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Preparation and characterization of stationary phase gradients on C8 liquid chromatography columns

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ABSTRACT

Continuous C8 stationary phase gradients are created on commercial Waters Symmetry Shield RP8 columns by strategically cleaving the C8 moieties in a time-dependent fashion. The method relies on the controlled infusion of a trifluoroacetic acid/water/acetonitrile solution through the column to cleave the organic functionality (e.g., C8) from the siloxane framework. The bond cleavage solution is reactive enough to cleave the functional groups, even with polar groups embedded within the stationary phase to protect the silica. Both the longitudinal and radial heterogeneity were evaluated by extruding the silica powder into polyethylene tubing and evaluating the percent carbon content in the different sections using thermogravimetric analysis (TGA). TGA analysis shows the presence of a stationary phase gradient in the longitudinal direction but not in the radial direction. Two different gradient profiles were formed with good reproducibility by modifying the infusion method: one exhibited an 'S'-shaped gradient while the other exhibited a steep exponential-like gradient. The gradients were characterized chromatographically using test mixtures, and the results showed varied retention characteristics and an enhanced ability to resolve nicotine analytes.

1. Introduction

Gradient separations have been a mainstay of liquid chromatography (LC) almost from the inception of the technique [1,2]. The use of a mobile phase gradient allows for the separation of complex samples that would otherwise not be possible under isocratic conditions. Also, mobile phase gradients can be used to shorten run times and separate multiple peaks more effectively. However, the use of gradient mobile phases is not the only approach that can improve the resolving power of a chromatographic system. Another approach involves the use of stationary phase gradients (SPGs) [2] either formed in a single column housing [3–6] or by serially connecting different columns having different stationary phase functionality [7,8]. The former approach has the advantage of being similar to a mixed-mode column allowing for varied functionalization in a single-column housing but the disadvantage of being challenging to make. The latter approach has the advantage of being easy to produce but the disadvantage of increased extra-column volume and longer elution times depending on how many individual columns are connected [2].

The focus of the present work is on the fabrication of a continuous, mixed-mode gradient stationary phase starting with a commercial, uniformly modified stationary phase, the Waters Symmetry Shield RP8 phase. Such SPGs contain two or more different functionalizations, such as C8 and bare silica, whose concentrations change across the length of the column [2]. The stationary phase packings thus formed are akin to mixed mode columns [9,10] except for the placement of the different functionalities that vary along the length of the column. Such variations potentially allow for varied retention characteristics and improved selectivity to resolve coeluting analytes [5,11,12]. A continuous gradient stationary phase can improve separation performance, particularly when coupled with a mobile phase gradient [11]. It has been theorized that these gradients can allow for selectivities that cannot be produced by mobile phase gradients alone [5,6,11,13].

Continuous, mixed-mode SPGs can be difficult to fabricate but recent work has shown they can be produced using different approaches [14,15]. One such method involves strategically bonding an organically functionalized silane to the stationary phase on a packed silica LC column in a gradient fashion [16]. This method involves pumping reactive organic functionalized silanes (e.g., hydrolyzed phenylbutyltrimethoxysilane) into prepacked silica columns, where they react with surface silanol groups in a time- or concentration-dependent process to form a gradient stationary phase [3,16]. Additionally other

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media have been used as the silica support such as thin-layer chromatography plates [17,18] and monolith columns [4] using (3-aminopropyl)triethoxysilane as the starting precursor.

Another approach involves removing the organic functional groups from a bonded-phase particle-packed column in such a fashion as to create a gradient stationary phase [5]. This method begins with the slow infusion of trifluoroacetic acid into the column at an elevated temperature to remove the functional groups in a gradient fashion through bond cleavage [19]. By carefully controlling the removal process, an SPG with both the normal phase character of silica and the reversed-phase character of the functionalized silica can be produced. In recent work by Cain et al., a SPG on commercial C18 columns was made using this approach [5]. The resulting removal of the organic functional groups creates silanols [20] and produces a stationary phase that can interact with an analyte species via a normal phase or HILIC (hydrophilic interaction chromatography) separation mechanism, as well as a reversed phase mechanism. The formation of multicomponent gradients on C18 columns has also been reported using a combination of bond-breaking and bond-forming processes [3]. Such gradient columns have been shown to yield new selectivities [2,3] and provided unique separation characteristics that differed from uniform standard C18 columns [5].

In the present work, we seek to better understand how to tailor the fabrication of silica-based SPGs using the bond-breaking strategy and to study its applicability for the formation of a C8 gradient stationary phase, starting with a commercial, carbamate-protected C8 column. Previous work focused only on C18 columns [3,5,6]. The goal is to not only characterize the longitudinal heterogeneity of the column (i.e., the gradient profile) but also evaluate radial heterogeneity/homogeneity, which is currently unknown for these columns. In addition, we show how the infusion parameters can be changed to form SPGs with different profiles and with good reproducibility. The column chosen for this work is the Waters Symmetry Shield RP8 phase, which includes a carbamate moiety between the silica and the long-chain hydrocarbon to limit the negative effects of silanols on the retention of basic analytes [21]. Both uniformly modified columns and SPG columns are evaluated chromatographically to understand the retention characteristics as they pertain to the separation of a test mixture of common neurotransmitters and amino acids. Finally, we show the separation of nicotine derivatives using these new C8 SPGs under HILIC conditions.

2. Experimental

2.1. Chemicals and columns

Isopropanol (IPA, 99.9 %, ACS grade), chloroform, and toluene were purchased from Fisher Scientific. Acetonitrile (ACN, UV grade), methanol (HPLC grade), sodium phosphate monobasic anhydrous, and sodium phosphate dibasic anhydrous were purchased from VWR. Ammonium formate (99 %) and formic acid (98 %) were purchased from Honeywell Fluka. Trifluoroacetic acid (TFA, 99 %) was obtained from Acros Organic. Uracil (98 %), l-tyrosine, l-tryptophan (98 %), tryptamine (98 %), and anthranilic acid (98 %) were acquired from Sigma-Aldrich. l-phenylalanine (99 %) was acquired from J. T. Baker. Water (18.2 MΩ cm) was attained from a Milli-Q system (Millipore). Cotinine, nicotine, anabasine, and trans-3-hydroxycotinine were acquired from Cerilliant. Anatabine was acquired from Toronto Research Chemicals. Waters Symmetry Shield RP8 columns [22] 4.6 × 150 mm LC columns were purchased from Ebay.

2.2. Hardware

All chromatographic testing was performed on an Agilent 1260 Infinity instrument equipped with a quaternary pump, autosampler, column oven at 40 °C and UV–Vis photodiode array detector (DAD), with spectra collected from 190 to 400 nm. All data was collected using ChemStation A.02.13 (Agilent Technologies, Santa Clara, CA). The data were exported using ACD/Spectrus Processor 2015, and an in-house multivariate curve resolution alternating least squares software (MCR-ALS) within MATLAB version R2021a (Mathworks, Natick, MA) was used to resolve overlapped peaks [23–25]. TGA analysis was performed on a TA Instruments TGA500 as well as a TA Instruments TGA Q5000. Data were processed in both TRIOS (TA Instruments, New Castle, DE) and Microsoft Excel. Functional group removal to create the gradient stationary phase was performed using an Agilent 1100 quaternary pump and column oven. Column extrusion was performed using a Shimadzu LC-20AB binary pump (Kyoto, Japan).

2.3. Column cleaning

All columns used in these studies were previously used and were obtained from eBay from a variety of vendors. Prior to conducting any testing, all columns were thoroughly cleaned to recondition the columns. This column cleaning was done using a dedicated LC pump (Shimadzu LC-20AB) that was not connected to a detector. The cleaning method entailed flushing the column with 10 column volumes (CV) of 50:50 MeOH/H_2O; then with 10 CV of MeOH; and then 20 CV of acetonitrile (ACN); and then with 10 CV of isopropanol (IPA) and finally with 20 CV of ACN. Toluene and uracil were used to test the columns to ensure that they had reasonable efficiencies before further experimentation. These results are shown in **Table S1**. The columns were then stored in ACN until use.

2.4. Pycnometry

For the estimation of the dead volume of the columns, pycnometry was used [26]. Columns were weighed, filled with chloroform using an LC pump, weighed, and the process was repeated with acetonitrile. The difference in weights allows for an estimation of dead volume based on the difference in density. The volume was then estimated using Eq. (1).

$$V_m = \frac{W_1 - W_2}{\rho_1 - \rho_2} \tag{1}$$

This value was then used to estimate column volumes (CV) but for the calculation of retention factors, uracil was used.

2.5. Chromatographic testing

The columns were characterized chromatographically using test mixtures before and after the C8 functionalities were removed from the column. These results are shown in **Table S1** for the individual columns studied herein. The peaks were resolved using MCR-ALS, software that capitalizes on the differences between the spectra of the analytes to resolve peaks [23–25]. It allows for complex test mixtures with coeluting peaks to be separated and characterized. For the primary testing of the columns, a test mixture containing neurotransmitters and amino acids (tyrosine, phenylalanine, tryptophan, tryptamine, and anthranilic acid) was used. Structures of the constituents in this test mix can be seen in the supplementary information in **Figure S1.** The mixture included a variety of well-retained and poorly-retained compounds when evaluated in reversed-phase mode LC. The primary characteristics of interest were the retention factor of the compounds (k), peak asymmetry (As), and the plate count (N). The retention factor (k) is given in Eq. (2).

$$k = \frac{t_R - t_M}{t_M} \tag{2}$$

2.6. Creation of the mixed-mode stationary phase gradient

This work explored two different approaches for the removal of the C8 functionality in a gradient fashion: a constant flow approach and a 5-on-5-off approach. All columns were first equilibrated with 27 column

volumes (CVs) of 90/10 H_2O/ACN prior to ligand hydrolysis. Consistent flow SPGs were fabricated by infusing 60 CVs of a trifluoroacetic acid (TFA) solution (50/45/5 TFA/H_2O/ACN) into the column at an elevated temperature. The exothermic nature of the acid mixing with the solvent leads to the formation of bubbles that can disrupt the flow through the column. To avoid this, the TFA solution was prepared ahead and allowed to rest until it returned to room temperature before use. This solution was pumped through the column at 0.5 mL/min with the mobile phase and column thermostatted at 68 °C. After the full 60 CV were pumped through the column, the column was disconnected and reconnected to the system with the flow direction reversed and immediately back-flushed using the cleaning procedure described earlier. The backflush steps were done primarily to halt the reaction and remove the reaction byproducts. After the cleaning procedure, the columns were left in ACN until further characterization was performed. Because of the observed gradient profile on these columns (vide infra), these columns were termed as the 'S' -shaped gradient columns.

The 5-on-5-off gradient SPG columns were created in a similar manner except the system was run at 0.1 mL/min for 5 min. The system then was stopped for 5 min, and then the cycle was repeated over three hours. This allowed for a similar total acid exposure time to the constant flow gradient procedure.

To test the stability of the SPG columns, a stability run of 500 injections of dopamine was used, as reported in earlier work [5,27]. An example of one of these stability experiments can be seen in the supplementary information in **Figure S2**.

2.7. Column extrusion

Upon the completion of the chromatographic characterization, the column packing was extruded, subdivided, and assessed via TGA. The column was solvated by pumping acetonitrile at 1 mL/min for 30 min before extrusion. The end fitting at the front of the column was removed from the column. The other end of the column was attached to the pump, and the packing was extruded with isopropanol, with flow rates ranging from 3 to 7 mL/min to ensure a consistent extrusion rate and limit the mixing of the silica. The flow rate was adjusted depending on the consistency of the silica that was extruded from the column. The flow rate was increased if the extruded silica appeared wetter and looser. The extruded silica was then carefully segmented into 0.5 cm segments for the first 2 cm and then every 1 cm afterward.

To evaluate radial heterogeneity, the extrusion method was altered to address the delicate nature of the extruded phase while sampling. The open end of the column was attached to a straight piece of polyethylene tubing with an inner diameter of 3/16″. The silica was then extruded into this rigid tubing in a manner similar to that described above. The tubing was cut into 0.5 cm segments and the center of the segment was removed using a disposable pipette with a 1 mm diameter for subsequent TGA analysis. The material surrounding this segment was also collected and assessed with TGA. These represent the 'inner' and 'outer' sections of the packing material extruded from the housing.

2.8. TGA analysis

After extrusion, the segments were first dried in a 40 °C oven overnight to remove residual solvent. Before testing, the segments were dried in a 120 °C oven for 1 hour to remove most of the remaining water. The segments were then placed on platinum pans and introduced into the TGA instrument. The weight loss of the stationary phase samples between 150 and 600 °C was used to determine the weight lost, which was then correlated to the% carbon (%C) in the column. This is achieved using Eq. (3) below.

$$\%\ Carbon = (weight\ loss) * \frac{MW\ of\ ligand}{MW\ of\ total\ carbon} * \frac{100}{100 - weight\ lost\ before\ 150^{\circ}C} \quad (3)$$

These results allow us to infer the relative amount of the remaining functional groups on the column after stripping with TFA. All TGA testing was done in duplicate. Previous studies have shown that %C measurements obtained from TGA are similar to those obtained by elemental analysis [28] and the manufacturer's values [29]. An example TGA can be seen in the supplementary information, **Figure S3**.

2.9. General procedure

Because the fabrication of the gradient stationary phase is a destructive process, the procedure always begins with the complete chromatographic testing of the cleaned, uniformly modified RP columns using the different analytes. Next, the gradient stationary phase is prepared via infusion of TFA as described above followed by back flushing, stability testing, and full chromatographic testing of the now modified commercial column. Final assessment and ultimate proof of the formation of the gradient stationary phase involves extruding the column packing and evaluating the %C on each section along its length. It is very important that all chromatographic experiments are completed before the column packing is extruded.

3. Results and discussion

3.1. Method optimization for gradient stationary phase fabrication

In prior work, we introduced a bond-breaking strategy as a promising method to fabricate continuous SPG columns [5]. A schematic of this process is shown in **Figure S4**. This earlier work focused on the proof-of-concept but many unanswered questions remained regarding the radial heterogeneity of the columns, adaptability of the method to other, more stable stationary phases, and the ability to tailor the shape of the gradient thus produced. In this work, we focused on the Waters Symmetry Shield RP8 columns and adapted the procedure to improve SPG formation; we also tested two procedures that produced two varied shapes of the gradient stationary phase.

First, the concentration of acid (TFA) was reduced to provide more water for the acid hydrolysis reaction [30]. This also allows for the reaction to be less dependent on the amount of water retained within the column; whereas previously, the method used pure TFA [5]. Second, instead of infusing the TFA into the column for a set period of time, it was infused for a consistent number of column volumes. This was done to ensure consistent quantities of acid were introduced into the column. The previous method's dependence on total time did not account for variability in flow rate caused by the exothermic reaction dynamics. Further, it was found that allowing the TFA solution to cool to room temperature after the initial dilution helped to reduce the number of bubbles in the system. A significant loss of the flow rate was noted when the mobile phase was still producing bubbles.

3.2. Chromatographic characterization

Initial work focused on studying the chromatographic properties of a test mixture **(Figure S1)**. These test molecules probe different properties of the column and a comparison of the chromatographic behaviors of the different molecules before and after the functional group was removed from the silica surface can provide valuable information. Observing the retention of compounds with differing polarity can hint at the level of functionalization within the column and whether the nonpolar C8 groups were removed via infusion of TFA. A loss in C8 groups should lead to reduced non-polar interactions with the stationary phase and a

change in retention.

The test mixture was analyzed at two pHs, 2.5 and 6 to evaluate the effects of the silanol groups. Silanols exist in different forms on the column (geminal, vicinal, isolated) and their pKa values range from 2–3 to 9–10 depending on the type and its environment, with the average pKa estimated to be around 7 [20,31–33]. Theoretical work suggests that the vicinal and convex geminal sites have low pKa sites whereas the higher pKa sites are mostly concave geminal and isolated sites [32]. At pH 2.5, most exposed silanols are not ionized, limiting ionic interactions. At pH 6 some silanols on the columns remain protonated; however, there will be enhanced retention due to ionic interactions from the sites that are deprotonated at this higher pH.

Fig. 1 shows the chromatograms of the test analytes before and after infusing the TFA/water solution through the column. The corresponding retention factors, based on uracil retention times, and selectivities for adjacent eluting analytes for these chromatograms are provided in Table 1. A decrease in the retention of compounds such as anthranilic acid, which is relatively non-polar, after infusion can be noted. During the stripping or removal of the organic group via acid hydrolysis by TFA [19], the siloxane bond that connects the C8 group to the silica is removed. Thus, the number of C8 groups on the column is reduced and the number of silanol groups increases. This leads to drastically decreased retention for those analytes that interact more strongly with the hydrophobic groups. Additionally, the changes in selectivity for different compound pairs show different patterns (see last column of Table 1). There is a loss of selectivity between anthranilic acid and tryptamine after cleavage of the functionalization as indicated by a negative % change in α. There is also indication of silanol interactions with the positive % change in α seen between tryptamine and tryptophan. These results indicate that the gradient column can provide differences in selectivity that cannot be explained by just a simple decrease in the concentration of C8 ligands on the stationary phase and suggests that the exposed silanols are playing a role in the retention of these analytes.

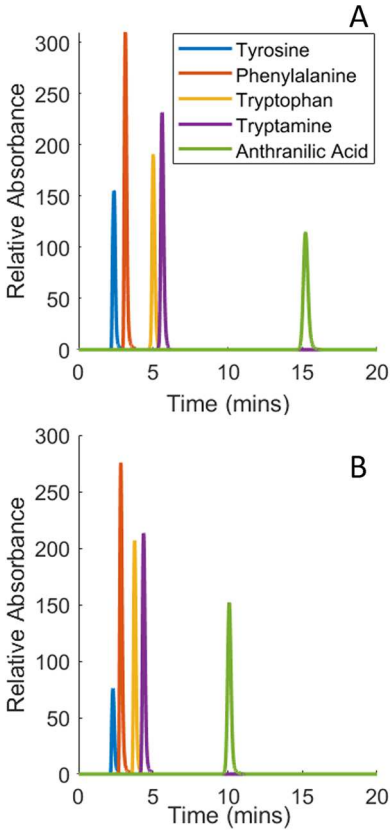

Fig. 1. MCR-ALS resolved peaks of representative chromatograms before (A) and after (B) exposure to TFA. The columns were run isocratic in a 95:5 10 mM phosphate buffer pH 2.50/ACN at 1 mL/min.

The difference in retention of anthranilic acid with pH of the mobile phase is shown in Fig. 2, which shows a chromatogram acquired at two different mobile phase pHs; the corresponding peak metrics (asymmetry (*As*), retention factor (*k*), and plate count (*N*)) are shown in Table 2. Increased tailing is observed at pH 6 due to the exposure of silanols within the column after TFA infusion. It is noteworthy that the *As* factor increases from 1.146 to 1.89, which indicates successful removal of some of the C8 functional groups in the column and replacement with silanols. The retention factor is higher at pH 2.50. It can also be seen that the plate count decreases from 5920 to 2860 upon increasing the pH from 2.50 to 6.00.

The dependence of the retention factor (*k*) on mobile phase composition at pH 2.5 is shown in Fig. 3. In this figure, the relationship between ln *k* vs. φ is depicted for tyrosine and anthranilic acid where *k* is the retention factor (Eq. (1)), and φ is the fraction of organic solvent in the mobile phase. For both columns, a relatively linear relationship between ln *k* vs. φ is noted and is indicative of the retention characteristics of a reversed-phase column [34]. This is to be expected as the silanols will be mostly inactive at pH 2.5. It can also be seen that the retention of each analyte is affected differently by the creation of the gradient. The more hydrophobic anthranilic acid shows a reduction in retention on the gradient column vs the uniform column, consistent with a loss of functionalization on the column. The reverse occurs for tyrosine (a zwitterion) with an enhancement in retention on the gradient column that can be attributed to interaction with the exposed silanol sites. It can also be seen that the effect of mobile phase composition varies as well. For anthranilic acid, the slope of the ln *k* vs. φ is relatively the same for both the uniform and gradient columns. For tyrosine, however, the slope is shallower after gradient formation. This indicates that the tyrosine is now less sensitive to mobile phase changes compared to a uniform column.

3.3. Gradient profile

In order to prove that a gradient was formed after infusion of TFA, the column was sacrificed by extruding the packing material and evaluating small sections along its length using TGA as described in Section 2.8. The weight loss between 150 and 600 °C was used to estimate the % carbon content remaining in each individual section. Fig. 4 shows these results for three different columns. The segments with low % carbon indicate a lower degree of functionalization while high % carbon indicates the opposite. Several features can be noted: (a) All three columns depict an unusual 'S' -shaped profile with the %C increasing during the first few centimeters followed by a steady decrease in %C from 3 to 12 cm; (b) all three gradient columns show lower %C than that stated by the manufacturer of 15% due to the long-term exposure to TFA [19]. It is possible that a small fraction of the C8 groups are cleaved quickly while the remaining part hydrolyzes slowly; (c) consistent gradient profiles were observed for 3 different columns, although the magnitude of the % C varied from column to column. This latter feature is likely due to the columns being purchased used each with a different history.

An interesting feature of these gradient stationary phases is the 'S' -shaped profile, which is different than what was observed in our prior work with C18 SPGs [3,5,6]. In previous work, [5] the gradient shape was a slow asymptotic curve across the length and is pictorially depicted in Fig. 5. Fig. 5**A** depicts a cartoon of an expected gradient column and the observed column. The red color represents segments with more C8 groups, while the blue color represents segments with more silanol groups. In Fig. 5**B** an overlay of an expected shape (orange curve) and the shape obtained in this work (blue curve). It was expected that more C8 groups would be cleaved at the point in the column where TFA is first infused (distance = 0 cm) and fewer at the point furthest away from this inlet. It was also expected that this change would be gradual. What was

Table 1
Retention factors (k) for test mixture analytes on a uniform and gradient column and selectivity (α) relative to the prior eluting analyte for the 'S'-shaped gradient columns.*

Analyte	Uniform C8 column, k	'S'-shaped gradient column, k	Uniform C8 column, α	'S'-shaped gradient column, α	Change in α,%
Tyrosine	0.40 ± 0.05	0.37 ± 0.03	—	—	
Phenylalanine	0.84 ± 0.07	0.68 ± 0.03	2.10 ± 0.07	1.82 ± 0.05	−13 ± 4
Tryptophan	1.9 ± 0.2	1.21 ± 0.07	2.26 ± 0.09	1.75 ± 0.04	−22 ± 5
Tryptamine	2.1 ± 0.2	1.54 ± 0.04	1.19 ± 0.01	1.32 ± 0.05	11 ± 4
Anthranilic acid	8.0 ± 0.4	4.93 ± 0.08	3.7 ± 0.2	3.22 ± 0.04	−12 ± 7

* The average values are obtained from columns 1A, 1B and 1C shown in Table S1.

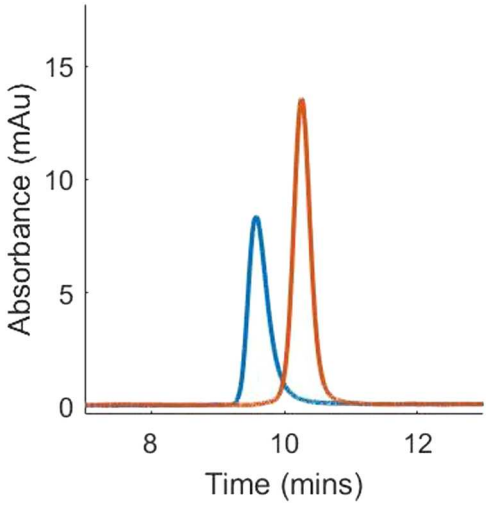

Fig. 2. Chromatograms (254 nm) of anthranilic acid at pH 2.5 (orange) and 6 (blue) on a gradient column. The mobile phase is 95:5 10 mM phosphate buffer/ACN at 40 °C.

Table 2
Comparison of retention factor (k), peak asymmetry (As) and plate count (N) for anthranilic acid with varied mobile phase buffer pH.

pH	k	As	N
2.50	6.15 ± 0.04	1.146 ± 0.003	5920 ± 50
6.00	5.77 ± 0.05	2.89 ± 0.02	2860 ± 90

observed in the first few centimeters is the opposite. The silica powder has ***more*** C8 groups at the inlet from $d = 0$ to ~3 cm. Near the 4 cm point, the system then behaves as expected showing a gradual increase in C8 groups the further it is away from the inlet. A slight decrease is observed in the last section.

At this point, we are not sure what causes this effect but one possibility could be related to thermal effects. When the TFA solution enters the column, which is located in a thermostatted Agilent 1100 column oven, the solution is at room temperature. After a certain point, the solution will adjust to its surroundings and approach ~68 °C. This temperature differential would reduce the rate of the hydrolysis reaction at the inlet relative to the rest of the column. The net result of this temperature differential could lead to a higher carbon loading in the packing material near the inlet of the column. Near the 4 cm point, the system then behaves as expected showing a gradual increase in C8 groups the further it is away from the inlet. A slight decrease is observed in the last section, which could also be due to thermal effects. Additional studies are needed to fully understand the process taking place in the column. As can be seen, this 'S' shape is consistent between different columns.

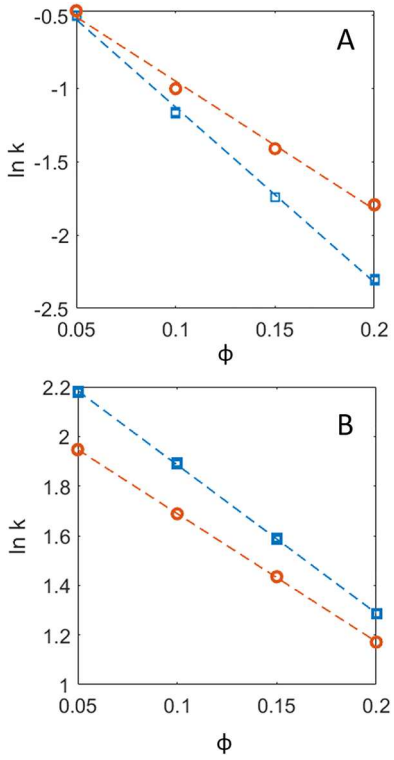

Fig. 3. Plot of ln k vs. φ for tyrosine (A) and anthranilic acid (B) at pH 2.5 for the uniform column (blue squares) and the gradient column (orange circles). Least squares fit shown by dotted line.

3.4. Characterization of radial homogeneity

Another possible cause that we investigated to help explain the unusual shape of this SPG is related to the radial heterogeneity of the stationary phase within the column, particularly at the column inlet where TFA is first introduced. Gritti and Guichon previously indicated that theoretically, an analyte flowing through a column may never reach the outer walls of the column [35]. It was thus rationalized that at the inlet TFA may only be reaching the center of the column and thus only the functional groups in the center would be cleaved. When the powder in each of these segments is collected, mixed, and analyzed via TGA, the segments located near the inlet would then appear to have a higher %C loading. This hypothesis led to an investigation on whether the inner part of the stationary phase underwent more bond cleavage than the outer portion.

To evaluate radial heterogeneity, the packing material needed to be extruded differently so that its radial profile could be maintained during the extrusion process. The method chosen involved extrusion of the packing material into a rigid polyethylene tube as illustrated in Fig. 6, followed by careful separation of the inner powder from the outer powder. This added rigidity helps to keep silica in place during extrusion. The polyethylene tube was then cut, and the degree of

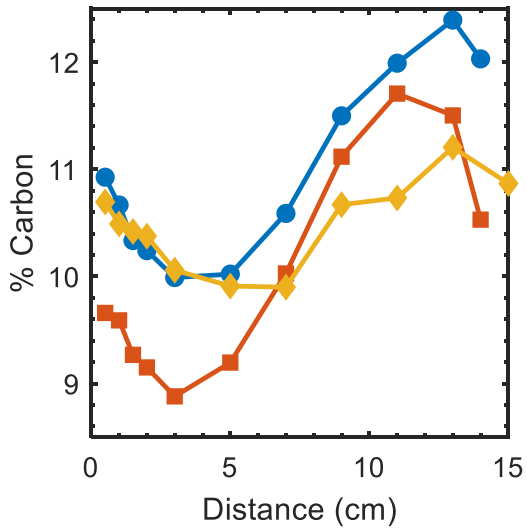

Fig. 4. Gradient profile after the constant infusion of TFA into three different C8 columns. The three lines – blue (circle), orange (square), and yellow (diamond)) represent the gradient profile of three different columns.

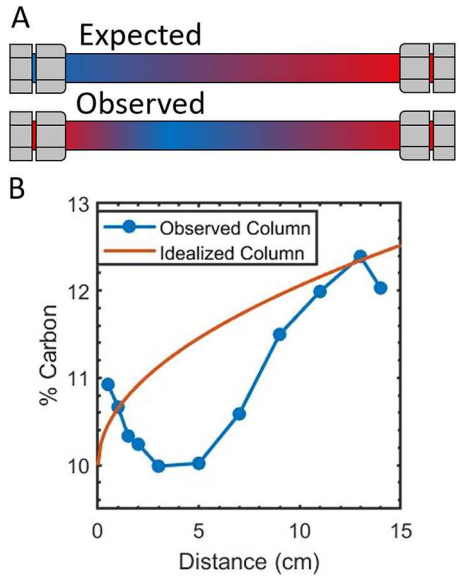

Fig. 5. (A) Cartoons of a gradient stationary phase expected based on previous work and that observed (obtained). The least functionalized regions are represented by blue and the more functionalized regions by red. (B) Gradient profile (% carbon vs distance along the length of the column) of the expected (blue line) and obtained (orange symbols and line) gradient profile. The segments with low % carbon indicate a lower degree of functionalization while high % carbon indicates the opposite.

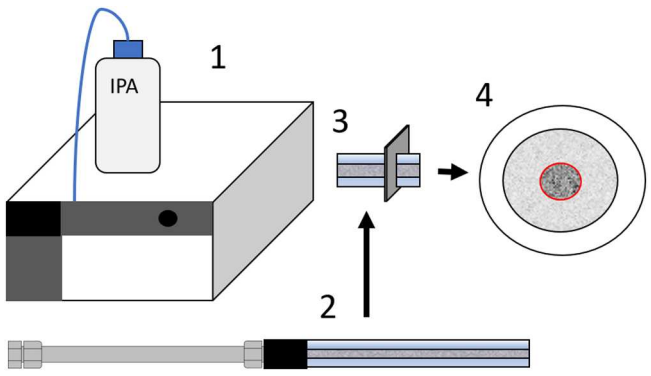

Fig. 6. Cartoon depicting the extrusion method for testing for possible radial heterogeneity in the gradient stationary phase. 1) IPA is pumped at rates between 3 and 7 mL/min to ensure consistent extrusion and prevent loss of structure. 2) The silica is extruded into polyethylene tubing of similar ID of the column. 3) the extruded segments are cut into well-defined segments for TGA analysis. 4) the inner section of each segment is separated from its outer edges as denoted by the red inner circle.

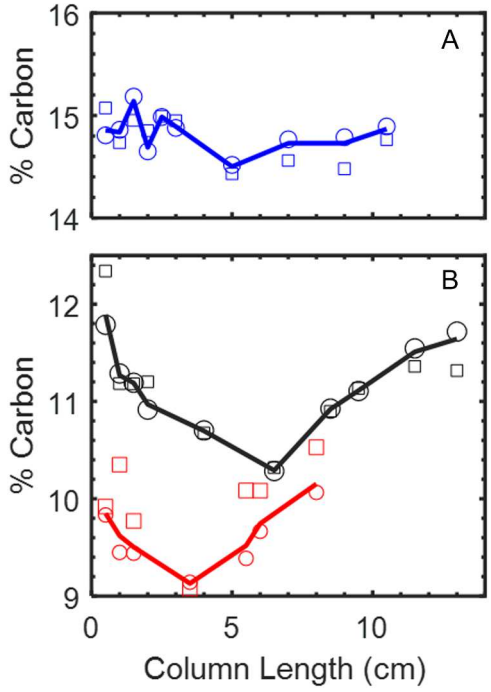

Fig. 7. Gradient profile of one uniform column (A) in blue and two gradient columns (B) in black and red. The interior (circle) and the exterior segments (square) are given for each. The lines represent a weighted average of the % carbon in the column.

functionalization (%C) at the center and outer edges of each segment and along the length of the column was independently characterized via TGA as described in Section 2.8. This experiment was designed to assess the radial homogeneity of the stationary phase functionalization by comparison to uniform, as-received columns.

The radial profile results are shown in Fig. 7 for a uniform (as-received) column and two SPG columns. (Note: both gradient columns were 15 cm in length, but for the second column, shown in red, the stationary phase collapsed into a slurry and could not be accurately sampled). The plot displays the percent carbon across the length of the column with interior and exterior segments depicted individually. The circles represent interior segments of the column; the squares represent the exterior segments and the line represents a weighted average for that segment of the column. The uniform column (Fig. 7**A**) shows a functionalization level of 14.8% carbon in agreement with the manufacturer's documentation. As expected, negligible differences between the degree of functionalization on the inner and outer portions of the uniform column packing were observed. The gradient column results are seen in Fig. 7**B**. The two gradient columns examined show some slight differences between the inner and outer portions of the column with the same variability as that observed in a uniform column. These differences are not large enough to explain the unexpected 'S'-shaped gradient profile. If anything, there appears to be greater loading within the interior of the column. This ran counter to the hypothesis that more ligand removal via exposure to TFA would occur at the center of the

column.

3.5. Gradient profile modification

In an attempt to more clearly understand and control the shape of the stationary phase gradient, the modification procedure was changed and an intermittent flow experiment was performed, as described in Section 2.6. The column had acid infused with an intermittent halting of flow every 5 min. This allowed ~ 1/3 of the column dead volume to be exchanged every 10 min. The stopping of the flow of the column allows the acid to spend more time in contact with the stationary phase functional groups at the head of the column. It also allows more time for the acid to diffuse to the edges of the column and reach a temperature of 68 °C. This should ensure there is little difference in chemistry in the radial direction and ideally reduce the 'S'-shape gradient profile observed in the original experiment.

Fig. 8 shows the profile of the gradient stationary phase (%C vs distance along the length of the column) for the 5-on-5-off gradient columns (N = 2). As can be seen, this on/off infusion experiment generated a very different overall gradient profile shape; the gradient formed a steep exponential curve as opposed to the 'S'-shaped profile noted under constant flow conditions. This variation in the final gradient profile between the two different infusion methods indicates a possibility of creating on-demand shapes. The column prepared by the on/off infusion experiment shows a sharp gradient in the first 1–2 cm of the column indicating the reaction occurs as soon as it enters the column. A lengthy plateau is quickly reached within 3 cm showing about 12 %C. In contrast, the gradient that exists using the continuous flow procedure is much less steep and a plateau is not reached until the ~10–12 cm mark. In both, there appears to be a minor loss of functionalization towards the end of the column, but that appears to be within the variance of the TGA and the fluctuations of the uniform columns.

These SPG columns were also evaluated chromatographically using the test mixture shown in **Figure S1.** Table 3 reports the retention factors, based on uracil retention times, and selectivities for adjacent eluting analytes on a uniform column and 5-on-5-off gradient columns. As observed with the 'S' -shaped gradient column, a decrease in retention factor is noted for the different analytes, with anthranilic acid showing the greatest change. Also, as observed on the 'S'-shaped SPG, changes in the selectivity for the different solute pairs show different patterns, again suggesting that the silanol groups play a role in retention.

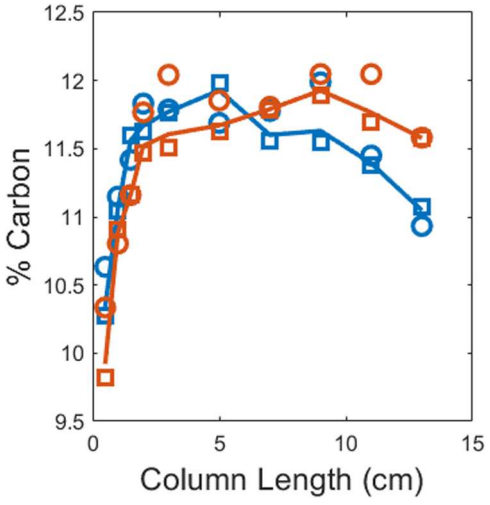

Fig. 8. Plot of % carbon vs. distance after infusing TFA into the column using the 5-on-5-off method. The two lines represent trials of two different columns. The interior (orange circles) and the exterior segments (blue squares) are similar with minor deviations in % carbon. The lines represent a weighted average of the % carbon in the column.

3.6. Nicotine separation

To evaluate the applicability of the gradient columns for the separation of analytes of practical interest, the separation of nicotine and nicotine derivatives was explored. Tobacco and the products produced through smoking produce upwards of 8700 separate compounds and the need to explore new methods to separate such complex mixtures is important [36]. Separations of particularly nicotine and cotinine have been of interest for years [37]. GC-MS has been used but more recently HPLC is of interest with both RP [38–40] and HILIC separations [41,42] being performed to achieve separations. Because these gradient columns have both silanol (HILIC) and C8 (RP) moieties available in the stationary phase, we evaluated the separation of a simple mixture of nicotine derivatives under HILIC conditions on a uniform and two different gradient columns (constant flow vs. 5-on-5-off).

Fig. 9 displays the chromatogram obtained for a mixture containing trans-3-hydroxycotinine (t3hc), cotinine (c), anatabine (at), anabasine (ab) and nicotine (n) obtained on the stopped-flow gradient column housing a steep chemical gradient, a uniformly modified column, and a gradient column with the 'S'- shaped profile prepared using continuous flow. The structures of these nicotine derivatives are provided in the supplementary information in **Figure S5.** On the uniform column, all the analytes elute within one messy peak. The column with a steep gradient shows some resolution of the analytes but not with baseline resolution. The gradient column with the 'S'- shaped gradient shows three peaks. The trans-3-hydroxycotinine and cotinine elute in the first peak while anatabine and anabasine elute in the second peak. The additional presence of the silanol sites improves the resolution of these more hydrophilic compounds.

Relative to the uniform column, both gradient columns provide improved resolution of these nicotine analytes. This indicates the silanol sites, which are exposed when the C8 groups are cleaved, lead to enhanced retention of nicotine and its alkaloids. The metabolites show only minor changes in retention. It can be seen that the 5-on-5 off column with a steeper gradient is more similar to the uniform column. That is of note as the level of functionalization for the uniform column is closer to the gradient columns. This column has a total functionalization level of 11.6%, whereas the 'S'-shaped gradient column average is 10.6%. This indicates that the additional exposure of silanol sites in the 'S' -shaped gradient column is very important for retention. The uniform column has a functionalization of 15% (see Fig. 7**A**).

The separation of the nicotine analytes can be further improved by utilizing a mobile phase gradient and the 'S'-shaped stationary phase gradient column. Fig. 10 shows the separation achieved with both the SPG column and a mobile phase gradient. The five solutes are nearly baseline resolved. This displays the enhanced resolving capability of these columns, particularly when coupled with a mobile phase gradient. The SPG column combines the retentive characteristics of both reversed-phase and HILIC separations and allows for the separation of these analytes with an appropriate mobile phase gradient.

4. Conclusions

A method has been developed to reproducibly produce SPGs on octyl-functionalized silica columns. By modifying the infusion method, gradient stationary phases with different shapes can be produced. In this work, one exhibited an 'S'-shaped gradient while the other exhibited a steep exponential-like gradient. There is a negligible difference in the ligand removal efficacy radially throughout the length of the column. Results also suggest that the temperature of the stripping solvent when entering the column may be important to controlling the overall gradient profile. A demonstrable change in retentive characteristics for these columns was observed under both isocratic and gradient conditions. The selectivity pattern was also different between the uniform

Table 3
Retention factors (k) for test mixture analytes on a uniform and gradient column and selectivity (α) relative to the prior eluting analyte for the 5-on-5-off gradient columns.*

Analyte	Uniform C8 column, k	5-on-5-off gradient column, k	Uniform C8 column, α	5-on-5-off gradient column, α	Change in α,%
Tyrosine	0.37 ± 0.09	0.44 ± 0.04	—	—	
Phenylalanine	0.8 ± 0.1	0.79 ± 0.06	2.1 ± 0.1	1.82 ± 0.05	−14 ± 6
Tryptophan	1.9 ± 0.3	1.6 ± 0.1	2.37 ± 0.02	2.12 ± 0.04	−11 ± 2
Tryptamine	2.1 ± 0.4	1.9 ± 0.1	1.10 ± 0.02	1.18 ± 0.03	8 ± 4
Anthranilic acid	7.2 ± 0.8	6.1 ± 0.2	3.5 ± 0.3	3.06 ± 0.08	−14 ± 9

* The average values are obtained from columns 2A and 2B shown in Table S1.

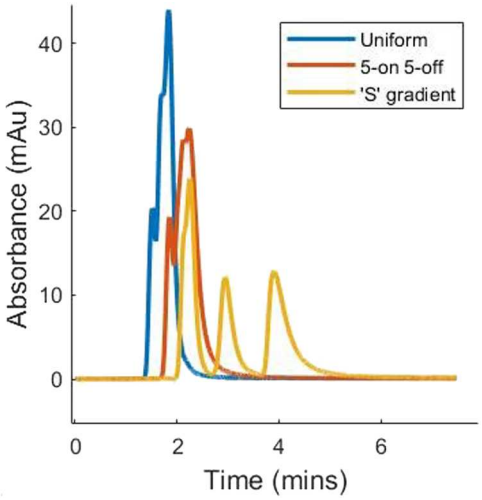

Fig. 9. Chromatograms (254 nm) of a mixture of trans-3-hydroxycotinine, cotinine, anatabine, anabasine and nicotine (eluting in that order). All separations were carried out using a mobile phase of 80:20 1:1 MeOH/ACN and 10 mM formate buffer pH 3.0. Uniform (blue), 5-on-5-off (red) and S gradient (yellow) column separations are overlayed.

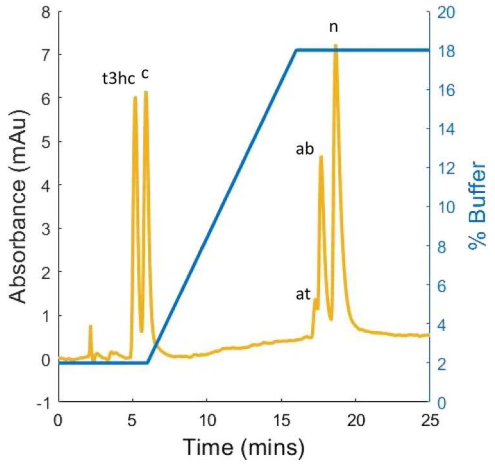

Fig. 10. Chromatogram (270 nm) of trans-3-hydroxycotinine, cotinine, anatabine, anabasine and nicotine separated on an 'S' -shaped gradient column. Analytes were separated using a mobile phase gradient from 2:98 10 mM formate buffer pH 3.0/ACN to 18:82 buffer/ACN.

columns and the 'S-shaped' and 5-on-5-off columns. These new gradient columns have shown improved resolution of nicotine analytes compared to a uniform C8 column. At this point in time and to the author's knowledge there are only two methods to produce ***continuous*** mixed-mode silica stationary phase gradients. By utilizing and repurposing older columns, the expense of making such columns can be considerably reduced. While the exposure of the silanol groups may create problems for the separation of some analytes such as basic compounds, these silanols may be beneficial in other applications by influencing selectivity and/or serving as a point to add additional functional groups on the columns [2,3]. Additional experiments and simulations are needed to fully understand the chromatographic performance of these columns.

CRediT authorship contribution statement

Thomas Cecil: Writing – original draft, Visualization, Methodology, Investigation, Conceptualization. **Judith Bautista:** Methodology, Investigation. **Maryanne M. Collinson:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Sarah C. Rutan:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2024.464974.

References

[1] L.R. Snyder, J.J. Kirkland, J.W. Dolan, Introduction to Modern Liquid Chromatography, John Wiley & Sons, 2011.
[2] S.L. Weatherbee, M.M. Collinson, Chapter 2: stationary phase gradients in liquid chromatography, in: N. Grinberg, P.W. Carr (Eds.), Adv. Chromatogr., CRC Press, 2021, pp. 75–120.
[3] C.N. Cain, S.L. Weatherbee, A.V. Forzano, S.C. Rutan, M.M. Collinson, Fabrication and characterization of a reversed-phase/strong cation exchange stationary phase gradient, J. Chromatogr. A 1623 (2020) 461177, https://doi.org/10.1016/j.chroma.2020.461177.
[4] V.C. Dewoolkar, L.N. Jeong, D.W. Cook, K.M. Ashraf, S.C. Rutan, M.M. Collinson, Amine gradient stationary phases on in-house built monolithic columns for liquid chromatography, Anal. Chem. 88 (2016) 5941–5949, https://doi.org/10.1021/acs.analchem.6b00895.
[5] C.N. Cain, A.V. Forzano, S.C. Rutan, M.M. Collinson, Destructive stationary phase gradients for reversed-phase/hydrophilic interaction liquid chromatography,

J. Chromatogr. A 1570 (2018) 82–90, https://doi.org/10.1016/j.chroma.2018.07.073.
[6] C.N. Cain, A.V. Forzano, S.C. Rutan, M.M. Collinson, Experimental- and simulation-based investigations of coupling a mobile phase gradient with a continuous stationary phase gradient, J. Chromatogr. A 1602 (2019) 237–245, https://doi.org/10.1016/j.chroma.2019.05.033.
[7] T. Alvarez-Segura, J.R. Torres-Lapasió, C. Ortiz-Bolsico, M.C. García-Alvarez-Coque, Stationary phase modulation in liquid chromatography through the serial coupling of columns: a review, Anal. Chim. Acta 923 (2016) 1–23, https://doi.org/10.1016/j.aca.2016.03.040.
[8] C. Ortiz-Bolsico, J.R. Torres-Lapasió, M.J. Ruiz-Ángel, M.C. García-Álvarez-Coque, Comparison of two serially coupled column systems and optimization software in isocratic liquid chromatography for resolving complex mixtures, J. Chromatogr. A 1281 (2013) 94–105, https://doi.org/10.1016/j.chroma.2013.01.064.
[9] K. Zhang, X. Liu, Mixed-mode chromatography in pharmaceutical and biopharmaceutical applications, J. Pharm. Biomed. Anal. 128 (2016) 73–88, https://doi.org/10.1016/j.jpba.2016.05.007.
[10] D. Sýkora, P. Řezanka, K. Záruba, V. Král, Recent advances in mixed-mode chromatographic stationary phases, J. Sep. Sci. 42 (2019) 89–129, https://doi.org/10.1002/jssc.201801048.
[11] F. Gritti, G. Guiochon, Band broadening along gradient reversed phase columns: a potential gain in resolution factor, J. Chromatogr. A 1342 (2014) 24–29, https://doi.org/10.1016/j.chroma.2014.03.025.
[12] S. Fekete, M. Lauber, Studying the possibilities of dual stationary phase gradients to explore alternative selectivities in liquid chromatography, J. Chromatogr. A 1681 (2022) 463492, https://doi.org/10.1016/j.chroma.2022.463492.
[13] S. Horváth, D. Lukács, E. Farsang, K. Horváth, Study of efficiency of capacity gradient ion-exchange stationary phases, Separations 10 (2023) 14, https://doi.org/10.3390/separations10010014.
[14] A. Ampe, E. Bandini, K. Broeckhoven, F. Lynen, On-column modification for the creation of temperature-responsive stationary phases, Anal. Chim. Acta 1283 (2023) 341961, https://doi.org/10.1016/j.aca.2023.341961.
[15] C.A. Pohl, Preparation of ion exchange columns with longitudinal stationary phase gradients, Heliyon 7 (2021) e06961, https://doi.org/10.1016/j.heliyon.2021.e06961.
[16] A.V. Forzano, C.N. Cain, S.C. Rutan, M.M. Collinson, In situ silanization for continuous stationary phase gradients on particle packed LC columns, Anal. Methods 11 (2019) 3648–3656, https://doi.org/10.1039/C9AY00960D.
[17] V.C. Dewoolkar, B. Kannan, K.M. Ashraf, D.A. Higgins, M.M. Collinson, Amine-phenyl multi-component gradient stationary phases, J. Chromatogr. A 1410 (2015) 190–199, https://doi.org/10.1016/j.chroma.2015.07.089.
[18] B. Kannan, M.A. Marin, K. Shrestha, D.A. Higgins, M.M. Collinson, Continuous stationary phase gradients for planar chromatographic media, J. Chromatogr. A 1218 (2011) 9406–9413, https://doi.org/10.1016/j.chroma.2011.10.075.
[19] H. Song, G. Desmet, D. Cabooter, Evaluation of the kinetic performance differences between hydrophilic-interaction liquid chromatography and reversed-phase liquid chromatography under conditions of identical packing structure, Anal. Chem. 87 (2015) 12331–12339, https://doi.org/10.1021/acs.analchem.5b03697.
[20] J. Nawrocki, The silanol group and its role in liquid chromatography, J. Chromatogr. A 779 (1997) 29–71, https://doi.org/10.1016/S0021-9673(97)00479-2.
[21] U. Neue, C. Niederlaender, J. Petersen, Liquid chromatography stationary phases with reduced silanol interactions, 5,374,755, 1994.
[22] M. Jezierska, I. Cendrowska, M. Markuszewski, R. Kaliszan, B. Buszewski, Comparative study of surface topography of high performance liquid chromatography columns in terms of hydrophobicity, Chromatographia 51 (2000) 111–118, https://doi.org/10.1007/BF02490704.
[23] S.C. Rutan, A. de Juan, R. Tauler, Introduction to multivariate curve resolution, in: S.D. Brown, R. Tauler, B. Walczak (Eds.), Compr. Chemom., Elsevier, Oxford, 2009, pp. 249–259, https://doi.org/10.1016/B978-044452701-1.00046-6.
[24] A. de Juan, J. Jaumot, R. Tauler, Multivariate Curve Resolution (MCR). Solving the mixture analysis problem, Anal. Methods 6 (2014) 4964–4976, https://doi.org/10.1039/C4AY00571F.
[25] E. Bezemer, S.C. Rutan, Analysis of three- and four-way data using multivariate curve resolution-alternating least squares with global multi-way kinetic fitting, Chemom. Intell. Lab. Syst. 81 (2006) 82–93, https://doi.org/10.1016/j.chemolab.2005.10.005.
[26] F. Gritti, Y. Kazakevich, G. Guiochon, Measurement of hold-up volumes in reverse-phase liquid chromatography: definition and comparison between static and dynamic methods, J. Chromatogr. A 1161 (2007) 157–169, https://doi.org/10.1016/j.chroma.2007.05.102.
[27] C. Ye, G. Terfloth, Y. Li, A. Kord, A systematic stability evaluation of analytical RP-HPLC columns, J. Pharm. Biomed. Anal. 50 (2009) 426–431, https://doi.org/10.1016/j.jpba.2009.05.028.
[28] M.R. Schilling, F. Preusser, G. Gutnikov, Analysis of high-performance liquid chromatography bonded stationary phases using thermogravimetry, J. Therm. Anal. 38 (1992) 2483–2490, https://doi.org/10.1007/BF01974626.
[29] B. Lumley, T.M. Khong, D. Perrett, The characterisation of chemically bonded chromatographic stationary phases by thermogravimetry, Chromatographia 60 (2004) 59–62, https://doi.org/10.1365/s10337-004-0329-2.
[30] C.J. Brinker, G.W. Scherer, Sol-gel science: the Physics and Chemistry of Sol-Gel Processing, Academic Press, Boston, 1990.
[31] A. Méndez, E. Bosch, M. Rosés, U.D. Neue, Comparison of the acidity of residual silanol groups in several liquid chromatography columns, J. Chromatogr. A 986 (2003) 33–44, https://doi.org/10.1016/S0021-9673(02)01899-X.
[32] M. Pfeiffer-Laplaud, D. Costa, F. Tielens, M.-P. Gaigeot, M. Sulpizi, Bimodal Acidity at the Amorphous Silica/Water Interface, J. Phys. Chem. C 119 (2015) 27354–27362, https://doi.org/10.1021/acs.jpcc.5b02854.
[33] M. Buszewska-Forajta, M.J. Markuszewski, R. Kaliszan, Free silanols and ionic liquids as their suppressors in liquid chromatography, J. Chromatogr. A 1559 (2018) 17–43, https://doi.org/10.1016/j.chroma.2018.04.002.
[34] L.R. Snyder, J.W. Dolan, High-Performance Gradient Elution: The Practical Application of the Linear-Solvent-Strength Model, John Wiley & Sons, 2007.
[35] F. Gritti, G. Guiochon, Perspectives on the evolution of the column efficiency in liquid chromatography, Anal. Chem. 85 (2013) 3017–3035, https://doi.org/10.1021/ac3033307.
[36] A. Rodgman, The Chemical Components of Tobacco and Tobacco Smoke, CRC Press, Boca Raton, 2009.
[37] S. Jin, W. Pang, L. Zhao, Z. Zhao, S. Mei, Review of HPLC–MS methods for the analysis of nicotine and its active metabolite cotinine in various biological matrices, Biomed. Chromatogr. 36 (2022) e5351, https://doi.org/10.1002/bmc.5351.
[38] P. Jacob, L. Yu, M. Duan, L. Ramos, O. Yturralde, N.L. Benowitz, Determination of the nicotine metabolites cotinine and *trans*-3′-hydroxycotinine in biologic fluids of smokers and non-smokers using liquid chromatography–tandem mass spectrometry: biomarkers for tobacco smoke exposure and for phenotyping cytochrome P450 2A6 activity, J. Chromatogr. B 879 (2011) 267–276, https://doi.org/10.1016/j.jchromb.2010.12.012.
[39] M.A. Kaisar, R.R. Kallem, R.K. Sajja, A.E. Sifat, L. Cucullo, A convenient UHPLC-MS/MS method for routine monitoring of plasma and brain levels of nicotine and cotinine as a tool to validate newly developed preclinical smoking model in mouse, BMC Neurosci. 18 (2017) 71, https://doi.org/10.1186/s12868-017-0389-5.
[40] V.V. Gholap, L. Kosmider, M.S. Halquist, A standardized approach to quantitative analysis of nicotine in e-liquids based on peak purity criteria using high-performance liquid chromatography, J. Anal. Methods Chem. 2018 (2018) 1720375, https://doi.org/10.1155/2018/1720375.
[41] I.A. Abdallah, D.C. Hammell, A.L. Stinchcomb, H.E. Hassan, A fully validated LC–MS/MS method for simultaneous determination of nicotine and its metabolite cotinine in human serum and its application to a pharmacokinetic study after using nicotine transdermal delivery systems with standard heat application in adult smokers, J. Chromatogr. B 1020 (2016) 67–77, https://doi.org/10.1016/j.jchromb.2016.03.020.
[42] M. Dobrinas, E. Choong, M. Noetzli, J. Cornuz, N. Ansermot, C.B. Eap, Quantification of nicotine, cotinine, *trans*-3′-hydroxycotinine and varenicline in human plasma by a sensitive and specific UPLC–tandem mass-spectrometry procedure for a clinical study on smoking cessation, J. Chromatogr. B 879 (2011) 3574–3582, https://doi.org/10.1016/j.jchromb.2011.09.046.