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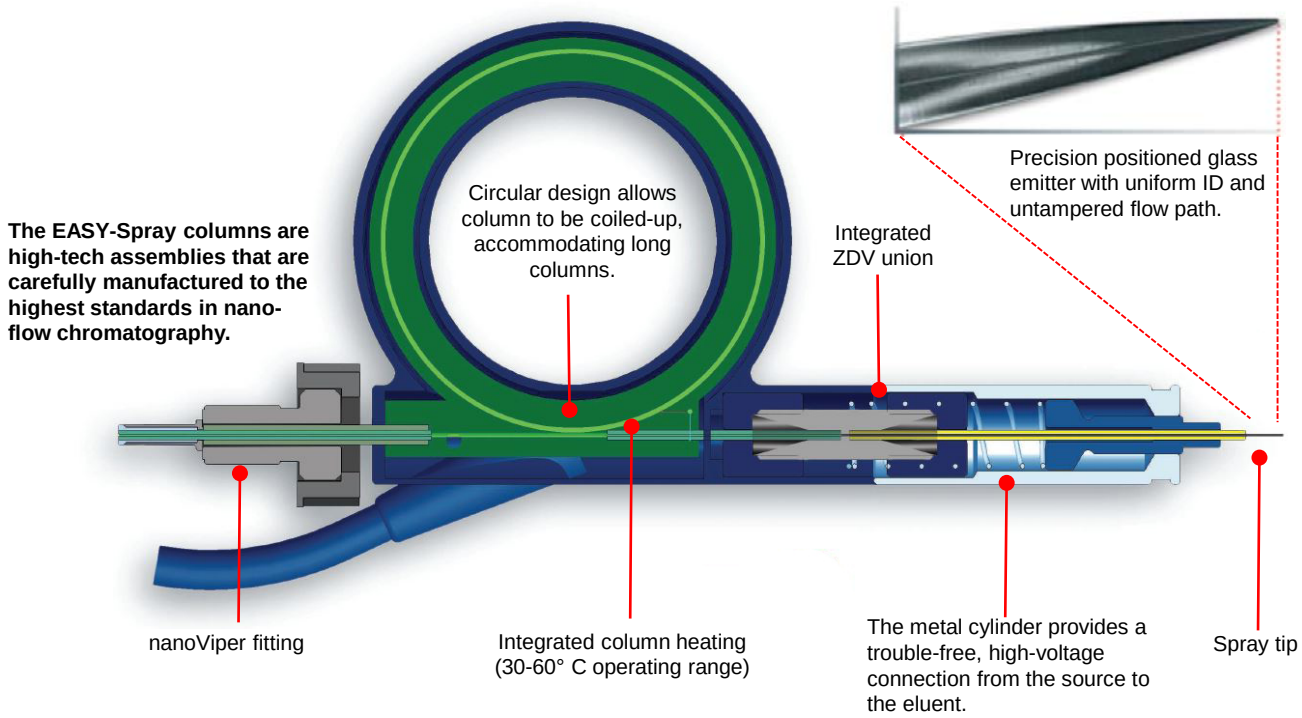
Thermo Scientific™ EASY-Spray™ LC Columns Optimization - Red flags to watch for and how to troubleshoot your workflow

Estee Toole

Unique design provides uncompromised performance and ease-of-use in nano and capillary LC-MS

- **Precision positioned glass emitters**
  - Uniform inner diameter delivers an exceptionally stable spray
- **Integrated zero-dead-volume (ZDV) unions**
  - Narrower peaks and maximized peak capacity
- **Integrated temperature control**
  - Better run-to-run reproducibility and allows the use of longer columns and/or smaller particle sizes since elevated temperatures lower eluent viscosity and overall backpressure
- **Thermo Scientific™ nanoViper™ fittings**
  - Easy-to-use fingertight fitting to 1000 bar eliminates column damage due to overtightening and experimental damage due to bad connections



To ensure the best performance and lifetime out of EASY-Spray columns, monitor performance of your LC system and column

Top Usage Tips:

1. Have a good standard and be able to identify warning signs of contamination or system malfunction
2. Save and log chromatograms before and after sample sets to see what type of changes occurred as a result of exposure to different experiments.
3. Have a consistent means of reviewing your LC system and results when introducing a new column to ensure that your column is not being contaminated by any existing system contamination.
4. Use a clear diagnostic standard (such as the digest of a purified protein) that allows the extraction of known peptide m/z. This allows peak shape symmetry and intensity to be examined. Complex digestion mixtures such as Hela lysate, are not ideal for pinpointing chromatographic issues.
5. Assessing eluting ions during cleaning cycles can indicate if residual contaminants are present on the column stationary phase.

Best Practice Guide: Performance Monitoring

1. Build comparative baseline of data.
   If you have a benchmark to compare to, expected performance can be easier to understand while diagnosing issues.

2. Save your chromatograms!
   Suspicious of column malfunction? Chromatograms and traces tell thorough stories.

3. Establish a practice of qualifying new columns using a standard protein digest and a standard gradient.
   Standard protein digests actually play an important role in cleaning the column and maintaining the life of the column.

4. Cleaning protocols and gradients tell valuable stories and are recommended in practice.
   Include gradients that contain extra cleaning cycles at the end of the chromatogram -
   This can make diagnostics easier.

Before You Start – Review All Instructions Before Attempting Column Installation

The first step to ensuring that your column is functioning properly is to set it up correctly

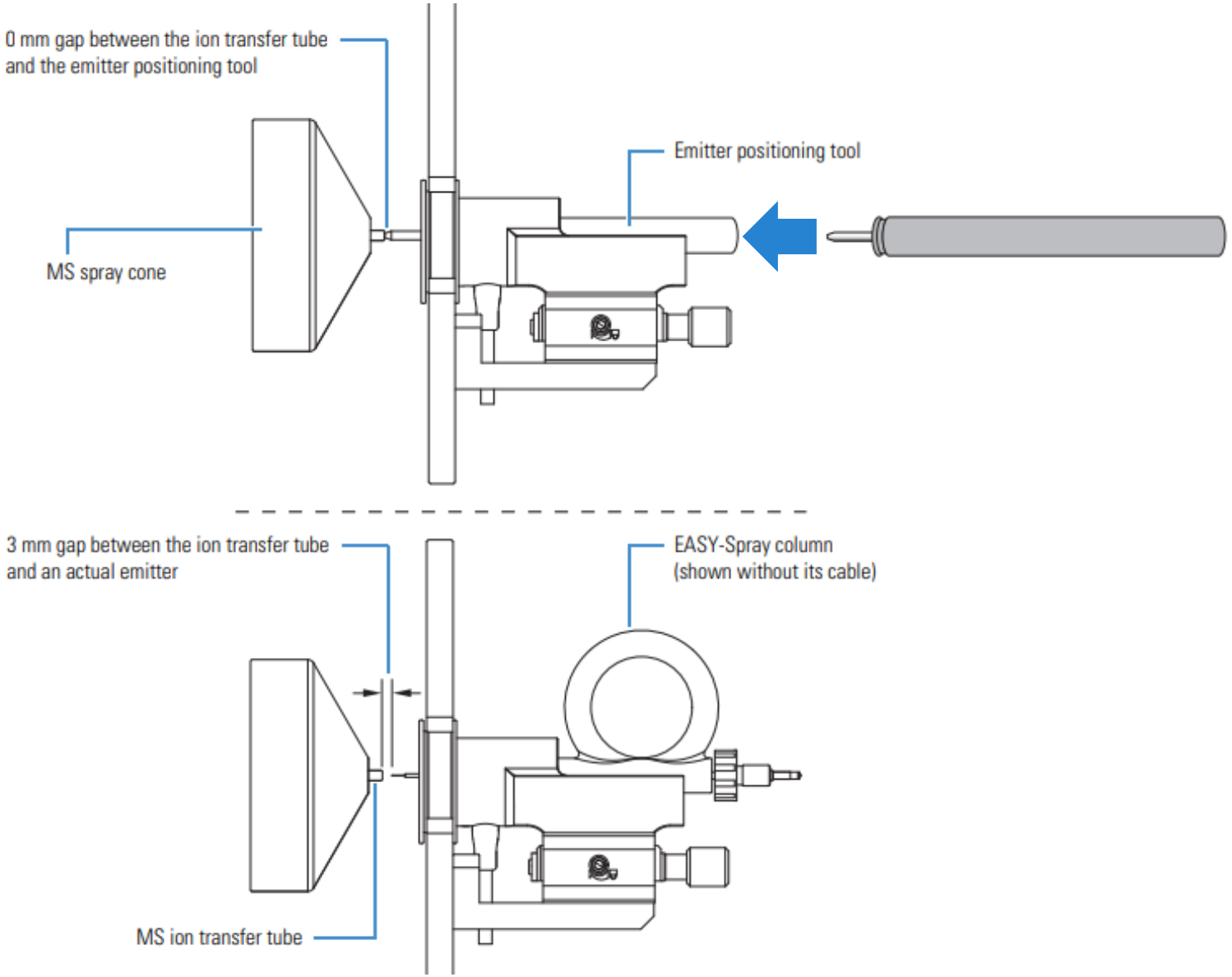
The next slide shows a schematic from our manual that shows how to position your source to ensure proper emitter placement

Is required to be done anytime the EASY-Spray source is removed and placed back onto the Mass Spectrometer to avoid breaking the emitter tip of the column.

Thermo Scientific EASY-Spray Column Setup – Use the Emitter Positioning Tool

1. Adjust the emitter position in the EASY-Spray source using Emitter Positioning Tool (PN ES232)

2. After following this step, you may mount the column into the source



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The best thing that you can do for your LC system is to keep it clean and use high quality reagents

Top Usage Tips:

1. Replace solvents regularly
2. Don’t add solvents to solvent bottles already in use
3. Don’t use premixed solvents

Chromatography Tip:

Regular replacement of solvents keeps them at correct ionic content (buffering capacity), pH (acid content) and organic solvent levels (important for volatile solvents). Variation in all or any of these can lead to changes in retention time, peak width and mass spectrometer ionization.

Use high-purity, high-quality, freshly made, and degassed.

Use of premixed solvents is not recommended

- Mobile Phase A (Pump A): Fisher Scientific™ Optima™ HPLC water 2% Acetonitrile 0.1% Formic Acid
- Mobile Phase B (Pump B): 80% Acetonitrile 0.1% Formic Acid
- Flush Solution: 10% Acetone 45% Acetonitrile 45% Isopropanol
  - *Caution – If using an EASY nLC system, do NOT pump this reagent as solvent, inject it through the sample loop*

Solvent	Part No.
Acetonitrile, Optima LC/MS Grade, Fisher Chemical	A955-500
Water, Optima LC/MS Grade, Fisher Chemical	W6500
Thermo Scientific™ Pierce™ Formic Acid	PI28905
Thermo Scientific™ ChromaCare™ LC-MS Biologics Flush Solution, 45% 2-Propanol: 45% Acetonitrile: 10% Acetone	MB124-X

Diagnose the Issue: Use a Simple Mixture to Evaluate

A simple mixture containing known peptide masses allows operators to extract known ions to assess chromatographic peak shape, ion resolution and elution profile

To assess column performance, the selection of a good sample is important. Selecting a simple mixture of peptides such as Peptide Retention Time Calibration (PRTC, p/n 88320) mix or a digest of a purified protein such as Bovine Serum Albumin (BSA) is recommended.

Using a simple mixture allows you to ask:

1. Are the hydrophilic peptides retaining as well as the hydrophobic peptides?
2. What is the peak width and symmetry?
3. Are all expected ions present missing?
4. Is the intensity/signal appropriate to amount injected?

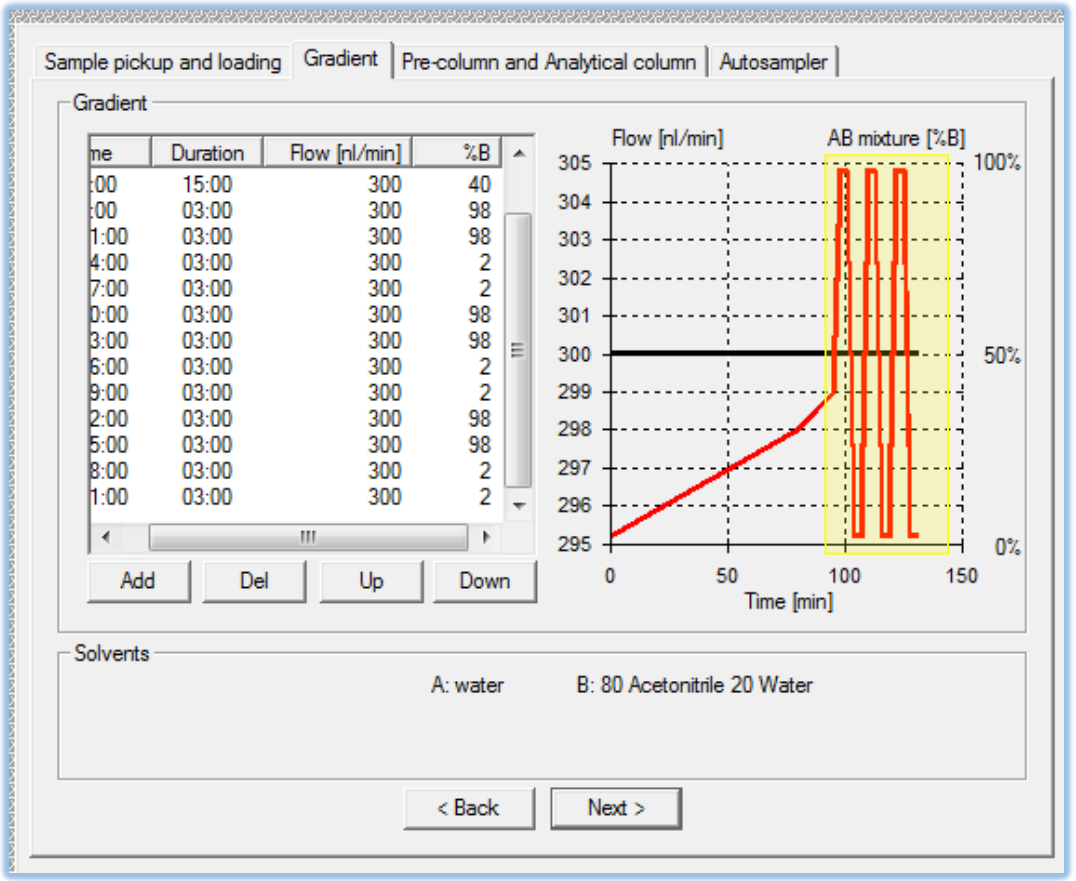
Tip:

Try adding organic/aqueous cycles at the end of the gradient, this will allow you to see if your column still has contaminants present at the end of the run and to what extent.

Simplify troubleshooting and design method parameters that will maximize information output as well as minimize run time.

How to design your column diagnostic experiment:

- Run your usual gradient
- Add three fast seesaw cycles of organic to aqueous (yellow box)
- What comes off the column?

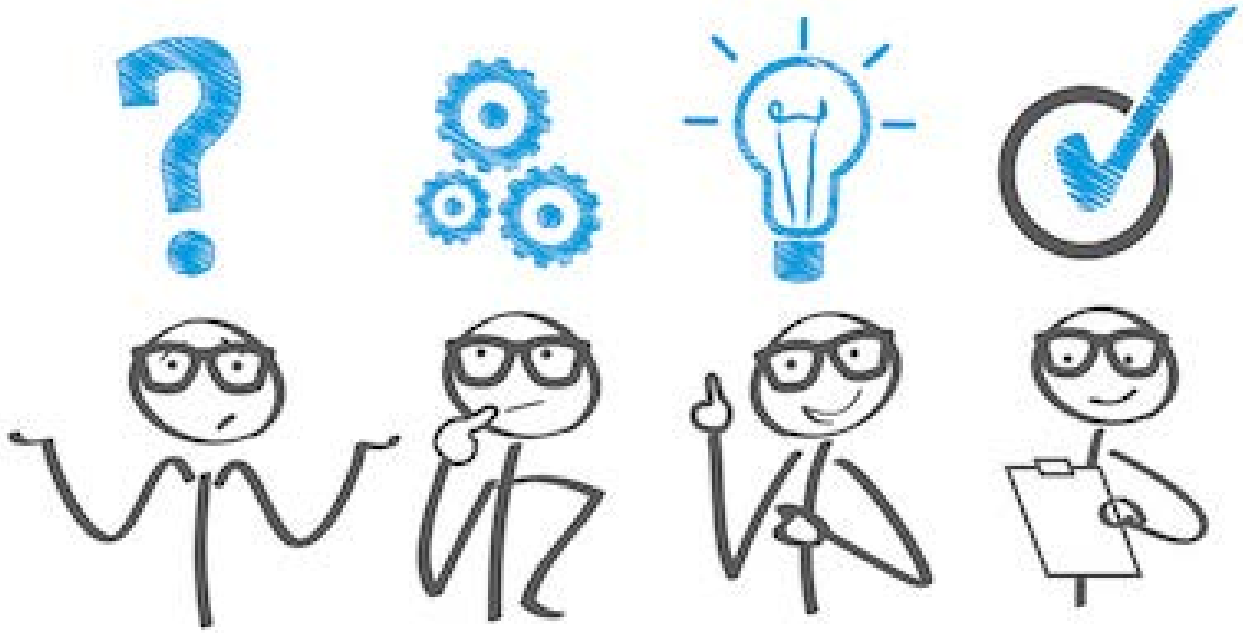


Real Examples from Customers: Common Complaints

Each one was a logged complaint described as “bad column”.

The examples were investigated thoroughly…….

The results were quite surprising…..



Example 1: General Column Contamination

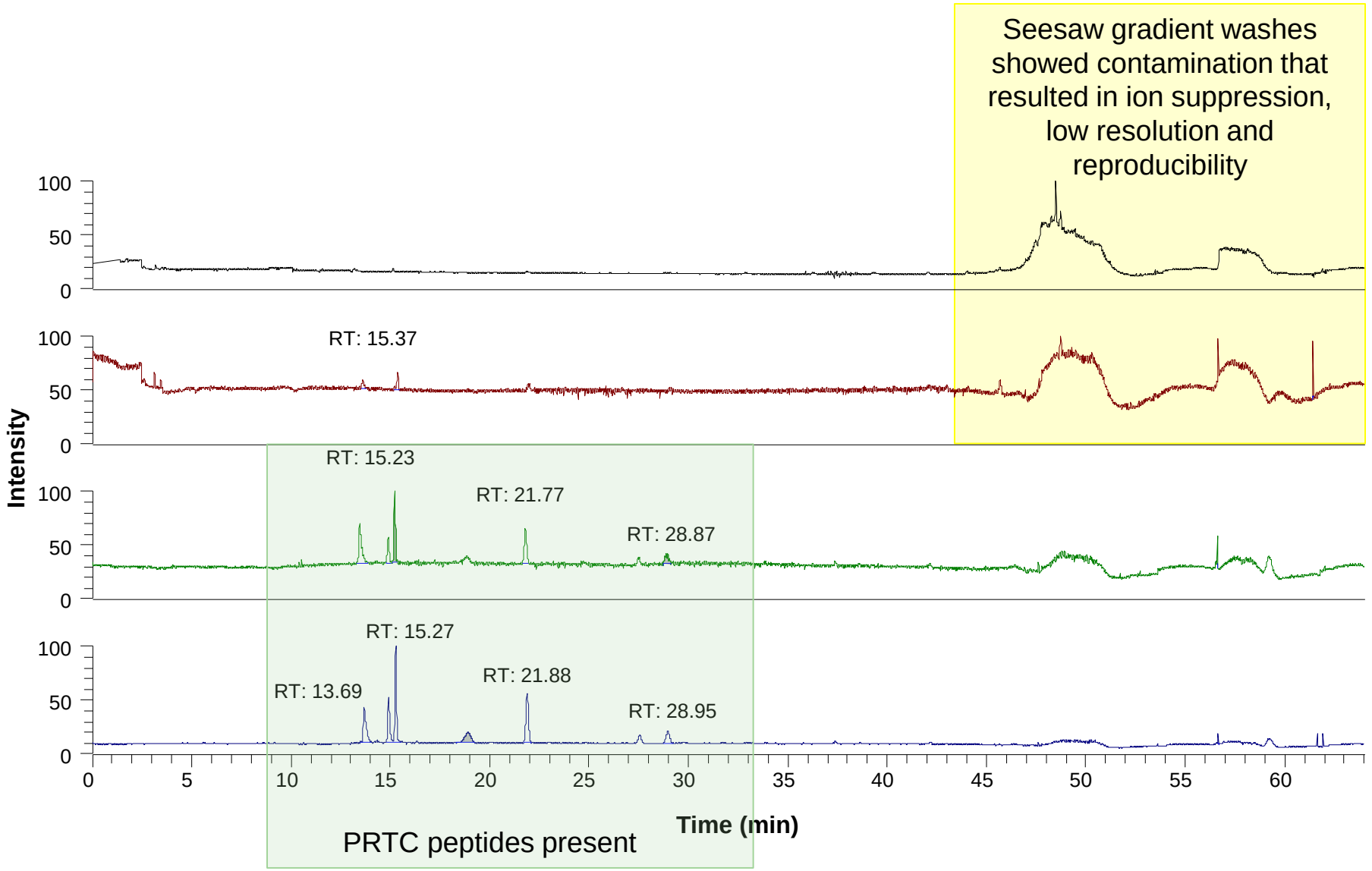
The example shown on the next page is a series of Total Ion Chromatograms (TIC) obtained during the analysis of PRTC (Pierce Retention Time Calibration Standard)

1. In the example, the samples were analyzed using a 2%-40% organic gradient followed by 3 seesaw gradient cycles as shown in slide 10, yellow box
2. The top 2 TIC traces show large elutions during the high organic segments of the seesaw gradient and low detection of PRTC peptides
3. The bottom 2 TIC traces (post cleaning via seesaw gradients) show these elutions during the high organic segments of the seesaw gradient are absent and that the PRTC peptides are abundant, reproducible in retention time and symmetrical.

This example demonstrates the presence of ion suppression, low resolution and reproducibility caused by column contamination.

- In this case a determination of the cause of the contamination is now needed.

Example 1: General Column Contamination Diagnosed by 3 “seesaw” Gradient Washes



Before Column Cleaning

After Column Cleaning

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Example 2: Sputtering and Poor Spray

The example shown on the next page is a Total Ion Chromatogram (TIC) obtained during the analysis of BSA Digest (top) and an extracted ion chromatogram of a known ion (bottom).

1. In the example, there are areas of no spray (yellow) and areas of unstable spray (green) where the baseline is irregular. Ideally, the baseline should remain relatively stable with changes only observed during analyte elution.
2. Sputtering is observed (blue), which usually arises from droplet formation at the emitter tip and leads to periods of unstable spray alternating with no spray.
3. A suspected large contamination peak is observed (pink) at the end of the TIC chromatogram
4. Finally, if you look at the orange box on bottom chromatogram, which was taken at the same time as the green/blue box in the TIC, it shows that it is not possible to extract a signal for a known ion

Often times this type of spray instability is caused by presence of contaminants on the column resulting in uneven flow, disruption in ion formation and ion suppression.

- In this case a determination of the cause of the contamination was needed

Example 2: Sputtering and Low or No Spray Caused by Presence of Contaminants

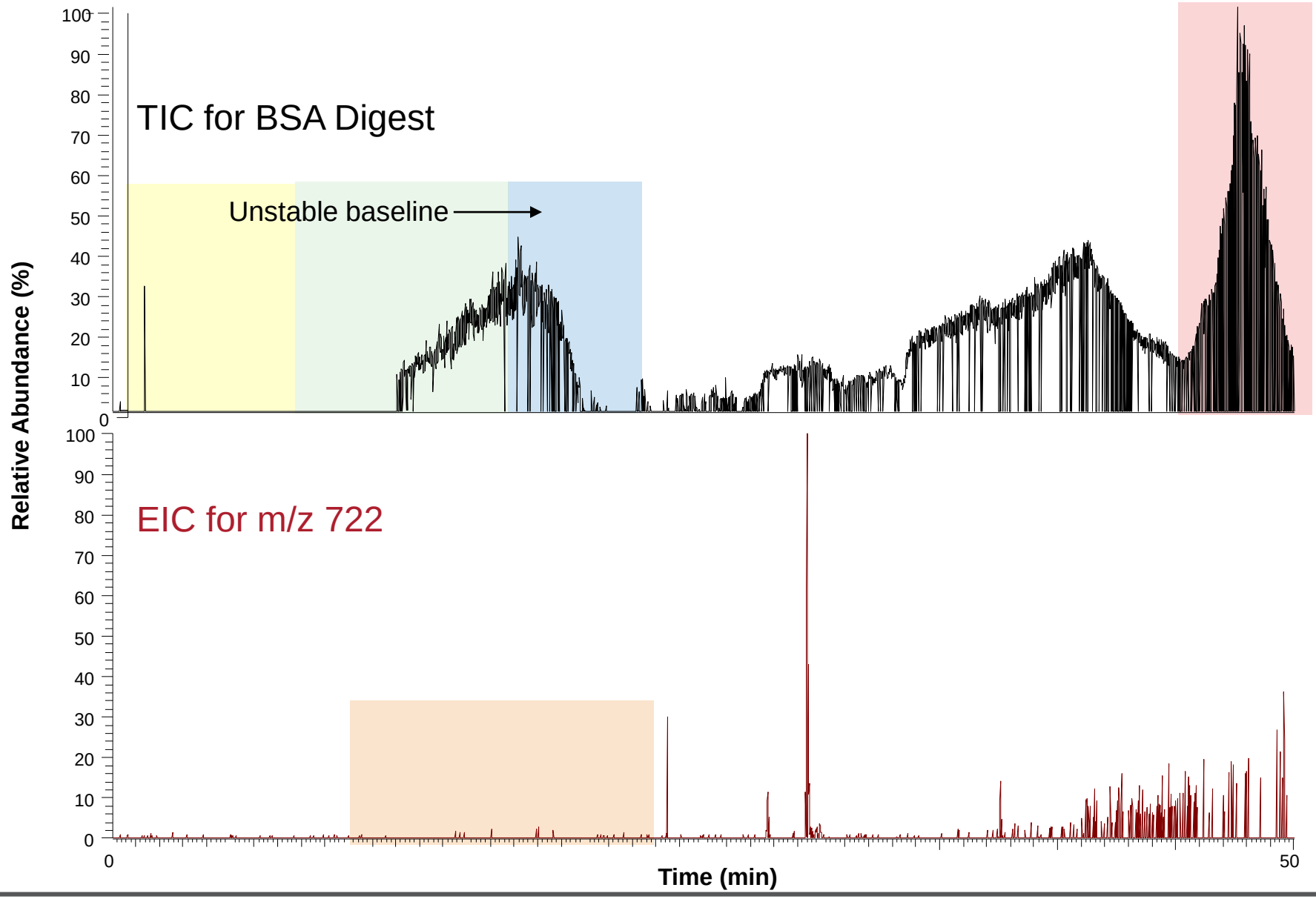
No spray

Unstable spray

Sputtering

Large Contamination Peak

Known ion standard not detectable



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Example 3: Background Interference and Noisy Baseline

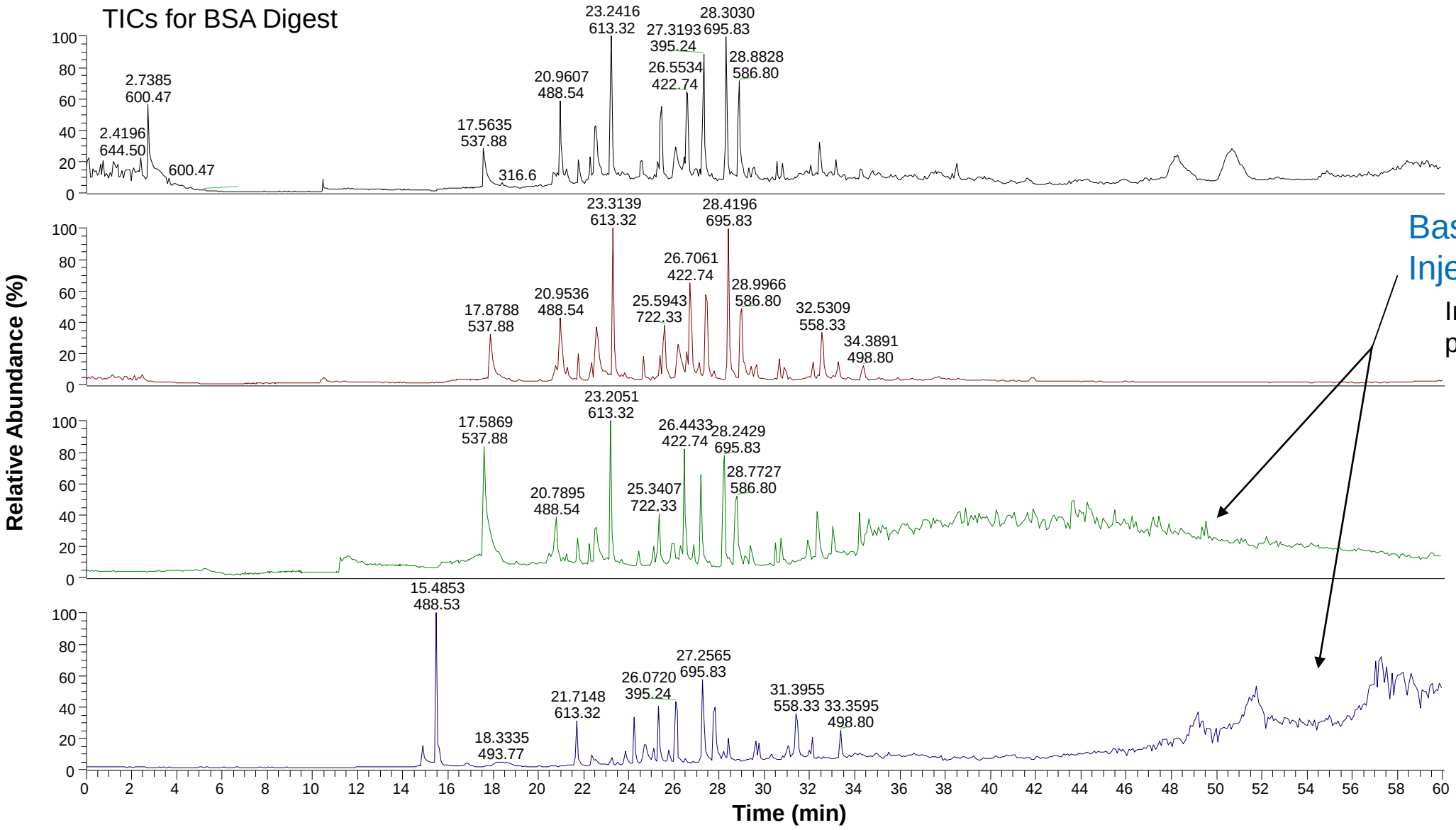
In the next example, we see a series of 4 TICs for BSA Digest Peptides (40 min gradient / 20 min wash) and this is a good example of LC contamination which causes column contamination.

1. Despite using a clean standard, the baseline and pressure are increasing in each run as the analysis progresses.
   - This type of slowly increasing baseline and pressure is indicative of a hydrophobic contaminant such as a lipid, or undigested protein.

2. In this example where there is a new column and a clean routinely used standard, it is recommended to investigate the presence of LC system contamination which is contaminating the column.
   - Common area to investigate are the mobile phases and the autosampler.

→ Carryover of contaminant OR elutes in high organic =
Reduced resolution and signal intensity AND/OR ion suppression

Example 3: Background Interference and Noisy Baseline



Baseline Increasing After Injection of Standards

Investigate the mobile phases and autosampler

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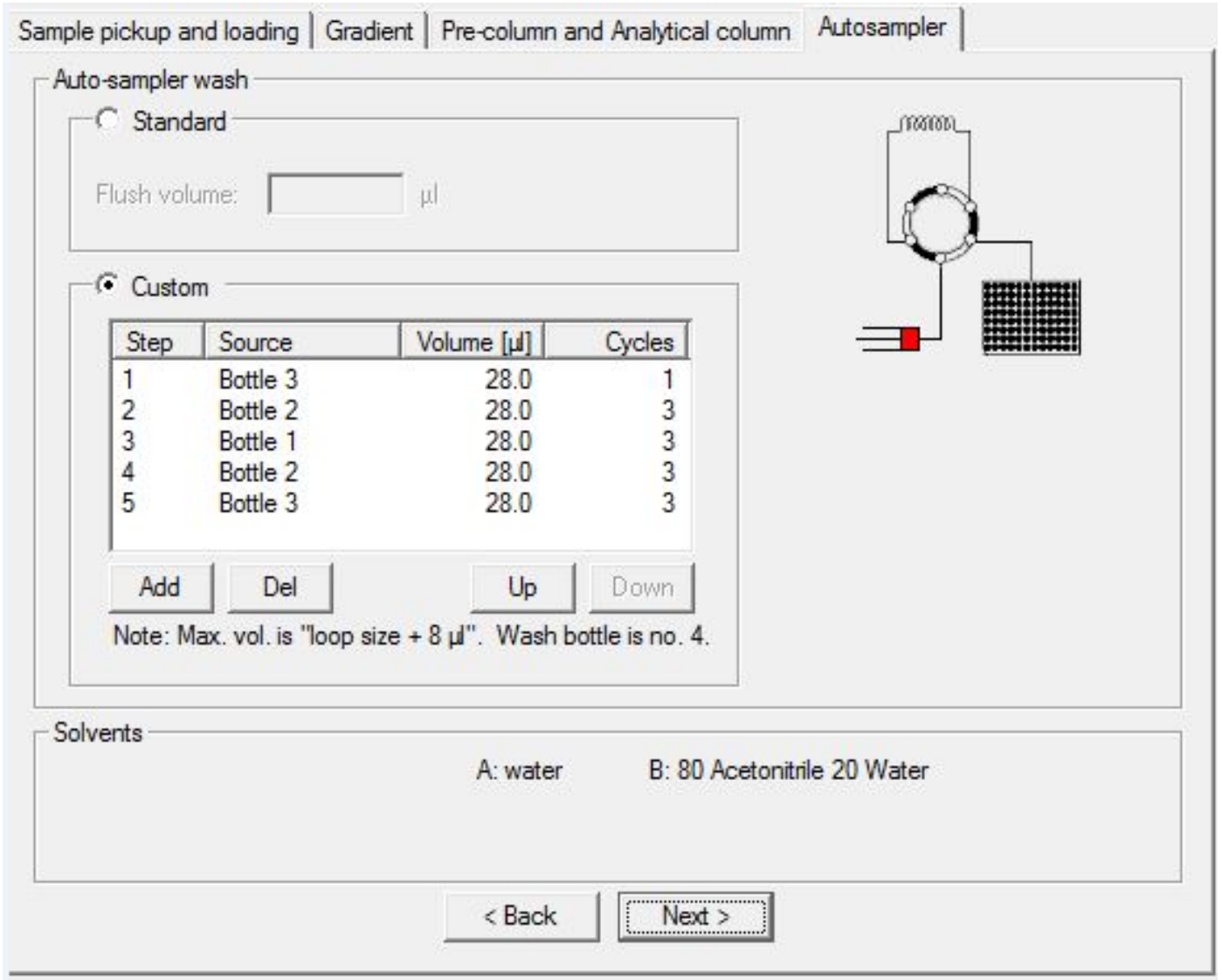
Example 3: LC Autosampler is a Common Area for Contamination



Autosampler Routine Maintenance

- Regularly check for contamination
- Sample loop should be changed when contamination is present

Bottle 1 - IPA
Bottle 2 - 50% ACN
Bottle 3 - Mobile Phase A

(For use with a 20 µL sample loop)

Example 4: High Pressure Errors

We have many examples of these, but generally speaking high pressure errors fall into two categories:

Error	Cause	Solution
Immediate High pressure error	Usually due to the presence of particulates in sample	Filter samples
Slow pressure increases as pressure progresses	Usually due to insoluble or hydrophobic contaminants on stationary phase	Address sample quality

Example 5: Impact of Ion Suppression on Signal Intensity

The customer complained of a high noise level and poor chromatography after injecting a number of samples. The response from the column appears to be exceedingly poor but our own stringent quality tests (every column is analyzed using a mass spectrometer in the factory) indicated no issue with the column before use.

1. Inject BSA sample using the same conditions used in our Factory (see Quality Assurance Report – QAR) before cleaning (top chromatogram)
2. Inject BSA sample again AFTER cleaning (bottom chromatogram) yields improved TIC

→ *Column had been contaminated due to sample quality*

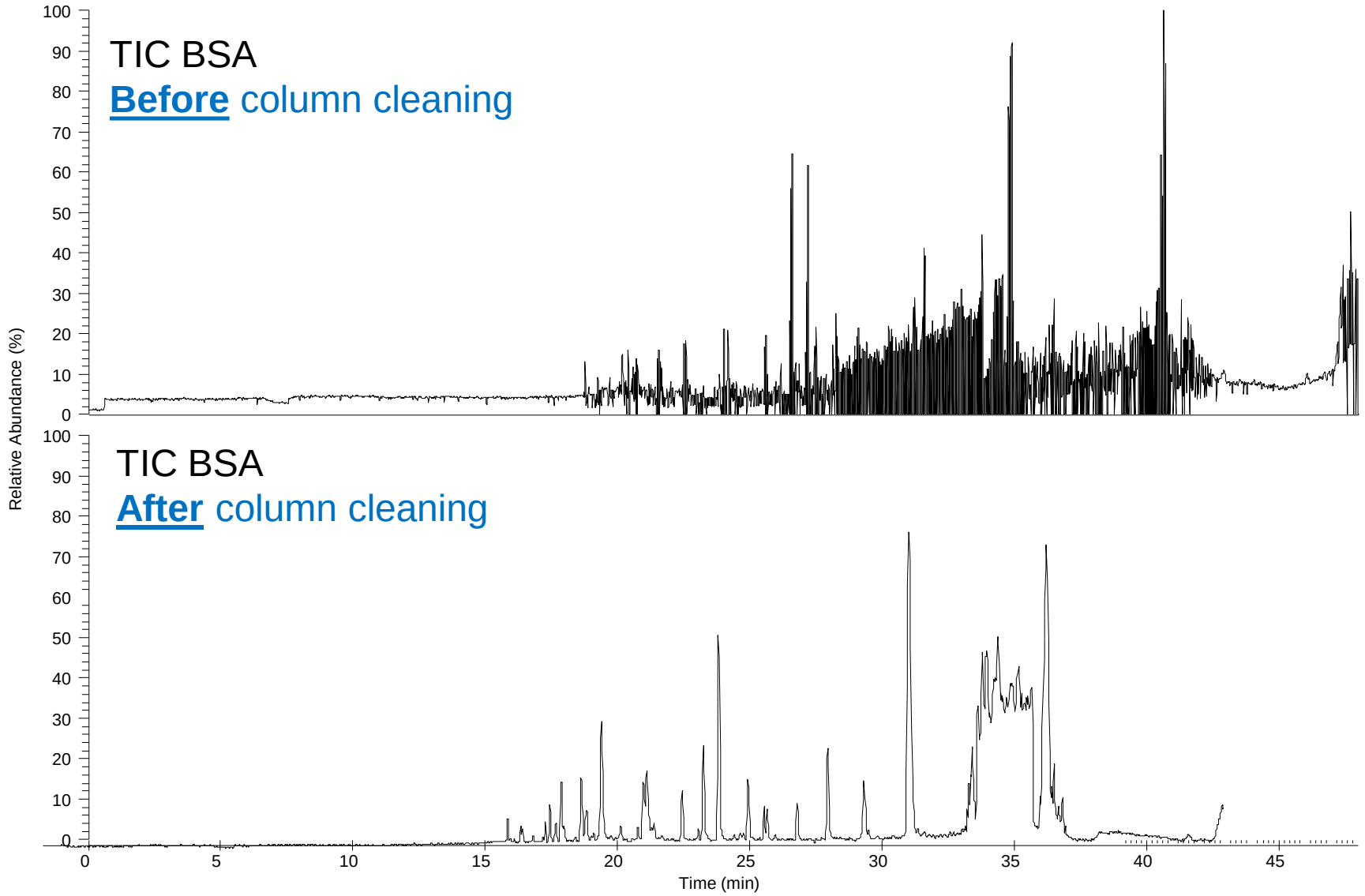
Tips to reduce contamination:

- *Improving the sample quality → use trap and /or SPE methods*
- *Implementing column cleaning protocols → use cleaning gradients and blanks between runs*

Example 5: Impact of Ion Suppression on Signal Intensity

Noisy Baseline

Clearly Defined Peaks



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Examples 6: Impact of Contamination on Resolution and Peak Shape

A customer complained of poor chromatography and resolution off a standard EASY-Spray column. The signal appeared compressed and suppressed with a suspicion of contamination.

- A series of injections of BSA digest peptides were analyzed, with 3 “seesaw” washes added to the end of each run

Observations:

1. Signal was compressed and suppressed but improved with washing
2. Resolution and peak intensity improved as contamination cleaned
3. Large contaminant peak at end of run decreased over subsequent run

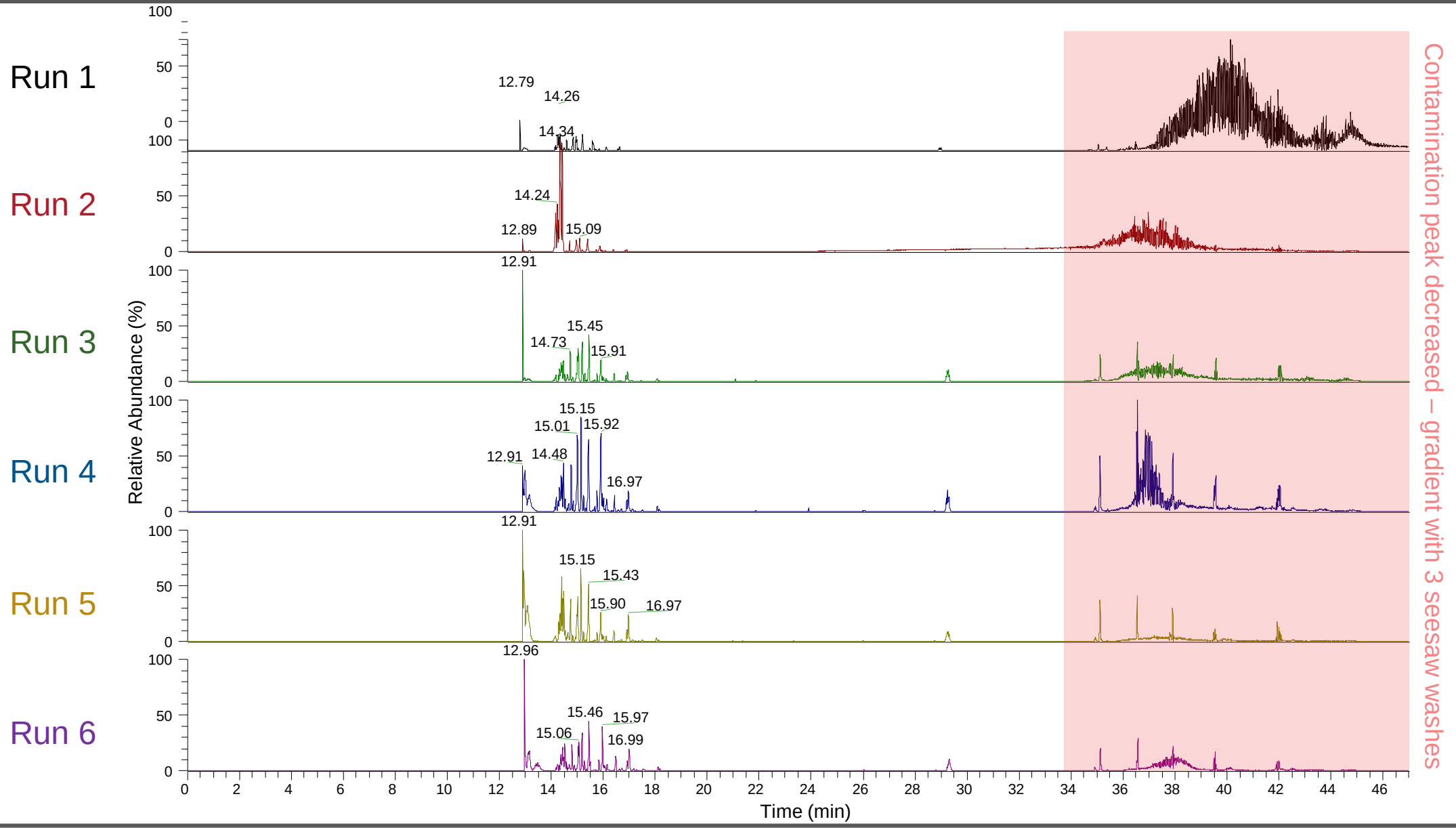
- *This case of a new column with poor performance and high contaminant peak was highly suggestive of LC system contamination*
- *The sample loop was contaminated and the contaminants in the loop were transferred to the column in the first run, then washed from the column with subsequent runs.*

Tips to reduce contamination in cases of complex samples:

Use a more rigorous autosampler cleaning protocol

- *Run a blank periodically (with a gradient) to screen for sample loop contamination.*

Examples 6: Impact of Contamination on Resolution and Peak Shape



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Example 7: Impact of Ion Suppression on Signal Intensity

In addition to testing the LC system for contamination, you should routinely test the column for contamination before, during and after a sample set.

Take a look at the following example, which is a chromatogram of sub fractionated and labeled HeLa peptides. The standard showed a low stable baseline with good response before samples were analyzed but poor response after sample analysis.

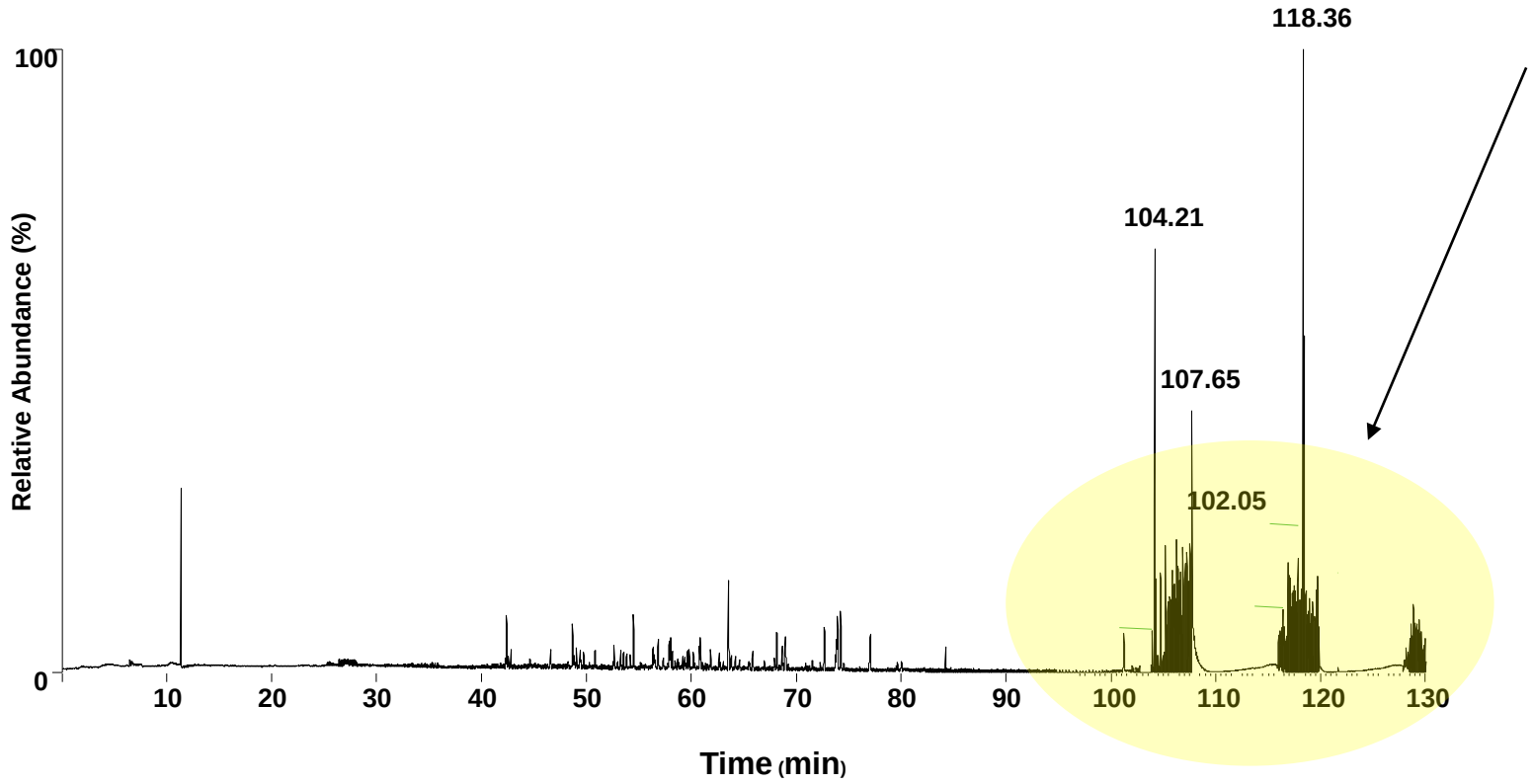
1. Impurities can be seen eluting during the high organic areas of the “seesaw” gradient (the yellow area).
2. “Seesaw” gradients clean column contamination.

→ Using the right SPE conditions on your sample can prevent the contamination from happening.

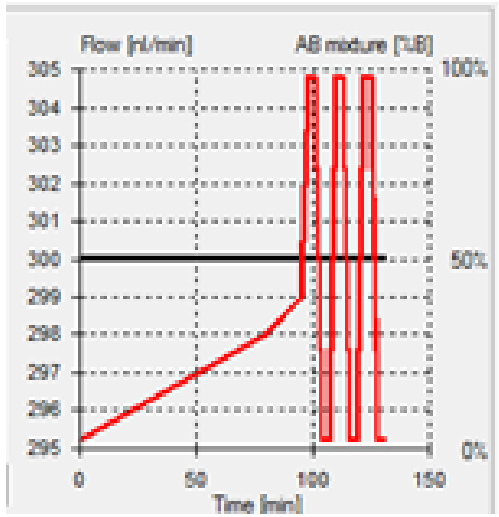
Tips for peptide elution:

- *Most peptides have eluted by 35 to 40% organic, most small molecules elute at 60-70% organic.*
- *Eluting your peptides or TMT labeled peptides at 50% organic leaves more hydrophobic contaminants bound to the SPE bed*

Example 7: Repeating Peaks and Polymeric Elutions



Impurities can be seen eluting during the high organic areas of the “seesaw” gradient



Hela-TMT-0 Lysate eluted from Thermo Scientific™ SOLA ™ SOLA SPE cartridge with 70% Acetonitrile 0.1% Formic Acid

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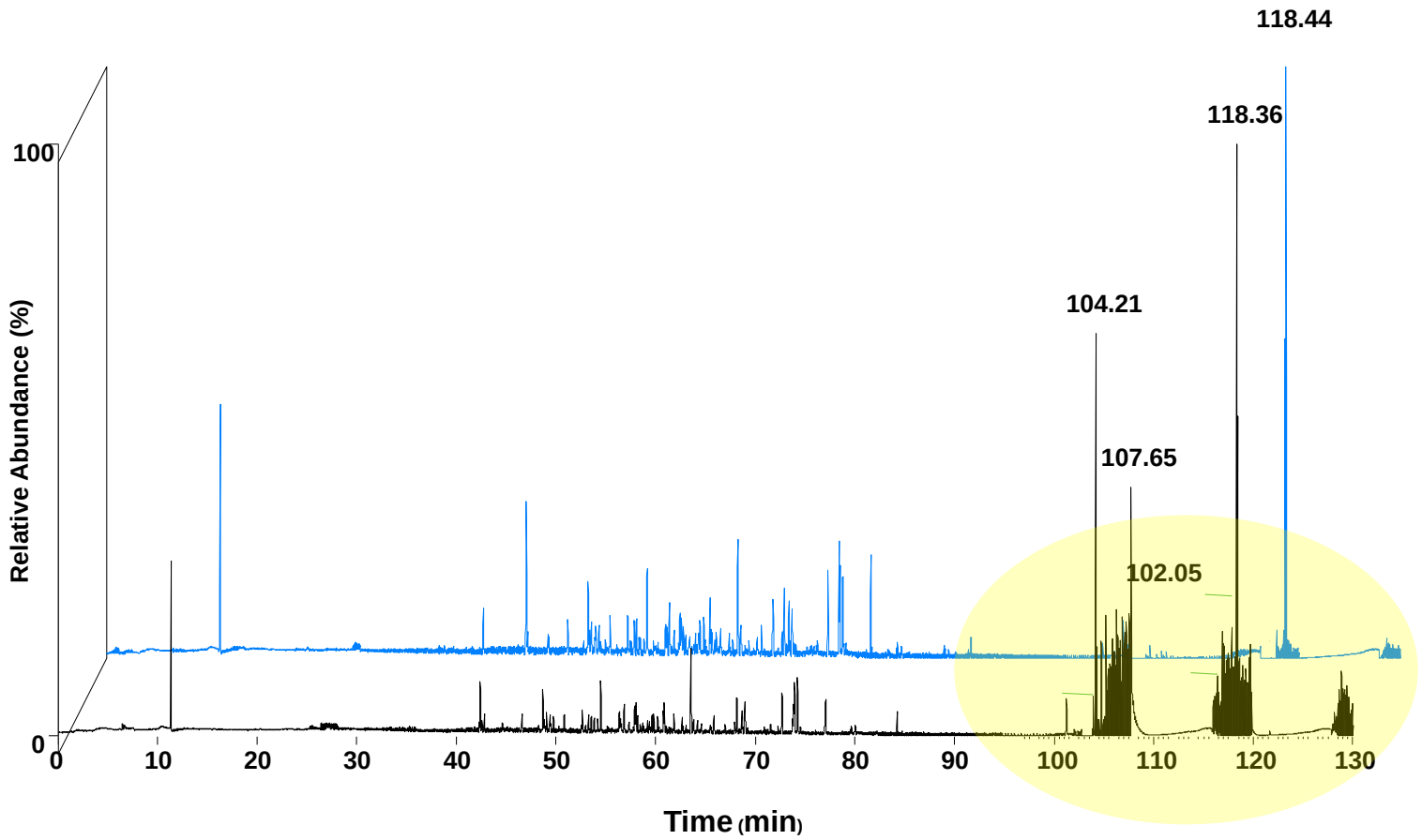
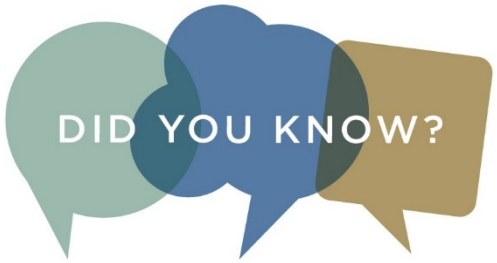
Example 7: Adjusting SPE Elution Conditions to be Peptide Specific



- Adjusting SPE Elution Conditions Leave more Hydrophobic Contaminant on SPE Sorbent
- Decreased intensity of hydrophobic impurities are observed present in the organic wash when **sample is eluted using 50% Acetonitrile**

Top : Hela Lysate-TMT0 eluted from Thermo Scientific™ SOLA ™ SPE cartridge with 50% Acetonitrile 0.1% Formic Acid
Bottom : Hela-TMT-0 Lysate eluted from Thermo Scientific™ SOLA ™ SOLA SPE cartridge with 70% Acetonitrile 0.1% Formic Acid

Learn to Love Your Thermo Scientific EASY-Spray Column



EVERY EASY-Spray column is thoroughly tested before it is sent to our customers?

Each of the previous examples were obtained from customers who all thought the issues they observed were due to a “bad column”.

But …

Don’t forget the basics of Chromatography

- Careful sample prep, sample quality, and sufficient column washing and equilibration are key to a successful analysis

Column Conditioning and Equilibration

Chromatography can be EASY but there are always a few things that need to be remembered:

1. All columns need to be conditioned anytime flow is introduced into a column that is at rest
2. nLC systems have scripts that you can run with these parameters already detailed
3. Other LCs must be conditioned manually, increasing flow in 50 nL/min increments until desired running flow rate is achieved

   → Slowly ramping up flowrate prevents over-stressing the column which can present as sputtering and elevated operating pressure

4. Column equilibration has a dramatic affect on run to run reproducibility.

Tip:

Use the tables below as guidelines for number of conditioning steps and equilibration volume for each EASY-Spray column.

Thermo Scientific EASY-nLC 1200 Column Conditioning Script Parameters

Column	Total Duration [min]	Max Pressure [bar]	No. of Steps
ES800A	20	500	10
ES801A	30	800	15
ES802A	40	1000	20
ES803A	40	1000	20
ES804A	30	800	15
ES805A	45	1200	22
ES806A	40	1000	20
ES810A	20	300	10
ES811A	30	800	15
ES812A	20	500	10

- This table is based on the “EASY-Spray Columns Guidance for column set up and installation Tips to maximize column lifetime”.
- For other LC’s, please follow the guidance for column conditioning contained in the guide i.e., “Start with a flow rate of 50 nL/min and increase stepwise in 50 nL/min steps until the maximum column pressure is reached or system pressure is reached. Each step should last for 2 minutes.”

Thermo Scientific EASY-Spray Column Equilibration Volume Guidelines

If not seeing acceptable reproducibility, check column equilibration volume!

The table below is a guide to the volume required at the start (or end) a gradient to equilibrate the column for the next injection. As a starting point use 5x column volume, but more may be required.

Column	Length [mm]	ID [µm]	Pressure [bar]	Volume [µL]	Column Equilibration Volume [µL]*
ES800A	150	75	500	0.66	3.5
ES801A	150	50	800	0.29	1.5
ES802A	250	75	1000	1.10	5.5
ES803A	500	75	1000	2.21	11
ES804A	150	75	800	0.66	3.5
ES805A	750	75	1200	3.31	16.5
ES806A	150	75	1000	0.66	3.5
ES810A	250	200	300	7.85	39.5
ES811A	150	75	800	0.66	3.5
ES812A	150	75	500	0.66	3.5

*Figures are rounded to the nearest 0.5 µL

Protocol: Column Conditioning and Equilibration

The protocols shown below can be used to clean a column that is contaminated, to clean a column prior to placing it into storage, or as a preventative measure.

- **Protocol 1:** Used on an Easy nLC or UltiMate 3000 RSLCnano system for running flush solutions prior to column storage or in cases of suspected contamination

- **Protocol 2:** Used at the end of every sample gradient as a preventative measure.
  - Implementing this as a preventative measure also gives a diagnostic view of how the column is doing over time, e.g. whether there is build up of contaminants over time.

Protocol 1: Cleaning Gradient for Running Flush Solution

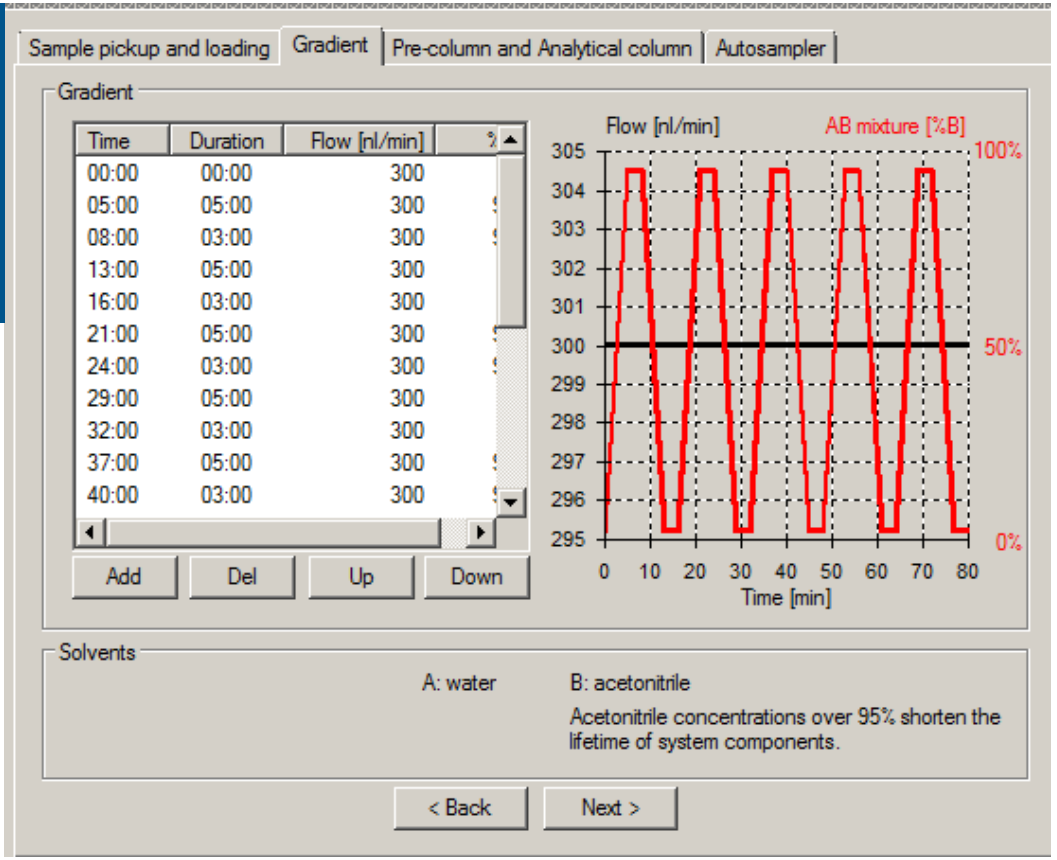
For EASY nLC and UltiMate 3000 RSLCnano LC Systems

This protocol is utilized in cases of:

1) Column contamination
2) Before putting column into storage

Inject ~15 µL Flush Solution (10% Acetone 45% Acetonitrile 45% Isopropanol: Fisher MB124-1).

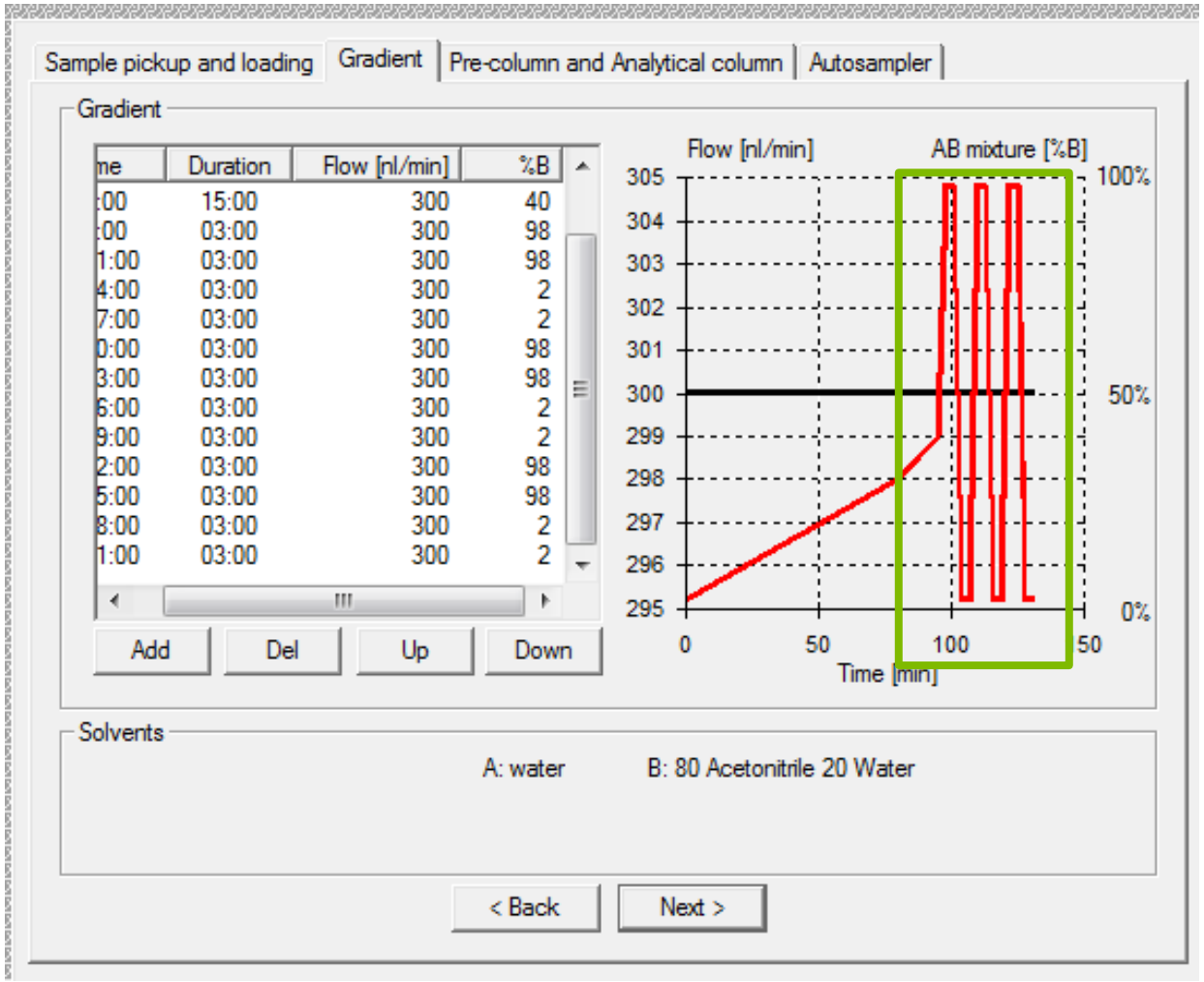
NOTE: Use flow rate that is appropriate for your column.



Time	Duration	Flow (nL/min)	%B
0	0:00	300	**2**
5	5:00	300	95
8	3:00	300	95
13	5:00	300	2
16	3:00	300	2
21	5:00	300	95
24	3:00	300	95
29	5:00	300	2
32	3:00	300	2
37	5:00	300	95
40	3:00	300	95
45	5:00	300	2
48	3:00	300	2
51	5:00	300	95
54	3:00	300	95
59	5:00	300	2
62	3:00	300	2
67	5:00	300	95
70	3:00	300	95
73	5:00	300	2
75	3:00	300	2
80	5:00	300	95
83	3:00	300	95
88	5:00	300	2
91	3:00	300	2
96	5:00	300	95
99	3:00	300	95
104	5:00	300	2
107	3:00	300	2

Protocol 2: Cleaning Gradient at the End of a Sample Gradient

Used at the end of every sample gradient as a preventative measure



- Add 3 seesaw cleaning cycles to the end of the sample gradient
  - Will help prevent carryover and column contamination.

- Adding the three cleaning cycles is recommended practice for all nano LC programs.

Summary: Tips and Tricks to Reduce the Risk of Column Contamination

Tip	Best practice
Do not use premade solvent	Always prepare fresh degassed solvents
Never add to solvent bottles already being used	Use fresh mobile phases in clean reservoirs
Change sample loop and injection needle periodically.	To rule out sample loop contamination run a blank followed by a no injection run
Use SPE, eluting your peptides with 50% ACN 0.1% formic acid (instead of 70% ACN 0.1% formic acid)	Reduce contaminants by using SPE - Cell lysate digests, plasma samples and peptide labeling are likely to produce contaminants
Run 3 cleaning cycles at the end of each run	This as a preventative measure that can reduce carryover and gives a diagnostic view of how the column is doing over time, e.g. whether there is build up of contaminants over time.
Run 12-24 hours of cleaning gradients with flush solution injections between sample sets or after use/prior to column storage	This as a preventative measure that can reduce carryover and extend the lifetime of your column

More Resources

Find more resources and learn more about EASY-Spray columns at

www.thermofisher.com/EASYspray

www.separatedbyexperience.com/chromexpert

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