Amine-Phenyl Multi-Component Gradient Stationary Phases

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Abstract:

Continuous multi-component gradients in amine and phenyl groups were fabricated using controlled rate infusion (CRI). Solutions prepared from either 3-aminopropyltriethoxysilane (APTEOS) or phenyltrimethoxysilane (PTMOS) were infused, in a sequential fashion, at a controlled rate into an empty graduated cylinder housing a vertically aligned thin layer chromatography (TLC) plate. The hydrolyzed precursors reacted with an abundance of silanol (Si-OH) groups on the TLC plates, covalently attaching the functionalized silane to its surface. The extent of modification by phenyl and amine was determined by the kinetics of each reaction and the exposure time at each point along the TLC plate. The local concentrations of phenyl and amine were measured using diffuse reflectance spectroscopy and X-ray photoelectron spectroscopy, respectively. The profile of the multi-component gradients strongly depended on the order of infusion, the direction of the gradient and the presence of available surface silanol groups. A slightly higher amount of phenyl can be deposited on the TLC plate by first modifying its surface with amine groups as they serve as a catalyst, enhancing condensation. Separation of water- and fat-soluble vitamins and the control of retention factors were demonstrated on the multi-component gradient TLC plates. Uniformly modified and single-component TLC plates gave different separation compared to the multi-component gradient plates. The retention factors of the individual vitamins depended on the order of surface modification, the spotting end, and whether the multi-component gradients align or oppose each other.

Keywords: gradient, functionally graded surfaces, sol-gel, organoalkoxysilanes, silane chemistry, planar chromatography

Introduction

A single-component gradient has one chemical component whose surface concentration changes in a predictable manner from one end of a substrate to the other. Such materials have been used to study cell adhesion and migration, directed transport, and protein adsorption among other phenomena as described in a number of reviews.[1-6] An one-component gradient, however, may not be sufficient for a number of applications, particularly those that rely on the interaction of an analyte species with more than one functional group located in close proximity to each other on a surface. Separation science and bifunctional heterogeneous catalysis are two areas where such interactions are vitally important. The presence of two or more components, each having different characteristics (e.g., charge, hydrophobicity), can provide an avenue for improved retention and selectivity, particularly if the two or more components act together in a synergistic fashion.

Multi-component gradients can be inherently more complex to prepare and characterize, and can also be more molecularly complex on the microscopic length scale. One popular method to fabricate multi-component gradients involves the selective removal of one component on a substrate followed by backfilling with another. An early example of this approach involved using scanning tunneling microscopy-based replacement lithography to remove and replace alkanethiols self-assembled on gold with a second functionalized thiolate present in solution.[7] Bohn and coworkers have also described an electrochemical gradient technique to form a multi-component surface whereby functionalized thiols adsorb at certain locations along a potential gradient on gold and then the remaining uncoated areas are backfilled with a second thiol. This approach can be followed by immobilizing a specific reagent such as an antibody to the surface via reaction with a terminal functional group on the gradient surface, thus extending applications.[8-12] A variation of this method has also recently been described that involves the electrochemical desorption/readsorption of surface modifiers while withdrawing the substrate from solution.[13] Other approaches to the formation of multi-component gradients include gradually exposing a suitable substrate to a modifying solution followed by full immersion,[14-16] creation of a gradient monolayer via microfluidic lithography followed by backfilling,[17] the use of a photo-mask followed by electrochemical oxidation and selective immobilization,[18] modulating the exposure time of a self-assembled monolayer on gold to a reducing agent[19] and cross diffusion of different organothiols through a sephadex gel on a gold slide.[20-22]

We have also fabricated two-component gradients using controlled rate infusion (CRI)[23-25] and infusion-withdrawal dip coating (IWDC).[26-28] These two methods involve a variation of the sol-gel process.[29-31] In CRI, a reactive organoalkoxysilane is gradually infused at a controlled rate into a reaction vessel where it reacts with Si-OH groups on the surface of a siloxane-coated substrate in a time-dependent fashion.[23-25] Gradient formation is governed by the rate of infusion, the rate of reaction, and the concentration of the silane. In IWDC, the siloxane-coated substrate is suspended in a sol prepared from tetramethoxysilane and synchronized syringe pumps are used to infuse an organoalkoxysilane into the deposition reservoir, while the mixed sol (and/or the slide) is withdrawn to produce a film.[26-28] In both of these approaches, one component is unreacted Si-OH and/or Si(OR)_x groups due to incomplete condensation and the other is the pre-determined organic modifier (e.g., CH₃, NH₂, C₆H₆ etc). Thus, a gradient in the organic modifier also contains a counter gradient in surface silanol (Si-OH) groups.

Here, we describe a simple method to generate continuous multi-component silane gradients using CRI with 3-aminopropyltriethoxysilane and phenyltrimethoxysilane as the organoalkoxysilane precursors. These multi- component materials have a gradient in amine and phenyl that either oppose or align with each other in a predictable fashion. Also present are surface silanol groups, whose concentration will vary along the surface, albeit in a more unpredictable manner depending on the extent of modification by the amine and/or phenyl moieties. We also demonstrate a practical application of such materials in the area of separation science with the separation of a mixture of vitamins on modified thin-layer chromatography (TLC) plates. We show that the retention factors (R_f) and the degree of separation are highly dependent on the manner and extent to which the TLC plates are modified.

Material and Methods:

Reagents: 3-Aminopropyltriethoxysilane (98%, APTEOS) and phenyltrimethoxysilane (97%, PTMOS) were purchased from Alfa Aesar. Vitamins B1 and C were purchased from Sigma Aldrich while vitamins D3 and K1 were purchased from Alfa Aesar. Ethanol (200 proof, USP/ACS grade) was purchased from Pharmaco-Aaper. Merck Silica gel 60 F₂₅₄ TLC plates (20 x 20 cm) were used. All plates were from the same batch. According to the manufacture, the

TLC plates have a specific surface area of 480-540 m²/g, a pore volume of 0.74-0.84 mL/g, and a layer thickness of 210-270 μm.

Fabrication of stationary phases: TLC plates were cut into pieces of 8 cm x ~1.8 cm and soaked in ethanol to first remove impurities that adhered to the surface and then dried in air. Once dried, they were placed in an oven at 110 °C for 10 min to drive off excess adsorbed water and then cooled to room temperature. The plates were used without modification, with uniform modification by amine (A), phenyl (P), or both (A+P, P+A) and with single gradients of A or P and dual gradients (A+P aligned, A+P opposed, P+A aligned and P+A opposed). The uniformly modified substrate was prepared by soaking the activated TLC plates in either APTEOS solution for less than a minute or in PTMOS solution for 12 min. A+P uniform TLC plates were fabricated by first soaking the activated TLC plates in amine solution (for less than a minute) followed by the phenyl solution (for 12 min) or in the reverse order for P+A uniform plates. The composition of the APTEOS solution was ethanol: APTEOS: distilled water (20:0.1:0.1 v/v/v) while the PTMOS solution was ethanol: PTMOS: 0.01 M HCl: 0.02 M NaOH (15: 3: 1: 1 v/v/v/v). The PTMOS solutions were prepared by first hydrolyzing PTMOS in 0.01 M HCl followed by addition of 0.02 M NaOH after 15 min. The solution was further stirred for another 15 min.

The multi-component gradients were prepared using controlled rate infusion (CRI).[23] In this method, the activated TLC plate was placed vertically in a 25 mL graduated cylinder and either the APTEOS or PTMOS solution was infused at a rate of 20.48 mL/min or 1.38 mL/min, respectively, using a syringe pump (New era NE-1000). Infusion was stopped ~ 0.5 cm from the top of the plate. All the TLC plates modified with the APTEOS solution were immediately rinsed with ethanol and air dried while those modified with PTMOS solution were directly air dried and placed in an oven for 1 hr at 160 °C to form a more stable coating. Both the steps were sequentially applied to produce the multi-component TLC plates. For aligned gradients, the TLC plates were placed in the graduated cylinder in the same direction each time, while for opposed gradients, the slide was inverted between depositions. It is important to note that the concentration of PTMOS in solution was significantly higher and the infusion time significantly longer than for APTEOS, leading to a much higher degree of modification of the TLC plates by phenyl in contrast to amine. **Figure 1** depicts a cartoon of the fabrication of amine-phenyl multi-component gradients.

Characterization of stationary phases: The extent of amine and phenyl modification across the length of the TLC plates (gradient profile) was assessed via X-ray photoelectron spectroscopy and by diffuse reflectance spectroscopy, respectively. XPS was performed with a ThermoFisher ESCA lab 250 imaging X-ray photoelectron spectrometer (Al KR (1486.68 eV)), using a 500 μm spot size, 50 eV pass energy, 0.1 eV step size. The TLC plate was first cut into five 1.5 cm length pieces, the powder scrapped off each piece, and mixed to form a uniform sample. Approximately 1 mg of powder was then firmly pressed on conducting tape on a 5 cm $\times$ 2 cm sample holder and XPS spectra were collected at three different points on each powdered sample. The mean and standard deviation are reported. Charge corrections of the peaks were done using the C1s peak at 284.6 eV. Area of the peaks under N1s was calculated using commercially available software (Avantage Version 4.4). For the phenyl modified TLC plate, the plate was cut into 5 pieces of ~ 1.8 cm $\times$ 1.5 cm along its length starting at ~ 0.5 cm from the top and each piece examined using a diffuse reflectance spectrophotometer (Agilent technologies, Cary Series 6000i UV-Vis-NIR). The reflectance relative to that of an unmodified TLC plate was converted using the Kubelka-Munk (K-M) equation and the K-M function plotted against wavelength. The average value at 260 nm obtained from three separate TLC plates was used to create the profile plots.

Separations: The separations of vitamins B1, C, D3, and K1 were carried out in triplicate on unmodified, amine and phenyl uniformly modified and gradient TLC plates. The TLC plates were spotted with 3 spots, which consisted of vitamins B1 (20 mg/mL) and C (10 mg/mL) in methanol, vitamins D3 (2 mg/mL) and K1 (0.6 mg/mL) in methanol, and a mixture of all four from left to right, respectively. The TLC plates were spotted 1 cm above the bottom edge and placed in a chamber pre-saturated with freshly prepared mobile phase consisting of chloroform: ethanol: water: glacial acetic acid (2: 8: 2: 0.5 v/v/v/v). The solvent front was allowed to travel 7 cm on each TLC plate. Once the mobile phase reached the predetermined mark, the TLC plates were removed from the chamber and air dried. The TLC plates were visualized under UV radiation at 254 nm and the retention factor (R_f) calculated by $R_f = \frac{\text{distance travelled by analyte}}{\text{distance travelled by the solvent front}}$. The average R_f factor and standard deviation obtained from chromatograms on three TLC plates separately prepared on a given day are reported.

Results and discussion

Fabrication of amine and phenyl modified TLC plates

In this work, amine and phenyl silane gradients were fabricated on silica TLC plates using CRI.[23-25] In this method, a solution containing either the amine or the phenyl silane is infused at a fixed rate into a graduated cylinder holding the vertically aligned TLC plate. The condensation of the hydrolyzed amine precursors to surface silanol groups proceeds fairly rapidly because of the self-catalyzing ability of the amine group.[16,24,32] An external catalyst is not necessary.[16] For the deposition of the phenylsilane, the condensation reaction with surface silanol groups and/or to each other is notably slower. As a result, an external catalyst (OH⁻) was added to the sol after first hydrolyzing PTMOS with acid. The immobilized amine could also act as a catalyst particularly when it is deposited first, as in the case of the A+P plates. Heating the modified plate at 160 °C was necessary to attach the phenyl moieties to the TLC plate and prevent them from being removed during rinsing or separation.

During infusion, the bottom of the TLC plate is exposed to the silane for a longer period of time than the top; hence the surface density of the organic group decreases from bottom to top. In contrast, the uniformly modified TLC plates should have a uniform coverage of amine (uniform A), phenyl (uniform P) or both amine and phenyl (uniform A+P, uniform P+A) while dual-component gradients should exhibit a surface gradient in amine (A) or phenyl (P) from top to bottom. For clarity, gradients prepared from either amine- or phenylalkoxysilanes are termed 'single-component gradients' and those prepared from both precursors in a sequential fashion are referred to as 'multi-component gradients'. In all cases, however, surface silanol groups will also be present, and their surface concentration will also vary from bottom to top depending on the extent of modification.

For multi-component gradients, the extent of modification will depend on the order of infusion (A+P or P+A) and the direction of the gradients (aligned or opposed). The expected dependence on order arises because the amine can act as a catalyst and promote the condensation of hydrolyzed organosilanes (e.g., phenylalkoxysilane) to surface silanol groups on the TLC plate, leading to greater coverage of phenyl on the A+P uniform and gradient plates vs P+A modified plates. Steric factors may also contribute. The presence of phenyl groups may limit the accessibility of hydrolyzed precursors to surface silanol groups resulting in lower coverage of amine groups in the P+A vs A+P uniformly modified and gradient plates. Likewise, for the

multi-component gradient plates, direction is expected to matter as the extent of modification depends on the surface density of functional groups (both NH_2 and/or Si-OH), which varies across the length of the substrate. A different extent of modification is thus anticipated when the gradients are aligned vs opposed.

Characterization of amine and phenyl gradient and uniformly modified TLC plates

The presence and extent of amine modification on TLC plates was evaluated using X-ray photoelectron spectroscopy. In this experiment, the TLC plates were cut into five pieces each 1.5 cm in length starting 0.5 cm from the end and the powder scraped off each piece. Approximately 1 mg of this powder was carefully and securely pressed on conducting tape and XPS data collected from three locations on each powdered sample for a total of 15 spectra per gradient plate.

Figure 2A shows the N1s XPS spectra acquired from each powdered sample removed from an aligned multicomponent gradient (P+A). The peak appears at ~400 eV, corresponding to the binding energy of the free amine.[23] Consistent with the formation of a gradient in amine, the peak increases in intensity from top to bottom. Likewise, the presence and extent of modification by phenyl groups was evaluated by diffuse reflectance spectroscopy in the UV region at 260 nm, as shown in **Figure 2B**. Consistent with the presence of a gradient in phenyl, the K-M function increases from top to bottom.

To best see how the extent of modification changes along the length of the TLC plate, profile plots were constructed from spectra such as those shown in **Figure 2**. The shape of the gradient profile depends on factors such as reaction kinetics, silane concentrations, infusion rate, order of infusion, and the presence and extent to which the plate has already been modified.[23-25] As expected, for the unmodified, as-received plates, negligible signal was observed (data not shown). Likewise, as expected, both the uniformly modified one-component and two-component TLC plates depict a near constant intensity/absorbance along their length, **Figure 3**. The magnitude of the intensity/absorbance depends on the number of components the plates are modified with as well as the order of modification for the multi-component plates.

For the A+P uniformly modified TLC plate, the surface silanol groups react first with the aminosilane followed by the phenylsilane. In this case, the surface immobilized amine can act as

a catalyst and enhance the condensation of phenylsilane precursors to the surface silanol groups, resulting in a slightly higher K-M function for phenyl at 260 nm (~ 4.5) on the multi-component plate (**Figure 3B**) compared to the phenyl-only TLC plate (~ 4 ; **Figure 3A**). For the P+A uniform plate (**Figure 3C**), the K-M function at 260 nm is slightly lower than that observed on the phenyl-only plate (**Figure 3A**), and can likely be attributed to a change in the reflectivity of surface with the addition of the amine.

For the TLC plates modified with amine groups, the area under the N1s peak is also constant with distance. The average N1s peak area on each plate was obtained by averaging the results from 15 spectra and is similar for the three surfaces: 3600 ± 110 , 3400 ± 110 , 3400 ± 98 CPS eV for uniform A, uniform P+A, and uniform A+P, respectively. On a uniformly modified phenyl plate, the N1s area was 690 ± 110 CPS eV, significantly smaller than that observed on the amine modified plates and attributed to adventitious nitrogen.[23]

In **Figures 4** and **5**, the profiles of the different gradient plates are shown. As expected, in all cases, the surfaces show gradually increasing concentrations with distance from top (0 cm) to bottom. The chemistry in this case is more complicated than that observed for the uniformly modified plates because of the continuous change in the surface concentration of silanes with distance, along with the change in infusion order (e.g., A+P or P+A) and direction (e.g., opposed vs aligned).

Figure 4 shows profile plots for both the single-component amine and phenyl gradients on the TLC plate and the aligned A+P and P+A multi-component gradient plates, where both amine and phenyl surface concentrations change from top to bottom. As can be seen for the single- and multi-component plates, the area underneath the N1s peak gradually increases with distance, consistent with a shallow amine gradient on the surface. There is not a lot of amine on the surface of the plates because its concentration is low in the deposition solution and the infusion time is fast (~ 1 min). For the single-component amine gradient, the area under the N1s peak at the high amine end (3500 ± 140 CPS eV, $N=6$) is similar to that observed for the uniformly modified plate (3600 ± 110 CPS eV, $N=3$), which is to be expected. It can also be observed that the area under the N1s peaks is slightly larger for the A+P plates vs the P+A plates at all positions along the plate. The signal is believed to be lower for the P+A gradients because of the reduction in surface silanol groups available to react with the aminosilane when phenyl is

deposited first. A small difference can also be noted in the uniformly modified plates (P+A vs A+P).

For phenyl, a slightly higher maximum signal is found on the A+P plate (~4.1) compared to the P+A (~3.8), consistent with the surface bound amine catalyzing the condensation of the phenylsilane precursors and/or a change in reflectivity of the surface when the layer of amine is on top, respectively. The shape of the phenyl gradient also depends on the order of modification. For the gradient A+P plate, the profile changes gradually with distance as compared to the P+A or single-component P gradients, which appear steeper. This difference is attributed to the presence of a gradient in catalyst (e.g., the amine group) along its length for the A+P plate but not for the P+A plates.

Figure 5 shows the opposed amine and phenyl multi-component gradients whose surface coverage change in opposite directions. The N1s signal is high when the phenyl signal is low and vice versa, a characteristic of an opposed gradient. Again, it can be seen that the N1s signal gradually increases with distance as expected for a shallow amine gradient. With regard to phenyl, the peak (or maximum) K-M function observed on the A+P gradient is similar to that observed on the P+A gradient. However, its shape is more gradual and comparable to that observed for the aligned A+P and single-component phenyl gradient, consistent with the presence of a gradient in amine (catalyst) on the surface as described earlier.

Separation of water- and fat-soluble vitamins

One application where multi-component gradients would be valuable is in the separation of a mixture that contains analytes with different hydrophobicities, an example of which is a mixture of water- and fat-soluble vitamins such as those shown in **Figure 6**. All these vitamins are essential for the body and play a role in human health.[33-36] The water-soluble vitamins (B1, C) have numerous oxygen and/or nitrogen atoms in their structures, which are capable of forming hydrogen bonds with tethered amine groups and surface silanol groups. The aromatic rings in their structure can exhibit $\pi - \pi$ interactions with phenyl-modified phases. The fat-soluble vitamins (D3, K1) lack heteroatoms and have long alkyl chains in addition to aromatic and aliphatic rings, thus making them more hydrophobic.

The separation of these four compounds will depend on the extent to which the TLC plates have been modified by the amine and phenyl groups, as defined by the factors described

earlier, most notably the order of infusion and the presence and extent to which the plate has been modified. Modification of the TLC plate with amine first will lead to a greater amount of phenyl on the plate and change the gradient profile due to the catalytic ability of the amine, as described earlier. In addition, the separation will also depend upon at which end of the plate the analyte mixture is spotted. For example, on an aligned gradient, if the vitamin mixture is spotted on the low-amine low-phenyl end, as it moves up the plate, the vitamins will face increasing surface concentration of amine and phenyl, but the reverse (e.g., decreasing surface coverage) when spotted on the high-amine high-phenyl end. It should then be possible to tweak retention factors and improve/diminish separation through subtle changes in degree of modification via the use of multi-gradient stationary phases.

To begin, we looked at the separation of the four vitamins on control TLC plates. In **Figure 7**, the separation of the four vitamins on the unmodified and single component amine- and phenyl-modified plates are shown. The phenyl-modified plate appears uniformly dark under UV radiation because phenyl diminishes the intensity of fluorescent indicator present in the TLC powder. Because the dark spots can be hard to see against the dark background, we adjusted the brightness and contrast of the image and circled the locations where the vitamin(s) appear on the plate. As can be seen in **Figure 7** in the column marked “All”, the vitamins are not well separated on either the as-received unmodified silica gel TLC plate or the uniform amine-modified plate, but do separate on a uniform phenyl plate. On the unmodified plate and uniform amine plate, vitamins B1 and C separate but vitamins D3 and K1 do not. The latter two more hydrophobic vitamins appear together near the solvent front, indicating that they have minimal interactions with the polar stationary phase. Upon modification with phenyl, vitamins D3 and K1 no longer travel together and thus separate. Since vitamin K1 has a longer aliphatic tail compared to vitamin D3, it will have stronger interactions with the phenyl groups and is more retained on the stationary phase. Also, modification of the TLC plate with phenyl reduces the interactions of the more hydrophilic vitamins (B1 and C) to the now more hydrophobic stationary phase and thus their retention factors are larger than that obtained on the unmodified or amine-modified plates. Collectively these results indicate that the vitamins are very sensitive to modification of the stationary phase with phenyl groups. Amine, being much lower in concentration than phenyl, does not have as strong of an effect on separation as phenyl. Both modifications,

however, do influence the number of residual silanol groups present on the surface, which play a role in the separation.

In **Figure 8**, photographs of P+A uniform and multi-component gradients on TLC plates are shown. For comparison, results from a phenyl gradient with the vitamin mixture spotted on the high phenyl end are also shown. The R_f values for the different vitamins are strongly influenced by (1) the end at which they are spotted and (2) whether the multi-component gradient is aligned or opposed. These values are reported in **Table I**. Consistent with that observed on the control plates, vitamin C travels near the solvent front while vitamin B1 interacts most strongly with the stationary phase and has the smallest R_f . The interaction of the basic amine groups on vitamin B1 with acidic silica sites leads to streaking, which is evident on both modified and unmodified plates. It may be possible to reduce some of this streaking via changes in the mobile phase, but this is beyond the goals of this study. Vitamins D3 and K1 fall near the middle to upper end of the plate and their R_f values can be tweaked by changing the degree of modification in gradient profile (aligned vs opposed) and by changing the spotting end.

The R_f values for vitamins D3 and K1 are the largest when spotted on the low phenyl-high amine (LPHA) end of an opposed gradient. These vitamins clearly interact most strongly with a more hydrophobic stationary phase consistent with that observed on the control plates. In this case, they move with the mobile phase until the concentration of phenyl is high enough near the upper half of the plate where they begin to spend more time in the stationary phase. These two vitamins (D3 and K1) do not fully separate on this plate, and they also now appear with vitamin C. Vitamin B1 is significantly retained on the TLC plate due to interactions with the amine groups and surface silanol groups, which are expected to be higher on the end of the plate that is least modified and least hydrophobic (e.g., LPHA and LPLA).

Very different results are observed when the vitamins are spotted on the HPLA end of an opposed gradient. The retention factor for vitamin B1, is larger on HPLA vs LPHA. The increased surface concentration of phenyl and/or decreased surface concentration of silanol groups reduces the interaction of vitamin B1 with the stationary phase. Likewise, the R_f factors for vitamins D3 and K1 are also very different on the two plates. The initial presence of a relatively high concentration phenyl at the spotting end (for HPLA) slows down the movement of the two vitamins along the plate, thus they become separated. The mixture of four vitamins is

separated. The separation observed on the HPLA multi-component gradient is similar to that noted on a single-component phenyl gradient when spotted on the high phenyl end.

For the two aligned gradients, spotting end is also important and influences separation. When the vitamin mixture is spotted at the high phenyl – high amine end (HPHA), the retention factors for vitamins B1, D3, and K1 are different enough from that observed on LPLA such that separation is achieved. For LPLA, the presence of surface silanol groups at the spotting end aids in the retention of vitamins B1 and C while reducing retention for the more hydrophobic vitamins. Thus, significant overlap is observed for vitamins C and D3. For HPHA, as observed for the opposed gradients, the initial presence of a relatively high amount of phenyl on the spotting end (for HPHA) aids in the separation of the more hydrophobic vitamins (C and D3). The separation on HPHA was very similar to that observed on the uniform P+A plate with the exception that vitamin B1 is retained more on the aligned gradient plate. Again separation is achieved on the gradient plate but not the uniformly modified plate. Retention factors are also very different for opposed gradients when they are spotted on different ends (LPHA vs HPLA) such that separation of the four vitamins is achieved on HPLA but not when spotted on the low phenyl/high amine end (e.g., LPHA).

The retention factors and thus the separation of the four vitamins can further be tweaked by independently changing the gradient direction of the two modifiers: aligned vs opposed. For example, the retention factors and the quality of the separation of the four vitamins are very different on the aligned gradient plates vs opposed (e.g., HPHA vs LPHA or LPLA vs HPLA). When spotted on the high phenyl-high amine (HPHA) end of the aligned gradient plate, for example, the presence of the high amount of phenyl counteracts the higher amount of surface-bound amine and vitamins D3 and K1 are retained more on the stationary phase and thus have a smaller R_f than when spotted on the LPHA end of the aligned gradient. For both the aligned gradients, separation of the four vitamins was not achieved while separation was possible on one of the opposed gradients (HPLA).

Figure 9 shows separations of the vitamin mixtures on A+P uniform and aligned and opposed gradients. For comparison, results from an amine gradient with the vitamin mixture spotted on the high amine end are also shown. Consistent with that observed for the P+A gradients, both the spotting end and infusion direction (aligned vs opposed) influence the separation of the four vitamins. When spotted on the side that is less modified (LALP), in

contrast to the HAHP end of the aligned gradient plate, for example, vitamin B1 is more strongly retained and vitamins D3, K1, and C appear near the top of the plate in close proximity to each other. The importance of infusion direction can be also noted in the gradient plates, e.g., HAHP vs HALP or LALP vs LAHP, most notably by differences in retention of vitamin B1 on the different gradient plates. Due to the presence of more surface silanol groups and a lower phenyl content, the retention factor for vitamin B1 is much lower when spotted on LALP vs LAHP. There are also subtle differences in the R_f factors for the more hydrophobic vitamins on HAHP vs HALP, which leads to a slightly better overall separation when the mixture is spotted on HAHP particularly in terms of the overlap between vitamins C and D3. On the uniform A+P plate, the spots observed for vitamins B1 and K1 overlap and diminish the separation of the four vitamins. Because the R_f for vitamin B1 is smaller while vitamin K1 is larger on the multi-component HAHP gradient, separation of all four vitamins in the mixture was possible.

The separation of the mixture of vitamins also depends on the infusion order (P+A) vs (A+P), though this dependence is perhaps not as strong and obvious as the other factors described above. As evident in the diffuse reflectance data, the phenyl gradient profiles are slightly different in that the gradient shape is more gradual for A+P relative to P+A. Also, the amount of amine on the surface of P+A is slightly lower than A+P as observed in the XPS data. Upon comparison of **Figure 8** (P+A) to **Figure 9** (A+P), it can be seen that the order of modification is most important for LAHP vs HPLA and HPHA vs HAHP plates. The retention factors for vitamins D3 and K1 are slightly higher and vitamin B1 slightly lower on LAHP vs HPLA opposed gradients. The net result is that vitamin C and D3 overlap diminishing the separation of the four vitamins on LAHP but not HPLA. The R_f values and pattern of separation of the four vitamins on the uniform A+P plate and uniform P+A plate and the low phenyl plates (LALP vs LPLA) are similar and less dependent on order of modification compared to the high phenyl multicomponent gradients.

Conclusion

Multi-component gradients for thin layer chromatography (TLC) have been successfully prepared using silane chemistry and controlled rate infusion and their applicability to the separation of a vitamin mixture has been demonstrated. This work focuses specifically on multi-component gradients prepared from amine and phenyl groups, but it should be recognized

that such gradients also contain residual SiOH groups due to incomplete condensation. The presence of a gradient in phenyl and amine on TLC plates has been confirmed using a combination of diffuse reflectance and X-ray photoelectron spectroscopy, respectively. The separation of a vitamin mixture containing vitamins C, B1, D3, and K1 was studied on 15 different uniformly modified and single- and multi-component gradient TLC plates. Both gradient direction (opposed vs aligned) and the end of the plate at which the analyte mixture was spotted influenced the separation. While multi-component gradients are a little more complex to make and characterize than single-component gradients, they do provide an avenue for cooperative interactions to take place between an analyte and multiple surface functionalities, which are undoubtedly important in governing analyte retention in chemical separations. This work shows that subtle changes in the gradient profile and hence surface chemistry lead to significant differences in retention factors (R_f) of the individual vitamins, and ultimately, the separation of the vitamin mixture. Although this work has focused on multi-component gradients prepared from phenyl and amine as proof-of-principle, the procedure can be extended to other organoalkoxysilanes and analyte mixtures. Future work will explore these opportunities.

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Figure Captions

Figure 1. Fabrication of multi-component gradient TLC plates by controlled-rate infusion (CRI)

Figure 2. (A) N1s XPS spectra collected from five sections along the length of a multi-component gradient plate from the top (red line) to the bottom of the gradient (green line). (B) Diffuse reflectance converted to K-M function for five sections of a gradient phenyl plate from top (purple line) to bottom (black line).

Figure 3. Area profile of amine and phenyl plots on (A) uniformly modified single amine and single phenyl TLC plates; (B) uniformly modified Amine + Phenyl TLC plates; and (C) uniformly modified Phenyl + Amine TLC plates. The average and standard deviation of three measurements acquired on a single powdered sample are shown.

Figure 4. Profile of (A) amine and phenyl single gradients and (B, C) multi-component aligned amine + phenyl (A+P) (B) and phenyl + amine (P+A) (C) gradients on TLC plates. The average and standard deviation of three measurements from two plates made on different days are shown.

Figure 5. Profile of multi-component opposed gradients (A) amine + phenyl (A+P) and (B) phenyl + amine (P+A) on TLC plates. The average and standard deviation of three measurements obtained from one (B) or two plates made on different days (C) are shown.

Figure 6. Water-soluble vitamins: B1 and C; Fat-soluble vitamins: D3 and K1

Figure 7. Photographs of TLC plates under UV radiation when spotted on unmodified and uniformly modified amine and phenyl TLC plates (blue: B1, yellow: C, red: K1, orange: D3).

Figure 8. Photographs of TLC plates under UV radiation when spotted at different ends of **P+A** aligned and opposed gradient TLC plates (blue: B1, yellow: C, red: K1, orange: D3).

Figure 9: Photographs of TLC plates under UV radiation when spotted at different ends of **A+P** aligned and opposed gradient TLC plates (blue: B1, yellow: C, red: K1, orange: D3).

Figure 1

FIGURES

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

B1

C

D3

K1

Figure 7

Figure 8

Figure 9

Table I. Average Rf values and their standard deviations (N=3).

Vitamins	B1	K1	D3	C
NC	0.07±0.01	0.89±0.02	0.89±0.02	0.60±0.02
Uniform A	0.11±0.01	0.90±0.01	0.90±0.01	0.56±0.02
A grad H	0.09±0.01	0.92±0.01	0.92±0.01	0.58±0.02
A grad L	0.09±0.01	0.87±0.03	0.87±0.03	0.60±0.01
Uniform P	0.30±0.04	0.55±0.03	0.67±0.04	0.79±0.02
P grad H	0.32±0.01	0.53±0.03	0.68±0.01	0.79±0.01
P grad L	0.19±0.01	0.72±0.01	0.72±0.01	0.72±0.01
A+P uniform	0.43±0.02	0.48±0.04	0.65±0.02	0.83±0.03
P+A uniform	0.51±0.03	0.52±0.03	0.67±0.03	0.80±0.01
A+P alig. HAHP	0.44±0.02	0.55±0.03	0.68±0.03	0.79±0.03
A+P alig. LALP	0.17±0.03	0.60±0.02	0.69±0.01	0.77±0.01
P+A alig. HPHA	0.40±0.02	0.48±0.01	0.64±0.02	0.82±0.02
P+A alig. LPLA	0.18±0.02	0.63±0.01	0.73±0.01	0.73±0.01
A+P opp. HALP	0.18±0.02	0.59±0.01	0.70±0.01	0.75±0.01
A+P opp. LAHP	0.25±0.03	0.61±0.04	0.76±0.04	0.75±0.02
P+A opp. LPHA	0.20±0.02	0.73±0.01	0.77±0.01	0.77±0.01
P+A opp. HPLA	0.40±0.07	0.52±0.03	0.68±0.02	0.79±0.01