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Reduction of purge and re-equilibration times following a gradient reversed-phase liquid chromatography method

Stevi Elise Parker

THE FLORIDA STATE UNIVERSITY

COLLEGE OF ARTS AND SCIENCES

REDUCTION OF PURGE AND RE-EQUILIBRATION TIMES FOLLOWING A GRADIENT REVERSED-PHASE LIQUID CHROMATOGRAPHY METHOD.

By

STEVI E. PARKER

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Abstract

Pancreatic islet cells secrete hormones that regulate glucose levels in the body; measuring the output of these hormones is of interest for the study of diabetes mellitus. A gradient method was optimized for analyzing peptides secreted from islet cells with liquid chromatography (LC). A solution of somatostatin and glucagon was used to find minimal purge and re-equilibration step times for this gradient. Once the gradient method was tested for precision, the purge and re-equilibration steps were shortened until the chromatograms showed an increase in peak area. Extending the flush time was explored in later experiments and was found to improve reproducibility between injections. Too short of a re-equilibration time led to increased variation in the area of the glucagon peak. The original purge time of 1 min was extended and the original re-equilibration time of 3.25 min was be reduced. This refined method will be used in future experiments with an automated microfluidic LC-mass spectrometry (MS) system.

1. Introduction

1.1 Islets of Langerhans

The pancreas has two main functions: digestion and blood glucose regulation. Diabetes mellitus, a condition that affects roughly 9.4% of Americans, is a direct result of improper glucose regulation.[1] Monitoring the hormone output of pancreatic cells, specifically the islets of Langerhans, is an established way to study this condition and possible treatments. Islet cells make up 2% of the pancreas and have four distinct designations based on the peptides they synthesize: α cells, which produce glucagon; β cells, which produce insulin (most common islet cell); δ cells that produce somatostatin; and F or PP cells that produce pancreatic peptide.[2]

After an oral glucose load, insulin is typically released from β cells in two phases, the first occurring within minutes and the second within an hour or two.[2] Insulin lowers glucose levels by stimulating either its conversion into glycogen or its break down into energy by glycolysis; glucagon stimulates the breakdown of glycogen and conversion back into glucose through gluconeogenesis for further use in the body. Glucagon is released in phases that contrast with that of insulin such that the two peptides keep the body at a low glucose level (~5 mM). In patients with type 2 or early type 1 diabetes patients, the first insulin phase is lost, disrupting the homeostasis between these peptides and raising glucose levels dramatically.[3] Analyzing the time-dependent relationships of the peptides released from islet cells would allow for a better understanding of the biological relationships between not just insulin and glucagon, but other islet-secreted peptides.

1.2 Chromatography

Chromatography is a process by which gas- or liquid-state chemical mixtures are separated into their individual components and measured. It is used for quantitative and/or qualitative analyses, which measure the amount of an analyte present and the physical qualities of an analyte, respectively. Liquid-state chromatography (LC) is used for the analysis of nonvolatile or temperature-sensitive compounds, namely organic and biological molecules.

In LC systems, analytes are injected and then carried through a column, where they are separated by diffusion through porous particles. After being separated by the column, analytes are detected and measured by a detector. The resulting chromatogram

will ideally show one peak for each analyte where each analyte has its own individual retention time, the time that it takes for the analyte to be detected after injection.

LC systems can be tailored to an experiment by changing components of the system, the first of which is the LC column. Columns differ by length, diameter, and stationary phase (the portion of the column that is responsible for separating the analytes). The two main types of stationary phase are polar (normal-phase) and non-polar (reversed-phase). The stationary phase of reversed-phase LC (RPLC) columns are comprised of nonpolar hydrocarbons bonded to fixed porous silica particles. The most common hydrocarbon to use for the stationary phase is an alkane chain of 18 carbons, abbreviated “C18”. The hydrophobicity of an analyte determines how long it is retained in the column; hydrophobicity is a measure of the interaction between the analyte and water molecules and changes with the chain length and surface area of that analyte.[4] Analytes will partition into the nonpolar stationary phase dependent on their hydrophobicity: analytes with a high hydrophobicity will spend more time in the stationary phase compared to those with a low hydrophobicity, causing them to leave the column, or elute, at different times. RPLC is preferred for the analysis of peptides because they are not degraded by the solvents and are retained in the column due to their hydrophobic carbon backbones.

An LC pump delivers mobile phase at a constant flow rate through the system. Mobile phases used in RPLC are often a two-component mixture, a combination of a polar “Mobile Phase A” (MPA) and nonpolar “Mobile Phase B” (MPB). Varying the composition of mobile phase will affect the time the analyte takes to elute. Higher compositions of MPB will create a more nonpolar environment within the column and cause the analyte

to diffuse back into the MP from the stationary phase and elute from the column. For the RP system, MPB is a strong eluent and MPA is a weak eluent. Some common mobile phases are water, acetonitrile, methanol, and tetrahydrofuran. Mobile phase is selected for a system based on eluent strength, cost, and how it interacts with the analyte.

1.3 Mobile Phase Gradients

Analytes in mixtures sometimes vary too widely in retention time to simply raise (or lower) MPB composition for an entire analysis. This problem is known as the general elution problem, which states that a single set of analysis conditions tends to be not ideal for complex mixtures.[5] As a solution to this problem, mobile phase gradients are used. Mobile phase gradients increase the percentage of MPB throughout the run to lower the retention time for later-eluting analytes without affecting analytes that elute early into the run.

An example of a gradient (plotted as %MPB over time) is shown in Figure 1.

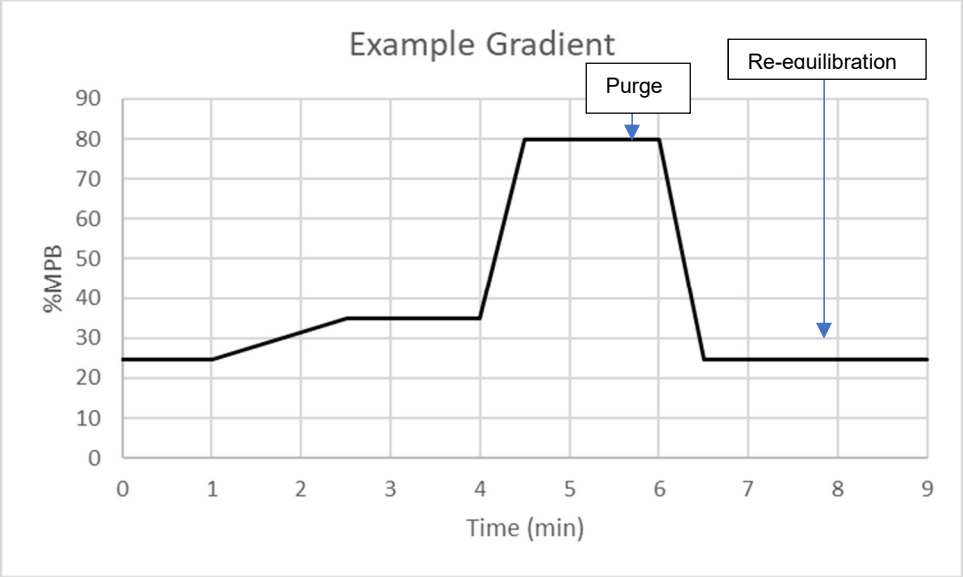

Fig. 1. *RP-LC mobile phase gradient. MPB composition is plotted over time for a 9-minute gradient method.*

Gradient methods typically end at a higher MPB than their starting conditions. If the next run begins before the system has been re-equilibrated to the starting conditions, the chromatogram will show inconsistency between runs. The optimum gradient will maximize resolution and sensitivity while producing similar results under controlled conditions.[6] One way to prepare the system for the runs to follow is to flush with a high MPB and then re-equilibrate at starting conditions. The gradient method in Figure 1 begins at 24.5% MPB (75.5% MPA) which is a weak MP composition. At 1 min, the %MPB increases for 1.5 min to 35%, a large enough increase in eluent strength from starting conditions to lower analyte retention times. The MP composition is held constant until minute 4 to allow for analytes to elute. At minute 4, the concentration is ramped to 80% MPB and held for 1 min to flush any remaining solutes from the column; this is known as the purge (or flush) step. At 6 min, the MPB reduces to 24.5% and held until 9 minutes to return the system to starting conditions; this is known as the re-equilibration step. Columns that have not been adequately flushed will retain analyte in the column that will cause variations in analyte peak areas measurements, altering the concentration of analyte found from quantitative analysis. Columns that have not been fully re-equilibrated may cause a shift in analyte retention time on subsequent runs, causing separation time to increase with each run. To optimize a gradient method for rapid consecutive runs, finding the minimum flush and re-equilibration volumes is critical.

1.4 Background Information

There are many ways to shorten re-equilibration time. Waters™ (Milford, MA) recommends calculating the minimum re-equilibration time by adding together five

column volumes and three system volumes.[7] The column volume can be calculated from length and radius of the column. For this system, the column was 50 mm long and 4.6 mm in diameter, so the calculated column volume (equation 1) was 0.830 mL. The system volume is the amount of liquid in the system between where solvent is mixed and where it enters the column; the system volume used in my research was approximately 0.5 mL. The minimum re-equilibration volume calculated from these two parameters was 5.7 mL (equation 2), so at 1 mL/min, it would take 5.7 minutes to re-equilibrate the system.

Equation 1. $$V_{column} = \pi R^2 L$$

Equation 2. $$V_{re-eq} = 3V_{sys} + 5V_{column}$$

One method to lower the re-equilibration time further is to mix additives into the solvents. Dorsey et al. found that re-equilibration time could be reduced up to 78% by simply incorporating 1-propanol as a mobile phase additive.[8] Our system already used formic acid as a mobile phase additive, so we opted not to add 1-propanol.

Another programmed element that can be used to reduce re-equilibration time is the flow rate. Schellinger found that by using a flow rate of 3 mL/min, the system was fully equilibrated within ten minutes; a flow rate of 1 mL/min was also tested, but it was found that the system was not fully equilibrated until about 20 minutes.[9]

Another research group, Coym et al., investigated the effects of raising column temperature on re-equilibration. Coym found that the temperature dependence of a column depends on the stationary phase: C_{18} had a 38% decrease in re-equilibration

volume with acetonitrile as the organic modifier when column temperature was raised from 20°C to 50°C.[10]

This background literature provides information that streamlined our purge and re-equilibration optimization process. Some of these techniques, like adding 1-propanol to MP and changing flow rate, will not be used in the rapid separations of peptides, while others such as the base re-equilibration time and column temperature were considered.

1.5 Thesis Goals

The goal of the project was to refine an LC gradient method for the rapid analysis of fractionated peptide samples from islets of Langerhans. Once optimized, this method will be used for successive separations, so it is critical to have as short of runs as possible to minimize the overall experimental time. While the method had been optimized in terms of changing the %MPB from time 0 – 4 min (as seen in Fig 1), the flush and re-equilibration times had not. As can be seen in figure 1, these times are longer than the actual separation time, so their optimization is critical to reducing the overall method duration. To perform this optimization, the variations in retention time, peak area, and peak height of sample peaks over multiple repetitions of the same gradient method were measured.

2 . Materials and Methods

2.1 Materials

HPLC-grade acetonitrile, formic acid, and glucagon were purchased from Sigma-Aldrich (St. Louis, MO). Somatostatin was purchased from BaChem (Torrance, CA). MPA was composed of deionized water with 0.1% formic acid as an additive and MPB was acetonitrile with 0.1% formic acid as an additive. Glucagon and somatostatin were

prepared in a 5 μM glucagon and 5 μM somatostatin solution with water. Blank injections on the LC were deionized water.

2.2 Instrumentation

All experiments were completed on a Beckman System Gold HPLC system (Beckman Coulter, Indianapolis, IN) containing a 127S solvent module and 166 UV-Vis detector. The solvent module mixed two mobile phases. All data was collected at a wavelength of 214 nm, which is absorbed by amide bonds in peptides. The HPLC column was a Waters Atlantis reversed-phase dC18 column (Waters Corp., Milford, MA). The dimensions were 50 mm by 4.6 mm with a 5 μm particle diameter and 100 Å pore size. The injection valve was a manual 6-port injector and had a sample loop of volume 20 μL.

2.3 Methods

32 Karat software controlled the LC system and collected all data. Peak areas and heights were calculated by the software. The flow rate was held at 1 mL/min for all experiments. Experiments were completed at room temperature. The original gradient employed: 24.5% MPB for 1 min, increase to 35% MPB over 1.5 min, hold for 1 min, 35%MPB to 80%MPB over 0.5 min, hold for 1 min, 80% MPB to 24.5% MPB for 0.5 min, hold until 8 min.

For analysis of chromatographic peak characteristics, values of the relative standard deviation (RSD) of either peak area or peak height less than 15% were considered reproducible. RSD was calculated as:

Equation 3. $$RSD = \frac{average}{standard\ dev.} * 100\%$$

3. Results and Discussion

As the goal of the project was to reduce the total method time for separation of peptide mixtures, a reliable method first had to be established. The gradient itself had already been determined, but the purge and re-equilibration steps needed to be optimized. First, the elution order for the two sample components was found. Then, an initial experiment was conducted that tested a 1-minute purge step and a 3.25-minute re-equilibration step to find the variation in measurements between consecutive runs of the same solution. From those results, both steps were altered systematically to achieve the lowest total time with data consistent between consecutive runs.

3.1 Elution Order of Glucagon and Somatostatin

While the main experimental goal of the project was optimizing a method, the elution order of the two sample peptides, glucagon and somatostatin, still had to be determined. To find their order, a single-component sample of glucagon was prepared and run five times; the data collected was used to calculate its average retention time. Since there are only two peptides used in this project, only one had to be tested because the remaining peak is guaranteed to be the other peptide. The elution part of the gradient method was not altered in any experiments, so the retention time of glucagon will remain consistent in all experiments. Figure 2 shows a chromatogram of the glucagon-only run, which has an injection peak around minute 1, an analyte peak around 3.5 minutes, and a peak due to the purge between minutes 5 and 7. The analyte peak has an absorbance of about 1. Table 1 contains the glucagon retention times for five consecutive runs.

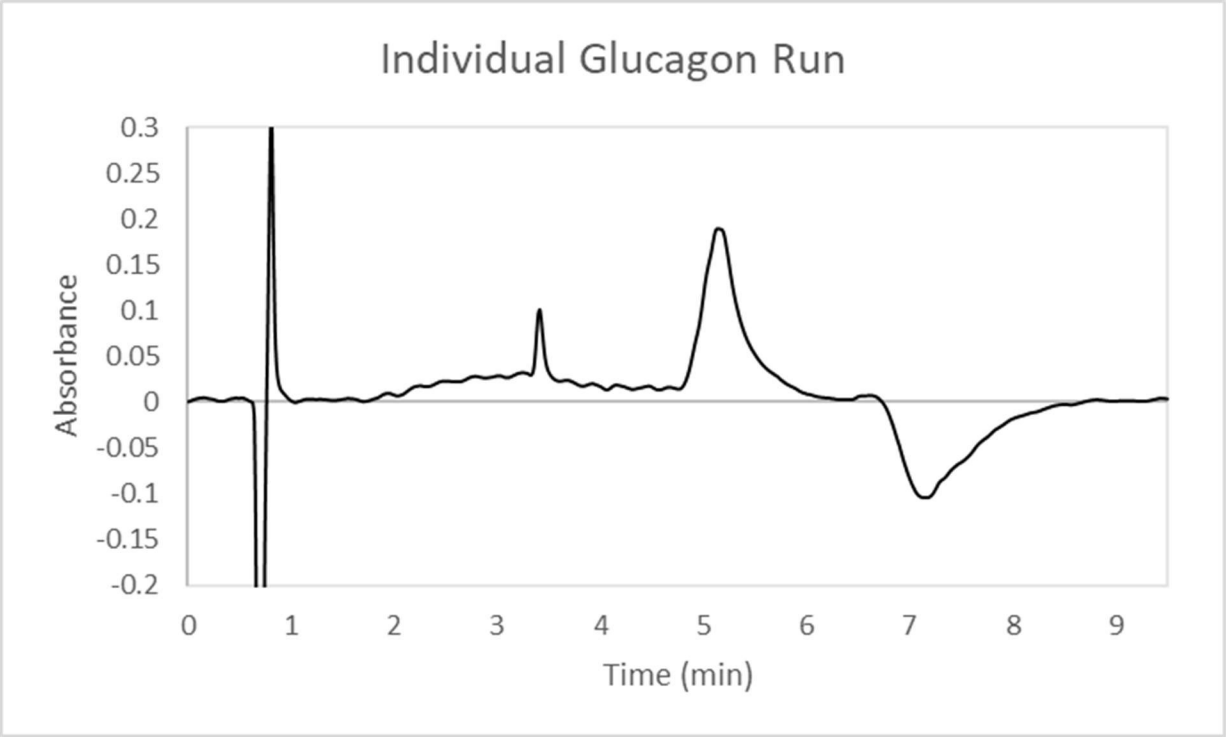

Figure 2: Glucagon Elution Run. A glucagon solution was run to determine its retention time. There is a sample peak between 3 and 4 minutes.

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Table 1: Retention Time Data for Glucagon

Run #	**Retention Time**
1	3.433
2	3.433
3	3.417
4	3.417
5	3.417
Average	3.423
Standard Deviation	0.007838
Residual SD	0.2290

The data in Table 1 shows that the average retention time for glucagon is 3.423 minutes. Looking forward, Table 2 shows the retention times for the two analyte peaks as 2.717 min and 3.417 min, so the first peak must be somatostatin and the second must be glucagon.

3.2 Establishment of a Method

First, a consistent gradient method had to be established. Four consecutive separations of somatostatin and glucagon were run with a 1-minute flush step at 80% MPB and a 3.25-minute re-equilibration step at 24.5% MPB. Figure 3 shows a chromatogram of the two analytes with both analyte peaks between 2.5 minutes and 3.5 minutes. There is a large injection peak before minute 1 and a solvent peak between minute 5 and minute 7. Table 2 shows the retention time, peak area, and peak heights for both analyte peaks over five separations (only four are plotted in Figure 3).

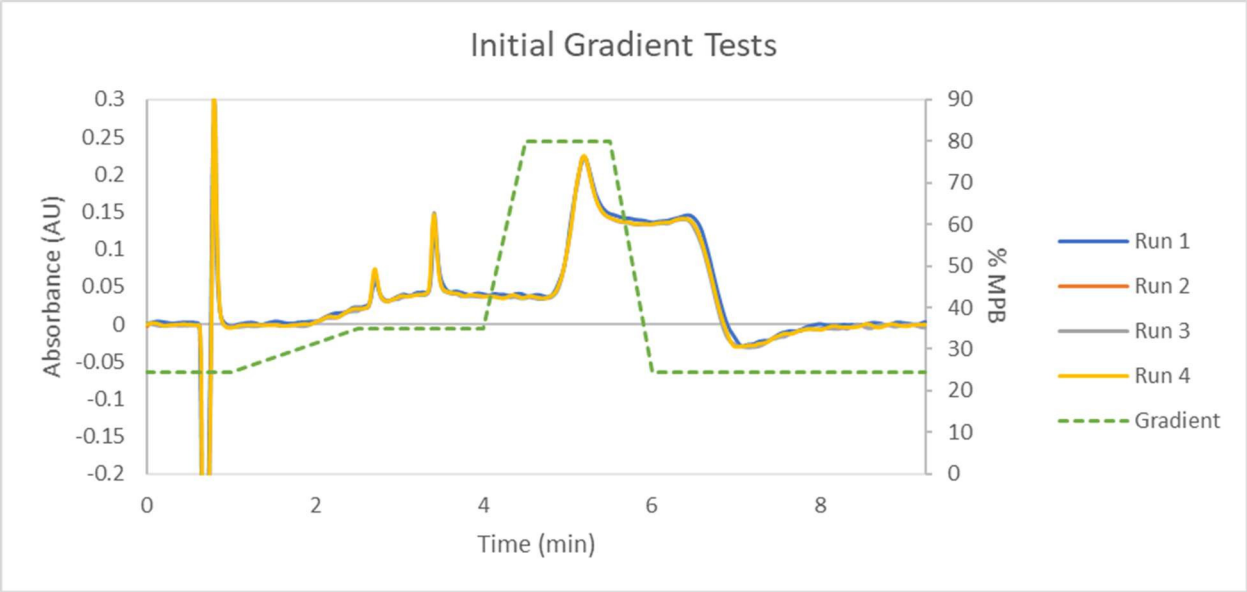

Figure 3: Initial Gradient Testing. Four consecutive separations are overlaid. There are two analyte peaks between minute 2 and minute 4, plotted as absorbance on the left y-axis. MPB percentage is displayed on the right y-axis and is shown as a dotted line over the separation.

Table 2: Chromatographic Data of the Analyte Peaks

	Somatostatin Peak			Glucagon Peak		
Run #	**Retention Time**	**Peak Area**	**Peak Height**	**Retention Time**	**Peak Area**	**Peak Height**
1	2.717	52026	15083	3.417	36805	18942
2	2.717	58252	16122	3.417	96105	32373
3	2.717	66714	20374	3.417	71956	31581
4	2.717	82611	24294	3.417	72603	32433
5	2.717	101656	28416	3.417	94627	36073
Average	2.717	72252	20858	3.417	74419	30280
Std. Dev.	0	20048	5589	0	23994	6573
RSD (%)	0	27.75	26.80	0	32.24	21.71

As can be seen in Table 2, analyte retention time did not vary between runs. This consistency implies that the re-equilibration time (3.25-minute) used in this method was sufficient to return the system to initial conditions before the next run began. In contrast to the retention time, the peak areas and heights had RSDs between 21.71% and 32.24%, which are significantly higher than the 15% limit. Peak areas and heights increased throughout the runs, which is a sign that the peptides were not being completely flushed from the system after each run.

3.3 *Extended Purge Time*

Because the original gradient showed an increase in analyte peak area after each consecutive run, the purge step was extended from 1 minute to 1.25 minutes. In the previous experiment, successive separations were run in order to determine whether the re-equilibration step was long enough to result in a shift in retention time. This experiment was altered to test the purge time, so instead of only sample separations, sample separations were alternated with blank injections. Figure 4 shows two overlaid traces, a chromatogram of a glucagon/somatostatin sample run and a baseline blank run. The two analyte peaks are between 2.5 minutes and 3.5 minutes.

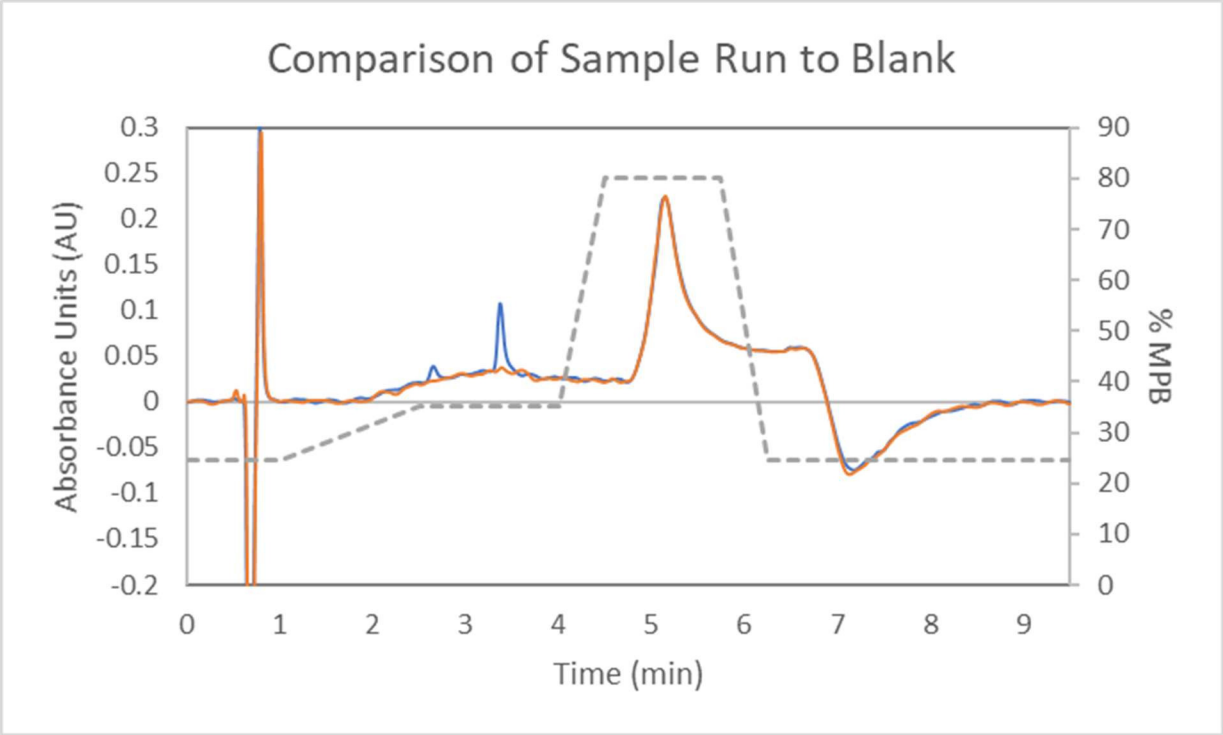

Figure 4: Comparison Between Sample and Blank Chromatograms. The line shown in blue represents the sample run and the line shown in orange represents the blank taken after the sample. The gradient method is shown on the same plot as a dashed line and the right y-axis is % MPB.

If the system was not fully flushed before the blank injection, a small peak of residual analyte would appear on the blank chromatogram. The sample peaks always fall into ranges of 2.527-2.741 min and 3.153 – 3.428 min, so these ranges were used to calculate the peak areas for somatostatin and glucagon, respectively. Once peak area was calculated for each analyte peak, the blank peak areas were compared to those of the sample separations. Each blank run was divided by the sample

immediately prior. This was done with the following equation, where the blank area is calculated as a percentage of the sample:

Equation 4. $$\% \, Area = \frac{area_{blank}}{are \ _{sample}} * 100\%$$

Table 3: Glucagon Peak Area Comparison

Run	Area (Blank)	% Area	Area (Sample)
0	390696	n/a	n/a
1	387213	11.52	3362509
2	313644	10.77	2911447
3	454528	15.63	2908349
4	310357	12.60	2463477
Average	371287.6	12.75	2911446
Std. Dev.	60404.04	1.85	367035
RSD (%)	16.27	14.51	12.61

As shown in Table 3, the area percent of the blank areas compared to the sample peak areas averaged to 12.75% with a standard deviation of 1.85%. This correlated to an RSD of 14.51%, which falls in the 15% range that is considered replicable.

Somatostatin was not included in this data because the software did not detect a peak in its range (2.527 – 2.741 min) for the blank injections. This shows that the analyte was being properly flushed from the system, which was the goal of the experiment.

To further test the capability of the system to flush out analytes after a separation, the flush time was extended from 1.25 minutes to 2 minutes. This

experiment was performed to test how much the area percentage could be lowered by flushing the system for a long time. Figure 5 shows the entire gradient method used in this experiment. The %MPB starts at 24.5% and holds for 1 minute, increases to 35 until 2.5 minutes, holds until 4 minutes, increases to 80% for 30 seconds, holds for 2 minutes, reduces back to 24.5% over 30 seconds and holds until 10.25 minutes. Table 4 contains the data from four separations of somatostatin and glucagon using this method.

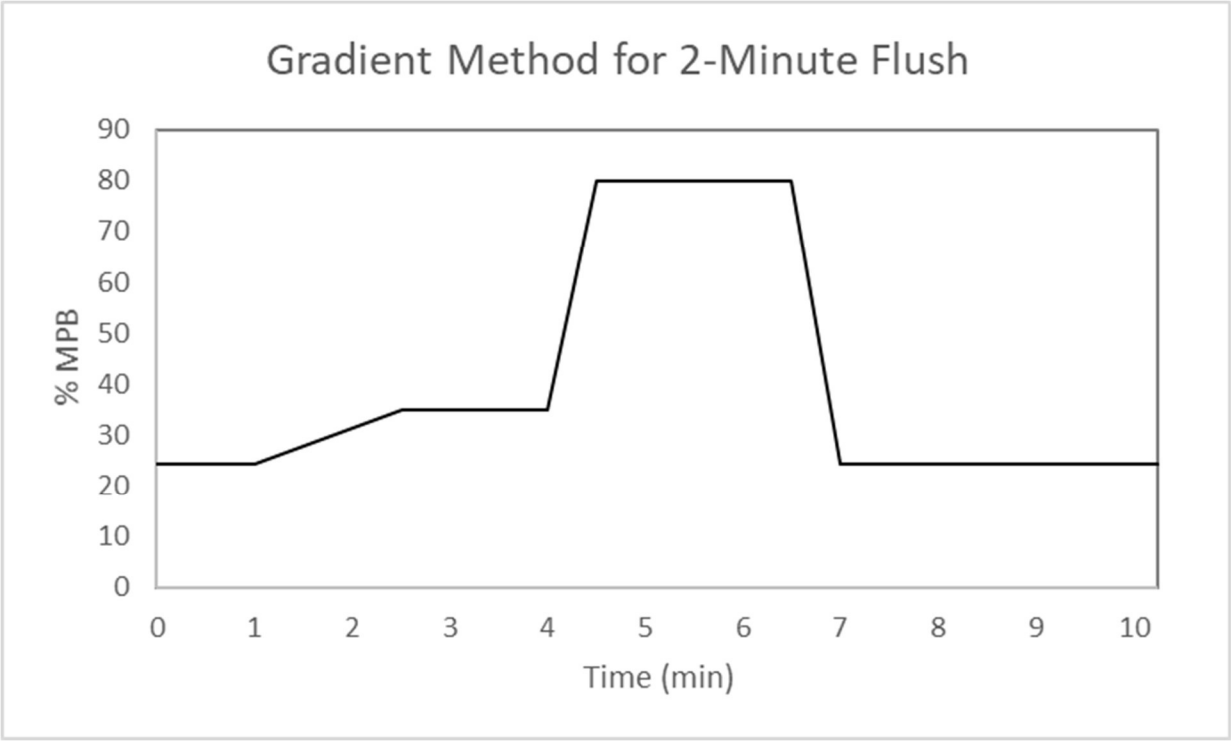

Figure 5: Gradient Method for 2-Minute Flush Test. The program for the gradient method is plotted as %MPB over time, a total separation time of 10.25 minutes

Table 4: Chromatographic Data of Somatostatin and Glucagon with 2-Minute Flush

	Somatostatin				Glucagon			
Run	**tR**	**Blank Area**	**% Area**	**Sample Area**	**tR**	**Blank Area**	**% Area**	**Sample Area**
1	2.533	52817	2.6092	2024282	3.383	303444	5.3138	5710538
2	2.583	144087	7.8314	1839867	3.383	213669	3.1199	6848481
3	2.667	108351	6.1726	1755355	3.383	282541	4.2550	6640264
4	**	**	**	**	3.383	346576	4.8901	7087300
ave	2.594	101752	5.5377	1873168	3.383	286558	4.3947	6571646

**No peak was detected in the somatostatin range on the fourth run likely due to somatostatin being fully flushed from the system.

As shown by the data in Table 4, the area percent was reduced from the previous experiment (12.75% average) with this increased flush time (4.39% for the glucagon peak). While this data shows that the amount of residual peptide in the column after each separation (described by area percent measurements) can be lowered by flushing the system for a longer amount of time, the area percentages for the 1.25-minute runs were reproducible (RSD<15%) so this flush time was used in further experiments.

3.4 Re-equilibration step reduction

After extending the purge time from 1 minute to 1.25 minutes, the re-equilibration time was then examined. The initial method experiment (shown in Figure 4) used a gradient method with a re-equilibration time of 3.25 minutes and because there was no change in retention time between successive separations, it was determined that the re-

equilibration time was enough to minimize deviation in retention time between runs. In the next series of experiments, the re-equilibration step was reduced and tested with the 1.25-minute flush step established in the previous experiments. Figure 6 shows the gradient method used for this experiment, where the first 4 minutes are unchanged (the separation steps). At 4.5 minutes, the %MPB is ramped to 80% over 30 seconds and held for 1.25 minutes, then reduced to 24.5% over 30 seconds and held for 2.75 minutes. Table 5 contains the retention times, peak areas, and peak heights for somatostatin and glucagon analyte peaks for five consecutive runs.

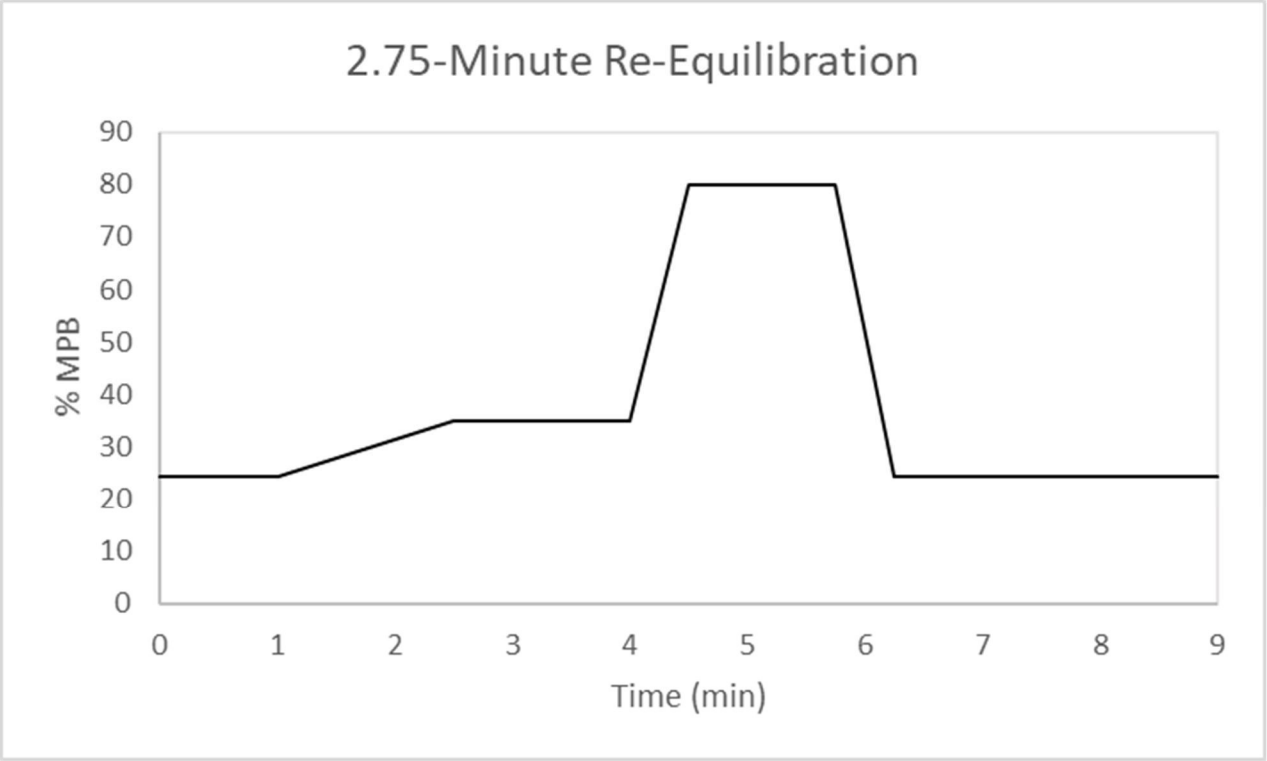

Figure 6: Gradient Method for the 2.75-Minute Re-Equilibration Experiment. A time program for a separation with a 1.25-minute flush step and a 2.75-minute re-equilibration step is shown.

Table 5: Chromatographic Data for Glucagon and Somatostatin with 2.75-Minute Re-Equilibration

	Somatostatin			Glucagon		
Run #	Retention Time	Peak Height	Peak Area	Retention Time	Peak Height	Peak Area
1	2.717	7361	39232	3.433	9775	52497
2	2.700	7921	41395	3.433	13335	78303
3	2.717	6890	37001	3.417	17396	97927
4	2.700	6178	36880	3.417	16028	95991
5	2.717	6065	36728	3.417	19099	103572
Average	2.710	6883	38247	3.423	15127	85658
STD	0.0093	786.1	2037.7	0.0088	3660	20808
RSD	0.3436	11.42	5.328	0.2560	24.20	24.29

While the retention times shown in Table 5 reman virtually unchanged (RSD ~0.3%), the glucagon peak area and height had an RSD of about 24% each. While the data in Table 3 had RSD values between 12% and 16% for the glucagon peak of sample and blank runs, respectively, reducing the re-equilibration time caused a significant increase (8 – 12%), meaning that less glucagon is being purged from the column after each run. This results in more analyte eluting in later runs and causing large deviations between each data set. Extending the flush step was shown in a previous experiment to reduce the RSD between peak area and height measurements (Table 4), so changing the flush time from 1.25 minutes to 1.5 minutes was explored as a means to reduce the glucagon peak RSD. As the overall goal of the project is to reduce the total method time, the re-equilibration time was reduced by 15 seconds to compensate for the flush extension, keeping the total method time to 9 minutes. Figure 7 shows a chromatogram of a glucagon/somatostatin separation with a new gradient. The new gradient has a 1.5-

minute flush time and a 2.5-minute re-equilibration time. Table 6 contains the retention times, peak areas, and peak heights for five consecutive runs of somatostatin and glucagon on this method.

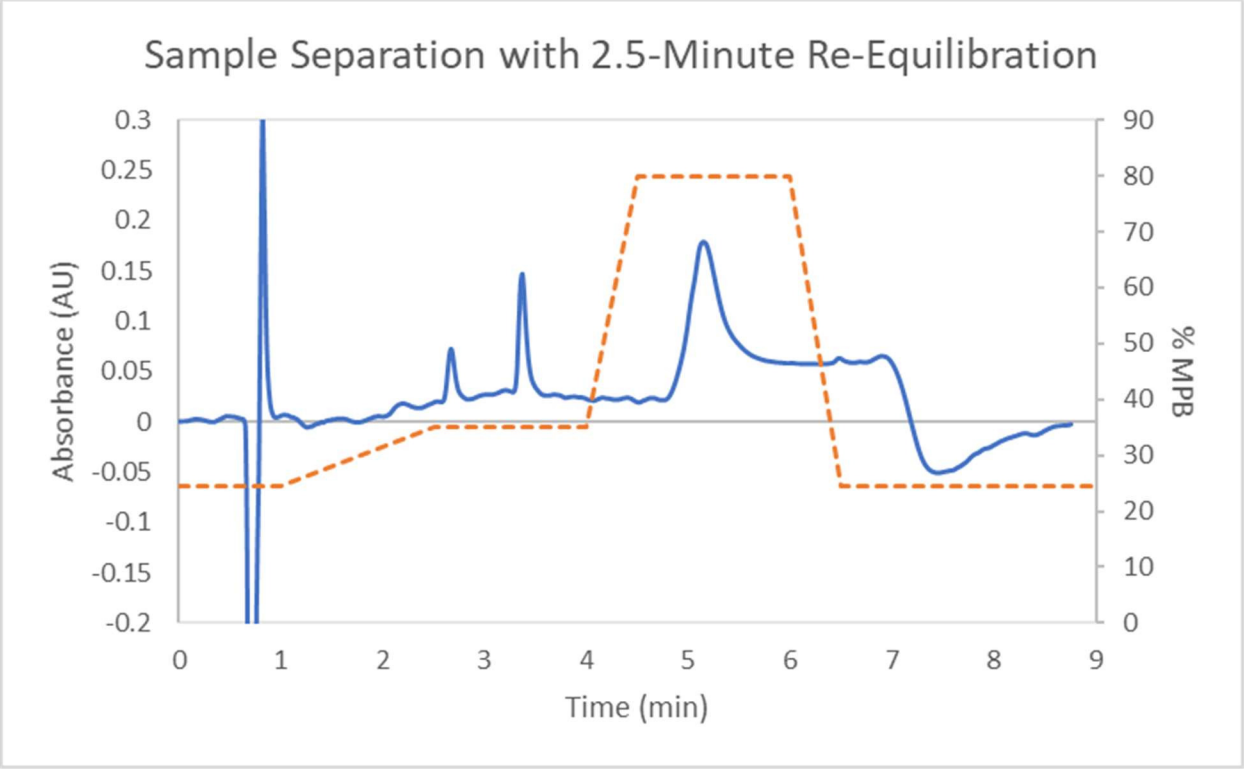

Figure 7: Sample Separation with Extended Flush and Reduced Re-Equilibration. A gradient method is overlaid with a sample separation of glucagon and somatostatin.

Table 6: Chromatographic Data for Glucagon and Somatostatin for 2.5-Minute Re-Equilibration

	Somatostatin			Glucagon		
Run #	**Retention Time**	**Peak Area**	**Peak Height**	**Retention Time**	**Peak Area**	**Peak Height**
1	2.7	220707	44223	3.383	817025	193986
2	2.683	235293	47756	3.367	742332	182594
3	2.717	194833	42471	3.383	796185	168782
4	2.7	206953	44025	3.341	**	**
5	2.667	209963	42427	3.383	719882	182460
Average	2.693	213550	44180	3.371	768856	181956
STD	0.01903	15254	2168	0.01835	45347	10311
RSD	0.7067	7.143	4.908	0.5443	5.898	5.667

**Software error resulted in no peak data other than retention time for the fourth run.

As shown in table 6, the glucagon peak area and height RSDs were reduced from 24% to about 5.8%. From these results, the re-equilibration time was reduced even further, from 2.5 minutes to 2.25 minutes. Figure 8 is a chromatogram for a somatostatin and glucagon separation overlaid with the gradient method that was used for the separations. The flush time is 1.5 minutes and the re-equilibration step is 2.25 minutes. Table 7 shows the retention time, peak area, and peak height data for the somatostatin and glucagon peaks from 6 consecutive separations.

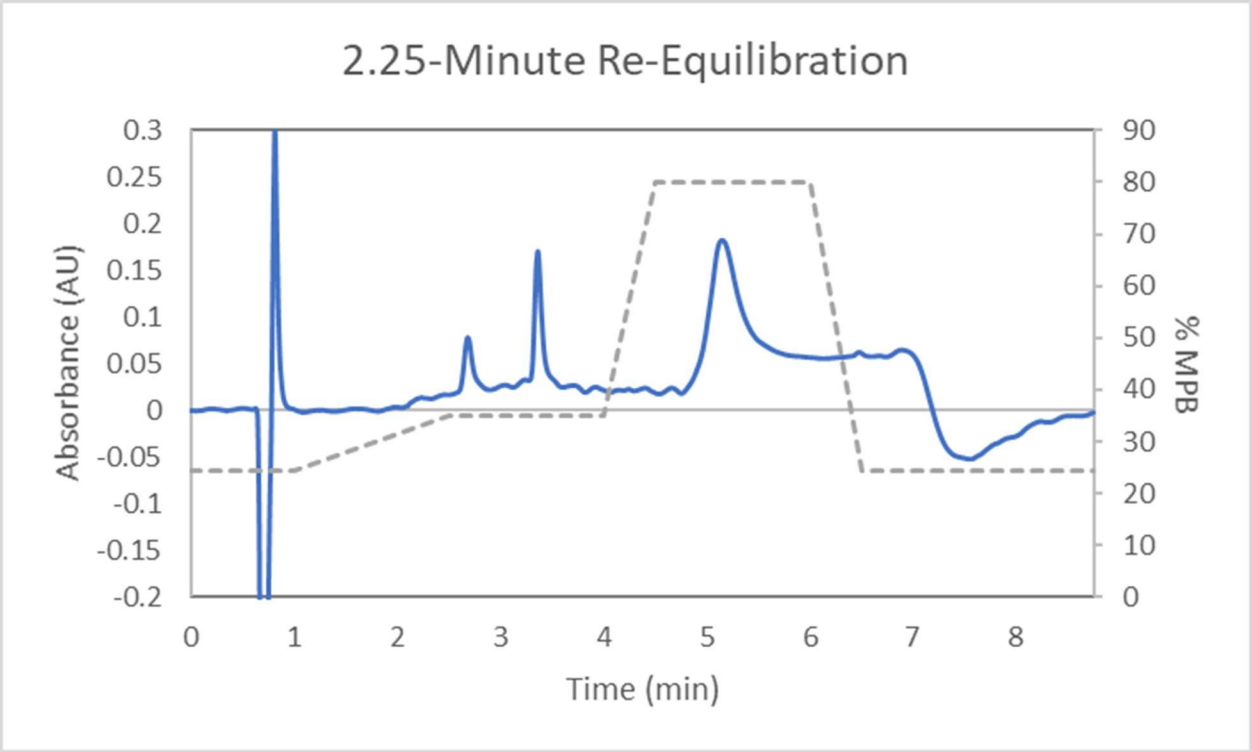

Figure 8: 2.25-Minute Re-Equilibration Run. Overlay of the reduced gradient with a 2.25-minute re-equilibration step and 1.5-minute purge step.

Table 7: Reduced Re-Equilibration Data

	Somatostatin			Glucagon		
Run #	**Retention Time**	**Peak Area**	**Peak Height**	**Retention Time**	**Peak Area**	**Peak Height**
1	2.733	163788	37082	3.383	858452	192002
2	2.700	229114	47345	3.383	982328	214075
3	2.650	196084	42102	3.400	1113722	235017
4	2.683	245983	48962	3.383	922289	192611
5	2.700	204777	40238	3.400	944961	198903
6	2.700	209081	42698	3.400	920377	188194
Average	2.694	208138	43071	3.392	957022	203467
STD	0.0272	28272	4426	0.0093	86725	17952
RSD	1.008	13.58	10.28	0.2745	9.062	8.823

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Reducing the re-equilibration time resulted in an increase in RSD for somatostatin retention time from 0.71% to 1%. Replicable retention time RSD values fall below 3%; while RSD did increase when the re-equilibration step was lowered from 2.5 minutes to 2.25 minutes, the system was re-equilibrated enough between separations to fall into this range. Lowering re-equilibration time further will increase the RSD, but because the current value is 1%, the RSD would still likely fall under 3%. Similarly, the peak area and height RSDs for both peptides were under the limit of 15%, so this flush time is ideal to produce consistent data.

4. Conclusions

The total time of a gradient method can be reduced without sacrificing data consistency. Too short of a purge time will cause peak area to vary between runs and too short of a re-equilibration time will cause the peaks to shift in retention time. Finding and maintaining the proper length for each of these steps is vital for the development of a method for rapid and successive separations. The purge step is notably sensitive to change and must be carefully tested to find the shortest time with the most precise results. For a two-component peptide mixture, the purge time had to be extended from 1 minute to 1.5 minutes to reduce the peak area RSD from 32% to 9% (glucagon peaks, Tables 2 and 7). While reducing the length of the re-equilibration step does cause an increase in retention time RSD, the increase in RSD is very gradual, so the re-equilibration time can likely be reduced from 2.25 minutes without increasing the RSD above the 3% limit. Through experimenting, the re-equilibration time was reduced from 3.25 minutes to 2.25 minutes with only a 1% increase in retention time RSD

(somatostatin peaks, Tables 2 and 7). The method shown in Figure 8 appears to be optimized for the flush and re-equilibration steps of peptide separations, but due to time constraints, this method was not fully evaluated and further reductions were not tested. Supplementary testing should be conducted to validate these findings. As the optimized method will be used in further experiments for the rapid separation and analysis of islet secretions, minimizing the length of the purge and re-equilibration steps was important. The total separation time was reduced from 9.25 minutes to 8.25 minutes and can likely be reduced further. This optimized method will be employed to resolve peptide mixtures in rapid and successive separations of islet cell secretions.

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