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Application Note

Optimized Chromatography for Mass-Directed Purification of Peptides

Jo-Ann M. Jablonski, Thomas E. Wheat

Waters Corporation

Abstract

This application note illustrates the development of general instrumental parameters and chemical conditions for mass directed purification of peptides in the range of 8 to 25 residues.

Introduction

Peptides play important roles in the physiology of organisms. They do, therefore, provide a major class of compounds for novel therapeutic products. Pure peptides are required to effectively study biochemical interactions in organisms as well as to understand structure-activity relationships in the development of peptide therapeutics.

Traditionally, preparative peptide separations are monitored using UV detection with the collection of many fractions over the course of the entire chromatographic program, necessitating labor-intensive post-run analysis and processing. Mass spectrometry (MS) has proven useful in quickly assessing peak identity and homogeneity

in complex chromatograms, increasing sample throughput. Mass-directed isolation of compounds uses the mass spectrometer to recognize the target peak and deposit it in a fraction collector tube, reducing the number of processing steps.

The chemistry of peptides poses unique chromatographic challenges, including the selection of the appropriate column chemistry, the selection and adaptation of the mobile phase, and the optimization of the mode of detection.

In this study, we illustrate the development of general instrumental parameters and chemical conditions for mass-directed purification of peptides in the range of 8 to 25 residues. A refined protocol for translating a rapid pilot separation to an optimal preparative separation is demonstrated. Peptide compound isolation is more efficiently and effectively obtained using optimized chromatography and mass-directed purification.

Experimental

The purification of peptides poses specific challenges to the chromatographer. The first challenge is achieving high throughput while maintaining the shallow gradients necessary for peptide separation. Variable impurities, such as side products, deletions, and remaining reagents, can complicate and interfere with separations. Finally, the multiple charging of peptides often makes it difficult to predict the most abundant species in a given MS experiment.

Waters Preparative Chromatography System

Throughout the experiments, a Waters Preparative Chromatography System consisting of a 2525 Binary Gradient Module, 2767 Sample Manager, Column Fluidics Organizer, ZQ Mass Spectrometer, 2996 Photodiode Array Detector, 2420 Evaporative Light Scattering Detector, two 515 Pumps (for makeup/buffer delivery), LC Packings Flow Splitter (20 to 100 mL, 1:10,000 split), MassLynx Software and its FractionLynx Application Manager was used.

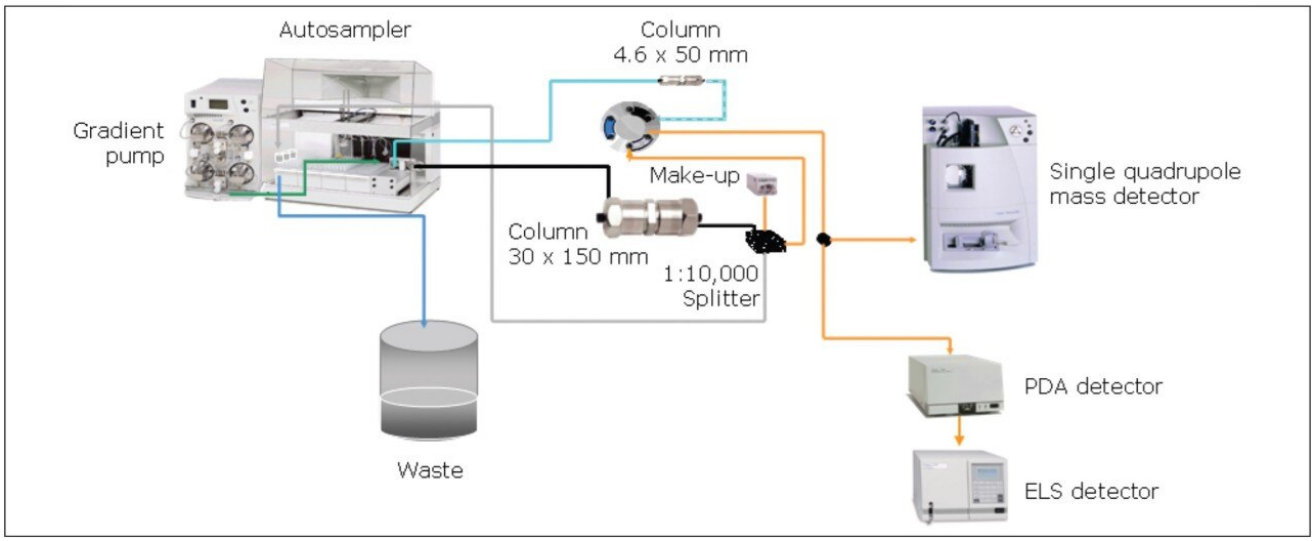

Figure 1. Configuration of the Waters Preparative Chromatography System used in these studies.

The analytical and preparative flow capabilities of the 2525 Binary Gradient Module allow the system to be used for both pilot and preparative-scale experiments.

- For pilot-scale studies, a 4.6 x 50 mm Waters Symmetry 300 C_{18} column with 5 µm particles is used.
- For the preparative experiments, a 30 x 150 mm Waters Symmetry 300 C_{18} column with 7 µm particles is used.

Special consideration must be given to detection in preparative chromatography. The highly concentrated peaks exceed the linear range of common detectors. In addition, the high flow rates are not tolerated by detector hardware. Finally, some detectors used in this study (MS and evaporative light scattering) are destructive. It is, therefore, necessary to use a technique that reduces the flow and concentration reaching the detectors while minimizing loss of desirable material. A passive splitter is commonly used in the high ratio split and dilute technique, as shown in Figure 2.

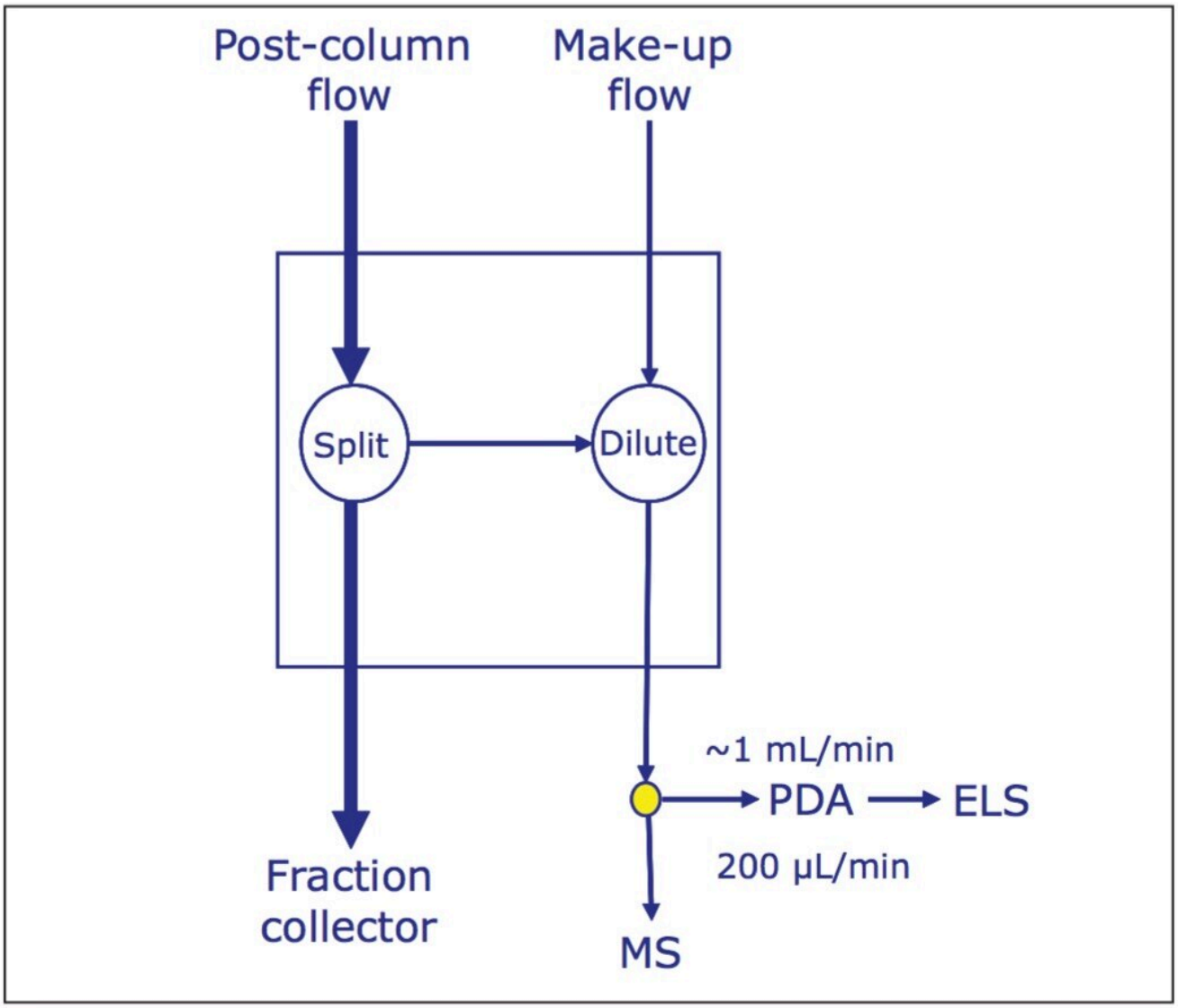

Figure 2. Flow path for the post-column sample flow. The passive flow splitter divides the prep flow 10,000:1, with 99.99% of the flow going to the fraction collector. The remaining 0.01% of the flow is transported to the detectors using the make-up flow.

Samples

The crude synthetic peptides were provided by Dr. Kelly Wasmund of Research Genetics, Inc. (Huntsville, AL, U.S.). The two peptides were:

- NH_2-ISQAVHAAHAEINEAGR-COOH (abbreviated as ISQA)

- NH_2-SIINFEKL-COOH (abbreviated as SIIN)

Purification strategy

The strategy for the purification of the peptides is based on the use of pilot runs to define preparative conditions. From the retention time of the pilot runs, the percent organic mobile phase (%B) needed to elute each peptide is estimated. Assuming that the pilot scale separation is satisfactory, the conditions for the preparative chromatography are scaled based upon the ratio of column volumes. A shallow preparative gradient is then defined, ranging from 5% below to 3% above the calculated %B needed for peptide elution. The masses of the expected ions for possible charge states are used as mass triggers. The fractions are analyzed for purity using the same chromatographic conditions as the pilot run, with multiple detection modes to ensure purity.

System calibration

The relationship between time and actual organic mobile phase delivered to the column on the pilot-scale system is established using uracil (an unretained, UV-absorbing compound) in the organic mobile phase (B). The conditions for the system calibration are given below:

Solvent A:	100% water
Solvent B:	100% acetonitrile with 0.01 mg/mL uracil
Column:	4.6 x 50 mm Symmetry 300, C_{18}, 5 µm

Time (min)	Flow Rate (mL/min)	%A	%B
0.00	1.35	95	5
20.00	1.35	20	80
21.00	1.35	0	100
24.00	1.35	0	100
25.00	1.35	95	5
30.00	1.35	95	5

Table 1. Calibration gradient. A constant 0.15 mL/min of aqueous 1% trifluoroacetic acid (TFA) is added to the gradient stream to make a total flow rate of 1.50 mL/min and final concentration of 0.1% TFA.

The absorbance trace resulting from the calibration run is given in Figure 3. From this run, the observed percentage of organic mobile phase (%B) is calculated using the following equation:[1]

$$\text{Observed \% B} = (\text{Observed Absorbance}/\text{Absorbance at 100\% Acetonitrile}) \times 95 + 5$$

Where:

- The absorbance at 100% acetonitrile is 600,000
- The observed absorbance is obtained from the absorbance trace at 258 nm
- Table 2 summarizes the results of the calibration.

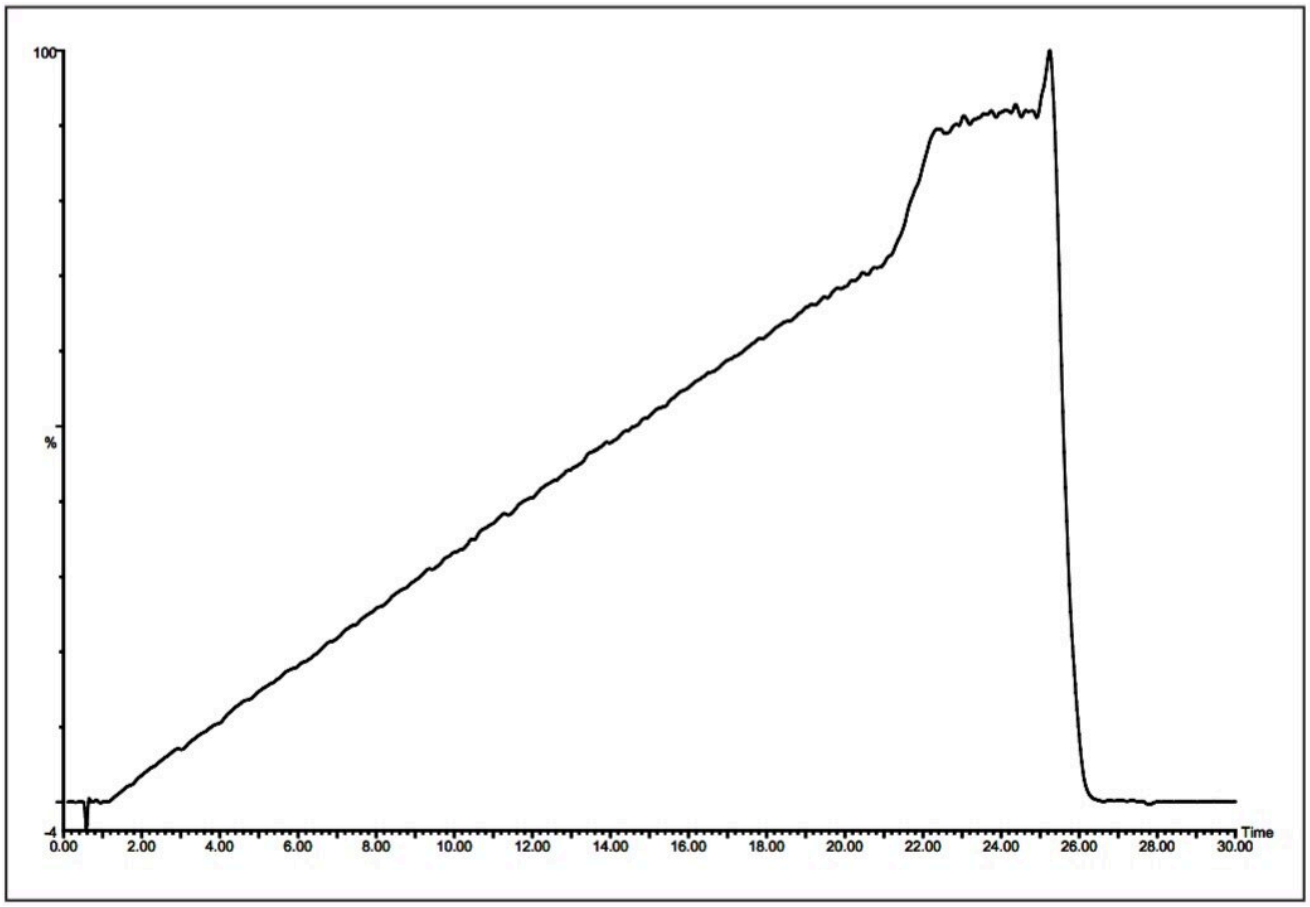

Figure 3. Absorbance trace for the system calibration.

Time	Observed Absorbance	Observed %B
0	1	
1	1	
2	24019	8.80
3	48966	12.75
4	70822	16.21
5	97858	20.49
6	121924	24.30
7	146816	28.25
8	172784	32.36
9	198040	36.36
10	221936	40.14
11	249464	44.50
12	272560	48.16
13	299896	52.48
14	320792	55.79
15	344572	59.59
16	370616	63.68
17	394568	67.47
18	416016	70.87
19	441728	74.94
20	460624	77.83

Table 2. Observed absorbance and calculated percentage of organic mobile phase with time.

Results and Discussion

Pilot-scale separations

Each peptide is individually wetted in 0.5 mL DMF and then diluted to 4.5 mL with water. The concentration of ISQA is estimated at 5 to 10 mg/mL and SIIN is estimated at 10 mg/mL. The conditions for the pilot-scale separations of each of the peptides are given in Table 3; the chromatographic results are presented in Figure 4.

Solvent A:	100% water
Solvent B:	100% acetonitrile
Injection vol.:	40 µL
Column:	4.6 x 50 mm Symmetry 300, C_{18}, 5 µm

Time (min)	Flow Rate(mL/min)	%A	%B
0.00	1.35	95	5
20.00	1.35	20	80
21.00	1.35	0	100
24.00	1.35	0	100
25.00	1.35	95	5
30.00	1.35	95	5

Table 3. Gradient conditions used in the pilot-scale separations of ISQA and SIIN. A constant 0.15 mL/min of aqueous 1% trifluoroacetic acid (TFA) is added to the gradient stream to make a total flow rate of 1.50 mL/min and final concentration of 0.1% TFA.

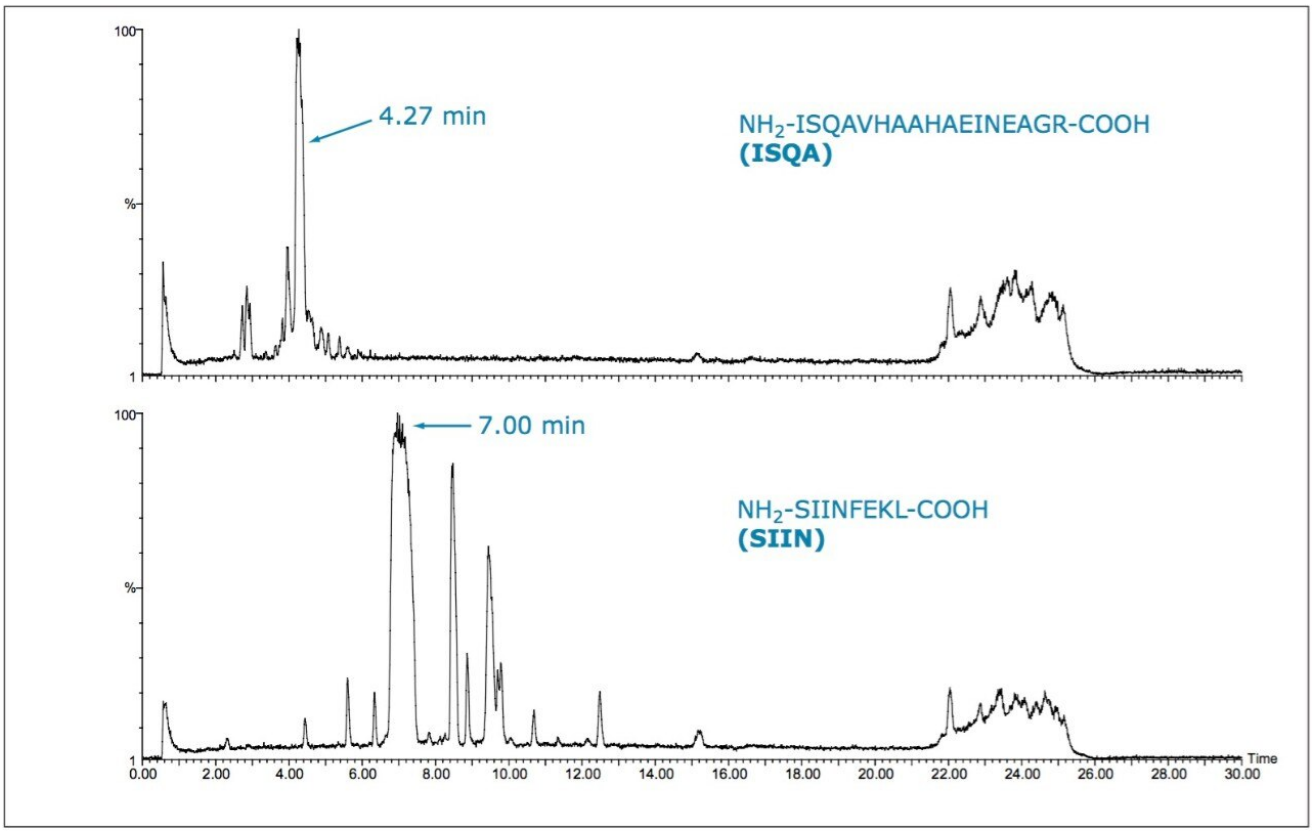

Figure 4. Total Ion Chromatograms (TIC) (ES^+) for the pilot-scale separations of ISQA and SIIN.

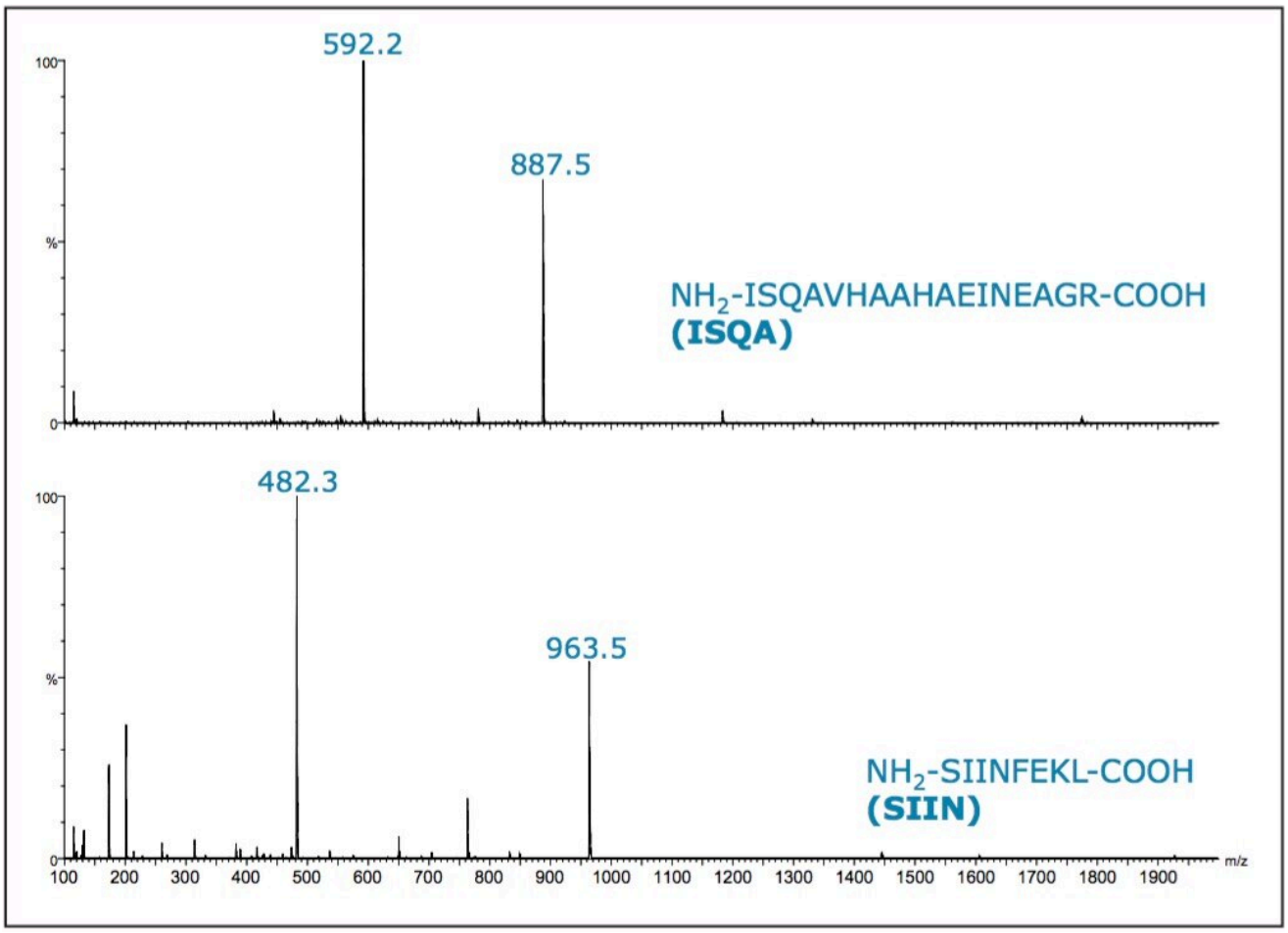

Figure 5. Mass spectra for ISQA and SIIN resulting from the pilot runs.

Defining the preparative gradient

Shallow preparative gradients create enhanced separations of the peptides from closely-eluting contaminants. In this case, a shallow gradient that ranges from 5% below to 3% above the calculated %B for peptide elution is used. The 4.27-minute retention time for ISQA indicates that it elutes near 17%B. This suggests that the preparative gradient for ISQA should start at 12%B and go to 20%B. Similarly, the retention time of SIIN at 7.00 minutes indicates that it elutes near 28%B. The shallow preparative gradient for this peptide should run from 23%B to 31%B.

Mass triggers

Target masses are selected to include the expected multiply-charged ions, Table 4.

NH_2-ISQAVHAAHAEINEAGR-OOOH (ISQA)	
Monoisotopic Mass	1772.9 Da
Target Ions for Charge States	m/z
$[M+H]^+$	1773.9
$[M+2H]^{2+}$	887.5
$[M+3H]^{3+}$	592.2
$[M+4H]^{4+}$	447.2
NH_2-SIINFEKL-COOH (SIIN)	
Monoisotopic mass	962.5 Da
Target ions for charge states	m/z
$[M+H]^+$	963.3
$[M+2H]^{2+}$	482.3
$[M+3H]^{3+}$	321.9

Table 4. Calculated target ions for various charge states of ISQA and SIIN.

Preparative chromatography

Purification of the ISQA and SIIN peptides is performed using shallow preparative gradients (Tables 5 and 6) for separation and fraction collection based on multiple target ions. The conditions for the preparative run of ISQA

and SIIN are given in Tables 5 and 6, and the resulting chromatograms are shown in Figures 6 and 7, respectively.

ISQA

Solvent A:	100% water
Solvent B:	100% acetonitrile
Injection vol.:	5 mL
Column:	30 x 150 mm Symmetry 300, C_{18}, 7 µm
Target masses:	$[M+H]^{+}$ = 1773.9, $[M+2H]^{2+}$ = 887.5, $[M+3H]^{3+}$ = 592.2, $[M+4H]^{4+}$ = 447.2

SIIN

Solvent A:	100% water
Solvent B:	100% acetonitrile
Injection vol.:	5 mL
Column:	30 x 150 mm Symmetry 300, C_{18}, 7 µm
Target masses:	$[M+H]^{+}$ = 963.5, $[M+2H]^{2+}$ = 482.3, $[M+3H]^{3+}$ = 321.9

Time (min)	Flow Rate (mL/min)	%A	%B
0.00	57.50	95	5
2.00	57.50	95	5
3.00	57.50	88.4	11.6
36.50	57.50	80.4	19.6
37.50	57.50	20	80
39.50	57.50	20	80
40.00	57.50	95	5
55.00	57.50	95	5

Table 5. Shallow preparative gradient used for the purification of ISQA. A constant 6.30 mL/min of aqueous 1% (TFA) is added to the gradient stream to make a total flow rate of 63.8 mL/min and final concentration of 0.1% TFA.

Time (min)	Flow Rate (mL/min)	%A	%B
0.00	57.50	95	5
2.00	57.50	95	5
3.00	57.50	77.1	22.9
36.50	57.50	69.1	30.9
37.50	57.50	20	80
39.50	57.50	20	80
40.00	57.50	95	5
55.00	57.50	95	5

Table 6. Shallow preparative gradient used for the purification of SIIN. A constant 6.30 mL/min of aqueous 1% (TFA) is added to the gradient stream to make a total flow rate of 63.8 mL/min and final concentration of 0.1% TFA.

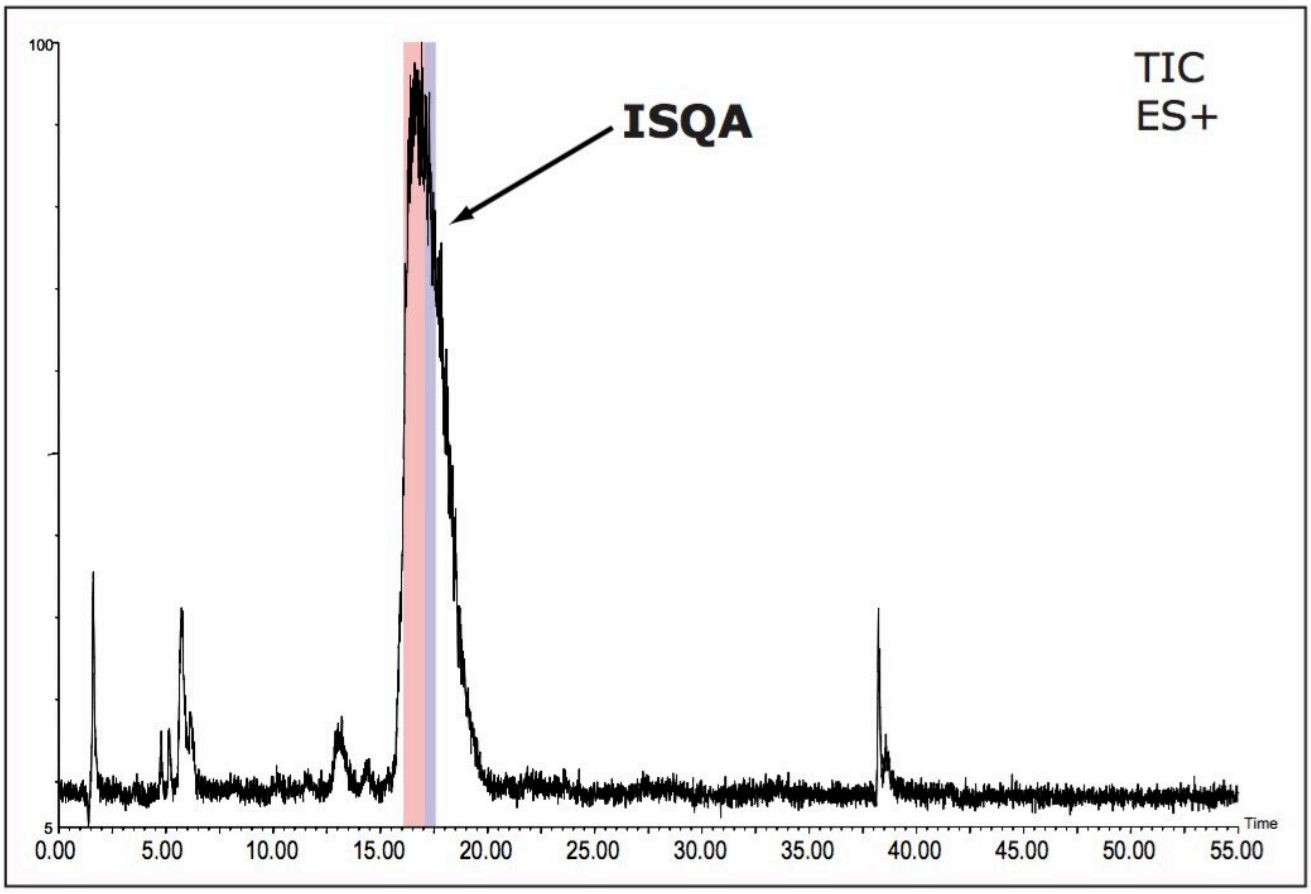

Figure 6. Preparative MS chromatogram for ISQA. The shaded bars represent fraction collection on m/z 592.2 in positive ion mode.

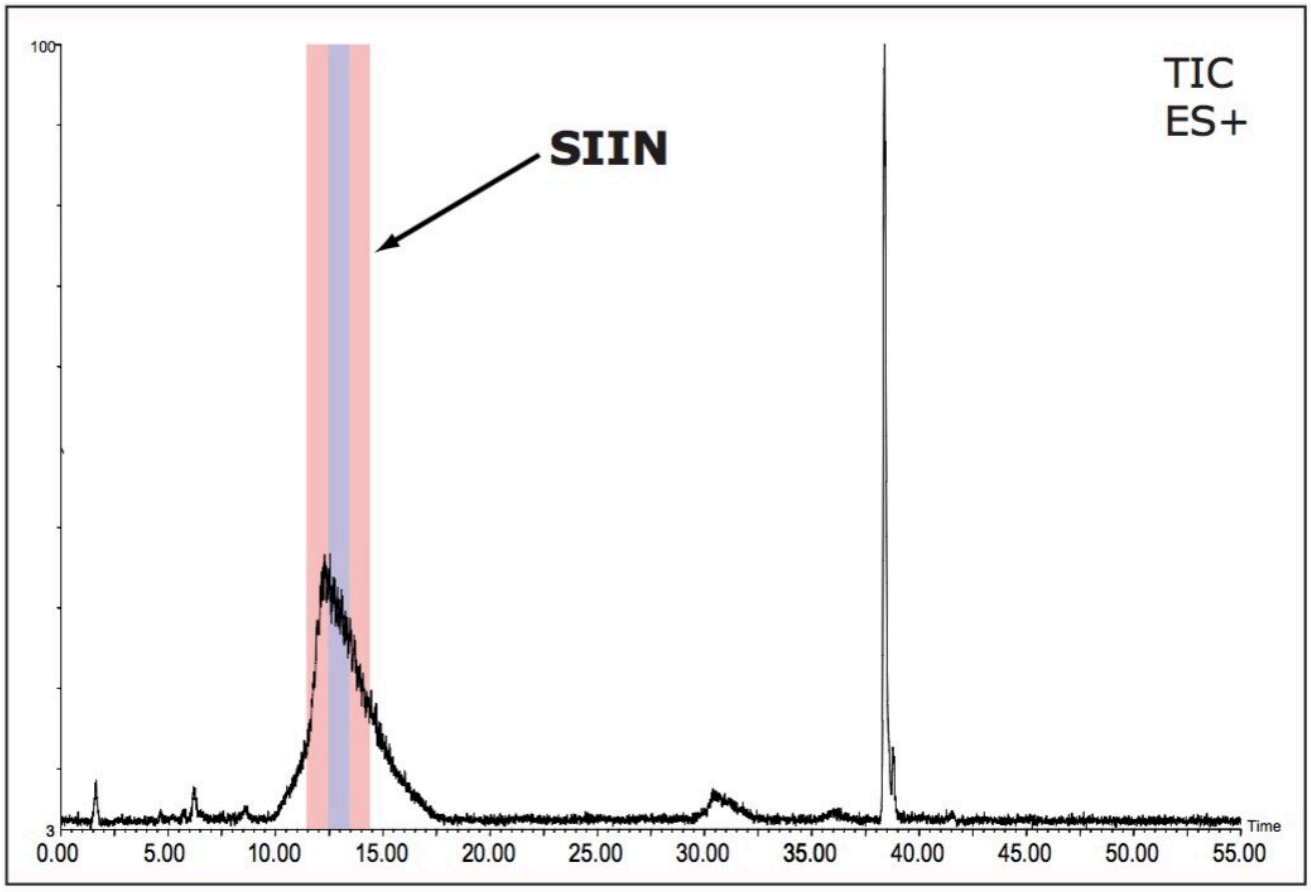

Figure 7. Preparative MS chromatogram for SIIN. The shaded bars represent fraction collection on m/z 482.3 in positive ion mode.

Fraction analysis

The fractions resulting from the preparative runs are analyzed to assess purity. Multiple detection channels are used to monitor the analysis to provide more complete characterization of the fractions. The fraction analysis of both ISQA and SIIN peptides use the separation conditions in Table 7. The results of the fraction analysis are shown for ISQA and SIIN in Figures 8 and 9, respectively.

Solvent A:	100% water
Solvent B:	100% acetonitrile

Column: 4.6 x 50 mm Symmetry 300, C_{18}, 5 µm

Time (min)	Flow Rate (mL/min)	%A	%B
0.00	1.35	95	5
20.00	1.35	20	80
21.00	1.35	0	100
24.00	1.35	0	100
25.00	1.35	95	5
30.00	1.35	95	5

Table 7. Gradient conditions for re-analysis of the ISQA and SIIN fractions. A constant 0.15 mL/min of aqueous 1% trifluoroacetic acid (TFA) is added to the gradient stream to make a total flow rate of 1.50 mL/min and final concentration of 0.1% TFA.

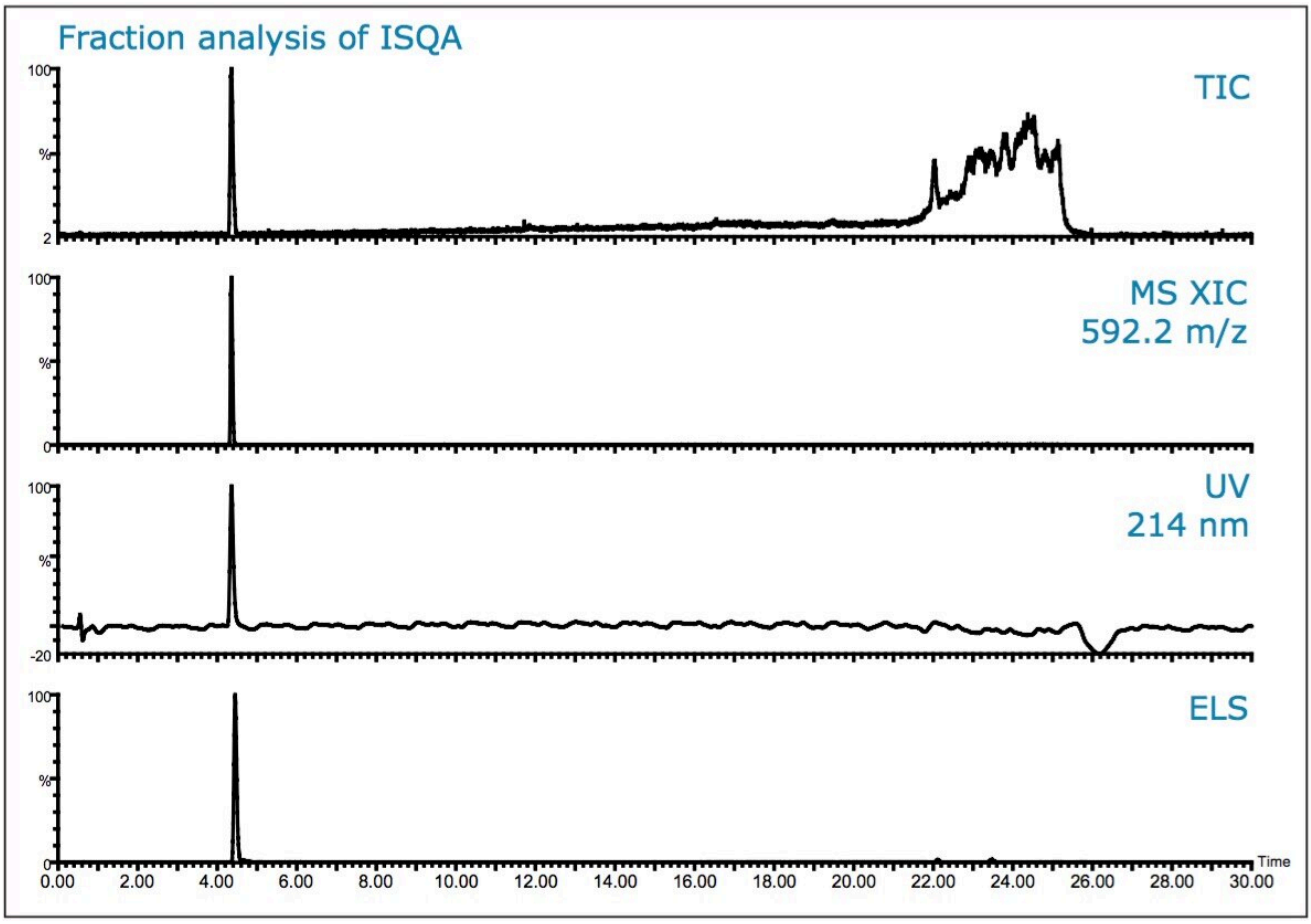

Figure 8. Fraction analysis of ISQA. The total ion chromatogram (TIC), the extracted ion chromatogram (XIC), the UV trace at 214 nm, and the evaporative light scattering chromatogram are each shown.

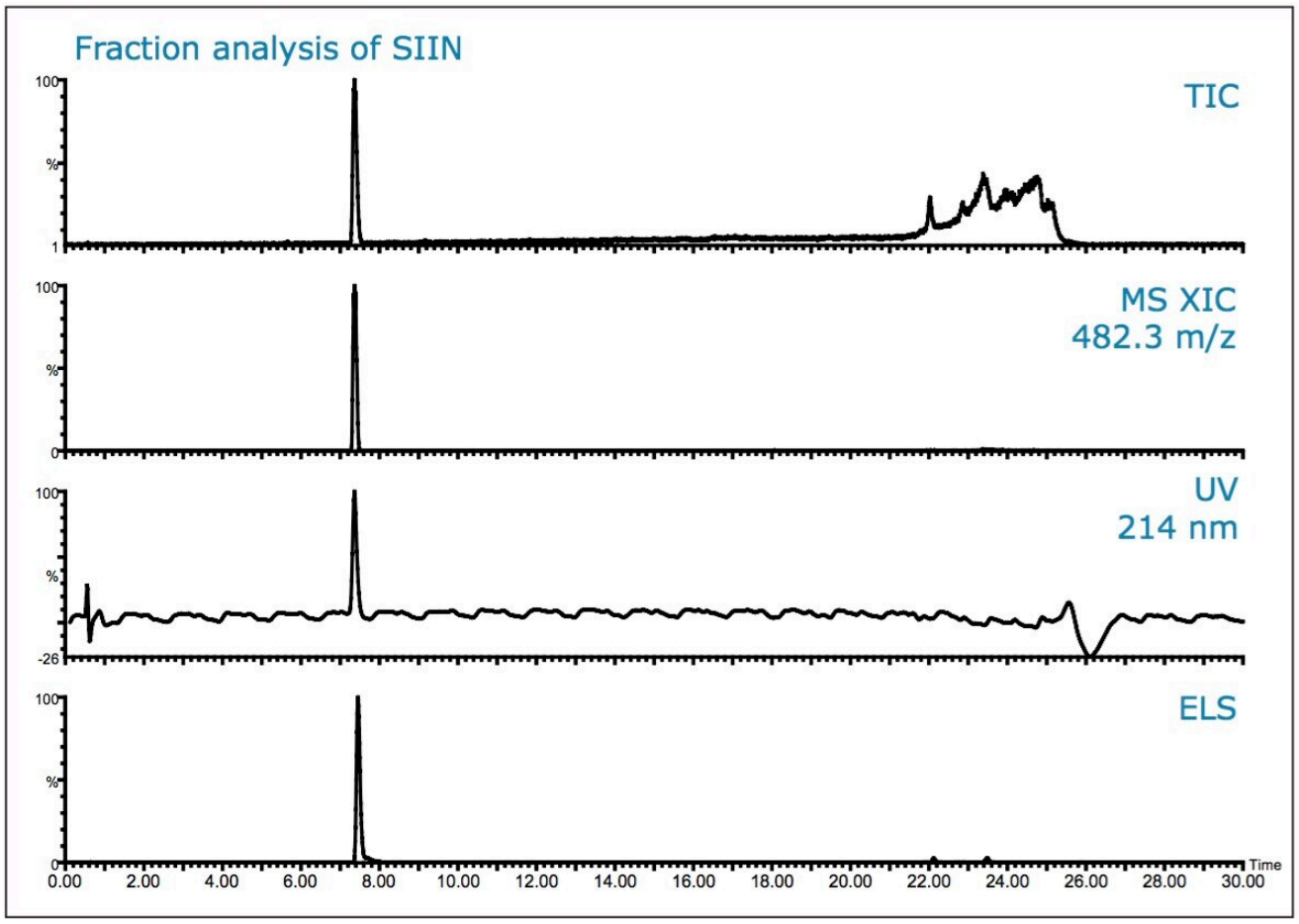

Figure 9. Fraction analysis of SIIN. The total ion chromatogram (TIC), the extracted ion chromatogram (XIC), the UV trace at 214 nm, and the evaporative light scattering chromatogram are each shown.

Conclusion

The use of fast, pilot-scale runs provides a useful prediction of conditions for shallow-gradient, high-resolution preparative chromatography.

Notes

1. The calibration gradient occurs over the 5% acetonitrile to 100% acetonitrile range. Five percent acetonitrile

has an absorbance of zero because the UV detector zeroes at the start of the gradient.

At the end of the gradient, where 100%B is reaching the detector, the observed absorbance is 600,000.

The interval between 5% acetonitrile and 100% acetonitrile is 95%. This corresponds to the range between 0 and 600,000 on the absorbance scale.

To calculate the observed percentage of B in the middle of the 5% to 100% range as a percentage of the total range (0 %B to 100%B), the observed absorbance divided by the absorbance at 100% acetonitrile is multiplied by 95. Five percent is added to the value to account for the offset of the curve at the start of the run.

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