 ± 0.7</td>
</tr>
<tr>
<td>Plate height H at z_f = 3 cm (μm)</td>
<td>345 ± 51</td>
<td>70 ± 5</td>
</tr>
<tr>
<td>R_f (z_f ≥ 3.0 cm)</td>
<td>0.109 ± 0.019</td>
<td>0.774 ± 0.007</td>
</tr>
<tr>
<td>Solvent System 2 (5:1:2 v/v 1-butanol:acetic acid:water)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Knox Constant B (± 0.5 SE)</td>
<td>157 ± 19</td>
<td>9.0 ± 0.8</td>
</tr>
<tr>
<td>Knox Constant C (± 0.5 SE)</td>
<td>92 ± 9.0</td>
<td>2.0 ± 0.6</td>
</tr>
<tr>
<td>Plate height H at z_f = 3 cm (μm)</td>
<td>348 ± 74</td>
<td>20 ± 5.7</td>
</tr>
<tr>
<td>R_f (z_f ≥ 3.0 cm)</td>
<td>0.311 ± 0.010</td>
<td>0.942 ± 0.005</td>
</tr>
</tbody>
</table>

The plate height (H) vs. solvent front (z_f) profiles for TLC separation of malachite green and fast green on diatom biosilica and silica gel for solvent systems 1 and 2 are compared in Figure 7. Generally, the plate height (H) for malachite green and fast green eluted on diatom biosilica was minimized at solvent front position between 2.5 and 3.0 cm, whereas H for fast green eluted on silica gel always decreased with increasing z_f. Values for H at a solvent front position of 3.0 cm are compared in Table 2. Values for plate height H were much smaller for diatom biosilica relative to silica gel, pointing to higher TLC separation efficiency. For solvent system 1, H was reduced by a factor of 5 for malachite green and 3 for fast green, whereas for the more polar solvent system 2, H was reduced by a factor of nearly 17 for malachite green and 11 for fast green. In Figure 7, at small solvent front migration distances near the beginning of the separation process, broadening of the initial analyte spot may have contributed the dispersion process, and hence affected the plate height.

The Knox constants (A, B, C) were obtained by fitting H vs. z_f data to the modified van Deemter equation (Equation (7)) by nonlinear regression, and best-fit estimates are provided in Table 2. Best-fit estimates for Knox constant A were negative or statistically zero, and so it was assumed that there was no channeling flow within the porous TLC layer. Best-fit estimates for Knox constant B, which represents dispersive mass transfer of the solute in the mobile phase, were at least 3 times higher on silica gel relative to diatom biosilica for each analyte. Best-fit estimates for Knox constant C, which represents convective mass transfer resistance of the solute between the mobile and stationary phases, were also at least 3 times higher for silica gel relative to diatom biosilica for each analyte.
3.4 Basis for improved TLC separation of Malachite green and Fast green FCF on diatom biosilica

This study has shown that a thin porous layer for conventional TLC composed of diatom biosilica significantly improved the separation of the triphenylmethane dyes malachite green and fast green relative to silica gel. The results suggest that the basis for improved separation is the unique nano- and microstructure of the diatom
biosilica thin porous layer, which consists of randomly deposited, ellipsoidal biosilica frustules isolated from cultured cells of the pennate diatom *Pinnularia*.

Relative to the silica gel porous layer, the diatom biosilica porous layer possessed an open structure both between and within the particles with a total void fraction of 0.96. Each biosilica frustule possessed a uniform array of 200 nm pores penetrating through the shell (Figure 2e). However, even with this open structure, the permeability factor for capillary flow was two times lower through diatom biosilica relative to silica gel. This result suggested the structure of the diatom biosilica promoted interaction of the mobile phase with the surface of the stationary phase. The increased interaction between the stationary and mobile phase on the diatom biosilica increased the mobility of both malachite green and fast green, enabling the chromatographic resolution of these molecules. This result was observed for both solvent systems 1 and 2.

The use of diatom biosilica significantly lowered the minimum plate height relative to silica gel for each analyte, suggesting that the diatom biosilica porous layer offered enhanced mass transfer relative to the silica gel porous layer. The six-fold decrease in Knox constant B for malachite green reflects a reduction in dispersive mass transfer. The structure of the diatom biosilica offers an open structure with lowered intraparticle diffusion resistance relative to microporous silica gel. Furthermore, the nearly three-fold decrease in Knox constant C for both analytes reflects a reduction of the convective mass transfer resistance for solute transfer from the mobile phase to the particle surface. The surface of diatom silica frustules is corrugated with a rectangular array of 200 nm pores. This surface topology would help disrupt the fluid boundary layer surrounding the particle, resulting in an improved convective mass transfer rate.

In conventional porous particle layers for TLC, capillary forces cannot generate sufficient flow to minimize the effects of zone broadening, and silica gel particle layers in particular suffer from inadequate mass transfer. Monolithic stationary phases for TLC with open internal pore structure offer improvements in performance. In this context, the ordered pore array structure on the diatom frustule
surface may promote mobile phase interactions with the stationary phase, leading to improved mass transfer and analyte resolution.

Previous work has used atomic layer deposition (ALD), plasma-enhanced chemical vapor deposition (PECVD), physical vapor deposition, and electrospinning to create nanostructured, silica-based thin films for ultra TLC. Both ALD and PECVD can precisely modify the stationary phase structure to enhance analyte mobility. Furthermore, ordered microstructures facilitate convective mass transfer, leading to enhanced TLC efficiency. However, these stationary phases were made through elaborate, top-down planar fabrication processes. The nanostructured diatom biosilica described in this study can be readily produced at scale through a cell cultivation process followed by chemical isolation of the diatom frustules. A simple drop-casting technique is then used to deposit a thin porous layer of diatom frustules suitable for TLC.
Chapter 4: Conclusion

This study showed that a stationary phase for conventional TLC composed of randomly-deposited biosilica frustules isolated from living cells of the diatom *Pinnularia* sp. significantly improved the separation performance of the triphenylmethane dyes malachite green and fast green relative to silica gel. The diatom frustules were highly porous, hollow shells with surface structure dominated by 200 nm pore arrays. The migration and separation of malachite green, a less polar, weakly cationic molecule, and fast green, a highly polar, anionic molecule, could be fully separated on the open structure of diatom biosilica for both solvent systems. However, silica gel, which possessed mesopores of 6 nm diameter, did not enable the migration of fast green in solvent system 1. Improved TLC performance on the biosilica layer was also demonstrated by reduced plate height for both analytes, particularly for the more polar solvent system 2. Further chromatographic mass transfer analysis suggested that the nanostructured surface of the frustule decreased boundary layer resistance and reduced dispersive mass transfer, leading to increased analyte flux. Overall, these analyses suggest that the basis for improved chromatographic performance is the unique nano- and microstructure of the diatom biosilica frustule. Therefore, nanostructured diatom biosilica frustules produced through cell culture of living diatom cells merit continued investigation as a new stationary phase for TLC.
Bibliography


APPENDIX
Procedure A-1: Diatom subculture

The following protocol is designed per 4 subculture flasks and can be scaled accordingly, based on the desired quantity of subculture flasks.

Equipment

- Autoclave
- Laminar Flow Hood
- Micropipettes with sterile tips (1-mL and 5-mL)
- Vacuum Line
- Incubator at 22˚C with array of 9W CFL bulbs (14:10 light:dark photoperiod)

Materials/Chemicals

- Diatom seed culture flasks (3 – 4)
- 500-mL Erlenmeyer flasks (4x)
- Foam stoppers with aluminum foil covers (4x)
- 1-L Kimax bottle
- 100-mL graduated cylinder
- Autoclave indicator tape
- Harrison’s and Guillard’s f/2 enrichment Artificial Seawater Medium (at least 360 mL)
- Silicon stock solution #4 (200 mM Na₂SiO₃·5H₂O, at least 1 mL)*
- Vitamin stock solution #6 (Harrison + f/2, at least 1 mL)*
- Sterile filter

*Previously sterilized via autoclavation or sterile filtration

Procedure

1. **Subculture vessel preparation**
   
   1.1. Prepare 4 subculture vessels by fitting each flask with a foam stopper and capping with an aluminum foil cover.
1.2. Autoclave subculture flasks, 1-L Kimax bottle, and 100-mL graduated cylinder on dry cycle for ~ 30 minutes, then allow these materials to cool to room temperature.

2. **Artificial Seawater Medium (ASM) preparation**
   
   2.1. Prepare at least 360 mL ASM (per 4 subculture flasks) according to the ASM Preparation protocol.
   
   2.2. Using proper aseptic technique in the Laminar Flow Hood, sterilize ASM by sterile filtering into autoclaved 1-L Kimax bottle.

3. **Diatom subculture** – All steps below should be performed *using proper aseptic technique in the Laminar Flow Hood* to preserve axenic cultures and prevent contamination.
   
   3.1. Combine 3 - 4 seed culture flasks into a single flask and swirl to evenly suspend the seed culture.
   
   3.2. Save 1 – 2 unused seed culture flasks as backup cultures.
   
   3.3. Remove foam stoppers/aluminum foil covers, then add 90 mL of sterile ASM into each of the 4 subculture flasks.
   
   3.4. Add the necessary volumes of stock solution #4 and stock solution #6 to each subculture flask, according to Table 1. Swirl each flask to ensure the culture medium is well mixed.
   
   3.5. Inoculate each subculture flask with 10 mL of homogeneously suspended seed culture and mix well by swirling for ~ 5 seconds.
   
   3.6. Replace foam stoppers/aluminum foil covers, then label each subculture flask using the following format: Species – Generation # - Initials – Date – Flask # (e.g. PI-197-JAK-3/24/18-F1)
   
   3.7. Place new subculture flasks in incubator at 22°C, near array of 9W CFL bulbs as a light source (14h:10h light:dark photoperiod). Try to keep the light intensity delivered to each flask as even as possible.
   
   3.8. Swirl each flask daily for ~5 – 10 seconds.
   
   3.9. Record subculture information in Diatom Subculture Log.
   
   3.10. Repeat subculture protocol every 21 days, keeping the protocol as consistent as possible.
<table>
<thead>
<tr>
<th>Species</th>
<th>Media [mL]</th>
<th>Vitamin Stock [mL]</th>
<th>Silica Stock [mL]</th>
<th>Seed Culture [mL]</th>
<th>Frequency [days]</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pinnularia sp.</em> (PI)</td>
<td>90</td>
<td>N/A</td>
<td>0.25</td>
<td>10</td>
<td>21</td>
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<tr>
<td><em>Cyclotella sp.</em> (CY)</td>
<td>90</td>
<td>0.25</td>
<td>0.25</td>
<td>10</td>
<td>21</td>
</tr>
<tr>
<td><em>Thalassiosira pseudonana</em> (Tp)</td>
<td>90</td>
<td>0.25</td>
<td>0.25</td>
<td>10</td>
<td>21</td>
</tr>
</tbody>
</table>
Procedure A-2: Diatom frustule isolation

Equipment
- Beckman Coulter Z2 Particle Counter
- Centrifuge (Thermo IEC CL30)
- Vortex Mixer

Materials/Chemicals
- Diatom cell culture
- 50-mL centrifuge tubes (usually 3 – 4 per isolation)
- Nanopure H₂O (~ 240 mL)
- 12.1 M (37 wt%) hydrochloric acid (HCl, ~ 14 mL)
- 30% v/v hydrogen peroxide (H₂O₂, ~ 60 mL)
- Anhydrous methanol (MeOH, ~ 120 mL)

Procedure
1. Obtain diatom cultures. If optimal nanopatterning of frustule pore array is desired, the cultures should be grown up to stationary phase.
2. Using the particle counter (see “Cell Density Measurement with Z2 Beckman Coulter Counter” protocol by JC), determine the cell density of the well-mixed culture(s).
   - For *Pinnularia* sp., measure particles above 6 µm.
   - For *Cyclotella* sp., measure particles between 6 µm and 19 µm.
3. Into a 50-mL centrifuge tube, dispense/consolidate the appropriate volume of diatom culture necessary to achieve a total cell count of $3 \times 10^8$ cells (*Pinnularia* sp.) or $6 \times 10^8$ cells (*Cyclotella* sp.). This will roughly translate to ~ 100 - 130 mg DW.
4. Centrifuge the culture suspension at 2500g for 20 min.
5. After removing the supernatant, completely re-suspend the cell pellet in 40 mL Nanopure H₂O via vortexing. Manually shaking may also help with this step.
6. Centrifuge the cell suspension at 2500g for 20 min.
7. Repeat steps 5 and 6 two more times for a total of 3 aqueous washes.
8. Remove the majority of the resulting supernatant, leaving behind a cell slurry in 5 mL H2O. Suspend the cell slurry as well as possible, breaking apart any large chunks of biomass.
9. Add 10 mL 12.1M HCl to the cell slurry in the centrifuge tube. Close the tube and gently mix by inverting 5 times. Leave the tube unsealed, but covered, for ~24 h.
10. Remove the majority of the supernatant, leaving behind 5 mL slurry.
11. Add 30 mL 30% (v/v) H2O2 to the centrifuge tube, followed by 2 mL 12.1M HCl. Close the tube and gently mix by inverting 5 times. Leave the tube unsealed, but covered, for ~72 h. During this time, gently mix by closing the tube and inverting 5 times once after 24h and again after 48 h.
12. After gravity settling, carefully remove the supernatant.
14. Completely re-suspend the crude frustule isolate in 40 mL Nanopure H2O and allow to gravity settle.
15. Remove the supernatant.
16. Repeat steps 14 and 15 two more times for a total of 3 post-treatment aqueous washes.
17. Completely re-suspend the crude frustule isolate in 40 mL MeOH and allow to gravity settle.
18. Remove the supernatant.
19. Repeat steps 17 and 18 two more times for a total of 3 post-treatment organic washes.
20. Completely re-suspend the final frustule isolate in ~20 mL MeOH, and store in the sealed centrifuge tube.
Procedure A-3: Fabrication of diatom biosilica TLC stationary phase

This protocol was developed to create frustule biosilica TLC stationary phases, with 75 x 3 mm substrate dimensions and a mass coverage of 500 µg/cm². If desired, mass coverage can be tuned by adjusting frustule suspension mass density.

Equipment
- Vortex Mixer
- Hot Plate
- 20 – 200 µL micropipette
- UV-Ozone Cleaner (Novascan PSD-UVT)

Materials/Chemicals
- Frustule isolate in MeOH
- 75 x 3 mm cleaned glass substrates
- Ruler

Procedure
1. Heat the hot plate surface to a steady state temperature of 45°C.
2. Adjust mass density of frustule suspension, in MeOH, to 11.25 mg/mL (11.25 mg frustules per mL of suspension volume).
3. Ensure the frustule suspension is completely suspended and homogenous throughout via vortex mixing.
4. Place a 75 x 3 mm cleaned glass substrate on the center of the hot plate surface.
5. Using a 20 – 200 µL micropipette, dispense 100 µL of the homogeneous frustule suspension evenly across the substrate.*
6. Allow the MeOH to evaporate. Evaporation should be quick (~ 15 – 30 seconds) but no bubble formation should be observed.
7. After complete solvent evaporation, carefully place the dry film(s) in the UV-Ozone Cleaner. Set the operating temperature to 90°C, ensure the UV light is
engaged, and treat the films for 24 h. This helps fix the frustules to the substrate surface and oxidatively eliminates any residual organic contaminants.

8. Store the TLC films in a desiccator until activation/use.

*This is difficult, thus takes practice to master. Some tips:

- A ruler positioned along the length of the substrate helps with homogeneous deposition of frustule suspension.
- A slow, steady, and continuous dispensing motion leads to highest quality/most uniform film.
- If the suspension drips off the sides of the substrate, some of the biosilica will deposit on the sides. This will most likely ruin a TLC experiment if not prevented or fixed, because sharp front wicking flow is compromised by mobile phase traversing the sides unevenly.
Procedure A-4: TLC experiments

This protocol was developed in tandem with the “TLC optimization modeling” procedure (Procedure A-7) for real-time measurement of key TLC experimental parameters (solvent front migration distance, analyte migration distance, analyte band width) needed to calculate/model TLC separation efficiency for a given analyte. TLC film substrate dimensions were 75 x 3 mm. Real-time measurement is only feasible if the analyte emits/reflects visible color.

Equipment

- Muffle furnace
- Ventilation hood
- 0.2 – 10 µL micropipette
- Video camera with sufficient storage capacity (e.g. Logitech C920 webcam connected to computer with QuickTime Player or similar video recording software)

Materials/Chemicals

- 75 x 3 mm TLC stationary phase films (e.g. film of frustule biosilica or silica gel)
- Analyte standard solution (e.g. 1000 ppm malachite green in ethanol)*
- Freshly prepared mobile phase**
- Binder clips
- VWR Ruler trimmed to the 12.7 cm tick mark (~12.8 cm total length).
- 250 mL glass bottle with cap
- Whatman filter paper (150 mm diameter, grade 1)
- Pasteur Pipettes with suction bulb

* The ideal concentration of the reference analyte will depend on its visibility and affinity for both stationary and mobile phases.
**The ideal chemistry of this liquid will depend on the target analyte(s) being separated/migrated. For example, a 9:1:1 (v/v) solution of 1-BuOH:EtOH:H₂O was optimized for the analyte malachite green.**

**Procedure**

1. Activate TLC stationary phase films in muffle furnace at 110°C for 15 – 50 min. Keep the activation time consistent for all studied films.
2. Cut a circular Whatman filter paper (Grade 1) into quadrants.
3. Place one of the filter paper quadrants into a 250 mL glass bottle. It should rest against the inner wall such that one of the straight edges is in constant contact with the bottom rim of the bottle. This will serve as the TLC developing chamber.
4. Using a Pasteur pipette, dispense about 4 full pipette volumes of mobile phase liquid into the bottom of the TLC developing chamber. Seal the chamber and let sit for 20 minutes to establish vapor-liquid equilibrium.
5. Fix a 75 x 3 mm TLC film to a trimmed ruler with a binder clip. The length of the film should be parallel to the axis of measurement, and the bottom of the film should be flush with the 5 mm mark. The measurement tick marks should be completely visible up to the 75 mm mark.
6. Using a 0.2 – 10 µL micropipette, dispense 0.2 µL of analyte standard solution onto the TLC film at the 10 mm mark on the ruler. This may need to be dispensed in increments to prevent the analyte spot from reaching the sides of the TLC film (this leads to erroneously high analyte band widening). Allow the solvent to completely evaporate off the film.
7. Carefully place the ruler/TLC film into the developing chamber and seal it. The ruler should snugly fit in the sealed chamber at an inclination angle of 70°. The mobile phase surface should be very close, but not contacting, the bottom of the TLC film. Let the system equilibrate for at least another 5 min.
8. Begin recording a video of the entire length of the TLC film.
9. Open the chamber and quickly pipette more mobile phase into the bottom of the chamber such that contact forms between the stationary/mobile phases.*
Capillary action will drive wicking mobile phase flow up the TLC film, commencing the experiment.

10. Cease recording after the solvent front has passed the 75 mm mark on the ruler.

11. If characterizing the TLC separation efficiency, proceed to Procedure A-7: TLC optimization modeling.

*Keep the majority of the chamber top covered with the cap to best preserve vapor-liquid equilibrium.
Procedure A-5: Wicking flow analysis

This protocol is intended as a guide to analyze TLC experimental data obtained using the “TLC experiments” procedure (Procedure A-4). After recording experimental data for solvent front height ($z_f$) versus elapsed time ($t$), the data is fitted to a math model (the Lucas-Washburn equation) to characterize time-dependent solvent front height and extrapolate flow velocity and permeability constants ($\chi$ and $k_o$), affected by the nanostructure of the TLC stationary phase.

Equipment

- Computer with the following applications
  - iMovie or similar video analyzing/editing software
  - Excel
  - Matlab, Statgraphics, or other software capable of linear curve fitting

Materials/Chemicals

- Video recordings obtained according to “TLC experiments” procedure (Procedure A-4)

Procedure

1. Compiling Experimental Data

1.1. Import video of TLC experiment to iMovie or similar video editing/analyzing software.

1.2. Locate the time frame at which the mobile phase and TLC film initially contact. The initial raw time value at this frame ($t_{raw_o}$) is used to calculate elapsed time ($t$) at any moment in the video as follows:

$$t = t_{raw} - t_{raw_o} \quad (A-1)$$

1.3. At the same time frame ($t_{raw_o}$) the initial raw solvent front height ($z_{f,o}$) should be observed at the 5 mm mark on the ruler. Since the TLC film
begins here, solvent front height \( (z_f) \) at any moment in the video is calculated as follows:

\[
z_f = z_{f,raw} - z_{f,o}
\]  \hspace{1cm} (A-2)

1.4. Use Equations A-1 and A-2 to construct a data set of \( z_f \) [cm] vs t [s] for \( 0 \leq z_f \leq 7 \) cm. Be sure to record enough data points during the initial moments of capillary rise to capture the linear regime of the \( z_f \) vs t relationship. For example, data points from \( 0 \) cm \( \leq z_f \leq 0.5 \) cm in 0.1 cm increments and from 1 cm \( \leq z_f \leq 7 \) cm in 0.5 cm increments should suffice.

1.5. Replicate data sets can be obtained from videos of replicate experiments by measuring new \( z_f \) data sets at t values identical to those derived in step 1.4.

1.6. Plot experimental \( z_f \) vs t data.

1.7. Plot experimental \( z_f^2 \) vs t data.

2. **Fitting Math Model to Experimental Data**

2.1. Export experimental \( z_f^2 \) vs t data to Matlab or other software capable of linear least-squares regression.

2.2. Use linear least-squares regression to fit the modified Lucas-Washburn equation (Equation A-3) to experimental \( z_f^2 \) vs t data.

\[
z_f^2 = 2k_o d_p \sigma \mu t = \chi t
\]  \hspace{1cm} (A-3)

2.3. Plot experimental and model \( z_f^2 \) vs t data, and ensure a good fit.

2.4. From the best-fit model equation, solve for the flow velocity constant (\( \chi \)) and the permeability constant (\( k_o \)).
**Procedure A-6: Measuring analyte migration during TLC experiments**

This protocol is intended as a guide for measuring analyte migration distance ($z_s$) and band width ($w_B$) from TLC experimental data obtained according to the “TLC experiments” procedure (Procedure A-4). This data is used in the “TLC optimization modeling” procedure (Procedure A-7) to measure plate height ($H$) versus solvent front migration distance ($z_f$) and gauge separation efficiency.

**Equipment**
- Computer with the following applications
  - iMovie or similar video analyzing/editing software
  - ImageJ
  - Matlab or other software capable of applying a best-fit Gaussian curve to an experimental mass distribution

**Materials/Chemicals**
- Video recordings obtained according to the “TLC experiments” procedure (Procedure A-4)

**Procedure**
1. Familiarize with parameters depicted in Figure A-1.
2. Import video of TLC experiment to iMovie or similar video editing/analyzing software.
3. At a known $z_f$ value of interest, export an image of the TLC system to ImageJ software. Record the $z_f$ value.
4. Calibrate the scale of the image using the tick marks on the ruler.
5. Using the rectangular selection tool, highlight the analyte band (see Figure A-2) and generate a grayscale intensity plot (see Figure A-3).
6. Record the position (distance from $z_o$) of the highlighted area’s leftmost boundary as $z_{so}$.
7. List the intensity plot data in table format. Export the data as a txt file to Matlab.
8. Translate the intensity values to the x axis by subtracting the maximum intensity value from the entire intensity data array.

9. Reflect the intensity values about the x axis by multiplying the entire intensity data array by -1. Steps 8 and 9 convert the raw data into a mass density distribution with arbitrary units (see Figure A-4). If desired, normalize the data by dividing the entire set by the maximum y value.

10. Add z_{so} to the entire distance array (x values) from the intensity plot data. This converts raw distance data to sample migration distance along the stationary phase.

11. Use Matlab to apply a best-fit Gaussian distribution to the data.

12. Extrapolate w_B (4\sigma) and z_s (distance at mean response) from the best-fit Gaussian equation, or graphically determine these values (see Figure A-5). 4\sigma can be determined based on the distance between x-intercepts of tangential lines from the best-fit Gaussian, and the mean response occurs where the slope of the curve is zero. Note that z_{so} must be added to the distance value at mean response to acquire the true z_s value (step 10).

13. Repeat steps 3 – 12 to obtain z_s and w_B measurements for each z_I value of interest.

Figure A-1. TLC experimental parameters, also listed on the following page.
$z_0$ = sample origin (cm)
$z_s$ = sample migration distance (cm)
$z_f$ = solvent front migration distance (cm)
$w_B$ = sample spot width (cm or mm)

Figure A-2. Highlighted analyte band in ImageJ software.

Figure A-3. Intensity plot of analyte band.
Figure A-4. Analyte mass density plot (after noise subtraction and reflection about x-axis).

Figure A-5. Determination of $z_s$ and $w_B$ based on plot of best-fit Gaussian curve.
Procedure A-7: TLC optimization modeling

This protocol is intended as a guide to analyze TLC experimental data obtained according to the “TLC experiments” procedure (Procedure A-4). Experimental data for plate height (H) versus solvent front migration distance (z_f) is fitted to a math model to characterize the TLC separation efficiency of the process. Modeling protocol adapted from “Quantitative Thin-Layer Chromatography: A Practical Survey” by Spangenberg, et al.

Equipment

- Computer with the following applications
  - iMovie or similar video analyzing/editing software
  - Excel
  - ImageJ
  - Matlab, Statgraphics, or other software capable of nonlinear curve fitting

Materials/Chemicals

- Video recordings obtained according to “TLC experiments” procedure (Procedure A-4)

Procedure

1. Compiling Experimental Data

1.1 Import video of TLC experiment to iMovie or similar video editing/analyzing software.

1.2 In Excel, record solvent front migration distance (z_f) versus elapsed time (t) as detailed in “Wicking flow analysis” procedure (Procedure A-5).

1.3 Plot z_f vs t and z_f² vs t as detailed in “Wicking flow analysis” procedure (Procedure A-5).

1.4 For all data points within the domain of z_o < z_f ≤ 7.5 cm, measure analyte migration distance (z_s) and band width (w_B) as detailed in the “Measuring analyte migration during TLC experiments” procedure (Procedure A-6).
1.5 For all data points within the domain of $z_0 < z_f \leq 7.5$ cm, calculate the analyte retardation factor ($R_f$) using Equation A-4.

$$R_f = \frac{z_s}{z_f - z_o} \quad (A-4)$$

1.6 For all data points within the domain of $z_0 < z_f \leq 7.5$ cm, calculate the theoretical plate number ($N$) using Equation A-5.

$$N = 16z_s \frac{(z_f - z_o)}{w_h} \quad (A-5)$$

1.7 For all data points within the domain of $z_0 < z_f \leq 7.5$ cm, calculate the observed average theoretical plate height ($H$=HETP) using Equation A-6.

$$H = \frac{z_f - z_o}{N} \quad (A-6)$$

1.8 Construct a plot of $H$ vs $z_f$.

2 Fitting math model to experimental data for $H$ vs $z_f$

2.1 Familiarize with Equation A-7. This modified form of the van Deemter Equation was developed for planar adsorption chromatography to model “observed average theoretical plate height” ($\overline{H}_M$) as a function of stationary phase nanostructure, mobile phase fluid properties, flow velocity constant ($\chi$), and the so-called Knox Constants A, B, and C. Its derivation is explained in the reference cited above.

$$\overline{H}_M = \frac{3}{2} A \left( \frac{d_p^4 \chi}{2 D_m} \right)^{1/3} \left( \frac{2}{z_f^3 - z_o^3} \right) + \frac{B D_m}{\chi} (z_f + z_0) + \frac{C D_m d_p^2}{2 D_m (z_f - z_o)} \ln \left( \frac{z_f}{z_0} \right) \quad (A-7)$$
2.2 Apply a best-fit line to the linear $z_f^2$ vs $t$ plot generated in step 1.3. The slope of this line is equal to the flow velocity constant $\chi$ [cm$^2$/s] as described by the relationship below.

$$\chi = \frac{z_f^2}{t} \quad (A-8)$$

2.3 If necessary, calculate or measure the average stationary phase particle diameter ($d_p$) and molecular diffusion coefficient of the analyte in the mobile phase ($D_M$). With $d_p$ and $D_M$ known, the only unknown parameters remaining are the Knox Constants (A, B, and C).

2.4 Export the experimental $H$ vs $z_f$ data (plotted in step 1.8) as a txt file to Matlab, Statgraphics, or other software capable of least-squares-based nonlinear curve fitting.

2.5 Fit the theoretical model equation (Equation A-7) to the experimental data (e.g. lsqcurvefit function in Matlab may be used to do this, alternatively nonlinear curve fitting in Statgraphics can be used).

2.6 Plot the experimental and theoretical $H$ vs $z_f$ data, and ensure a good fit.

2.7 Determine the Knox Constant values and their confidence intervals from the fitted model. Compare experimental vs theoretical minimum $H$ values, and their corresponding $z_f$ values. Recall minimization of $H$ indicates optimization of the TLC separation efficiency for the characterized process.