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Radial multi-stationary phase thin-layer chromatography for the field-ready fingerprinting of herbal material

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Abstract

The growing international demand for herbal-based products has prompted the need for more stringent quality control methods to detect substandard and adulterated herbal plant materials. However, most prescreening methods, such as thin-layer chromatography (TLC), remain inaccessible to herbal producers in resource-limited settings. Here, we report a proof-of-concept demonstration of a multi-stationary phase thin-layer chromatography (MSP-TLC) method for the preliminary on-site identification and evaluation of herbal materials. Our method is based on a unique TLC plate design that features multiple phenyl- and octyl-modified silica gel stationary phases configured as radial sectors. The modified stationary phase patterns were fabricated by screen printing organosilane solutions onto commercial silica gel TLC plates. Radial elution with an ethanol-water mobile phase from the center of the MSP-TLC plate generates multiple chromatographic profiles simultaneously for a single sample extract. To facilitate the interpretation of the multiple TLC profiles, the MSP-TLC system was coupled with image analysis and chemometric pattern recognition to classify a sample as "within-specifications" or "off-specifications" for a given herbal plant species. Application of the system to *Blumea balsamifera* and *Vitex negundo* demonstrated sensitivity and specificity rates that range from 73.1 to 95.1% compared to the respective standard Pharmacopeia TLC methods. The presented method holds considerable promise as a cost-effective, user-friendly on-site prescreening tool for herbal materials in resource-limited settings.

Keywords: *thin-layer chromatography, multi-phase chromatography, herbal medicine, pattern recognition, on-site analysis, screen printing*

Introduction

The herbal-based product industry is a growing market in international trade.[1] For many economies, this is also a source of opportunity for inclusive economic growth, where farming cooperatives supply bulk herbal material to pharmaceutical manufacturers.[2–4] However, compliance with the strict quality requirements by the manufacturers are often not met because of quality concerns such as incorrect plant variety, contamination with other plant materials, improper preprocessing, mislabeling, or mishandling.[5] This results in lost opportunity for both supplier and manufacturer, and is a major concern in the supply chain.

To improve the sustainability of the herbal supply chain, in-process quality control technologies should be made available to these community-based suppliers so that raw materials can be prescreened prior to transportation to manufacturers. In-field prescreening can lead to improved material preprocessing and in turn minimize supply rejections.[6] For herbal materials that have been dried, pulverized and homogenized, quality assessment *via* morphological characteristics alone is impractical. Instead, herbal material should be assessed of its chemical profile. In the laboratory, this would be achieved by techniques such as infrared spectroscopy, high-performance liquid chromatography (HPLC), or thin-layer chromatography (TLC).[7] These methods, however, are not suitable and are impractical for field use, and thus, a reliable, cost-effective method in the form of a test kit should ideally be available so that quality of herbal materials can be prescreened on-site, even by users with limited technical background.

To address the need for user-friendly screening tests, there have been recent efforts to develop simple and low-cost TLC-based field test kits, such as Speedy TLC Kit,[8,9] Global Pharma Health Fund (GPHF)'s MiniLab,[10] and Field Forensic Inc.'s microTLC.[11] There have also been reports of the integration of a smartphone to automate the interpretation of the TLC profiles.[12,13] Our group previously reported the development of a TLC kit coupled with a smartphone app for the image analysis and chemometric classification of herbal medicinal materials.[6] All these TLC kits are miniaturized or simplified versions of the lab-based TLC method with vertical linear development and organic mobile phases.

While TLC has been previously adapted for on-site analysis, some practical aspects of the method can be improved. From our experience, using organic solvent systems in a field TLC method for the analysis of herbal medicinal material can be cumbersome in terms of chemical transport, stability, storage, waste, and safety because these solvents are highly flammable and volatile. Additional challenges can arise when different types of samples are analyzed. Each herbal product would have its own optimized organic mobile phase system, thereby increasing the range of reagents included in the test kit. From a practical viewpoint, TLC-based kits should ideally minimize the use of organic solvents and the number of reagents needed for multiple sample types. Since TLC is also known to be especially sensitive to external variables such as temperature, humidity, and user technique, the ruggedness of the method should also be improved for analytical conditions in the field.

To establish TLC as a more practical field method, we propose an alternative approach to conventional TLC. To minimize the range of reagents required, a general set of mobile phase and stationary phase parameters can be used as a universal system for the analysis of a wide range of sample types. While this general system may not be optimized for a given sample, the system or a subset of the system can be used to generate characteristic fingerprint profiles that can discriminate different levels of sample quality. A straightforward design for this approach is to use independent, multiple developments with multiple mobile phases and a silica gel stationary phase (**Fig. 1A**). Alternatively, to minimize the use of different solvents, the general system can comprise multiple stationary phases and a single mobile phase, ideally one which is less volatile and less hazardous, such as ethanol-water (**Fig. 1B**). A disadvantage of this approach, however, is the need to have multiple, separate development runs, which can be time consuming and tedious. To circumvent this drawback, we propose an innovative design where different stationary phases are integrated onto a single TLC plate as radial sectors. Simultaneous multiple developments through the different stationary phases can be achieved by eluting a single mobile phase using radial development mode. Our proposed multi-stationary phase TLC (MSP-TLC) system is illustrated **Fig. 1C**. Radial development for simultaneous multi-phase TLC was previously reported using multiple mobile phases on a single type

of stationary phase[14], but to our knowledge, there have been no previous reports of simultaneous multi-phase TLC using multiple stationary phases.

The fabrication of the MSP-TLC plate can be achieved using printing methods. There have been recent reports of using 3D-printing to fabricate planar chromatography stationary phases in different shapes.[15,16] Alternatively, organosilane modifiers can be printed on precoated silica gel TLC plates to create the desired modification patterns. Printing technologies such as ink-jet printing[17] and contact printing[18] have been previously applied to modify silicon oxide with organosilane precursors, but there has been no report of printing organosilane precursors onto high surface area substrates such as silica gel TLC plates. Another 2D-printing method, screen printing, has yet to be explored to deposit greater volumes of organosilane modifiers in the desired radial pattern.

Here, we developed a field-ready TLC protocol based on our proposed MSP-TLC system. To realize our proposed concept, we demonstrate the feasibility of a screen printing technique to fabricate patterned modifications on precoated silica gel TLC plates. We also present the design of a custom, horizontal development chamber to achieve radial elution on MSP-TLC plates. The application of the MSP-TLC system is demonstrated with two widely-commercialized herbal medicinal plants in the Philippines, *Blumea balsamifera* and *Vitex negundo*. Using resulting MSP-TLC profiles, samples are assessed as either "within-specifications" (WS) or "off-specifications" (OS) using a custom image analysis algorithm and chemometric novelty detection models. We also compare our system with the respective standard Pharmacopeia TLC protocol as a screening test for herbal materials. At manufacturer facilities, herbal materials are typically screened using the Pharmacopeia TLC method. Thus, it is our aim that the proposed MSP-TLC system achieves a similar or improved classification performance relative to the Pharmacopeia protocol, while being user-friendly for the on-site analysis of herbal materials in resource-limited settings.

Experimental

Materials

Glacial acetic acid and concentrated H_2SO_4 were purchased from Thomas Scientific (Swedesboro, NJ, USA). *p*-anisaldehyde and poly(ethylene oxide) with average MW ~1,000,000 (PEO-1M) were purchased from Sigma-Aldrich Pte. Ltd. (Singapore). Silica gel 60 F254 TLC plates, phenyltrimethoxysilane (PTMS), octyltriethoxysilane (OTES), and absolute ethanol were purchased from Merck (Darmstadt, Germany). Polyester monofilament fabric of mesh size 350 was purchased from TULCO Screen Printing Supply, Inc. (Quezon City, Philippines). Murakami Aquasol ER photo emulsion and photo hardener were purchased from Murakami Co. Ltd. (Tokyo, Japan). Calibrated 5 μL microcapillary tubes were purchased from Drummond Scientific Company (Broomall, PA, USA).

Collection and Preparation of Herbal Samples

The sample set used in this study is the same as that used in our previous work.[6] The sample set includes twenty-two *B. balsamifera* and fifteen *V. negundo* preprocessed pure leaf samples, which were collected from various farms across the Philippines and were authenticated with the respective standard Pharmacopeia TLC protocols.[19,20] These samples served as "within-specifications" (WS) samples. "Off-specifications" (OS) samples were previously prepared with various stress treatments such as high heat drying, fermentation, and humid storage conditions to simulate improper handling or preprocessing. Mixtures were also prepared at varying weight ratios of *B. balsamifera* and *V. negundo* to simulate accidental mixing of the two materials in a processing center that may be handling both products. All mixtures and OS samples were evaluated using the respective Pharmacopeia TLC protocols.[6]

Preparation of Stencils

Stencil patterns were designed in Adobe Photoshop CS and transferred to screen printing screens using the photo emulsion method. Thin, even coatings of photo emulsion were applied onto hand-stretched screens and were dried in a dark cabinet for 12 hours. Stencil patterns were printed on ink-jet printable transparencies and were transferred to emulsion-coated screens by selective UV-light exposure. Exposure time was optimized at 25 minutes using a 366 nm UV lamp placed 35 cm above the screens. After exposure, the unexposed portions of the emulsion coatings were washed out to form the stencils. To improve solvent-resistance, the stencils were further exposed to UV light for 1 hour on each side and were treated with photo hardener for another 12 hours. Stencils were washed and rinsed with DI water and 95% ethanol prior to printing.

Preparation of Organosilane Solutions

Varying concentrations of PTMS and OTES solutions were prepared in 4:1 (V/V) ethanol-DI water with 5% wt. acetic acid, and were allowed to hydrolyze for 2 hours and 21 hours, respectively, to maximize the grafting potential of each organosilane.[21] PEO-1M was added to improve the screen printability of the solutions. The minimum amount of PEO-1M needed for satisfactory printing was determined by checking the resulting pattern integrity of screen printed PTMS solutions prepared with 0.25%, 0.50%, 0.75%, 1.00%, and 1.25% wt. PEO-1M. The viscosities of these solutions were measured using a Brookfield DV3T Rheometer.

Screen Printing Organosilanes on Silica Gel TLC Plates

Prior to modification, TLC plates were cut into 10 x 20 cm sheets, rinsed with 95% ethanol, air dried, activated at 105 °C for 15 minutes, and stored in a desiccator at a relative humidity of < 30%. Screen printing was performed on-contact by aligning the TLC plate directly under the stencil. Organosilane solution "ink" was applied onto the non-patterned areas of the stencil, and a flat-blade polyurethane

squeegee was manually swept across the stencil at an approximate rate of 3 cm/s to push the ink through the stencil and onto the TLC plate substrate (**Fig. ESI-1**). The stencil was lifted from the substrate and excess solution was immediately wiped off from the screen with organosilane solvent to prevent rapid self-condensation of excess organosilane, which can result in stencil clogging. The stencil was finally rinsed with organosilane solvent and allowed to air dry before the next print run.

The procedure was repeated using three other stencils using different organosilane inks to produce the final MSP-TLC radial plate modification. The screen printed TLC plates were cured at 120 °C for two hours, washed with 95% ethanol, air dried, and cut into individual 4 x 4 cm plates. The plates were also visualized under UV 254 nm to check for printed pattern integrity.

Radial TLC Development Chamber

A custom, radial TLC development chamber was designed using AutoDesk 123D Design. Parts were 3D-printed using Flashforge Creator Pro 3D Printer with polylactic acid (PLA) filament, while a clear cover was fabricated from a laser-cut 3-mm acrylic sheet. A 27-Ga 1/4" blunt-tip syringe needle served as mobile phase dispenser. When the chamber is closed, the needle tip descends onto the TLC plate exactly the center at a fixed height.

MSP-TLC Sample Analysis

Finely cut dried leaf samples were extracted using 95:5 (V/V) ethanol-water at a ratio of 10 mL solvent per 1 g leaf sample. The leaves were soaked in solvent with occasional shaking for 30 minutes and the extract was decanted into a fresh microtube. Using a calibrated 5 µL capillary tube, 15 µL of extract was spotted onto the center of the MSP-TLC plate. Once completely air-dried, the plate was slid into the custom horizontal chamber and 110 µL (approximately 5 drops) of mobile phase was placed into the chamber needle. Fingerprints were visualized by immersing the developed plate with an ethanol-based *p*-anisaldehyde-H_2SO_4 reagent, followed by heating at 105 °C on a hotplate for 2-3 minutes.

Selection of MSP-TLC System

To select the stationary phases to be printed onto the final plate design, 0.50 *m* (molal), 1.0 *m*, 1.5 *m* and 2.0 *m* solutions of PTMS and OTES were screen printed onto the different sectors of the TLC plate. The sectors are numbered 0-4 counterclockwise, with Sector 0 as an unmodified silica gel sector. *B. balsamifera* and *V. negundo* reference samples were developed on the screen-printed plates using 60:40, 50:50, and 40:60 (V/V) ethanol-water mobile phases. Based on the resulting MSP-TLC profiles of the reference samples, a mobile phase and four stationary phases were selected for the final MSP-TLC system. Selection was based on the combination of mobile and stationary phases that yielded the most diverse resolution of both reference samples. The final MSP-TLC system was then used to analyze all samples in triplicate.

Image Analysis and Data Preprocessing

Images of sample MSP-TLC profiles were captured using an Asus Zenfone Go 5.0 smartphone camera at a resolution of 4:3 3 MP. To ensure consistent imaging conditions, images were captured using a custom plate holder with a fixed 60 W fluorescent light source. All images were saved as .JPG files for image analysis.

Image analysis was conducted in ImageJ 1.49v.[22] Images were manually cropped to a size of 565 x 565 pixels and rotated to ensure that the orientation of the sectors were consistent across all images. Since the configuration of the MSP-TLC profile is unique, a custom macro was written to obtain the RGB channel densitograms of each sector. Results were saved as CSV files and exported to R version 3.3.2[23] for further preprocessing. The RGB profiles of each sector were aligned with respect to a reference profile using variable penalty dynamic time warping algorithm, implemented with R VPdtw package version 2.1-11.[24] The MSP-TLC profiles were then unfolded to vectors of 3750 intensity values, normalized to unit norm, and mean centered and scaled prior to any modeling and testing.

Chemometric Analysis

The *B. balsamifera* and *V. negundo* WS classes were modeled using an approach similar to our previous work.[6] WS class models for each plant species were constructed using Soft Independent Modeling of Class Analogies (SIMCA) with 95% Q and T^2 Hotelling limits for outliers and single value decomposition method using Multivariate Data Analysis for Chemometrics[25] (mdatools) R package version 0.8.2. The models were tuned and evaluated using an iterated nested *k*-fold cross-validation procedure (iterations = 40, $k = 5$).[26] During the inner loop tuning stage, the optimum combination of MSP-TLC sectors and the number of principal components (PCs) used for modeling were selected. Sensitivity (true WS classification rate) and specificity (true OS classification rate) were used as model performance metrics. The outer loops of nested CV were used to estimate the performance of the final models and to generate multiple models for aggregation. Class predictions from the outer CV loops were aggregated by 2/3 majority vote.[26]

Results and Discussion

MSP-TLC Plate Design

For an initial proof-of-concept study, five sectors were incorporated into the MSP-TLC plate design to accommodate and explore two different levels of modification for each organosilane modifier used in this study (phenyltrimethoxysilane and octyltriethoxysilane), while retaining another sector as unmodified silica gel. In principle, however, fewer or more sectors can be incorporated into the design for a given application. The size of the plate was limited to 4 cm x 4 cm to minimize the required development time and materials cost for field use.

A bare silica gel sampling center was also included in the design to allow uniform, manual sample application as a circular spot (**Fig. 2A-B**). The diameter of this bare silica gel sampling center was

determined based on the typical sample spot size of an ethanolic plant extract on silica gel, as shown in **Fig. 2B**. A uniform sample spot ensures even distribution of the sample onto the different stationary phases once the mobile phase is dispensed from the center. It was noted in preliminary work that if this circular-shaped gap is not included in the pattern, and the sample spot is applied at the intersections of the different stationary phases, the ethanolic extract would tend to distribute itself based on the polarity of the respective stationary phase even before mobile phase is applied. For our plate design, however, it was desired that the chromatograms developed on each stationary phase are independent of each other so that the stationary phase sectors can be varied without affecting the entire system.

Radial Development Chamber

To achieve radial elution of samples on the MSP-TLC plates, we designed a compact horizontal developing chamber shown in **Fig. 2C-E**. Approximately 110 µL of mobile phase just needs to be dropped into the needle hub (**Fig. 2D**), and the mobile phase elutes radially from the end of the needle at a consistent rate. The process is driven by gravity and capillary action. A gentle tap might be necessary to overcome the initial surface tension of the mobile phase, but once the mobile phase starts to flow, the development continues until all the solution in the needle hub is consumed. Elution using 110 µL of mobile phase takes about 4-5 minutes for a development distance of 1.5 cm.

Radial development mode has several advantages over conventional vertical linear development mode used in the standard Pharmacopeia methods and other field-ready TLC methods. In vertical linear development, the bottom of a TLC plate is submerged in a pool of mobile phase and any excess mobile phase is typically discarded once development is complete. In our previous field TLC method, for example, approximately 1 mL of mobile phase was required for each sample development.[6] The excess mobile phase after development also produced organic solvent waste that can be cumbersome to dispose. In our current system, on the other hand, only 110 µL of mobile phase needs to be placed into the needle hub of our radial

horizontal development chamber. The mobile phase is then used directly for elution, and no excess mobile phase needs to be disposed.

Radial elution is also beneficial for miniaturization, since it can yield greater separation quality compared to the separation achieved using vertical linear development.[27] Using a 4 x 4 cm plate, the radial development distance is only 1.5 cm from the center of the plate, but even at this short distance, components are readily separated into fine bands. The same separation quality can be difficult to achieve using vertical linear development at the same development distance and with manual, circular sample application.

Fabrication of MSP-TLC Plates

To fabricate the stationary phase sectors of the MSP-TLC plate design, we explored different approaches for the patterned deposition of organosilanes on commercial TLC plates with a precoated 210-270 μm silica gel layer. One of the first approaches we explored in preliminary work was inkjet printing using a modified commercial piezoelectric printer. However, we found that the volume of organosilane solution deposited using our set-up was too limited and tended to accumulate on the surface of the TLC plate, resulting in uneven cross-sectional modification of the silica gel layer. Sample development on these TLC plates with inkjet-printed modification resulted in uneven retention of components, so bands on these plates appeared to be blurred. Inkjet printing may be adapted to modify thinner sorbent layers, but for thicker layers, it is necessary to deposit greater volumes of organosilane solution all at once so that the solution can penetrate the entire silica gel layer for the uniform distribution of sorbent modification.

An approach that has yet to be explored for silica gel TLC plate modification is screen printing. The technique was considered because it can be adapted to print greater volumes of ink by varying the mesh size of the screen.[28] A requirement for screen printing inks, however, is a relatively high viscosity (100-50,000 cP).[29] On the other hand, organosilanes used for grafting are typically dissolved in ethanol-water solvents. A 0.50 *m* PTMS solution dissolved in 4:1 (V/V) ethanol-water with 5% wt. acetic acid, for

example, has a viscosity of 1.89 ± 0.02 cP, which is very much below the typical viscosity range for screen printing inks.

Despite this apparent limitation in ink compatibility, we tested the feasibility of the screen printing approach but with some modifications. In conventional screen printing applications, the high viscosity prevents the ink from readily permeating the stencil mesh. Application of shear force from squeegee motion causes a rapid decrease in the ink viscosity, which then allows the ink to flow through the stencil mesh to form a thick film on the surface of the substrate. For our application, on the other hand, the screen printing technique was adapted so that the squeegee pushes the ink through the stencil mesh and onto a substrate not to form a thick film on the surface, but to be absorbed through the porous silica gel substrate. To further ensure screen printability with very low viscosity ink, mesh 350 was used for the stencil fabrication. Higher mesh counts translate to smaller mesh openings and are typically used for lower viscosity inks.[28] To test this set-up, a solution of 0.50 *m* PTMS was initially screen printed using a test stencil pattern. However, the ink still proved to be too thin and difficult to control, resulting in very low pattern fidelity. When the solution was pushed through the stencil openings using a squeegee, the solution appeared to "bleed" or flow outside the printed area faster than its absorption through the substrate.

To improve screen printability of the organosilane solutions, PEO-1M was added to increase the viscosity, which can slow the rate of bleeding. PEO-1M was selected since it can increase the viscosity of a solution when added in very small amounts. The least amount of additive is desired since it is likely that it will remain on the silica gel after screen printing, therefore becoming part of the stationary phase. It was determined that a minimum of amount of 1.00% wt. PEO-1M (viscosity 19.18 ± 0.06 cP) was sufficient to produce good pattern integrity with minimal bleeding (**Fig. 3**). While the contribution of PEO-1M to the chromatographic properties of the stationary phase still has to be studied more extensively, its effect in our current application appears to be minimal, as the stationary phase interactions are dominated by contributions from the silica gel silanol groups or organosilane modifiers (**Fig. ESI-2**). Using this ink formulation, application of 70 μL of organosilane ink per MSP-TLC plate sector was determined to be

enough to print a complete pattern while forming a uniform distribution of silica gel modification (**Fig. ESI-3**).

The screen printing method has several advantages as a fabrication method for MSP-TLC plates. For one, the method is highly amenable for batch fabrication. Using our stencils shown in **Fig. 4**, 10 MSP-TLC plates can be printed simultaneously, which takes about 10-12 minutes. The materials used for screen printing are also commercially available at low cost. Thus, even if stencils tend to wear out with extensive use, replacements can be prepared readily at minimal cost.

MSP-TLC System

To determine the combination of phases to be incorporated into a general MSP-TLC system for *B. balsamifera* and *V. negundo*, we prepared several modified stationary phases and evaluated the resulting TLC profiles when reference samples were developed with different mobile phases. The silica gel modifiers investigated in this study were phenyltrimethoxysilane (PTMS) for moderate hydrophobic and π-π interactions, and octyltriethoxysilane (OTES) for hydrophobic alkyl-group interactions. Investigated mobile phases, on the other hand, were limited to ethanol-water systems to ensure safety and portability of the mobile phase once the technique is transferred for field use.

Sets of TLC plates were printed with the same organosilane but at increasing concentrations per sector (Sector 1: 2.00 *m*, Sector 2: 1.50 *m*, Sector 3: 1.00 *m*, Sector 4: 0.50 *m*) to vary the extent of silica gel modification. Since each sector was screen printed with the same volume of solution, the concentration of the organosilane solution was expected to determine the extent of modification: higher concentrations of organosilane solution would leave fewer hydrophilic silanol groups unmodified, resulting in a more hydrophobic stationary phase.

The profiles of the *B. balsamifera* and *V. negundo* reference samples developed using the different mobile phases and screen printed stationary phases are shown in **Fig. 5**. As the ethanol-water mobile phases are very polar, all the stationary phases behave as reverse-phase, as reflected in the shifting of all the bands

to lower retention factors (*Rf*) when the polarity of the mobile phase is increased with increasing ratio of the aqueous component. It is also notable that there is increased band broadening with increasing ratio of mobile phase aqueous component throughout all the different stationary phase sectors. In our MSP-TLC system, the mobile phase velocity is difficult to control and is determined primarily by system characteristics. We observed that mobile phase elution tended to proceed at a lower rate with increasing aqueous component, which is likely due to the strong interaction of the aqueous component with unmodified silanol groups on the stationary phases. The lower mobile phase velocity may have contributed to the band broadening observed with these mobile phases.

As for the stationary phases, higher concentrations of organosilane solutions used for screen printing indeed resulted in increased silica gel modification. With increasing hydrophobicity of the stationary phase, components were more retained on the stationary phase, resulting in shifting of bands to lower R_f values. It is noted, however, that a maximum modification point was likely reached when stationary phases were prepared with 1.00 or 1.50 *m* organosilane solutions, as there were minimal differences between the TLC profiles developed on stationary phases printed with higher solution concentrations. Using the evaluated inks, only two levels of modification were ultimately prepared using 0.50 *m* and 1.50 *m* PTMS, and 0.50 *m* and 1.00 *m* OTES.

Generally, while not all components of the leaf extracts are fully resolved in each profile, characteristic patterns are nevertheless apparent. From the *B. balsamifera* profiles, there are colorful characteristic sets of bands that are resolved with the phenyl- and octyl-modified stationary phases. *V. negundo* samples are not as well-separated, however. There are components that remain on the other edge with roughly the same R_f despite the varying polarity of the stationary phases. Since these components are not retained significantly, these components must be very polar. The resolution of these components may require functional groups of different selectivity such as amino- or cyano-modifications especially if an ethanol-water system is still used as the mobile phase. This can also be explored in future studies.

Based on the prepared stationary phases, 60:40 (V/V) ethanol-water was selected as the universal mobile phase since it yielded the most diverse *V. negundo* and *B. balsamifera* profiles with minimal band broadening. **Fig. 6** shows the profiles of the representative *B. balsamifera* and *V. negundo* samples developed using the final MSP-TLC plate design (Sector 1: 0.50 *m* OTES, Sector 2: 1.00 *m* OTES, Sector 3: 1.25 *m* PTMS, Sector 4: 0.50 *m* PTMS) with 60:40 (V/V) ethanol-water mobile phase. The MSP-TLC profiles for the full sample set are shown in **Table ESI-1**. While the degree of separation achieved using the current stationary phase sectors may not be drastically different from one another, the prepared multi-stationary phase system nevertheless demonstrates the feasibility of the MSP-TLC concept.

Image Analysis and Preprocessing

Development of samples using the MSP-TLC method yields complex profile patterns that can be analyzed using multivariate pattern recognition methods. Although there are visually evident differences between the profiles of each sample class (**Fig. 6**), image analysis and chemometric classification can be applied to minimize subjectivity.

The image analysis approach used in this study is similar to the method reported in our previous work in which we converted colored, linear TLC profiles to RGB densitometric profiles.[6] However, since the configurations of the MSP-TLC sector profiles are unique, we wrote a custom ImageJ macro that adapts a polar coordinate system to generate the RGB profiles of each sector. The algorithm is illustrated in **Fig. 7A**. Setting the center of the MSP-TLC profile as the origin, a line of fixed pixel length is drawn from the origin (e.g., the red line drawn in **Fig. 7A**), with a specified angle relative to the abscissa. We then obtain the RGB intensity values of each pixel along this line. The process is repeated by drawing new lines at 1° increments across the angle range of each sector, and the average RGB intensity values per line pixel is calculated to smoothen the densitograms. A buffer of 20° was added between sectors to account for possible printing errors. Resulting RGB intensity profiles per sector are shown in **Fig. 7B.**

Additional preprocessing steps were performed to correct method-related variations in the MSP-TLC profiles. Aside from normalization and centering and scaling, another major preprocessing step is alignment using variable penalty dynamic time warping (VPdtw), which shifts the position of profile peaks relative to the peaks of a preselected reference profile.[30] For our application, the variable volume of mobile phase used for elution is the major source of variation in the position of MSP-TLC solvent front. Nevertheless, the alignment algorithm can correct these resulting shifts in band positions (**Fig. ESI-4**). The RGB channel densitograms of each sector were then unfolded into a single vector as shown in **Fig. 7C**. The principal component analysis (PCA) scores plots of the MSP-TLC profiles of all samples and their technical replicates before and after alignment are shown in **Fig. 8**. After alignment, the sample classes become clustered more closely together, suggesting that intra-class sample variability related to profile development is minimized.

Chemometric Analysis

Using the MSP-TLC profile vectors as input variables, we constructed SIMCA models for the WS class of each plant species using a similar approach to the method used in our previous study.[6] The classification approach is a novelty detection method where we used only the WS samples for a respective plant sample to train a model, which can then predict whether a sample is part of the WS class or not (OS). Prepared OS samples were used as validation or test samples to estimate the specificity rates of the models. Since an MSP-TLC sample profile yields five separate chromatograms, not all data may contribute positively to the performance of a pattern recognition model. During the inner tuning stage, we also included a feature selection step in which we estimate the classification performance using all possible combinations of the MSP-TLC sectors (**Tables ESI-1** and **ESI-2**). To evaluate the MSP-TLC system as a screening test for herbal materials in the supply chain, we compared the classification performance of the system to the performance of the standard Pharmacopeia TLC method for the respective plant species.

***B. balsamifera* analysis.** For the *B. balsamifera* WS class, only Sector 3 profiles were used for the final model. The final estimated performance of the aggregated model was 95.1% sensitivity and 91.7% specificity (**Fig. ESI-5**). This is an improvement over our previously reported smartphone-based miniaturized Pharmacopeia-based TLC method, which demonstrated a classification performance of 90.2% sensitivity and 86.2% specificity (full comparison of classification performances are summarized in **Table ESI-4**).[6] The final MSP-TLC classifier can also correctly identify a sample spiked with 5% wt. *V. negundo* as anomalous, or "off-specifications." Detection of this level of foreign plant material can be challenging to achieve with our previous miniaturized TLC method and even with the standard Pharmacopeia TLC protocol, which can misclassify a *B. balsamifera* sample mixed with as much as 50% (w/w) *V. negundo* as acceptable.[6]

***V. negundo* analysis.** The final *V. negundo* WS class was modeled using the profiles of Sectors 2 and 3. Compared to the performance of our previously reported smartphone-based miniaturized Pharmacopeia-based TLC method (81.4% sensitivity and 92.0% specificity),[6] the *V. negundo* model did not perform as well, demonstrating an estimated performance of only 74.4% sensitivity and 73.1% specificity (**Fig. ESI-6**). The model was also limited in its capability to correctly classify samples spiked with *B. balsamifera* as foreign plant material: only samples mixed with 40% wt. *B. balsamifera* were correctly classified as anomalous. The performance of the MSP-TLC *V. negundo* model can be attributed to the limitations in resolving *V. negundo* components using the current MSP-TLC system. As discussed earlier, improved resolution of *V. negundo* extracts can be explored with different stationary phases or mobile phases.

Based on the current MSP-TLC system and chemometric modeling, future MSP-TLC plates for this application can be prepared using only the stationary phases in Sector 2 and 3 to minimize fabrication costs. Nevertheless, the other stationary phase sectors in the current MSP-TLC system may prove useful for resolving other non-specific adulterants. This is also the focus of another study.

Outlook

The MSP-TLC system developed in this work is a promising method for the on-site screening of herbal materials. For *B. balsamifera* analysis, the MSP-TLC system demonstrated comparable classification performance relative to the standard Pharmacopeia TLC method, which is typically used as a screening test for preprocessed herbal materials at manufacturers' laboratory facilities. Further work, however, can be done to improve the performance of the existing system, especially for *V. negundo* analysis, through the exploration of other stationary and mobile phase systems.

On the other hand, the MSP-TLC system is much faster, uses minimal mobile phase, and is more readily adapted for on-site prescreening compared to the standard Pharmacopeia TLC method. Since the MSP-TLC system is miniaturized, the materials and equipment costs required for our system are also much lower compared to the cost of the Pharmacopeia method. Indeed, the method reported in this work addresses the feasibility of performing TLC development for on-site analysis. However, to fully translate the method for the field, other steps, especially the band visualization step that involves the use of corrosive reagents, also need to be translated to a more user-friendly format. This can be the focus of another study.

Overall, the MSP-TLC platform has many opportunities for further development. For proof-of-principle, we demonstrated the fabrication and application of miniaturized MSP-TLC plates with phenyl- and octyl-modified silica gel stationary phases. To fully realize the potential of the proposed platform, other silica gel modifiers and mobile phases can be explored to diversify the MSP-TLC system. A more diverse MSP-TLC system can resolve a wider range of mixture components and can potentially serve as a general field analysis method for a variety of sample types. The MSP-TLC plate can also be designed to accommodate fewer or more stationary phase sectors as a given application requires. If greater resolution is required, larger plate sizes can also be prepared.

We also demonstrated screen printing as a feasible technique to fabricate the MSP-TLC plates. However, further work can be done to improve the preparation of the plates. The screen printing method used in our current study was done manually, so it is possible that there will be person-to-person variation

in plate preparation. Automation of the process would be a promising avenue for further development to guarantee reproducibility. Full characterization of the modified silica gel layer can also be investigated, especially in studying the cross-sectional distribution of the silica gel layer modification. In this work, the extent of layer modification was determined indirectly based the separation quality achieved using the stationary phases, but physico-chemical characterization can be useful to address possible inhomogeneities. The screen printing approach also requires PEO-1M ink additive to produce sharp printed patterns. Since it is very difficult to remove once deposited on silica gel, any deposited PEO-1M essentially becomes part of the stationary phase. While the effect of PEO-1M appeared to be minimal for our current application, it may have a more noticeable effect for other samples and applications. To avoid the addition of the PEO-1M additive, other fabrication techniques can be investigated such as 3D-printing[15] of pre-modified silica gel, or spray-on application of organosilane precursors on precoated silica gel TLC plates.

Conclusions

In this work, we presented the first proof-of-concept demonstration of a multi-stationary phase thin-layer chromatography (MSP-TLC) system. We also presented the first demonstration of a screen printing approach for the fabrication of patterned modification on silica gel TLC plates. For this study, the MSP-TLC system was applied for the fingerprinting of herbal materials, but the system can be readily adapted to analyze other complex mixtures.

The MSP-TLC system is especially promising to apply as a miniaturized field TLC method because of its ease-of-use, minimal use of reagents, avoidance of organic mobile phases, and minimal waste generation. The advantage of the MSP-TLC method over conventional TLC is that a sample can be analyzed with multiple chromatographic systems simultaneously, yielding more sample information in less time and with minimal solvent use. The technique was further translated into an easy-to-use format using a custom, 3D-printed radial elution chamber. Subjectivity in the interpretation of the multiple MSP-TLC profiles was also minimized with the use of image analysis and chemometric pattern recognition algorithms that can be

readily automated with a smartphone app. With further development and optimization, the MSP-TLC platform can serve as a low-cost, simple, and accessible analytical tool for the preliminary quality assessment and identification of herbal material. The accessibility of the method can also translate to practical cost-savings for users through the mitigation of herbal material supply rejection rates and reworking costs. While we developed the MSP-TLC platform with the herbal material supply chain in mind, the method can be adapted for other applications, such as the rapid screening of finished herbal or food products. Improved screening of herbal raw materials and finished products even in resource-limited settings can help ensure the safety of consumers as well as the sustainability of the herbal industry.

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Conflicts of interest

There are no conflicts of interest to declare.

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Figures

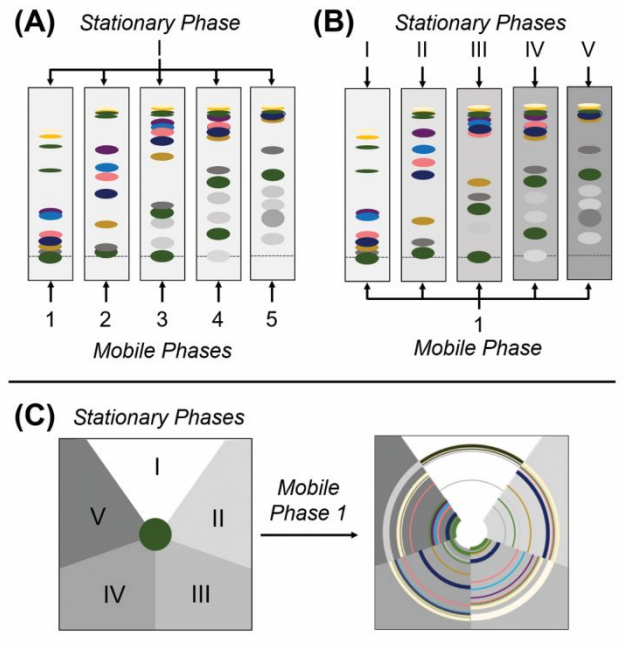


Fig. 1. Different approaches to independent multiple developments in TLC. (A) Multiple mobile phases and one stationary phase. (B) One mobile phase and multiple stationary phases. (C) Our proposed approach of simultaneous developments via radial elution of one mobile phase on multiple stationary phases.

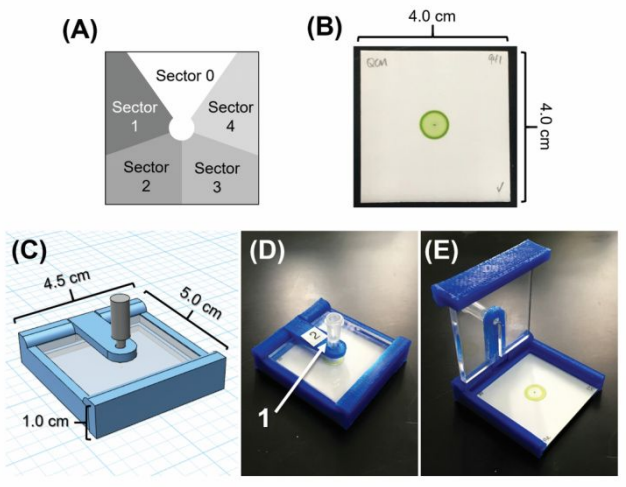


Fig. 2. (A) MSP-TLC plate design featuring a sample application center. (B) MSP-TLC plate with an ethanolic plant extract manually applied at the center. (C) 3D rendering of custom horizontal radial TLC chamber generated in Autodesk 123D Design. (D) Chamber in closed position to allow dispensing of mobile phase from needle hub (1). (E) Chamber in open position to allow insertion or removal of an MSP-TLC plate shown here with a partially-eluted sample spot.

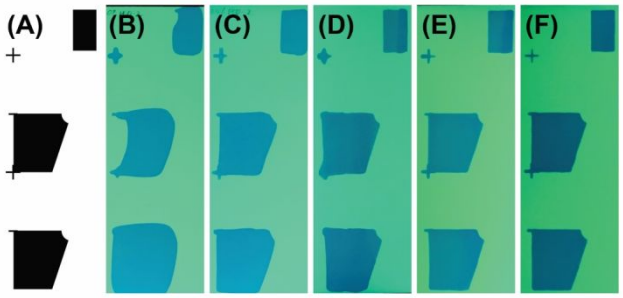


Fig. 3. Evaluation of screen printability of organosilane inks. (A) Test stencil pattern. Shown are screen printed TLC plates viewed under 254 nm UV light using 0.50 *m* PTMS inks with (B) 0.25%, (C) 0.50%, (D) 0.75%, (E) 1.00%, and (F) 1.25% wt. PEO-1M.

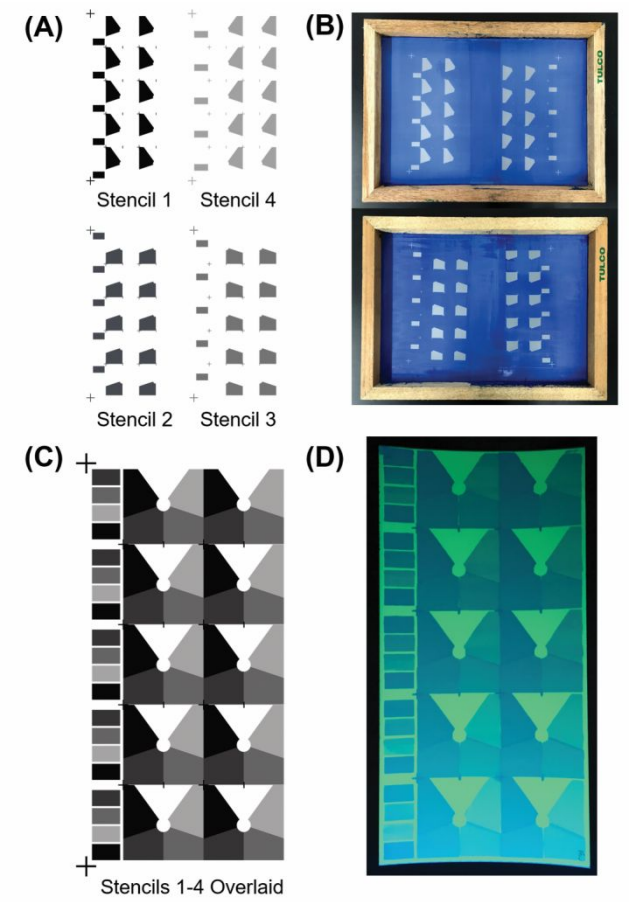


Fig. 4. (A) Digitally designed stencil patterns that were transferred to (B) screen printing screens using the photo emulsion method. (C) Final pattern when all stencils are overlaid. 10 MSP-TLC plates can be printed using this stencil. (D) Screen printed TLC plate viewed under UV 245 nm. TLC plate was printed with varying concentrations of PTMS.

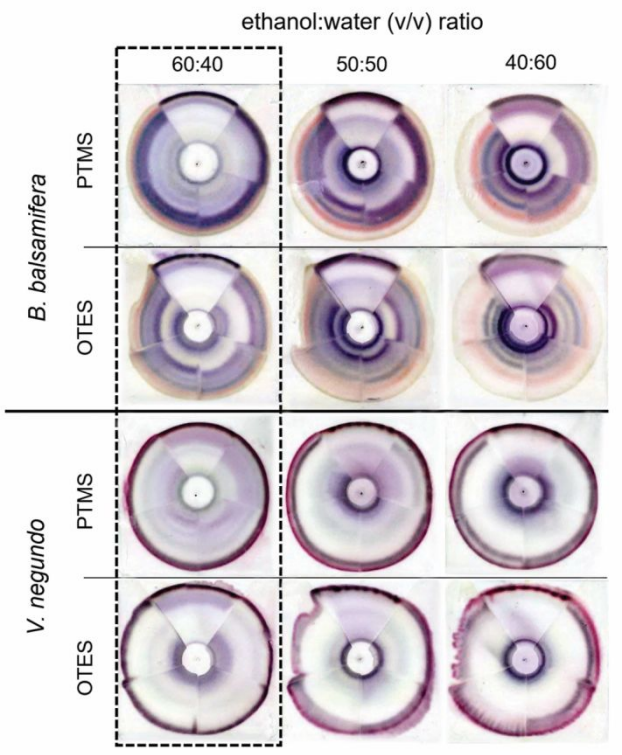


Fig. 5. MSP-TLC profiles of *B. balsamifera* and *V. negundo* using different ethanol-water ratios as mobile phases and stationary phases printed with increasing concentrations of PTMS and OTES. *Note*: contrast was increased in profile images to improve clarity.

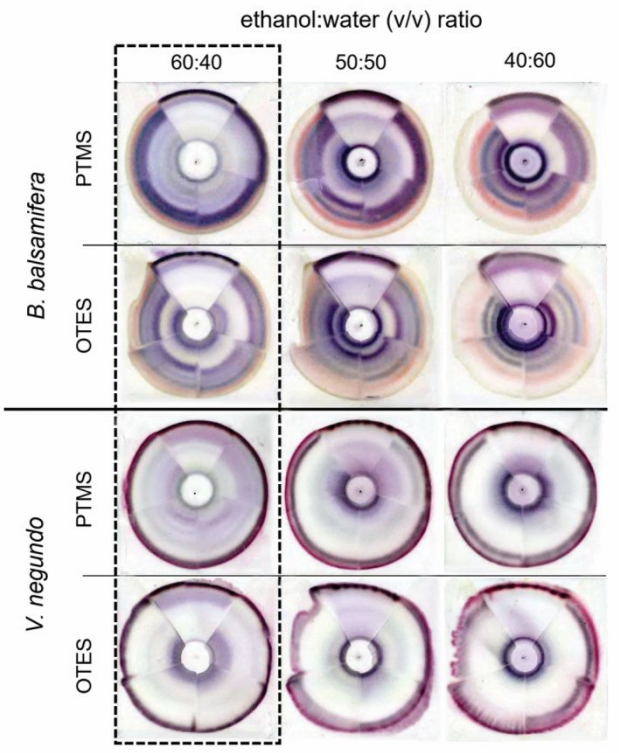


Fig. 6. MSP-TLC profiles of representative within-specifications (WS) and off-specifications (OS) *B. balsamifera* and *V. negundo* samples using the final MSP-TLC system. Also shown are profiles of *B. balsamifera/V. negundo* mixtures. *Note*: contrast was increased in profile images to improve clarity.

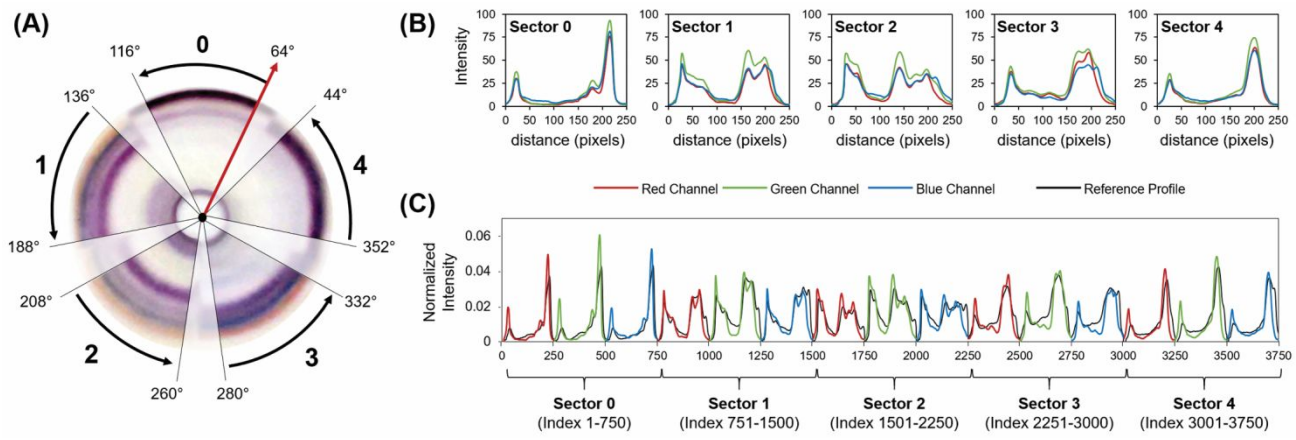


Fig. 7. Summary of the image analysis and chemometric preprocessing algorithm for MSP-TLC profiles. (A) Illustration of the ImageJ macro used to obtain (B) RGB channel densitograms per MSP-TLC sector. (C) Densitograms were aligned with respect to a reference profile, unfolded, and normalized.

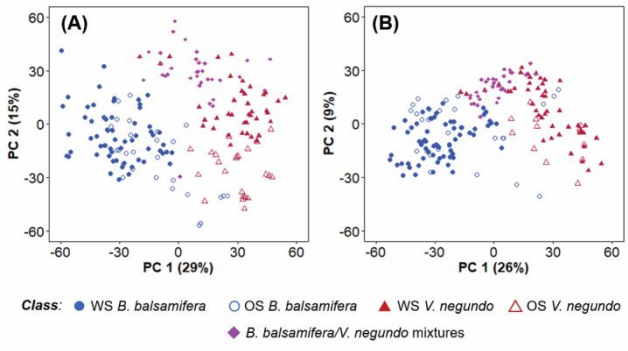
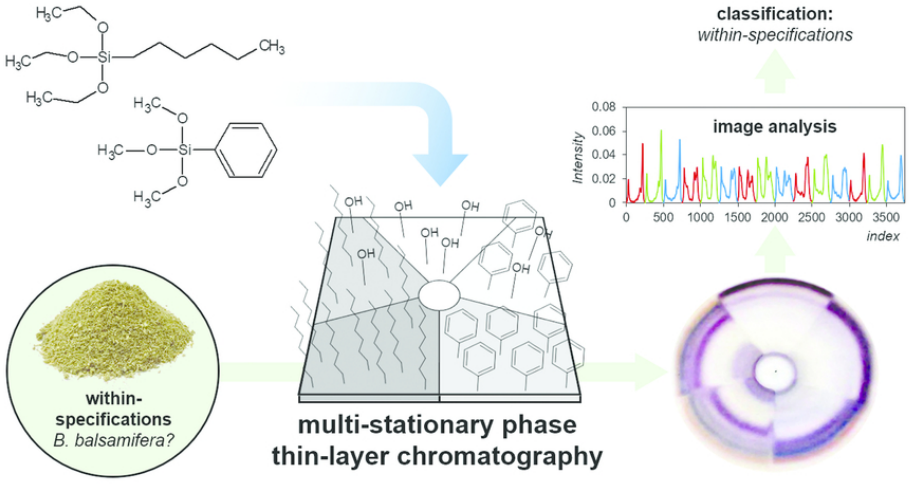


Fig. 8. Principal component analysis (PCA) scores plots of normalized, center and scaled MSP-TLC sample profiles (A) before alignment and (B) after alignment.



A multi-stationary phase thin-layer chromatographic fingerprinting method was developed for the on-site screening of raw herbal medicinal materials.

79x39mm (300 x 300 DPI)