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A multi-location peak parking protocol was developed as a non-destructive assessment of the axial heterogeneity of \textit{in-situ} modified monoliths. This was tested on a column designed specifically with a surface coverage density gradient along the length of the monolithic rod. Qualitative changes in band broadening was observed and are consistent with theoretical studies.
Non-destructive test to assess the axial heterogeneity of in-situ modified monoliths for HPLC

Danielle N. Bassanesea, Arianne Solivenb,c, Xavier A. Conlanb, R. Andrew Shallikerb, Neil W. Barnettb, and Paul G. Stevenson*a,*

aCentre for Chemistry and Biotechnology, School of Life and Environmental Science, Deakin University, Geelong, Vic. 3216, Australia

bAustralian Centre for Research on Separation Science (ACROSS), School of Science and Health, University of Western Sydney (Parramatta), Sydney, NSW, Australia

cDepartment of Natural Products and Pharmacognosy, Faculty of Chemistry, Universidad de la República, Montevideo, Uruguay

*Corresponding author:

Dr Paul G. Stevenson

Deakin University, Geelong Waurn Ponds Campus

75 Pidgons Road, Waurn Ponds, Victoria 3216 Australia

E-mail: paul.stevenson@deakin.edu.au

Office: +61 3 5227 2241

Fax: +61 3 5227 1040
Abstract

This paper describes a non-destructive “peak parking” protocol in order to assess the axial heterogeneity of an in-situ modified monolithic column for high performance liquid chromatography; a “gradient stationary phase” was designed whereby the ligand density decreases along the length of the rod in the “forward flow” configuration. Results of multi-location peak parking demonstrated a consistent increase in peak variance from the 1 cm position of the column to the 9 cm location. This increase in band broadening supported the theory of a decreasing ligand density along the length of this gradient column. This is consistent with efficiency measurements performed in both the forward and reverse flow directions, with an improved efficiency (15% increase in $N \text{ m}^{-1}$) in the reverse direction. These results are consistent with theoretical investigations into stationary phase gradients.

Keywords: Gradient ligand density; peak parking; multi-location; monolith modification; non-destructive column characterisation
1. Introduction

Despite the advantages to high performance liquid chromatography (HPLC) that analytical silica monolithic columns present\textsuperscript{1,2}, only three different stationary phases are commercially available – bare silica, C8 and C18. This limitation to selectivity is a major drawback for separation scientists, particularly because the preparation of column technology is heavily protected by a number of patents\textsuperscript{3–6}. Attempts to synthesize silica monoliths comparable in performance to those commercially available has not been completely mastered by academic researchers; partially because certain aspects of the preparation process (i.e. the drying and cladding steps) are particularly difficult to control and often result in voids or cracks in the rods\textsuperscript{7,8}.

To overcome these limitations, researchers have used \textit{in-situ} silylation reactions to modify commercial bare-silica monoliths with a desired moiety\textsuperscript{9–15}. However, this modification procedure is not always reproducible\textsuperscript{9}. While a number of different chromatographic tests can elucidate separation performance\textsuperscript{16–21} the surface coverage of the bonded ligands cannot be measured without destroying the column\textsuperscript{22–24}. Ideally, the homogeneity of the stationary phase surface coverage should be measured after the \textit{in-situ} modification procedure and using a technique that is not destructive to the column bed\textsuperscript{25}.

Gritti and Guiochon\textsuperscript{26} recently discussed the concept and potential advantages of gradient stationary phases that assumed a linear increase in retention factor along the column by evaluating the resolution between a pair of compounds that were difficult to separate from a theoretical viewpoint. They proposed the effect on peak width under 3 different conditions: (1) possible band compression is neglected (Giddings model)\textsuperscript{27}, (2) band compression is taken into account (Poppe model)\textsuperscript{28} and (3) both band compression and extra-column effects are considered. It was hypothesised that when band compression due to a positive differential between the front and rear parts of the peak is taken into account (the Poppe model), gradient stationary phases could be as effective as classical mobile phase gradients. However, these results are purely from a theoretical viewpoint and have yet to be confirmed \textit{via} laboratory experiment. These authors proposed that the simplest way to test this theory would be to prepare a silica monolithic column with a gradient of bonded ligand density along its length and assess the gradient of retention factors.

This paper suggests the use of a multi-location peak parking technique to characterise
the axial heterogeneity of the bonded material, where an analyte is allowed to disperse at selected locations along the stationary phase by halting the flow of mobile phase. The experimental setup is straightforward and involves injecting a sample band and letting it migrate to the approximate halfway point of the column before stopping the flow of the mobile phase. The band is allowed to diffuse for a specific amount of time and the flow is resumed. Knox and McLaren 29 first introduced this technique in 1964 to measure the diffusion coefficient and obstructive factors in gas chromatography. Peak parking has since been used to study the mass transfer kinetics in a number of gaseous 30-32 and liquid 33-39 phase systems. In 2006, Gritti and Guiochon 40 used the peak parking technique on five C18 particle packed columns with different carbon loadings and noted that a higher surface coverage led to slower axial diffusion. In 2011 41, they extended this research by adopting a multi-location peak parking technique to measure the axial diffusion at different locations along the length of packed columns as a way of searching for possible axial heterogeneities within the column bed.
2. Experimental

2.1. Chemicals

Deionised water was obtained in-house (Continental Water Systems, Victoria, Australia) and filtered through a 0.45 µm membrane filter (Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia) prior to use. HPLC grade methanol was purchased from Ajax Finechem Pty. Ltd. (Taren Point, NSW, Australia); HPLC grade isopropanol and heptane were purchased from Merck Pty. Ltd. (Kilsyth, Victoria, Australia). Heptane was dried by reflux over sodium. Chloro-dimethyl 3-cyanopropyl silane, chloro-dimethyl propyl phenyl silane and chloro-trimethyl silane were obtained from Gelest (USA). Thiourea was obtained from BDH Chemical Ltd. (Poole, England). Ethylbenzene, riboflavin and phosphorous pentoxide were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia).

2.2. Instrumentation

Chromatographic analysis was performed with an Agilent 1260 system (Agilent Technologies, Mulgrave, Victoria, Australia), incorporating a quaternary pump with solvent degasser, an auto-sampler, a thermostated column compartment and a DAD module which monitored the absorbance at 225 nm, 237 nm, 254 nm and 260 nm. Chromatographic data were obtained and processed with Agilent ChemStation software. The temperature of the column was held isothermally at 25 °C and all injections were 2 µL. A 2 position, 4 port switching valve was configured to “close” the system to maintain pressure while the mobile phase flow was halted.

2.3. Columns

The in-situ modified monolithic stationary phase was prepared according to the modification procedure outlined previously and is summarised here for completeness. A neat silica monolith (Onyx, 100 × 4.6 mm, 130 Å pore size) was purchased from Phenomenex (Lane Cove, NSW, Australia) for modification. Prior to surface modification, dried heptane (50 mL) was pumped through the monolith. A 1% v/v solution of chlorodimethyl propyl phenyl silane was used as the phenyl ligand bonding silane solution and a 1% v/v solution of chloro-(3-cyanopropyl)dimethyl silane in dried heptane was used as the cyano ligand bonding silane. End-capping was achieved using a 1% v/v solution of chlorotrimethyl silane. Column modification was undertaken using 100 mL of each silane solution that was pumped through
the monolith at 30 min intervals (at flow rates of up to 4 mL min⁻¹) using each time five column volumes of the silane solution. Between each pump cycle the silane solution was allowed to sit static within the column for 30 min. This step was completed once, in the forward direction, firstly for the phenyl silane solution, then repeated with the cyano silane solution and finally the end-capping silane solution. It was expected that a gradient ligand density would be achieved by modifying in the forward direction only¹⁴. These solutions were pumped through the monolith using a Waters 501 HPLC pump thermostatted at 80 °C using a HPLC column heater (Thermasphere TS-130, Phenomenex). An Onyx Monolithic C18 (100 × 4.6 mm) was also purchased from Phenomenex.

2.4. Methodology

2.4.1. Efficiency measurements

The plate height (H) of the modified column was measured according to van Deemter’s equation ¹⁶ whereby performance was measured by calculating the peak variance via the moments method ⁴³,⁴⁴ of ethylbenzene at 22 different flow rates between 0.3 and 3 mL min⁻¹. Ethylbenzene was injected into an isocratic mobile phase of 40% methanol in water.

2.4.2. Peak parking

Peaks were “parked” at five locations along the length of the column: 1, 3, 5, 7 and 9 cm from the column inlet by stopping the flow of the mobile phase when the peaks were predicted to be at these locations. As multiple thiourea samples were to be sequentially injected and parked on the column in one chromatographic run, the frequency of injection and the elution time to the parking locations needed to be determined by the following process:

1. Retention time of the selected compound was determined at either 0.2 mL min⁻¹ (thiourea) or 0.5 mL min⁻¹ (riboflavin).
2. The HPLC system dead time for the appropriate flow rate was recorded and subtracted from the retention time of the selected compound to find the corrected retention time.
3. As there were five parking locations along the column, this divided the column into six sections (Figure 1), the corrected retention time was divided by six to establish the wait time required between injections.
4. To calculate the ‘stop’ time for when the flow was to be halted in order to commence the parking, the injection time determined in step 3 was multiplied by five (to represent the last parking location on the column).
Finally the system dead time was added to the ‘stop’ time to account for the volume between the auto sampler and the column inlet.

Eight different values of residence times were used for each column: 0, 5, 10, 15, 30, 60, 120 and 180 min. The parking experiments were performed in both the forward and reverse flow directions.

The peak parking experiments conducted with thiourea were completed with a mobile phase of 25% aqueous methanol at a flow rate of 0.2 mL min\(^{-1}\). An injection program, outlined in Table 1 was used in order to place five peaks along the column length in one chromatographic run with analyte plugs with a volume of 2 µL.

The monolithic column displayed weak retention with a reversed-phase mobile phase, thus peak parking experiments with riboflavin were completed in hydrophilic interaction chromatography (HILIC) mode with an isocratic mobile phase of 98% aqueous acetonitrile and a flow rate of 0.5 mL min\(^{-1}\). The peak width of riboflavin was much broader after parking than that of thiourea and it was not possible to park peaks at all locations along the column in one chromatographic run without severe peak overlap. Instead, a single 2 µL injection was performed and parked at each location (1, 3, 5, 7 and 9 cm) separately. This was completed for all residence times.

2.4.3. Surface coverage

The homogeneity of the surface coverage was determined by measuring the percentage of carbon (%C) of sequential column sections with elemental analysis. The in-situ modified monolith was cut into sections ~1.25 cm in length (8 sections in total) and the rod was easily removed from the casing with minimal pressure prior to measurement of the carbon load. The surface coverage of the modified monolith was calculated according to Berendsen 45:

\[
\text{Ligand density} = \frac{10^6 \cdot \%C}{1200n_c - \%C \cdot (M_w - n_x)} \cdot \frac{1}{S_{\text{BET}}}
\]  

(1)

where %C is the percentage carbon content, \(n_c\) is the number of carbon atoms per ligand (in this case \(n_c\) was equal to the number of carbons in all bonded ligands), \(M_w\) is the molar mass of the silane ligand (i.e. propyl phenyl, cyano and methyl), \(n_x\) is the number of reactive groups on the silane ligand(s) and \(S_{\text{BET}}\) is the specific surface area of the unmodified silica (according to the manufacturer the \(S_{\text{BET}}\) for this column, prior to modification, was 300 m\(^2\) g\(^{-1}\)). The ligand density is measured in µmol m\(^{-2}\).
Elemental analysis for carbon and nitrogen was completed by CMAS Chemical and Microanalytical Services Pty. Ltd. (Highton, Victoria, Australia). Prior to analysis the column was washed with pure methanol for 25 minutes at a flow rate of 4 mL min\(^{-1}\). The end fittings were removed from the column, and the column was carefully cut into ~1.25 cm sections resulting in 8 samples. The silica rod sections were expressed from the PEEK tubing and dried in an oven at 50°C for 16 hours and then vacuum dried over phosphorus pentoxide until a constant mass was reached.

2.5. **Data analysis**

Measurements for plate heights; plate numbers and peak variances were performed with Wolfram Mathematica 9 (distributed by Hearn Scientific, South Yarra, Victoria, Australia) using algorithms written in-house. Plate counts were determined using the second peak moment (variance) method. Peak variance is related to the standard deviation of a Gaussian peak and is a good measurement of band dispersion. Variance is measured by observing changes in the recorded signal relative to the predicted baseline of ideal peaks\(^{43}\).
3. **Results and discussion**

Based on Gritti and Guiochon’s prediction that a positive ligand density differential will improve separation efficiency just such a silica monolithic column was created. To the best of our knowledge, this is the first example of an analytical scale silica monolith with the density of the bonded ligands applied deliberately in a gradient fashion along the axial length of the column. An investigation into the selectivity of this column can be found in reference 42.

3.1. **Column efficiency**

Column efficiency was measured to test the hypothesis that the ligand density had been bonded as a gradient along the length of the column. This was completed with thiourea and ethylbenzene. The performance of the column was measured in both the forward flow direction – from the column inlet to the outlet – and in the reverse flow direction – from the outlet to the inlet. The resulting van Deemter plot for ethylbenzene is displayed in Figure 2. The efficiency improved in the reverse flow direction, with a 15% increase in the number of theoretical plates (74,835 \( N \) m\(^{-1}\)) when compared to the efficiency in the forward flow direction (65,222 \( N \) m\(^{-1}\)). This increased efficiency is a result of band compression; as the peak band migrates along the column length, the front of the band profile encounters an area of higher ligand density and it begins to slow (more retained) which allows the rear part of the band profile to move faster. This results in a compressed band profile, which in turn gives a lower plate height resulting in a more efficient separation. This would suggest that the *in-situ* modified monolith does indeed have a higher ligand density in the 1 cm position than at the 9 cm location.

3.2. **Peak parking**

A non-destructive technique is required to determine the surface coverage of modified monolithic columns, particularly those modified *in-situ*. Gritti and Guiochon \(^{40}\) used a peak parking technique to measure the axial diffusion, an important kinetic property of HPLC columns. They tested five C18 columns with different carbon loadings and noted that a higher surface coverage led to slower axial diffusion of the parked peaks. Using this concept and the multi-location peak parking technique, also described by Gritti and Guiochon \(^{41}\), a protocol was designed to measure the degree of axial diffusion at five locations along the length of the *in-situ* modified monolith. If the gradient bonding density had been applied to the column surface, as intended, a difference in the degree of axial diffusion should be seen.
3.2.1. Unretained compound

It was found that the position of an unretained marker, thiourea, shifted in accord with parking time (not shown). This was overcome by “closing” the system via placing a switching valve between the detector and the waste container. The valve position was switched when solvent flow was halted to prevent the mobile phase from siphoning into the collection vessel. An injector program was designed so that thiourea was simultaneously “parked” at five different locations along the axis of the monolith (i.e. at 1, 3, 5, 7 and 9 cm from the column inlet in the “forward flow” configuration). Note: notwithstanding careful consideration in designing the injection program to park these peaks it is impossible to be absolutely certain that they reside at these exact locations.

Multi-location parking experiments were completed on the in-situ modified monolith in both the forward and reverse flow direction for residence times of 0, 5, 10, 15, 30, 60, 120 and 180 min, the results are illustrated in Figure 3. The measured variances for selected residence times (in the forward flow direction) are illustrated in Figure 4. These graphs show a distinct trend of increasing variance as the peak is parked further away from the column inlet. This was observed for all residence times tested and the overall variance between peaks increased with residence time. This is a strong indication that there may be a bonding density gradient along the column surface, as the peaks are axially diffusing at different rates in the various locations. The results also support the hypothesis that there is a higher bonding density at the inlet of the column (i.e. at 1 cm residence) than at the outlet, with less variance seen here than at the 9 cm position.

Peak parking experiments were also completed on the column in the reverse flow direction. In this direction, the 9 cm park position is now the column inlet and the 1 cm park position is the column outlet (see Figure 1). The measured variances in the reverse flow configuration show the same trend as in the forward flow mode, see Figure 5. It is noteworthy that in the reverse flow direction the difference between the lowest and highest measured variance for each park location is smaller than that measured in the forward flow direction, see Table 2. This may be due to the effect of compression on the band profile that was seen in the column efficiency tests and predicted by Gritti and Guiochon.

Multi-location peak parking experiments were also conducted on a commercial C18
monolith in both the forward and reverse flow directions to determine the axial diffusion at different locations along the column length. It was expected that the axial diffusion would be similar at the five park locations as these monoliths are designed and manufactured in such a way that the bonding density along the column should be homogenous \(^8,46\). The variance was calculated for each peak over the eight residence times and select residence times (in the forward flow direction) are displayed in Figure 6. The results of the parking experiments on the C18 monolith suggest a uniform surface coverage along the entire length of the column, with only very slight differences observed in the measured variances at the different parking locations. These results are supported by the findings reported by Soliven \textit{et al.} \(^{25}\) with regards to a homogeneous surface coverage of C18 monoliths determined by elemental analysis.

Parking experiments were also completed on the C18 monolith in the reverse flow direction and the results further indicated uniform surface coverage. The degree of variance recorded at each residence time matched well between the forward and reverse flow directions and the difference between the lowest and highest measured variance was very similar for each park location (see Table 2), further supporting uniform surface coverage on the C18 monolith.

### 3.2.2. Retained compound

The multi-location peak parking experiments were also completed on the \textit{in-situ} modified monolith using a retained compound. Ideally, a compound with a retention factor between 3 and 5 was required to ensure there was sufficient interaction with the stationary phase. However, after extensive testing using a variety of compounds and mobile phase compositions no analyte was found to give an adequate retention factor and maintain a suitable peak shape in reversed phase mode.

The cyano ligand can be used in the hydrophilic interaction chromatography (HILIC) mode thus a small number of compounds were tested by injecting into an isocratic mobile phase of 98% aqueous acetonitrile. Riboflavin was found to have adequate retention factor \((k' = 3.44)\) whilst maintaining an ideal Gaussian peak shape. The peak width prevented parking at multiple locations simultaneously without substantial overlap of the peak profiles, thus making it impossible to measure peak variance \textit{via} the moments method \(^{43,44}\). As such, a single peak was parked at the different locations in individual chromatographic injections. This was completed
in both the forward and reverse flow direction for all residence times. The measured variances for select residence times in the forward flow direction are displayed in Figure 7. The observations made from thiourea were reproduced with riboflavin as a smaller variance was measured at the column inlet (at the 1 cm residence position) than at the column outlet (9 cm). In the reverse flow direction (Figure 8) there was a larger measured variance at the 9 cm residence position when compared to the 1 cm position. This trend is again seen across all residence times and matched the results for the parking experiments of thiourea completed in this flow direction.

When comparing the difference between the lowest and highest measured variance for each park location, there is less variance in the reverse flow direction than in the forward flow direction (see Table 2). This is similar to what was observed for the thiourea parking experiments and again adds support to the hypothesis that compression of the band profile is taking place when the column is operated in the reverse flow direction.

3.3. Surface coverage

The results of the peak parking experiments strongly suggest that the in-situ modified monolith possessed a gradient ligand bonding density increasing along the column length. To determine the actual ligand density the column needed to be destroyed to measure the amount of stationary phase bonded to the surface via elemental analysis at various locations along the column. The surface coverage results calculated with Equation (1) are listed in Table 3; unfortunately however, these results were inconclusive. The measured carbon load was much lower than expected in comparison to previous modifications performed by Soliven et al. with an average surface coverage of 0.15 μmol m⁻² across different column sections, compared to a ligand density of between 2 and 3 μmol m⁻². It is impossible to get a measurements of each of the three ligands with carbon measurements alone. Elemental analysis of nitrogen would at least give us an accurate reading for the CN ligand, but given the suspected low bonding density and the presence of only 1 nitrogen per ligand these results were below the recommended limits of the instrumentation and an accurate measurement could not be obtained. This lack of stationary phase explains the poor retention in reversed phase mode and consequently improved retention when operating HILIC conditions.
Conclusions

A non-destructive protocol to assess the axial heterogeneity of the monolithic surface coverage has been developed. It was found that the peak variance increased along the length of the column. This correlates to band broadening which could be caused by a less dense coverage of the ligands on the stationary phase along the length of the monolithic rod. This was observed with unretained and retained markers in both the forward and reverse flow directions. It has been proposed that narrower peaks will be observed when there is a positive change in the ligand density across the chromatographic peak, which was observed in the reverse flow configuration that showed an improvement to plate height of 15%. This suggests the *in-situ* modified monolith had a decreasing surface coverage gradient from the 1 cm residence location to the 9 cm position.

Acknowledgements

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References


Figure Captions

**Figure 1:** Schematic of the different parking locations on the *in-situ* modified monolith. In the forward flow direction, the 1 cm park location is near the column inlet and the 9 cm park location is at the column outlet. In the reverse flow direction, the 9 cm park location is near the column inlet and the 1 cm park location is at the column outlet.

**Figure 2:** Height equivalent to a theoretical plate with ethylbenzene in the forward (blue squares) and reverse flow direction (red circles).

**Figure 3:** Overlay of the 5 thiourea bands parked at 5 locations (1, 3, 5, 7 and 9 cm) on the chromatographic bed for various residence times (0, 5, 10, 15, 30, 60, 120 and 180 min) on the *in-situ* modified monolith. This overlay is for the parking experiments completed in the forward flow direction.

**Figure 4:** Calculated variance (min²) of the modified monolith for the 5 park locations (1, 3, 5, 7 and 9 cm) for the residence times a) 0 min, b) 10 min, c) 30 min and d) 180 min in the forward flow direction.

**Figure 5:** Calculated variance (min²) of the modified monolith for the 5 park locations (1, 3, 5, 7 and 9 cm) for the residence times a) 0 min, b) 10 min, c) 30 min and d) 180 min in the reverse flow direction.

**Figure 6:** Calculated variance (min²) for the 5 park locations (1, 3, 5, 7 and 9 cm) for the residence times a) 0 min, b) 10 min, c) 30 min and d) 180 min in the forward flow direction on the C18 monolith. (It should be noted that these plots have the same range on the Y-axis as the variance plots for the *in-situ* modified monolith in Figure 4).

**Figure 7:** Calculated variance (min²) of the modified monolith of riboflavin for the 5 park locations (1, 3, 5, 7 and 9 cm) for the residence times a) 0 min, b) 10 min, c) 30 min and d) 180 min in the forward flow direction.

**Figure 8:** Calculated variance (min²) of the modified monolith of riboflavin for the 5 park locations (1, 3, 5, 7 and 9 cm) for the residence times a) 0 min, b) 10 min, c) 30 min and d) 180 min in the reverse flow direction.
Figures

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Figure 4: Calculated variance (min$^2$) of the modified monolith for the 5 park locations (1, 3, 5, 7 and 9 cm) for the residence times a) 0 min, b) 10 min, c) 30 min and d) 180 min in the forward flow direction.
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Figure 6: Calculated variance (min$^2$) for the 5 park locations (1, 3, 5, 7 and 9 cm) for the residence times a) 0 min, b) 10 min, c) 30 min and d) 180 min in the forward flow direction on the C18 monolith. (It should be noted that these plots have the same range on the Y-axis as the variance plots for the in-situ modified monolith in Figure 4).
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Figure 8: Calculated variance (min$^2$) of the modified monolith of riboflavin for the 5 park locations (1, 3, 5, 7 and 9 cm) for the residence times a) 0 min, b) 10 min, c) 30 min and d) 180 min in the reverse flow direction.
Tables

Table 1: HPLC injector program for injecting multiple peaks.

<table>
<thead>
<tr>
<th>Step</th>
<th>Function</th>
<th>Parameter</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Draw</td>
<td>Draw 40 µL from sample vial</td>
</tr>
<tr>
<td>2</td>
<td>Needle</td>
<td>Move Needle into seat</td>
</tr>
<tr>
<td>3</td>
<td>Valve</td>
<td>Switch valve to ‘Bypass’</td>
</tr>
<tr>
<td>4</td>
<td>Eject</td>
<td>Eject 2 µL into seat</td>
</tr>
<tr>
<td>5</td>
<td>Repeat</td>
<td>Repeat 5 times</td>
</tr>
<tr>
<td>6</td>
<td>Valve</td>
<td>Switch valve to “Main Start”</td>
</tr>
<tr>
<td>7</td>
<td>Wait</td>
<td>Wait 0.01 min</td>
</tr>
<tr>
<td>8</td>
<td>Valve</td>
<td>Switch valve to ‘Bypass’</td>
</tr>
<tr>
<td>9</td>
<td>Wait</td>
<td>Wait 1.31 or 1.17 min*</td>
</tr>
<tr>
<td>10</td>
<td>End Repeat</td>
<td>End Repeat</td>
</tr>
</tbody>
</table>

*Wait time of 1.31 min was for the *in-situ* modified monolith and the wait time of 1.17 min was for the C18 monolith.
Table 2: Range in measured variance (min²) for each parking location, column, flow direction and analyte probe.

<table>
<thead>
<tr>
<th>Parking location (cm)</th>
<th>In-situ modified monolith</th>
<th>C18 monolith</th>
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<tr>
<td></td>
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<td>Riboflavin</td>
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<td></td>
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<td>Reverse</td>
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<td>7</td>
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<tr>
<td>9</td>
<td>0.0848</td>
<td>0.0857</td>
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Table 3: Elemental results measuring surface coverage at different locations along the column axis.

<table>
<thead>
<tr>
<th>Column Section</th>
<th>Phenyl, Cyano &amp; Endcapping Ligands (μmol m⁻²)</th>
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<tbody>
<tr>
<td>1</td>
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<tr>
<td>2</td>
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